The role of the chemokine receptor CXCR4 in oral squamous cell carcinoma metastasis

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The Role of the Chemokine Receptor CXCR4 in Oral Squamous Cell Carcinoma Metastasis

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Thesis submitted for the degree of Doctor of Philosophy
Abstract

Oral Squamous Cell Carcinoma (OSCC) is the sixth most common cancer worldwide. The greatest cause of mortality and surgical morbidity is due to the common spread of tumour cells from the primary lesion to the lymph nodes of the neck. This pathway is not unique to cancer: under physiological conditions, specific chemokine receptors on the surface of leukocytes mediate cell “homing” to tissues defined by gradients of complimentary chemotactic cytokines (chemokines). The chemokine receptors CXCR4 and CCR7 mediate leukocyte “homing” to secondary lymphoid tissue and have been demonstrated on the surface of a number of carcinomas of the aero-digestive tract. The aim of this project was to determine the role of chemokine receptor expression and function in OSCC metastasis.

CXCR4 mRNA expression (microarrays and semi-quantitative RT-PCR) and surface protein production (flow cytometry and immunocytochemistry) was significantly increased in some (but not all) established OSCC cell lines compared with primary oral keratinocytes grown in culture. Examination of clinical samples using a novel, quantitative immunohistochemistry methodology demonstrated a positive association between CXCR4 staining of the cell membrane in primary OSCC lesions and histological evidence of lymph node metastases. CXCR4 over-expression in a constitutively low expressing OSCC cell line (H357) was produced using stable transfection with the CXCR4 insert. Stimulation of CXCR4-bearing OSCC cells with the ligand SDF was shown to mediate statistically significant increases in OSCC cell proliferation in-vitro using a number of complimentary techniques. No effect on apoptosis was demonstrated. The SDF/CXCR4 axis also mediated significant increases in tumour cell chemokinesis, chemotaxis and invasion as measured by a range of in-vitro assays.

These results demonstrate a potential role for CXCR4 as part of a panel of prognostic markers for OSCC. Therapeutic strategies aimed at the SDF/CXCR4 axis may be clinically beneficial if the problems of systemic toxicity can be overcome.
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<td>AIP4</td>
<td>Atrophin-Interacting Protein-4</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
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<tr>
<td>BCC</td>
<td>Basal Cell Carcinoma</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAF</td>
<td>Carcinoma-associated Fibroblast</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony Forming Units</td>
</tr>
<tr>
<td>CISK</td>
<td>Cytokine Independent Survival Kinase</td>
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<tr>
<td>CLN</td>
<td>Cervical Lymph Nodes</td>
</tr>
<tr>
<td>CMIR</td>
<td>Cancer Mortality to Incidence Ratios</td>
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<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<tr>
<td>DAPI</td>
<td>4',6-Diamidino-2-phenylindole dimethylsulfoxide</td>
</tr>
<tr>
<td>DEPC</td>
<td>Diethyl pyrocarbonate</td>
</tr>
<tr>
<td>ECACC</td>
<td>European Collection of Cell Cultures</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal cell Growth Factor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial-Mesenchymal Transition</td>
</tr>
<tr>
<td>EPC</td>
<td>Endothelial Progenitor Cell</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence-activated Cell Sorting</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal Calf Serum</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
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<tr>
<td>HGF</td>
<td>Human Gingival Fibroblast</td>
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<tr>
<td>HHV</td>
<td>Human Herpes Virus</td>
</tr>
<tr>
<td>HIF-1α</td>
<td>Hypoxia-inducible Factor-1α</td>
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<td>HNSCC</td>
<td>Head &amp; Neck Squamous Cell Carcinoma</td>
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<tr>
<td>HpGF</td>
<td>Hepatocyte Growth Factor</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
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<td>HPV</td>
<td>Human Papilloma Virus</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>HTLV-1</td>
<td>Human T lymphocyte Virus Type-1</td>
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<td>IL-1α</td>
<td>Interleukin-1α</td>
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<tr>
<td>ICAM</td>
<td>Intercellular Adhesion Molecule</td>
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<tr>
<td>IHC</td>
<td>Immunohistochemistry</td>
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<tr>
<td>IMRT</td>
<td>Intensity Modulated Radiotherapy</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
</tr>
<tr>
<td>LCMD</td>
<td>Laser Capture Micro Dissection</td>
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<tr>
<td>MFV</td>
<td>Mean Fluorescence Values</td>
</tr>
<tr>
<td>MMLV-RT</td>
<td>Moloney Murine Leukaemia Virus Reverse Transcriptase</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix Metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic Acid</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl tetrazolium bromide</td>
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<tr>
<td>NEV</td>
<td>Normalised Expression Value</td>
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<tr>
<td>NIH</td>
<td>National Institute for Health</td>
</tr>
<tr>
<td>NOK</td>
<td>Normal Oral Keratinocyte</td>
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<tr>
<td>NRF-1</td>
<td>Nuclear Respiratory Factor-1</td>
</tr>
<tr>
<td>NSCLC</td>
<td>Non-Small Cell Lung Carcinoma</td>
</tr>
<tr>
<td>NSK</td>
<td>Normal Skin Keratinocyte</td>
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<tr>
<td>ONPG</td>
<td>o-nitrophenyl-β-D-galactopyranoside</td>
</tr>
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<td>OSCC</td>
<td>Oral Squamous Cell Carcinoma</td>
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<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PET</td>
<td>Positron Emission Tomography</td>
</tr>
<tr>
<td>PET/CT</td>
<td>Combined Positron Emission Tomography with Computed Tomography</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphoinositide 3-kinase</td>
</tr>
<tr>
<td>RLB</td>
<td>Reporter Lysis Buffer</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>SDSDB</td>
<td>Sodium-diphosphate spectrophotometry dilution buffer</td>
</tr>
<tr>
<td>SCC</td>
<td>Squamous Cell Carcinoma</td>
</tr>
<tr>
<td>SDF</td>
<td>Stromal cell-derived Factor</td>
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<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>SLT</td>
<td>Secondary Lymphoid Tissue</td>
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<tr>
<td>siRNA</td>
<td>Small Interfering Ribonucleic Acid</td>
</tr>
<tr>
<td>SNB</td>
<td>Sentinel Node Biopsy</td>
</tr>
<tr>
<td>sqRT-PCR</td>
<td>Semi-quantitative Reverse Transcription Polymerase Chain Reaction</td>
</tr>
<tr>
<td>TGF-β1</td>
<td>Transforming Growth Factor-β1</td>
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<tr>
<td>TNFα</td>
<td>Tumour Necrosis Factor-α</td>
</tr>
<tr>
<td>tRNA</td>
<td>Total Ribonucleic Acid</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase Plasminogen Activator</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular Endothelial Growth Factor</td>
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<tr>
<td>VHL</td>
<td>Von Hippel-Lindau</td>
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<tr>
<td>WHIM</td>
<td>Warts, Hypogammaglobulinaemia, Immunodeficiency and Myelokathexis syndrome</td>
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<tr>
<td>X-Gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside</td>
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<td>YY-1</td>
<td>Yin Yang-1</td>
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Acknowledgements

I would like to thank my principal supervisor Professor Paula Farthing for her continued support and encouragement during my MPhil/PhD studies. Her balance and counsel has been greatly appreciated during the time of my ambitious academic plans. I would also like to thank my collaborators at Charterhouse Square, particularly Dr Julia Wilson (Cancer Research UK Translational Oncology) for her guidance on quantitative PCR and FACS analysis, and Mrs Tracey Chaplin (Medical Oncology) for her help with hybridising and scanning my microarray samples. Special mention also goes to the staff at the Department of Clinical and Diagnostic Oral Science and particularly Professor Farida Fortune for her support as Head of Department and Dean of the Dental School.

This work has been supported by grants from The British Society of Oral and Maxillofacial Surgery, The British Society of Oral and Maxillofacial Pathology and Cancer Research UK. I was also supported by clinical training fellowships from The Charitable Trust of St Bartholomew’s and The London School of Medicine and Dentistry and The Royal College of Surgeons of England.

I must thank my wife Sophie who has been a constant source of support during my studies, and to Lucy, Alexander and Sam who have brought us such joy, happiness (and tiredness!). Finally, this work is dedicated to my mother Angela.
Chapter 1: 
Introduction and 
Literature Review
1.1 Oral Squamous Cell Carcinoma

1.1.1 Definition

Squamous cell carcinomas of the head and neck region are malignant tumours derived from the squamous epithelia of the upper aero-digestive tract. Such a pathological definition is attractive in its simplicity. However, it fails to address the exact anatomical location of disease and its considerable impact on presentation, diagnosis, treatment and prognosis. Epidemiological observations have demonstrated a significant difference in Cancer Mortality to Incidence Ratios (CMIR) between, for example, carcinoma of the lip (0.065) and carcinoma of the upper oesophagus (0.96) which are both squamous cell carcinomas by pathological classification (Office of National Statistics 2006).\(^1\) One hypothesis is that this difference may be explained by clinical factors alone (such as delays in diagnosis and stage of disease at presentation). However, it is also possible that squamous cell carcinomas from different anatomical sites vary in their tumour biology. Therefore, in order to minimise the effect of this potentially confounding variable, it is necessary to be more specific in the definition of oral squamous cell carcinoma (OSCC).

OSCC accounts for over 90% of all oral mucosal malignancies (Daley and Darling 2003). For the purpose of this thesis I have defined OSCC as squamous cell carcinomas arising from the tongue, gum, floor of mouth, palate, oropharynx, tonsil, piriform sinus and hypopharynx. These match the anatomical areas as defined in the diagnostic codes C01-06, C09, C10, C12 and C13 from the International Classification of Disease (Tenth Revision, 2007 Version) (ICD-10) outlined in Table A1.1, Appendix 1 (World Health Organisation 2007). By this definition I have excluded carcinomas of the lip, salivary glands, nasopharynx, oesophagus, larynx and trachea (ICD-10 codes C00, C07, C08, C11, C14, C15, C32 and C33) although the metastatic patterns of squamous carcinoma of the lip will be compared briefly with OSCC using immunohistochemistry. Overall, approximately 40% of head and neck squamous carcinomas occur at sites defined “oral” as above (Stewart and Kleihues 2003). Further

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\(^1\) Cancer Mortality to Incidence Ratios (CMIR) are calculated by dividing the number of patients registered as dying of a particular disease by the number diagnosed with the same condition within the same year. United Kingdom data from 2003 are presented in the example.
subdivisions of OSCC by aetiology, tumour stage and prognosis are used to inform the clinical decision-making process and these are discussed below.

1.1.2 Epidemiology

Oral cancer and its treatment is a significant cause of morbidity and mortality amongst the population both in the United Kingdom and worldwide. In Asia and the Indian subcontinent, OSCC is the most common malignancy with a lifetime prevalence approaching 20-30% (Parkin, Pisani et al. 1993). Although accurate data derived from cancer registries are not available from these countries, the World Health Organisation has estimated that squamous carcinoma of the head and neck is the sixth most common malignancy worldwide (Stewart and Kleihues 2003; Warnakulasuriya 2009). In the United Kingdom, OSCC is much less common: the most recent epidemiological data derived from cancer registries show that 4,337 patients were diagnosed with the disease in 2005. However, two worrying trends in the United Kingdom incidence of OSCC have been described. Firstly the incidence within the immigrant Asian population is disproportionately high (Warnakulasuriya, Johnson et al. 1999). Secondly the incidence in younger adults has nearly doubled over the last three decades (Figure 1.1) (Llewellyn, Johnson et al. 2001; Warnakulasuriya 2009). Furthermore, similar changing patterns of incidence in younger patients (defined as adults under 45 years of age) have been demonstrated in Scotland (Macfarlane, Sharp et al. 1996; Warnakulasuriya 2009), Europe (Levi, La Vecchia et al. 1995; Annertz, Anderson et al. 2002) and North America (Davis and Severson 1987; Schantz and Yu 2002). Based on these epidemiological observations it is tempting to hypothesise that these trends are restricted to the Developed World. However, data from developing countries suggest that this is not the case as similar observations have been made in both the indigenous and migrant populations from the Indian Subcontinent (Gupta 1999; Warnakulasuriya, Johnson et al. 1999). This suggests that the factors influencing incidence rates are not geographically related. More recent evidence suggests that this trend may be due to OSCC following exposure to Human Papilloma Virus (HPV) (D’Souza, Kreimer et al. 2007).

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2 The precise global incidence and prevalence of OSCC is uncertain primarily due to poor registration data in the highly endemic areas of South Asia.
3 Data derived from http://info.cancerresearchuk.org/cancerstats/types/oral/incidence/.
OSCC is a highly lethal condition. Its calculated CMIR for the United Kingdom in 2005 was 0.33 for men and 0.36 for women. There are two principal reasons for the high lethality of this disease. Firstly; a significant proportion of patients present late with advanced disease. In these cases important anatomical structures may have been breached or tumour cells spread to multiple distant organs with the consequence that curative treatment is not possible. Secondly, for those patients that present early, treatment often fails to achieve disease control. The commonest cause of treatment failure is recurrent disease, either at the site of the original (primary) lesion or at a distant (secondary) site. If widespread dissemination has occurred then disease control is often impossible and further treatment may only be palliative. Consequently, in order to affect a cure in patients presenting early, it is vital to identify and address all the disease that is present, however small. In the diagnostic phase

\[\text{Figure 1.1: Percentage change in incidence rates of oral cancer in British men, 1975-6 / 2004-5.}\]
\[\text{Source: http://info.cancerresearchuk.org/cancerstats/types/oral/incidence/}\]

clinicians continually face the uncertainty that stems from the fact that small volumes of tumour cells (at either primary or secondary sites) are very difficult to detect.5

1.1.3 Clinical features

OSCC often presents as a painless white patch or ulcer within the oral mucosa. The most commonly affected areas are the floor of the mouth and both the lateral border and ventral surface of the tongue (Shah 1990). These are often referred to as the “sump” areas and are thought to be at particular risk because of the combined effect of gravity increasing the concentration of carcinogens in saliva and the thin oral mucosa. The typical clinical features of OSCC are of a primary lesion arising from within the squamous epithelium (oral mucosa) with a wide spectrum from mild dysplasia through to frank carcinoma. It should be stressed that, clinically, many OSCC lesions fail to demonstrate sequential progression from dysplasia to carcinoma unlike the well documented sequence of adenoma to carcinoma progression in colorectal adenocarcinoma. Carcinoma in situ is defined as full thickness (or almost full thickness) architectural disturbance in the viable cell layers accompanied by pronounced cytological atypia while invasive carcinoma is defined by penetration of the basement membrane below the basal layer of keratinocytes (Barnes, Everson et al. 2005; Gandolfo, Pentenero et al. 2006; van der Waal 2009). OSCC, like any carcinoma, has the potential to spread both locally (by direct invasion) and to distant sites (metastasis). Distant metastasis may involve the local lymph nodes or more remote body tissues. Oncological principles governing curative treatment determine that disease at any of these locations must be addressed adequately. However, the significance of subclinical micrometastases on clinical symptoms and overall survival remain debatable (vide infra).

5 In the absence of clinically or radiologically detectable metastatic disease, the indication for primary treatment (surgery or radiotherapy) of the ipsilateral neck is set an arbitrary threshold of 20-40% (Weiss, Harrison et al. 1994; Okura, Aikawa et al. 2009).
1.1.4 The impact of tumour metastases on patient prognosis and treatment

OSCC can spread locally (by the direct invasion of adjacent tissues), regionally to the cervical lymph nodes (via lymphatic channels) or to distant sites via the bloodstream. It is clear from epidemiological data that metastatic involvement of the cervical lymph nodes (CLN) is the single most important prognostic indicator of survival for patients with OSCC (Shah 1990; Persky and Lagmay 1999; Woolgar, Rogers et al. 1999; Rogers, Brown et al. 2009). Data from Liverpool (UK) demonstrate a disease-specific five-year survival rate from presentation from primary disease alone, CLN metastases within the capsule and extra-capsular CLN metastases of 81%, 64% and 21% respectively (Woolgar, Rogers et al. 1999; Rogers, Brown et al. 2009). Data from the same area has shown that the presence of distant metastasis reduces the treated five-year survival to less than 20% (Jones, Roland et al. 1994).

The main modalities for curative treatment of OSCC are surgery and radiotherapy. These may be used to treat lesions in the mouth and metastatic deposits in the neck. Both treatment modalities carry significant morbidity and risk of complications. Consequently, the treatment recommended should be sufficient to control the disease but avoid over-treatment and unnecessary suffering. However, incomplete removal of tumour cells will result in the failure of curative treatment. OSCC recurrence in the neck may occur if tumour deposits in the cervical lymph nodes remain outside the primary treatment field. Therefore, in order to offer appropriate treatment, it is critically important that clinicians are accurately able to determine the extent of disease dissemination at the time of diagnosis.

1.1.5 Diagnosis of OSCC metastases

Current detection strategies for metastatic deposits are limited to clinical and radiological examination. The critical determinant of the clinical usefulness of each diagnostic test is its sensitivity. Sensitivity is defined as the number of true positive results divided by the combined number of true positives and false negatives. A highly sensitive test will return few false negative results and consequently prevent the clinician from under-treating the disease in the neck.
1.1.5.1 Clinical history and examination

Primary OSCC lesions are frequently symptomatic although features may be subtle (Marshall and Mahanna 1997). However, OSCC metastases are frequently asymptomatic. For example, pain is poorly predictive of lymphatic (Shah 1990) and distant (Carlson and Ord 2002) tumour spread. Cervical lymph nodes are readily accessible to clinical examination by digital palpation. It might be predicted that the examination of the neck by skilled clinicians would provide accurate detection of metastases. This is not the case. Clinical examination has a false negative detection rate of between 30 to 50% when compared with subsequent histological examination of the surgical resection specimen (Woolgar and Scott 1995; Hodder, Evans et al. 2000; Greenberg, El Naggar et al. 2003). There is considerable variability in reported findings between clinicians, even when clearly palpable disease is present in the neck (Alderson, Jones et al. 2001). What is required is a reliable non-invasive method for the detection of small cervical lymph node metastases.

1.1.5.2 Radiological assessment

Radiological assessment marginally improves diagnostic sensitivity in those patients with impalpable lymph nodes (Woolgar 1999). The technique of ultrasound-guided fine needle aspiration cytology (FNAC) produces the highest sensitivity (Hodder, Evans et al. 2000; Stuckensen, Kovacs et al. 2000). However, there are three significant limitations in the methodologies of these series. Firstly, when treatment of the neck is based on the ultrasound findings, a considerable proportion of patients with no detectable CLN metastases either clinically (cN0) or radiologically (rN0) do not undergo elective neck dissection preventing the comparison with histological findings. Secondly, the cohort of patients with no clinical or radiological evidence of lymph node metastases (cN0 and rN0) is seldom compared to lymph node positive patients preventing specific analysis of the former, clinically important, group. Finally, prospective studies looking at survival data do not take into account the implications of other factors (such as tumour thickness) on prognosis and thus it is possible that their results may be explained by other factors associated with the tumour (Rogers, Brown et al. 2009). Consequently, it is likely that the quoted sensitivity for ultrasound-guided FNAC (85-90%) is not representative of the accuracy of the technique in the cN0 patient. Furthermore, it should not be forgotten that for every hundred cN0
patients staged using this technique, at least ten will have their neck metastases missed. This is an unacceptable rate of treatment failure.

Conventional radiological techniques (ultrasound, CT and MRI) focus on the detection of anatomically abnormal lymph nodes. With the advent of Positron Emission Tomography (PET) scanning it has been possible to investigate functional changes in cellular glucose metabolism that may be associated with high cell turnover such as that associated with tumour growth. Unfortunately initial results have been disappointing with sensitivities reaching only 70% (Stuckensen, Kovacs et al. 2000). The role of PET/CT in the management of first presentation OSCC is therefore restricted to detection of unknown primary lesions and staging advanced tumours (Wong and Batty 2009).

The accurate detection of OSCC tumour metastases is fundamental for treatment planning and patient prognosis. There are significant limitations to the use of current clinical and radiological screening modalities due to their high false-negative detection rates. Considering that metastatic cells are derived from the primary tumour it is possible that a better understanding of the molecular mechanisms of tumour metastasis may enable the scientific assessment of the primary tumour to aid the clinical prediction of distant dissemination.

1.2 The observed clinical behaviour of OSCC metastases

1.2.1 OSCC spread to cervical lymph nodes.

The most frequent site of OSCC spread is to the CLN. Overall, approximately 45% of patients presenting with OSCC have CLN metastases at the time of diagnosis (Shah 1990; Woolgar 1999; Okada, Mataga et al. 2003). Two predominant patterns of OSCC dissemination to the CLN have been described by Woolgar based on the histological examination of surgical resection specimens (Woolgar 1997; Woolgar 1999; Woolgar 2007). Firstly, the majority of tumours appear to spread to the “first echelon” of the CLN and then sequentially down the deep CLN chain by a progressive “overflow” mechanism (Woolgar 1999). According to this model the pattern of lymphatic dissemination is predictable and treatment can be targeted once the lowest lymph node
basin has been determined. However, a second pattern of tumour spread has also been observed from clinical specimens in which lymphatic deposits were seen to spread in a non-sequential manner and “skip” lymph nodes (Woolgar 1999). In a series of 277 patients with OSCC, CLN “skipping” was demonstrated in 15.8% of cases (Byers, Weber et al. 1997). Other studies suggest this is an overestimate with only 2/89 (2.2%), 5/192 (2.6%), 21/526 (3.9%) and 14/226 (6.2%) patients demonstrating skipping (De Zinis, Bolzoni et al. 2006; Dias, Lima et al. 2006; Woolgar 2007; Lodder, Sewnaik et al. 2008). Despite the technical limitations of these studies, the clinical observation of lymph node skipping supports the hypothesis that CLN metastasis does not follow a sequential path and may be a common event in the early pathogenesis of certain OSCCs. This has significant implications not only for our understanding of tumour biology, but also for patient outcomes based on sentinel node biopsy and elective surgical treatment of the N0 neck, both of which are currently undergoing clinical investigation.

The reason for this dichotomy is not clear. One possible hypothesis is that the pattern of lymph node metastasis is entirely determined by the position of the primary lesion within the mouth. OSCC-derived skip metastases to the lower CLN levels have been demonstrated with primary lesions arising in the tongue (Byers, Weber et al. 1997; Woolgar 2007). In a series of 339 patients Dias et al identified skip metastases in 7/222 (3.1%) tongue lesions but none out of 117 floor of mouth lesions (Dias, Lima et al. 2006). It is possible that metastatic deposits entering the lymphatic channels from the tongue may bypass the upper cervical lymph nodes. However, clinical evidence does not support this hypothesis. The location of the first (sentinel) lymph node within the first CLN basin for OSCCs can be determined using lymphoscintography. For all primary tumours (including tongue tumours) the sentinel lymph node was located in upper cervical chain (Stoeckli, Pfaltz et al. 2002). Consequently, the variable patterns of cervical lymph node metastasis may not be attributable to differences in primary tumour location. However, it is possible that the observed patterns of lymph node metastasis are due to other features of the primary tumour including size (horizontal diameter) or thickness (vertical depth of invasion). The hypotheses that attempt to

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6 The detection of micrometastases is limited by factors including surgical lymph node yield and assiduous histological examination of multiple fine microscope sections. Completely accurate investigation would require analysis with molecular methods of tumour cell detection.
7 Sentinel European Node Trial (SENT) and Selective Elective Neck Dissection (SEND) studies respectively.
explain the association between these attributes of the primary tumour and the observed patterns of metastasis are outlined below.

Cervical lymph node metastases are more commonly associated with larger primary tumours (Okada, Mataga et al. 2003). The correlation between increasing vertical invasion and an increased incidence of lymph node metastasis in OSCC has been demonstrated by several groups (Mohit-Tabatabai, Sobel et al. 1986; Spiro, Huvos et al. 1986; Yuen, Lam et al. 2002; O-charoenrat, Pillai et al. 2003; O'Brien, Lauer et al. 2003; Sheahan, O'Keane et al. 2003). In melanoma, the depth of invasion of the primary tumour correlates with both lymph node metastasis and survival (Breslow 1970). In OSCC, a histological depth of invasion greater than 5mm was associated with an increased risk of cervical metastasis (relative risk 2.4) and decreased disease specific survival (O-charoenrat, Pillai et al. 2003). As yet there are no published data investigating the relationship between depth of invasion and skip metastases in OSCC.

One hypothesis, based on anatomical principles, would suggest that more host tissue structures are encountered as tumour invasion progresses in both horizontal and vertical directions. These structures include arteries, veins and lymphatic channels. Their relative density increases with vertical distance from the mucosal surface. In theory, invasion of any of these vascular structures would provide a possible conduit for the passage of malignant cells to distant sites. However, clinical evidence does not support this theory in OSCC. Direct vascular invasion is uncommon, occurring in just over 5% of cases (Fagan, Collins et al. 1998). Perineural invasion is more common (occurring in approximately 25-50% of cases) and is associated with lymph node metastasis, extracapsular spread, distant metastasis and disease-specific mortality (Fagan, Collins et al. 1998; Rahima, Shingaki et al. 2004). Nevertheless, nerve fascicles are not hollow and do not provide a direct conduit for the passage of malignant deposits to distant sites. Therefore, what this may represent is a manifestation of a more aggressive tumour phenotype (with the ability/predilection to spread along nerves) rather than a direct causal relationship.
1.2.2 OSCC distant metastasis to distant body sites.

Data based on the incidence of symptomatic lesions indicate that the incidence of distant metastases is approximately 1% (Crile 1906; Ferlito, Shaha et al. 2001; Carlson and Ord 2002). However, it is possible that this may be a significant underestimate of the overall prevalence of distant metastases as a large proportion of lesions remain asymptomatic. The small amount of data available in the literature suggests that this is the case. An exhaustive histological examination of all body tissues made at autopsy demonstrated that the overall incidence of distant metastases in patients with OSCC was shown to vary between 24 and 47% (Kotwall, Sako et al. 1987; Slootweg, Hordijk et al. 1996; Jennings and Bradley 2002). This suggests that distant metastatic deposits are common in OSCC but do not ubiquitously assume a clinically significant size. This finding has two important consequences. Firstly, metastases, too small to cause symptoms, often fall below the size detection threshold of clinical and radiological examinations. As a result, distant metastatic tumour burden may be frequently underestimated. Secondly, it appears that distant metastatic deposits may frequently become established in OSCC, but often fail to grow, unlike those in the cervical lymph nodes. One possible explanation is that the development of metastatic lesions is dependent on the host tissue microenvironment. Further clinical evidence supporting this hypothesis comes from the observation that the distribution of OSCC distant metastases is not random. A pattern emerges that favours the lungs (56-80%), bone (16-31%), liver (10-31%) and mediastinal lymph nodes (34%) (Kotwall, Sako et al. 1987; Shingaki, Suzuki et al. 1996).

The clinical behaviour of OSCC metastases is an intriguing paradigm: the growth of OSCC deposits is favoured in the cervical lymph nodes more than certain distant sites. In roughly a third of tongue tumours, the metastatic distribution in the cervical lymph node chain is not sequential despite the uniformity of the location of the sentinel node. These observations suggest that tumour spread is not entirely determined by anatomical considerations. It is possible that the interaction between the tumour cell and the host tissue microenvironment plays a key role in determining metastatic progression.

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8 It has been estimated from one series that, although diagnosed with advanced head and neck cancer, approximately a third of these patients did not die of their disease (Jennings and Bradley, 2002).
1.3 Hypothetical models of metastatic dissemination

Historically, the potential interaction between malignant cells and their host has been the subject of much consideration. Two dominant theories of metastatic dissemination have emerged and each contributes to the modern understanding of metastatic mechanisms.

1.3.1 “Mechanisms of Circulation” hypothesis

Until the 1970s it was widely believed that metastatic dissemination was primarily determined by the anatomical structure of the vascular system. This “Mechanisms of Circulation” hypothesis was originally proposed by James Ewing in 1928 and outlined how the delivery of malignant cells was channelled by the existing lymphatic and vascular channels in the adjacent tissue (Ewing 1928).

On first inspection, it appears that the clinical evidence from OSCC would support this theory: invasion of the lymphatic channels by OSCC primary lesions is more common than vascular invasion and this accounts for the greater incidence of lymph node metastases compared with distant dissemination (Shah 1990; Fagan, Collins et al. 1998; Ferlito, Shaha et al. 2001). However, the “Mechanisms of Circulation” hypothesis does not explain several features of OSCC metastasis based on clinical observations. Firstly, it does not explain the occurrence of “skip” metastases in the cervical lymph node chain found in up to 30% of patients (Byers, Weber et al. 1997; Woolgar 1999). Secondly, it does not explain the relative preponderance (16-30%) of distant bony metastases compared with the fraction (5%) of the circulatory flow received by the bone as determined experimentally (Tothill and MacPherson 1986; Kotwall, Sako et al. 1987; Shingaki, Suzuki et al. 1996). These observations in OSCC are consistent with clinical and experimental evidence in other tumours that suggests that metastatic dissemination cannot be explained by mechanical factors and random distribution alone (Hart and Fidler 1980).
1.3.2 “Soil and Seed” hypothesis

The “Soil and Seed” theory of metastatic dissemination was originally proposed by Stephen Paget in 1889 (Paget 1889). He recorded the disparity between organ blood flow and the incidence of distant metastases in breast cancer and concluded that the development of a tumour metastasis at a specific location was due to an interaction between the disseminated tumour cells (“seed”) and the local tissue microenvironment (“soil”). Clinical observation of epithelial tumours such as breast, bronchus, renal, thyroid and prostate carcinomas reveals that they commonly metastasise to bone (Coleman 1997). The incidence of bony metastases in these tumours is disproportionately greater than the volume of the circulatory blood flow directed to bone.

By inference, Paget’s theory predicts that distant metastases will not develop if the interaction between the tumour and local tissue is not favourable (Paget 1889). Consequently, the “Seed and Soil” theory predicts that the metastatic process is not 100% efficient. Indeed, there is compelling clinical and experimental evidence that carcinoma metastasis is highly inefficient. Experimental evidence comes from the injection of viable tumour cells into the circulation of nude mice. These results demonstrate that less than 0.1% of tumour cells are still viable 24 hours after injection and under 0.01% of these tumour cells form metastatic deposits (Fidler 1970). Clinical evidence includes the palliative treatment of ovarian carcinoma by peritoneo-venous shunting. Using this technique, malignant ascites is delivered directly into the jugular vein and, via the right heart, to the pulmonary capillary bed. However, patients treated in this way only rarely demonstrate metastases outside of the peritoneal cavity at autopsy (Tarin, Price et al. 1984).

Observational studies in OSCC indicate that only a minority of distant metastases develop into lesions of clinically significant size. At autopsy the incidence of microscopic distant metastases approaches 50% (Kotwall, Sako et al. 1987). However, only 1% of distant metastases become large enough to cause symptoms (Crile 1906; Carlson and Ord 2002). These findings suggest that the vast majority of the metastatic deposits that initially invade at distant sites fail to proliferate substantially to form clinically significant lesions.
1.3.3 Contemporary perspectives on metastatic dissemination.

Paget’s and Ewing’s original hypotheses were amalgamated by Sugarbaker (1979), based on a review of clinical cases. He proposed that regional metastasis could be attributed to anatomical factors (such as venous or lymphatic drainage), but distant metastases were site specific and supported a “seed/soil” hypothesis (Sugarbaker 1979).

However, it may be that this dichotomy between the mechanisms underpinning lymphatic and distant metastases is over-simplistic. The “mechanical” delivery of malignant cells to the local lymph nodes does not circumvent the possibility of interaction between the tumour cell and the host microenvironment at this point. One of the principal functions of peripheral lymph nodes is to present foreign antigen to the immune system in order to coordinate a response. Therefore tumour cells within the peripheral lymph nodes must evade the host immune response and undergo significant proliferation in order to produce the lymphatic deposits observed clinically.

Metastases are formed by a complex sequence of cellular events initiated by the delivery of primary tumour fragments to distant locations by the circulation. This multi-step process has been summarised by Fidler and is listed in Table 1.1 (Fidler 2003). This parallels Darwin’s theories of natural selection: each tumour cell is required to “adapt” to each new tissue environment. The “selective pressure” from each environment results in an overall “positive selection” of only those tumour cells capable of survival at each stage. The overall low “efficiency” of metastasis parallels that of the natural selection of species.

It is unlikely that tumour cells develop all of these cellular processes de novo (Fidler 2003; Zlotnik 2004). The sequential progression through dysplasia to invasive carcinoma is caused by an accumulated genetic instability rather than the sudden development of entirely novel signalling pathways. These observations support the hypothesis that tumour cells subvert physiological homeostatic mechanisms to achieve survival and dissemination. Indeed, tumour cells are not unique in their affinity for particular body tissues. There are considerable similarities between steps (5) to (7) of the metastatic process listed above and the pathways of physiological leukocyte “homing” to distant tissue sites including peripheral lymph nodes. These parallels
suggest that it is reasonable to investigate the validity of the mechanisms of leukocyte “homing” as a model for metastatic dissemination.

Table 1.1: Multi-step process of metastatic dissemination

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Proliferation &amp; angiogenesis of the primary tumour</td>
</tr>
<tr>
<td>2</td>
<td>Detachment &amp; invasion of local vascular structures</td>
</tr>
<tr>
<td>3</td>
<td>Malignant embolisation</td>
</tr>
<tr>
<td>4</td>
<td>Transport through the circulation</td>
</tr>
<tr>
<td>5</td>
<td>Arrest in distant organs</td>
</tr>
<tr>
<td>6</td>
<td>Adherence to vessel wall</td>
</tr>
<tr>
<td>7</td>
<td>Tumour extravasation &amp; invasion</td>
</tr>
<tr>
<td>8</td>
<td>Establishment of tumour microenvironment</td>
</tr>
<tr>
<td>9</td>
<td>Metastatic tumour proliferation &amp; angiogenesis</td>
</tr>
</tbody>
</table>

Adapted from (Fidler 2003).

1.4 Physiological pathways of lymphocyte homing and dendritic cell uptake by lymph nodes

1.4.1 Physiological lymphocyte homing

Peripheral lymph nodes play a pivotal role in the coordination of the immune response. This is not due to random chance encounters between participating leukocytes but rather due to a carefully coordinated sequence of molecular events. The targeted circulation and re-circulation of leukocytes to specific tissues is known as “homing”. The mechanisms controlling the “homing” phenomenon are best
understood in lymphocytes. Butcher and Picker (1996) describe the “homing” process as “a multi-step sequential engagement of adhesion and signalling receptors” and their proposed model of lymphocyte-endothelial interaction has gained wide acceptance. This is summarised in Table 1.2 and outlined in Figure 1.2.

Table 1.2: Multi-step pathway of lymphocyte homing

<table>
<thead>
<tr>
<th>Step</th>
<th>Mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Transient adhesion to the endothelial surface</td>
</tr>
<tr>
<td>2</td>
<td>Lymphocyte rolling on the endothelial surface</td>
</tr>
<tr>
<td>3</td>
<td>Lymphocyte activation</td>
</tr>
<tr>
<td>4</td>
<td>Firm adhesion</td>
</tr>
</tbody>
</table>

Adapted from (Butcher and Picker 1996)

The initial contact between the circulating lymphocyte and the target tissue occurs at the endothelial cell surface of the post-capillary high endothelial venules. The delivery of the lymphocyte to this location is determined by the circulatory flow. The transient adhesion of this initial contact is mediated via weak binding between “homing receptors” expressed on the lymphocyte cell surface and “addressin” molecules on the endothelial cell surface (Figure 1.2a). Homing receptors belong to the selectin or integrin families of adhesion molecules and the expression of both homing receptors and addressins is tissue specific (Alon and Feigelson 2002).

1.4.1.1 Addressins and homing receptors mediating the initial stage of lymphocyte homing

Unstimulated, naïve T-lymphocytes preferentially re-circulate to secondary lymphoid tissue (SLT) (von Andrian and Mempel 2003). SLT comprises of the peripheral and mesenteric lymph nodes, spleen and Peyer’s patches of the small intestine. The surface homing-receptor L-selectin (CD62L) is found on naïve T cells and mediates transient adhesion at the post-capillary endothelial surface of the lymph node by binding to
Peripheral-Node Addressin (PNAd) (Table 1.3). Intravital microscopy in mice reveals L-selectin-mediated rolling of naïve T-lymphocytes on subcortical lymph node capillaries expressing PNAd (von Andrian 1996). In oral mucosal-associated lymphoid tissue, lymphocyte homing appears to be mediated by L-selectin / PNAd (Csencsits, Jutila et al. 2002). Naïve lymphocytes expressing L-selectin may also bind to carbohydrate residues on MAdCAM-1 (Bargatze, Julita et al. 1995). MAdCAM-1 is the addressin expressed on gut endothelium although naïve T-lymphocytes are not preferentially taken up at this site. This suggests that there is a second mechanism that also confers a degree of tissue specificity downstream of the homing receptor/addressin interaction.

### Table 1.3: Known Lymphocyte Homing-receptor and Endothelial Addressin interactions by tissue type.

<table>
<thead>
<tr>
<th>Tissue Type</th>
<th>Tissue Type</th>
<th>Endothelial addressin</th>
<th>Lymphocyte Homing Receptor</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral lymph node</td>
<td>Oral-associated lymphoid tissue</td>
<td>PNAd</td>
<td>L-selectin</td>
<td>(Salmi, Hellman et al. 1998)</td>
</tr>
<tr>
<td>Secondary Lymphoid</td>
<td></td>
<td></td>
<td></td>
<td>(Csencsits, Jutila et al. 2002)</td>
</tr>
<tr>
<td>Skin</td>
<td>E-selectin</td>
<td>CLA</td>
<td></td>
<td>(Hwang 2001)</td>
</tr>
<tr>
<td>Gut</td>
<td>MAdCAM-1</td>
<td>Integrin α4β7</td>
<td></td>
<td>(Briskin, Winsor-Hines et al. 1997)</td>
</tr>
<tr>
<td>Oral mucosa</td>
<td>Unknown</td>
<td>Integrin αeβ7</td>
<td>CLA</td>
<td>(Walton, Macey et al. 1998)</td>
</tr>
</tbody>
</table>

The homing-receptor/addressin mediated adhesion is transient and weak. Under physiological flow conditions, vascular shear stress propels the lymphocytes along the endothelial surface (Figure 1.2b). This greatly enhances the likelihood of the rolling T cell encountering chemokines ligands produced on the endoluminal surface. Endothelial cell-derived chemokines bind to specific receptors expressed on the lymphocyte surface (Figure 1.2c). The release of chemokines by endothelial cells is tissue specific. Similarly, the expression of chemokine receptors by lymphocytes defines the chemokines they will respond to. Consequently this mechanism determines a second level of specificity on the lymphocyte / endothelium interaction.

Peripheral lymph node endothelial cells constitutively express the chemokine CCL21 which binds to its receptor CCR7 expressed on the surface of naïve T-lymphocytes (Blades, Manzo et al. 2002; Phillips and Ager 2002; Stein, Soriano et al. 2003). The endothelial chemokine/chemokine receptor interactions that control lymphocyte homing to the skin and gut have also been characterised (Table 1.4). It appears that the chemokine receptor binding is the key determinant of tissue specificity. However, the chemokine receptors involved in lymphocyte homing to the oral mucosa are, as yet, unknown.

Naïve T cells express low levels of the integrin heterodimers αLβ2 (LFA-1), α4β1 (VLA-4) and α4β7 (LPAM), which bind ICAM-1, VCAM-1, and MAdCAM-1, respectively (Springer 1995). Of these, the interaction of αLβ2 with ICAM-1 is particularly important for T cell entry into pLN and T cell interactions with antigen-presenting cells (APCs).

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Endothelial Chemokine</th>
<th>Lymphocyte Chemokine Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peripheral lymph node</td>
<td>CCL21</td>
<td>CCR7</td>
</tr>
<tr>
<td>Skin</td>
<td>CCL17</td>
<td>CCR4</td>
</tr>
<tr>
<td>Gut</td>
<td>CCL25</td>
<td>CCR9</td>
</tr>
</tbody>
</table>
Chemokine mediated lymphocyte activation is mediated by cell-surface G-protein-coupled chemokine receptors. Tissue specific chemokines / chemokine receptor binding induces a conformational change in the expression of integrins on the surface of the lymphocyte and firm (but reversible) adhesion to endothelial integrins (Figure 1.2c) (Stein, Rot et al. 2000). The firm activation-dependent binding of the lymphocyte to the endothelial cell surface is a pre-requisite for the lymphocyte to leave the circulation (extravasation) and enter the tissue stroma (diapedesis).

Following activation in SLT, effector T-lymphocytes develop the capability to home selectively to non-lymphoid tissues including epithelial surfaces. This expedites antigen recognition. Activated T cells decrease expression of the L-selectin homing molecule and the chemokine receptor CCR7. It has been postulated that differential expression of integrins defines the homing properties of activated T lymphocytes to non-lymphoid tissue. The integrins α4β1 and α4β7 appear to be reciprocally expressed (Erle, Briskin et al. 1994). The homing receptor / addressin pairs for memory T-lymphocyte homing to the skin (E-selectin/Cutaneous Lymphocte Antigen [CLA]) and the gastrointestinal tract (MAdCAM-1/α4β7) have been determined (Table 1.3). The lymphocytes that home to the oral mucosa have been characterised and shown to express the integrin αeβ7 and the homing-receptor CLA (Walton, Macey et al. 1998). However, the addressin involved is, as yet, unknown.

The pattern of lymphocyte homing to peripheral lymph nodes and other specific sites is dependent on the combination of a number of complimentary molecular mechanisms. Failure at any one stage results in the return of the lymphocyte to the circulation. The stimulation of functional lymphocyte chemokine receptors by tissue-specific chemokines constitutively expressed by the endothelium is a key component of the homing mechanism. The uptake of lymphocytes from the circulation in this way mirrors the delivery of metastatic tumour cells to distant organs. The possibility that this “homing” mechanism shares molecular similarities with the metastatic process of OSCC and other carcinomas has prompted further investigation (Sections 1.6 and 1.5 respectively).
Figure 1.2: Schematic representation of the four principal steps of lymphocyte homing. Initially transient binding of the T-lymphocyte homing receptor to complementary addressins on the endothelial surface occurs mediating transient arrest (panel a). Tissue specific addressin / homing receptor binding is outlined in Table 1.3. Vascular shear force is resisted by transient binding of the T-lymphocyte homing receptors to complementary endothelial addressins allowing the lymphocyte to “roll” along the endothelial surface (panel b). Endothelial chemokines bind to complementary chemokine receptors on the lymphocyte surface resulting in G-protein activation and a change in the expression profile of cell adhesion molecules. Activated chemokine receptors on the lymphocyte surface mediate a change in the expression profile of cell adhesion molecules. These mediate firm adhesion to the endothelial surface (panel c). Tissue specific chemokines / chemokines receptor binding is outlined in Table 1.4. Legend: T-Lφ = T lymphocyte, HR = homing receptor, Ad = addressin. Adapted from (Butcher and Picker 1996).
1.4.2 Dendritic cell homing to peripheral lymph nodes

Dendritic cells are bone marrow-derived leukocytes that are specialized in the capture of antigen and its presentation to lymphocytes via a multi-step pathway (Table 1.5). In this way dendritic cells play a key role in the initiation of the primary immune response. Dendritic cells present in the epidermis are known as Langerhans cells and are the best studied dendritic cell population. Immature Langerhans leave the peripheral circulation and enter the epidermis. If antigen capture is successful then the Langerhans cells undergo a functional maturation and move to the peripheral lymph nodes cells in order to present antigen to naïve T-lymphocytes. It has been shown that Langerhans cells continuously leave the epidermis in the afferent lymphatics. However, local activation by inflammatory stimuli considerably increases the number of cells released (Brand, Hunziker et al. 1992). This suggests that the multi-step pathway involved with dendritic cell release is controlled by active mechanisms.

Immature Langerhans cells (ILCs) homing to the skin use a mechanism that mirrors the homing-receptor / addressin / chemokine pathway described in Section 1.4.1 above. Langerhans cell maturation is triggered by the capture of antigen and is mediated by the pro-inflammatory cytokines TNFα and IL-1. Upon maturation, dendritic cells significantly alter their chemokine receptor expression profile. They downregulate the chemokine receptors CCR6, CCR1, CCR5 and CXCR1; while significantly upregulating the chemokine receptors CCR7 and to a lesser extent CXCR4 and CCR4 (Lin, Suri et al. 1998; Sallusto, Schaerli et al. 1998; Forster, Schubel et al. 1999). The hypothesis that CCR7, CXCR4 and CCR4 all play an equal role in the physiological homing of dendritic cells to peripheral lymph nodes is not supported by experimental evidence. Only in the CCR7 -/- knockout mouse do dendritic cells lack the ability to home to peripheral lymph nodes (Dieu, Vanbervliet et al. 1998; Sallusto, Schaarli et al. 1998; Forster, Schubel et al. 1999). This suggests that CCR7 plays the most important role the homing of mature dendritic cells to peripheral lymph nodes.

Immature Langerhans cells (ILCs) and skin-homing lymphocytes constitutively express an isoform of the homing-receptor P-selectin glycoprotein ligand-1 (PSGL-1) that contains the CLA epitope (Kieffer, Fuhlbrigge et al. 2001). Intravital microscopy demonstrates that the CLA isoform of PSGL-1 mediates rolling on the addressins E-selectin and P-selectin which are expressed on dermal HEVs (Fuhlbrigge, King et al.
ILC precursors constitutively express the chemokine receptors CCR6, CCR1, CCR5 and CXCR1 (Jakob, Ring et al. 2001). ILCs can access the epidermis in the inflamed and un-inflamed states. Uptake of ILCs to the uninflamed epidermis is mediated by the stimulation of CCR6 by the constitutive chemokine MIP-3α (CCL20) (Charbonnier, Kohrgruber et al. 1999). However, at inflammatory sites, ILCs may access the epidermis independent of PSGL-1 and are recruited along gradients of the inflammatory-type chemokines. These including IL-8 (CXCL8) binding to CXCR1, RANTES (CCL5) to CCR5, MIP-1α (CCL3) to CCR1 and CCR5, and MCP-3 (CCL7) to CCR1 (Jakob, Ring et al. 2001; Pendl, Robert et al. 2002).

Table 1.5: Multi-step pathway of dendritic cell recruitment by lymph nodes

<table>
<thead>
<tr>
<th>Step</th>
<th>Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Attraction to inflammatory sites</td>
</tr>
<tr>
<td>2</td>
<td>Antigen capture</td>
</tr>
<tr>
<td>3</td>
<td>Maturation &amp; chemokine receptor switch</td>
</tr>
<tr>
<td>4</td>
<td>Reduction of adhesion</td>
</tr>
<tr>
<td>5</td>
<td>Migration</td>
</tr>
<tr>
<td>6</td>
<td>Uptake by lymph node</td>
</tr>
</tbody>
</table>

The chemokine receptor CCR7 binds two chemokines: Secondary Lymphoid-tissue Chemokine (SLC, CCL21) and the EBV-induced Chemokine-1 (ELC, CCL19) that bind with equal affinity to induce chemotaxis (Yoshida, Imai et al. 1997; Campbell, Bowman et al. 1998; Yoshida, Nagira et al. 1998). SLC is constitutively produced by the lymphatic endothelial cells in peripheral tissue and by the stromal cells of the peripheral lymph node T-cell zones. ELC is produced by dendritic cells and stromal
cells in the T-cell areas of lymphoid tissue. There is good evidence that CCR7 plays a key role in the recruitment of Langerhans cells to peripheral lymph nodes in the inflamed and un-inflamed states (Ohl, Mohaupt et al. 2004).

Blockade of SLC activity with neutralising monoclonal antibodies inhibits the migration of Langerhans cells to the draining lymph nodes (Saeki, Moore et al. 1999). Investigation of the spontaneous mouse mutant named PLT (paucity of lymphoid tissue) reveals no measurable SLC expression in their secondary lymphoid tissue (Gunn, Kyuwa et al. 1999). Interestingly, despite no SLC expression, these mice demonstrated normal entry of Langerhans cells into lymphatic vessels although reduced accumulation in the regional lymph nodes. The explanation for this observation was found to be a second gene in the mouse genome that encodes a SLC-like protein which is predominantly found in the lymphatic endothelium of non-lymphoid tissue. The PLT mice lack the SLC gene but demonstrate normal production of the second SLC-like protein (Vassileva, Soto et al. 1999). In sites of chronic inflammation, Langerhans cells that are not fully mature may only be recruited to peripheral lymph nodes if CCR7 expression has been induced (Geissmann, Dieu-Nosjean et al. 2002). The migration of T-cells and DCs from non-lymphoid tissue to draining lymph nodes appears to be more dependent on CCR7 expression than integrins (Forster, Schubel et al. 1999; Ohl, Mohaupt et al. 2004; Lammermann, Bader et al. 2008). However, it has been shown recently that blockade of the sphingosine 1-phosphate receptor 1 (S1P1) prevented migration of T cells into afferent lymphatics by increasing firm adhesion mediated by both αLβ2 and α4β1 integrins (Ledgerwood, Lal et al. 2008).

The role of CXCR4 in dendritic cell migration is less clear. Functional CXCR4 expression has been demonstrated on both immature and mature dendritic cells, (Sallusto, Schaerli et al. 1998; Delgado, Finkel et al. 1998). Mature dendritic cells express CXCR4 and are responsive to its ligand SDF (Lin, Suri et al. 1998; Delgado, Finkel et al. 1998; Tchou, Misery et al. 2001). However, it is not clear whether this is due to an upregulation of expression following stimulation with TNFα and IL-1β (Lin, Suri et al. 1998; Sallusto, Schaerli et al. 1998), granulocyte-macrophage colony stimulating factor (Tchou, Misery et al. 2001) or due to the persistence of expression levels present in the immature state (Delgado, Finkel et al. 1998). SDF is constitutively produced by fibroblasts in the bone marrow, lymph node, and lungs (Blades, Manzo et
al. 2002; Ratajczak, Majka et al. 2003). One interpretation of the experimentally-derived data regarding the response of CXCR4-expressing dendritic cells to SDF is that this chemokine/receptor interaction may play a role in the spatial co-ordination of antigen presentation within the lymph node substance (*vide infra*).

Dendritic cells entering the afferent lymphatics are transported to the subcapsular sinus of the draining lymph node. Both SLC and ELC are constitutively expressed within the T-cell zone of secondary lymphoid tissue. In addition ELC is produced by dendritic cells resident in the paracortical T-cell zone and it has been suggested that this may facilitate the correct positioning of newly arriving migratory dendritic cells recruited from peripheral tissues (Jakob, Ring et al. 2001). SLC is also expressed by inter-digitating dendritic cells and high endothelial venules of secondary lymphoid tissue where it promotes adhesion and chemotaxis of naïve T lymphocytes (Gunn, Ngo et al. 1998; Gunn, Tangemann et al. 1998).

CCR7 phenotype defines subsets of T cells that mediate two distinct aspects of immunological memory. CCR7-negative memory cells (TEM, effector memory T cells) express receptors for migration to inflamed tissue and have immediate effector function. CCR7-positive memory cells (TCM, central memory T cells) express lymph node homing receptors, efficiently stimulate dendritic cells and differentiate into TEM cells on secondary stimulation (Sallusto, Le nig et al. 1999). Interestingly, central-memory T-lymphocytes may access lymph node compartments by a SDF-mediated mechanism independent of CCR7 expression (Scimone, Felbinger et al. 2004).

In summary, the mechanism of physiological lymphocyte homing (Section 1.4.1) is a good anatomical analogue for the dissemination of distant metastases. However, it does not accurately represent the route of tumour spread to the peripheral lymph nodes. Dendritic cells mirror this process more closely as they home to peripheral lymph nodes via afferent lymphatic channels (Section 1.4.2). It has become apparent that, although the delivery of dendritic cells to the draining lymph nodes is defined by anatomical pathways, their uptake there is not a random process. Dendritic cell homing is carefully controlled by the interaction between chemokines and their receptors. Evidence is accumulating that CCR7 and CXCR4 play specific roles in this process.
1.5 Chemokines and their receptors

The role of chemokines in lymphocyte and dendritic cell homing has been outlined above. In this section an overview of the chemokine superfamily is presented and evidence for their role in tumour metastasis is discussed. The superfamily of chemotactic cytokines (commonly abbreviated to chemokines) bind to specific cell surface seven-transmembrane domains of G-protein coupled receptors of the rhodopsin family (Lindley, Westwick et al. 1993). Since their first identification in 1977, over forty human chemokines and eighteen chemokine receptors have been described (Walz, Wu et al. 1977). Their initially confusing nomenclature was simplified by the Nomenclature Committee of the International Union of Pharmacology (Murphy, Baggiolini et al. 2000; Zlotnik and Yoshie 2000; Murphy 2002). This classification is based on chemokine structure.

1.5.1 The classification & nomenclature of chemokines and their receptors

The majority of chemokines have four conserved cysteine residues and have been classified according to their number and spacing (Murphy, Baggiolini et al. 2000; Zlotnik and Yoshie 2000; Murphy 2002). There are two major groups: firstly the C-C group (28 members) with the first two cysteine residues adjacent, and secondly the C-X-C group (14 members) with the first two cysteines separated by one variable amino acid. The remaining chemokines form the C group (two members) and the C-XXX-C group (one member). Chemokine ligands are denoted with an “L” and a specific number. The classification of chemokine receptors mirrors that of their ligands. Chemokine receptors are denoted with an “R” and a specific number (Figure 1.3).

A dichotomous functional classification for chemokines has also been proposed comprising an inflammatory (or inducible) group and a homeostatic (or constitutive) group. Inflammatory chemokines tend to bind promiscuously to multiple receptors, while homeostatic chemokines tend only to bind to other homeostatic chemokine receptors located on the same chromosome. Interestingly the human genes for chemokines in the inflammatory group cluster to the chromosomes 4 (C-X-C group)
and 17 (C-C group) while those for the homeostatic group are widely scattered throughout the genome.

**Figure 1.3: Human chemokines and their receptors.** A schematic representation of the binding characteristics of human chemokines and their receptors. Some receptors demonstrate promiscuous binding to multiple ligands while others are more specific. The former can be described as inflammatory (inducible) and the latter as the homeostatic (constitutive) groups. Reproduced with permission from (Balkwill 2004).
### 1.5.2 The structure and function of chemokines and their receptors

With the exception of CX3CL1, chemokines lack transmembrane domains and are secreted by cells. However, chemokines are able to bind to cell membrane glycosaminoglycans that allows them to form chemotactic gradients. It is important to note that chemokines are the secreted messengers while their receptors are the effector units. Consequently, the chemokines secreted by a cell will mediate a paracrine effect on other cells. Meanwhile, the cell’s response to external chemokine stimuli is determined by its chemokine receptors expression pattern and the associated G-protein elements. Significant interaction between chemokine receptors at the secondary messenger level explains the ability of chemokines to play a role in a variety of cellular processes. Stimulation of differing chemokine receptors co-expressed on the same cell by different chemokine ligands can result in the same response. Conversely, a single chemokine receptor may sort signals from different ligands to different signalling pathways (Zhang, Youn et al. 1999).

#### 1.5.2.1 CXCR4 structure, function and associated signalling pathways

CXCR4 is a protein of 352 amino acids (molecular weight 42 kDa) that belongs to the superfamily of seven-transmembrane G-protein coupled receptors (Figure 1.4). Its only known ligand is Stromal Cell-Derived Factor-1 (SDF) also known as CXCL12 according to the universal nomenclature for chemokines and their receptors (Murphy, Baggliolini et al. 2000; Murphy 2002).

Under homeostatic conditions, transcriptional control of CXCR4 expression is predominantly regulated by the positive transcription factor Nuclear Respiratory Factor-1 (NRF-1) (Moriuchi, Moriuchi et al. 1997) with a potential role for a second, positive transcription factor SP-1 (Wegner, Ehrenberg et al. 1998). NRF-1 activity is increased by interaction with the human T lymphocyte virus type 1 (HTLV-1) transactivator protein (Tax) that may, in turn, increase the subjects susceptibility to HIV infection via CXCR4 binding (Moriuchi, Moriuchi et al. 1999). The principal negative regulatory factor in basal CXCR4 expression thought to be Yin Yang-1 (YY1) (Moriuchi, Moriuchi et al. 1999). Patients infected with human herpes virus-6 (HHV-6) show decreased surface CXCR4 levels, possibly due to decreased association between
c-Myc and YY-1 resulting in increased YY-1 binding (Hasegawa, Yasukawa et al. 2001).
In pathophysiological states a significant number of signalling molecules have been shown to have an effect of CXCR4 transcription including calcium, cyclic AMP, interleukins (1β, 2, 4, 7, 10 and 15), growth factors (including TGF-1β, EGF, VEGF and basic fibroblast growth factor) and pro-inflammatory mediators such as TNF-α and IFN-γ (Busillo and Benovic 2007). Some or all of these mediators of CXCR4 transcription control may play an important role within the tumour/host microenvironment (Section 1.5.5) (Rubin 2009).

Figure 1.4: Schematic representation of the structure of the CXCR4 receptor. Legend: ** Asn11 glycosylation site for post-translational control; shaded residues represent 25 potential phosphorylation sites, * denote cysteine residues involved in disulphide bonding; IC = site of initial SDF/CXCR4 interaction; SBP = secondary binding pocket created by a conformational change in CXCR4 following initial SDF binding; UZ = Ubiquitination zone mediating internalisation; GPBS = G-protein binding site (contains multiple potential phosphorylation sites). Adapted from (Alkhatib 2009)
The post-translational modification and control of CXCR4 have been extensively investigated. Two potential glycosylation sites have been identified within the extracellular domain of CXCR4 although only one of these (Asn11) appears to be involved in mammalian cells (Figure 1.4) (Zhou and Tai 1999; Chabot, Chen et al. 2000). Glycosylation of CXCR4 is important for SDF binding as mutation at the Asn11 site has been shown to reduce ligand association and decrease downstream signalling (Wang, Babcock et al. 2004). Tyrosine sulphation is another important post-translational modification that has been shown to affect SDF binding (Farzan, Babcock et al. 2002). Three extracellular tyrosine residues have been shown to undergo sulphation by tyrosyl protein sulphotransferase situated in the trans-golgi apparatus. However, their functional significance has yet to be determined.

Members of the G-protein coupled receptor superfamily typically undergo either homo- or hetero-dimerisation within the cell membrane. This may occur with or without ligand binding and, although extensively studied (particularly within the field of HIV research), the exact functional significance of this oligomerisation is still unclear. However, it seems most likely that homodimerisation may play a role in non-classical activation of signalling pathways (such as JAK/STAT) independent of G-protein function.

Interaction between SDF and CXCR4 is thought to occur in two stages. Initially, contact between SDF (residues 12-17) and CXCR4 (residues 2-36) results in a conformational change in the receptor (Figure 1.4) (Huang, Shen et al. 2003). This, in turn, allows interaction between SDF (residues 1-8) and an exposed binding pocket formed by a combination of the second and third extracellular loops (Figure 1.4). Cleavage of the N-terminal residues of either SDF or CXCR4 by proteases associated with the inflammatory response will disrupt ligand binding (Valenzuela-Fernandez, Planchenault et al. 2002). Inflammatory mediators may also play a role in increasing CXCR4 binding sensitivity. This is thought to occur through changes in the distribution of the receptor in the cell membrane, predominantly by incorporation in lipid rafts (Wysoczynski, Reca et al. 2005).

CXCR4 signalling has been shown to occur through G-protein dependent and independent mechanisms. However, the majority of CXCR4-mediated cell functions are pertussis toxin sensitive, suggesting the G-protein based signalling pathways
predominate. Ligand binding results in a conformational change in the cytoplasmic domain of CXCR4 and association with intracellular heterotrimeric G-proteins. Upon ligand binding the Gα protein subunit exchanges GDP for GTP. This, in turn, induces a conformational change in the Gα subunit exposing the interacting domains for the Gβγ subunits and initiating signalling. CXCR4 is able to bind multiple Gα isoforms although the functional significance of this is still unclear (Maghazachi 1997). However, chemotaxis and inhibition of adenylyl cyclase by CXCR4 are pertussin toxin sensitive suggesting a role for the Gaq and Gap subunits. CXCR4-mediated chemotaxis has been shown to be dependent on the activation of Rho-GTPases via Gaq subunits (Hart, Jiang et al. 1998). The Gβγ subunits also play an important role in CXCR4 signalling through their effects on multiple cascades including adenylyl cyclase, ion channels, signalling kinases, phospholipase-Cβ and direct activation of phosphoinositide 3-kinase (PI3K). The protein p101 regulates PI3K through association with Gβγ subunits resulting in transcription control of several pro-apoptotic and pro-survival genes. Interestingly PTEN, the negative regulator of PI3K, is frequently deleted or mutated in cancer (Salmena, Carracedo et al. 2008).

CXCR4 signalling is regulated by a combination of desensitisation, internalisation and degradation. Homologous desensitisation is mediated by G protein-coupled receptor kinase phosphorylation of serine or threonine residues. Twenty-one serine and threonine residues predominantly within the C-terminal tail and third intracellular domain act as potential phosphorylation sites (Figure 1.4) (Marchese, Paing et al. 2008). Following receptor activation, signal transduction is reduced (desensitisation) by phosphorylation of these residues. This permits binding with arrestin-2 and arrestin-3 (Figure 1.5). This effectively uncouples the receptor from further G protein activation and targets it for internalisation (Krupnick and Benovic 1998). Truncation of the C-tail of CXCR4 containing the potential phosphorylation sites enhances receptor activity and reduces internalisation (Haribabu, Richardson et al. 1997). However, it appears likely that arrestin binding may not only play a role in signal truncation, but also in the co-ordination of signalling cascades, particularly the G-protein independent pathways involving p38 and ERK (Lefkowitz and Shenoy 2005). CXCR4 also undergoes heterologous desensitisation mediated by a second messenger dependent protein kinase such as protein kinase-C (PKC). Potential PKC phosphorylation sites include multiple serine residues in the C-tail. Activation of PKC by phorbol esters increases phosphorylation and internalisation of CXCR4 (Signoret, Oldridge et al. 1997).
CXCR4 undergoes ligand-dependent degradation mediated by the E3 ubiquitin ligase atrophin-interacting protein-4 (AIP4). Endosomal sorting of CXCR4 is inhibited by cytokine independent survival kinase (CISK) that favours receptor recycling (Figure 1.6). AIP4 is required at multiple steps of the degradation process (Marchese, Paing et al. 2008). Ubiquinated CXCR4 is collected within HRS-positive microdomains together with AIP4. Ubiquination of one of three lysine residues in the C-tail appears to mark the internalised receptor for degradation. Mutation of these lysine residues appears to reduce degradation without affecting internalisation (Marchese and Benovic 2001).

Figure 1.5: An outline of the signal transduction pathways and regulation of CXCR4. SDF/CXCR4 interaction leads to the activation of multiple G-protein dependent signalling cascades. Two G-protein independent signalling pathways have also been demonstrated (JAK/STAT and p38/ERK). Adapted from (Busillo and Benovic 2007).
Originally two isoforms of SDF were described: the predominantly expressed SDF-1α (89 amino acids) and the less common SDF-1β (93 amino acids). Both SDF-1α and SDF-1β bind CXCR4 with similar affinity (Kd equals 7.5nM and 13.7 nM respectively) (Hesselgesser, Liang et al. 1998). More recently an additional four SDF isoforms (δ, ε, φ and γ) have been described (Yu, Cecil et al. 2006). For example SDF-1γ has been shown to be a weaker CXCR4 agonist than SDF-1α but more potent at blocking HIV binding due to its efficient internalisation of CXCR4 (Altenburg, Broxmeyer et al. 2007). These functional isoforms are differentially distributed across tissues although their biological significance has yet to be fully determined.

Figure 1.6. CXCR4 trafficking within the endosomal-lysosomal system. Activated CXCR4 undergoes ubiquitination by the E3 ubiquitin ligase atrophin-interacting protein-4 (AIP4). Cytokine independent survival kinase (CISK) inhibits endosomal sorting of CXCR4 and favours receptor recycling. Legend: ESCRT = endosomal-sorting complex required for transport; MVB = multivesicular bodies; Ub = ubiquitin; Vps4 = vacuolar protein sorting. Adapted from (Marchese, Paing et al. 2008; Alkhatib 2009).
1.5.3 Chemokine receptor distribution and function on normal non-haematopoietic cells

Chemokine receptors are mainly distributed on the surface of leukocytes. However, they have also been demonstrated on a wide range of normal cell types including endothelium (Pablos, Amara et al. 1999), neurones (Gupta, Lysko et al. 1998) and keratinocytes (vide infra). The significance of chemokine receptor expression by non-haematopoietic cells is unclear. One possibility is that they play a role in developmental processes due to their involvement in directed cell migration. Indeed, there is good evidence for chemokine receptor involvement in organogenesis, vascularisation and neural development (Tachibana, Hirota et al. 1998; Zou, Kottmann et al. 1998; Van Der Meer, Goldberg et al. 2001). Chemokine receptor knockouts display phenotypes of variable severity. CXCR4 is the only chemokine receptor whose murine knockout is fatal in the perinatal period due to ventricular septal defects, defective gastric vasculogenesis, abnormal cerebellar development, abnormal bone marrow myelopoiesis and defective B-cell (but not T-cell) lymphopoiesis (Zou, Kottmann et al. 1998). This suggests that this chemokine receptor is critical in the normal development of the vasculature as well as its haematopoietic components. Evidence supporting this hypothesis has been demonstrated for development of the intestinal microcirculation (Heidemann, Ogawa et al. 2004).

In addition to its role in vasculogenesis CXCR4 surface protein has been demonstrated in certain normal human epithelia. Low levels of CXCR4 protein has been shown in all layers of normal oral epithelium except for the lamina propria (Jotwani, Muthukuru et al. 2004). Other authors investigating normal oral epithelia as controls have demonstrated similar findings (Dilibasi, Okura et al. 2004; Moutsopoulos, Nares et al. 2007), although not universally so (Katayama, Ogino et al. 2005). Positive CXCR4 staining of has been shown in a proportion of specimens of normal breast epithelia (Salvucci, Bouchard et al. 2006) but this finding is poorly reproducible (Muller, Homey et al. 2001; Kato, Kitayama et al. 2003). Similar contradictory findings have been demonstrated for prostatic (Sun, Wang et al. 2003), ovarian (Scotton, Milliken et al. 2001) and colonic (Jordan, Kolios et al. 1999; Dwinell, Eckmann et al. 1999; Ottaiano, di Palma et al. 2005) epithelia. The reasons for these discrepancies are not clear although the presence of inflammation in these “normal” specimens may be a confounding variable due to ingress of CXCR4-labelled leukocytes or the upregulation of CXCR4
within the lamina propria. The latter has been demonstrated in specimens representative of chronic periodontitis (Jotwani, Muthukuru et al. 2004).

There is some experimental evidence of chemokine receptor expression on normal skin keratinocytes. CCR3 expression was demonstrated on normal skin keratinocytes in-vitro but not in-vivo (Wakugawa, Nakamura et al. 2001). However, strong upregulation of CCR3 (Wakugawa, Nakamura et al. 2001), CCR4 (Zheng, Nakamura et al. 2003) and CCR6 (Homey, Dieu-Nosjean et al. 2000) expression was seen in inflammatory skin conditions such as psoriasis and atopic dermatitis. The inference that the expression pattern of oral keratinocytes is similar to that of their skin counterparts suffers from a lack of direct experimental evidence. Instead data come indirectly from the investigation of HIV infectivity in the oral mucosa. It has been shown that the R5 strain and the X4 strain of HIV (using CCR5 and CXCR4 as co-receptors respectively infected oral gingival cells in-vitro (Feng, Broder et al. 1996; Liu, Zha et al. 2003; Moore, Rahemtulla et al. 2003). Treatment with alcohol increased the surface expression of CXCR4 and increased X4-HIV strain infectivity (Chen, Zha et al. 2004).

In summary, chemokine receptors are expressed on a variety of haematopoietic cells and contribute to the co-ordination of the immune response. They are also expressed by normal non-haematopoietic cells including keratinocytes, particularly under inflammatory conditions where they signal via a multitude of pathways. The expression of functional chemokine receptors by carcinoma cells would make them potentially responsive to chemokine gradients defined by the inflammatory process including those that mediate leukocyte homing to secondary lymphoid tissue.
1.5.4 Evidence for the role of chemokine receptors in the chemoattraction of tumour metastases.

Direct evidence for the expression of chemokine receptors by normal epithelia is scarce. However, chemokine receptor mRNA expression and protein production has been demonstrated by a number of carcinoma systems, including breast, prostate, pancreas, lung, ovarian and OSCC (see below). Cumulative experimental and clinical evidence indicates that the involvement of chemokine receptors in neoplastic progression can be divided into three broad categories (Zlotnik 2004). Firstly there is compelling evidence for the involvement of two chemokine receptors (CXCR4 and CCR7) with metastatic dissemination in a number of carcinoma systems. Secondly, there are some chemokine receptors (such as CCR10) that may be involved with tissue-specific metastasis. Thirdly, there are some chemokine receptors (such as XCR1 and CCR9) that appear not to be associated with metastasis at all. These observations are compatible with the “chemoattractant” hypothesis of metastasis. The extrapolation of evidence from physiological homing mechanisms and the observed behaviour in clinical specimens suggest that chemokine receptors may play a role in the metastasis of carcinoma cells to specific tissues. However, considering the multi-step model of metastatic dissemination previously outlined in Section 1.1.3 (Fidler 2003) it is possible that chemokine receptors may be implicated in other mechanisms that contribute to metastasis. These include tumour angiogenesis, proliferation and invasion. However, the evidence for the role of chemokine receptors in these pathways is limited to in-vitro observations while the overwhelming majority of in-vivo data supports the “chemoattractant” hypothesis.

In neoplasia it is possible that chemokine receptor activation may be achieved in a number of ways: mutations within the promoter region or changes in transcription factor levels may result in the deregulation of CXCR4 mRNA expression. Alternatively single nucleotide polymorphisms (SNP) within the CXCR4 coding sequence may confer changes in protein function (Petersen, Glashoff et al. 2005). An association between one or more abnormal CXCR4 SNP containing allele and an increased risk (Odds Ratio 2.66) of Stage III/IV OSCC disease (Teng, Liu et al. 2009). Finally, tumour cells expressing wild-type chemokine receptor may bind chemokines produced within the host/tumour microenvironment. These chemokines may be derived in an autocrine or paracrine fashion. Autocrine production of chemokine by tumour cells
will also exert an effect on those neighbouring host cells that bear a complimentary receptor. The production of the chemokines by tumour cells forms a complex tissue microenvironment in which the host immune response may be modulated (Mosser 2003; Mantovanni, Allavena et al. 2004). However, it must be remembered that it is the expression of functional chemokine receptors that determines the responsiveness of the tumour cell to the microenvironment. The primary focus of this project is the expression of chemokine receptors by OSCC tumour cells and consequently the delineation of their response to factors that may be present in the host/tumour microenvironment. The production of chemokines by OSCC tumour cells would be an interesting subject for investigation but falls outside the scope of this project.

1.5.4.1 Evidence for the role of chemokine receptors in lymphatic metastasis.

The chemokine receptors most commonly over-expressed by carcinomas are CCR7 and CXCR4 (Zlotnik 2004). CCR7 plays a key role in the physiological homing of lymphocytes and dendritic cells to peripheral lymph nodes. Consequently it may be hypothesised that the lymphatic spread of tumour cells is mediated by the expression of CCR7. There is some in-vitro data and observational evidence to support this theory. Investigation of carcinoma cell lines in-vitro has demonstrated an increase in the expression of the chemokine receptor CCR7 in the majority of breast, gastric and oesophageal carcinoma cell lines compared with primary cultures of epithelial cells (Muller, Homey et al. 2001; Mashino, Sadanaga et al. 2002; Ding, Shimada et al. 2003). The CCR7 receptors expressed by these cell lines were functionally active: demonstrating calcium flux, actin polymerisation, migration and invasion in response to the chemokine SLC (CCL21).

Immunohistochemical analysis of clinical samples from the digestive tract reveals that CCR7 expression is not ubiquitous. CCR7 protein was shown to be expressed by 90% of oesophageal carcinoma cells (Ding, Shimada et al. 2003) and 22 to 66% of gastric carcinomas (Mashino, Sadanaga et al. 2002; Kwak, Hur et al. 2005). The literature on the clinical correlations of CCR7 expression by gastric tumours remains unclear. Mashino et al (2002) found that primary gastric tumours staining positive for CCR7 were more likely to be associated with lymphatic invasion, lymph node infiltration and reduced patient survival. Kwak et al (2005) found no association between CCR7
expression and lymph node metastasis and, indeed, demonstrated that patients with CCR7 positive primary tumours had a better overall survival compared with their CCR7 negative counterparts. Examination of colorectal carcinomas found no association between CCR7 staining and lymph node metastasis or patient survival (Schimanski, Schwald et al. 2005). These contradictory findings may be due to experimental factors such as methodological inconsistencies, small study numbers or poor clinico-pathological correlation. Alternatively, it is also consistent with the hypothesis that CCR7 expression alone does not determine the metastatic potential of gastric carcinomas.

It has been shown that CCR7 mRNA expression by non-small cell lung carcinoma (Takanami 2003) and oesophageal (Ding, Shimada et al. 2003) carcinoma cells, positively predicts lymph node metastasis. Again, for gastric carcinomas, results from the literature are conflicting regarding this association (Yan, Zhu et al. 2004; Kwak, Hur et al. 2005). However, a methodological flaw of all of these studies was that the detection of CCR7 was based on the RT-PCR of homogenised tissue samples. Therefore it is possible that this difference is due to the recruitment of CCR7-positive host leukocytes into the tumour microenvironment and not due to increased CCR7 expression by the tumour cells. In the investigation of chemokine receptor expression in tissue samples it is important to separate tumour cells from host inflammatory infiltrate prior to analysis. Consequently, despite the methodological flaws in some of these studies, it seems unlikely that CCR7 expression is uniquely responsible for the mediation of lymphatic metastases in carcinomas of the upper aero-digestive tract. A role for other chemokine receptors is possible. One that is associated with physiological leukocyte homing to lymph nodes is CXCR4.

The role of CXCR4 in metastatic progression has been studied in a number of carcinoma systems. Renal, breast and lung carcinoma cells showed significantly higher expression of CXCR4 compared to adjacent normal tissue (Muller, Homey et al. 2001; Schrader, Lechner et al. 2002; Takanami 2003). Breast carcinomas with higher expression of CXCR4 showed more extensive lymph node metastasis (Kato, Kitayama et al. 2003; Liu, Lang et al. 2009). Interestingly, in the study by Takanami (2003), both CCR7 and CXCR4 expression was associated with lymph node metastasis, although only CCR7 mRNA expression was an independent predictive factor. These data suggest that CCR7 and CXCR4 may play a dual role in lymphatic metastases in some
tumour types. Further evidence for this theory is provided by investigation of lymphoma subtypes. Hodgkin’s disease of the classical type locates predominantly in the inter-follicular areas of the lymph node and was found to express both CXCR4 and CCR7, whereas the nodular lymphocyte-predominant type is regularly associated with the follicular structures and expressed CXCR4 but not CCR7 (Hopken, Foss et al. 2002). Lymphoid infiltration by Adult T-cell leukaemias appears to be CCR7 mediated (Hasegawa, Nomura et al. 2000).

1.5.4.2 Evidence for the role of chemokine receptors in distant metastasis

Clinical observation of carcinomas of the breast, bronchus, kidney and prostate reveals their affinity for metastasis to the lung, liver and bone. One hypothesis is that this affinity could be mediated by the CXCR4/SDF axis. Supporting evidence comes from the demonstration that SDF is preferentially expressed in human lymph nodes, lung, liver and bone marrow at the mRNA and protein levels (Muller, Homey et al. 2001; Phillips, Burdick et al. 2003). Experimental data show that primary human osteoblastic cell cultures express SDF (Taichman, Cooper et al. 2002). As stated earlier cell lines derived from breast, prostate and renal carcinomas express CXCR4. Interventional studies reveal that the CXCR4/SDF axis does play a role in tumour metastasis. Treatment of nude mice with neutralising anti-CXCR4 monoclonal antibodies significantly reduced the metastatic burden of CXCR4-positive breast carcinoma cells to the lung and peripheral lymph nodes (Muller, Homey et al. 2001) and CXCR4-positive prostate carcinoma cells to the bone (Sun, Schneider et al. 2005). Abrogation of distant non-small cell lung carcinoma metastases in nude mice was achieved by blockade with anti-SDF monoclonal antibodies (Phillips, Burdick et al. 2003).

However, experimental evidence has shown that the role of CXCR4 in the development of distant metastases is not purely related to its chemoattractive properties (see Section 1.5.5 for further discussion). The development of pulmonary metastases following intravenous injection of colonic carcinoma cells was studied in nude mice (Zeelenberg, Ruuis-Van Stalle et al. 2003). They observed that, 24 hours after inoculation, CXCR4-negative tumour cells colonised the lungs with the same efficiency as CXCR4-positive cells. However, CXCR4-negative cells failed to proliferate and form macrometastases while CXCR4-positive cells grew exponentially...
from approximately day six. These findings suggest that CXCR4 does not mediate the arrest of tumour cells in the pulmonary vasculature but does promote subsequent proliferation of the secondary tumour deposits. This group used an interesting technique to prevent CXCR4 surface expression. They transfected wild-type colonic carcinoma cells that produced CXCR4 with a vector containing SDF tagged with a KDEL sequence that anchored it to the nuclear membrane. Consequently the free bioactive end was able to bind CXCR4 preventing its association with the cell membrane. It is possible that the overexpression of SDF may have altered the cell phenotype in a way that alters its metastatic potential.

Studies using an alternative technique of gene silencing supports the theory that CXCR4 mediates metastatic tumour progression in breast carcinomas (Lapteva, Yang et al. 2005; Liang, Yoon et al. 2005). Both groups used small-inhibiting RNA (siRNA) to induce CXCR4 knockdown and they demonstrated that pulmonary metastases were reduced in mice inoculated with CXCR4-deficient tumour cells. However, these experiments did not differentiate between the chemoattractant and non-chemoattractant mechanisms of CXCR4 action.

A role for chemokine receptors has been postulated in other tumour types. Melanoma cells express the chemokine receptor CCR10 as well as CCR7 and CXCR4 \textit{in-vitro}. CTACK (CCL27), the ligand for CCR10, is preferentially expressed in the skin (Muller, Homey et al. 2001). The common sites for melanoma metastasis include satellite lesions to the skin. These may be mediated by CCR10 expression by melanoma cells.

1.5.5 The role of CXCR4 in mechanisms of tumour metastasis not involving chemoattraction.

Experimental data in ovarian, renal, prostate, colon and breast carcinoma cell lines have demonstrated that CXCR4 mRNA is not ubiquitously translated \textit{in-vitro}. (Muller, Homey et al. 2001; Scotton, Milliken et al. 2001; Taichman, Cooper et al. 2002; Zeelenberg, Ruuis-Van Stalle et al. 2003; Schimanski, Schwald et al. 2005). In these cell lines, SDF-mediated signalling has been shown to result in ligand-induced receptor endocytosis, intracellular calcium mobilisation, actin polymerisation, chemotaxis,
increased cell-to-cell adhesion, trans-endothelial migration and invasion of the extracellular matrix. However, as outlined above (Section 1.5.4.2), direct in-vivo evidence of a role for CXCR4 in the pulmonary dissemination of colonic carcinoma cells via a non-chemoattractive mechanism has been described (Zeelenberg, Ruuis-Van Stalle et al. 2003). Given the multistage process of tumour metastasis (Table 1.1) it is possible that chemokine receptors may play a role in a multitude of these steps (Fidler 2003). This is discussed below.

1.5.5.1 The role of CXCR4 in tumour proliferation and apoptosis.

Over-expression of CXCR4 has been shown to mediate the proliferation of prostate carcinoma cells in-vitro and in-vivo (Darash-Yahana, Pikarsky et al. 2004). Subcutaneous injection of transfected prostate carcinoma cells containing the CXCR4 insert into NOD/SCID mice yielded primary and secondary tumours significantly larger than controls lacking the CXCR4 insert. Similar experimental evidence has demonstrated a similar pro-proliferative effect of CXCR4 in anaplastic thyroid carcinoma (De Falco, Guarino et al. 2007), salivary mucopidermoid carcinoma (Wen, Zhu et al. 2007), breast (Wendt, Cooper et al. 2008) and epithelial ovarian carcinoma (Kajiyama, Shibata et al. 2008). Pathways thought to be involved include MAP/Erk kinase (Darash-Yahana, Pikarsky et al. 2004; Yang, Lee et al. 2007), ERK1/2 and AKT (De Falco, Guarino et al. 2007). However, direct empirical testing of the precise pathways involved is limited by the relatively crude pharmacological antagonists available and the complex interaction of the multiple downstream signalling pathways activated following SDF binding to CXCR4.

Net tumour growth is the outcome of cell proliferation and cell death. While cell necrosis can occur due to anoxia, the ability to evade programmed cell death (apoptosis) is another possible mechanism by which tumours may expand. Two reports have recently demonstrated a regulatory role for CXCR4 in bcl2-mediated apoptosis in thymic dendritic cells (Hernandez-Lopez, Valencia et al. 2008) and prostatic carcinoma cell lines (Porvasnik, Sakamoto et al. 2009). However, Pan and colleagues found no effect of SDF-blockade on renal cell carcinoma apoptosis (or proliferation) either in-vitro or in-vivo (Pan, Mestas et al. 2006). The role of
SDF/CXCR4 has been examined in anoikis (Wendt, Cooper et al. 2008). They found that autocrine SDF production inhibited tumour formation in non-adherent colorectal carcinoma cells by the induction of caspase 3/7-mediated apoptosis. Their data suggest that endogenous (but not exogenous) SDF stimulation yields tonic ERK1/2 phosphorylation leading to apoptosis. In this way their findings differ from those of others by suggesting that it is the suppression of autogenous SDF production that is the key step in metastatic progression for the colorectal cells examined. Without further work to determine if this effect can be demonstrated in other cell lines, it is impossible to tell if these findings are limited to the cell system examined.

The exact role of the SDF/CXCR4 axis in tumour proliferation and apoptosis is not as clear-cut as these data from different carcinomas suggest. The limitations of in-vitro findings are that manipulation of individual variables may not accurately reflect the complex tumour/tissue microenvironment found in-vivo. The limitation of animal model data is that the primary endpoint measured (relative tumour size) is inevitably a result of a combination of factors of which proliferation is one. Supportive evidence of a role of the SDF/CXCR4 axis in other components of the metastatic pathway should be sought.

1.5.5.2 The role of CXCR4 in tumour angiogenesis

The effect of CXCR4 stimulation on tumour growth has been reported in a number of carcinoma systems. As discussed in Section 1.5.5.1, continued cell proliferation requires the development of a new blood supply (neo-angiogenesis). SDF stimulation results in increased vascular endothelial growth factor (VEGF) production by CXCR4-overexpressing prostate carcinoma cells (Darash-Yahana, Pikarsky et al. 2004). One possible mechanism by which CXCR4 upregulation might be controlled in response to tissue hypoxia is via hypoxia-inducible factor-1α (HIF-1α) mediated signalling in a number of different cell types including endothelial and tumour cells (Schioppa, Uranchimeg et al. 2003). HIF-1α mRNA is stabilised under hypoxic conditions whereas under conditions of normal oxygen tension HIF-1α mRNA is degraded via the von Hippel-Lindau (VHL)-E3 ubiquitin ligase complex (Staller, Sulitkova et al. 2003).

9 Anoikis is the term used to describe programmed cell death due to lack of contact with the extracellular matrix.
Through this mechanism, mutations in the VHL protein have been shown to be associated with high CXCR4 expression and poor prognosis in renal cell carcinoma (Staller, Sulitkova et al. 2003; Pan, Mestas et al. 2006).

HIF-1α mediated control of CXCR4 expression is not the only role of the SDF/CXCR4 axis in tumour angiogenesis. Activated carcinoma-associated fibroblasts (CAFs), bearing a myofibroblast phenotype, have been shown to promote the growth of breast carcinoma cells by the recruitment of endothelial progenitor cells (EPCs) via a mechanism at least partly mediated by SDF (Orimo, Gupta et al. 2005). A role for EPC recruitment in tumour angiogenesis has been demonstrated in-vitro and in-vivo (Lyden, Hattori et al. 2001). By the activation of fibroblasts it is possible that tumour stroma parallels the physiological response of tissues to injury including the release of SDF (De Falco, Porcelli et al. 2004). Unlike the response to injury, CAFs remain permanently activated, for example by establishing autocrine stimulation by TGF-β. However, the role of CAFs in tumour progression is not limited to neo-angiogenesis and, recently, a direct effect on carcinoma cell proliferation has been described. This is outlined below.

It has been shown that the tumour/tissue microenvironment plays an important role in the initiation and promotion of carcinogenesis. Transforming Growth Factor-β (TGF-β) signalling in fibroblasts has been shown to control the oncogenic potential of adjacent epithelia in squamous cell carcinoma of the forestomach (Bhowmick, Chytil et al. 2004). This is in addition to the autocrine activation loop outlined above and was initially thought to be achieved through a multitude of fibroblast-derived growth factors including the transforming-β, hepatocyte-β, insulin-like, epithelial- and fibroblast-growth factor families (Bhowmick, Neilson et al. 2004).

More recently a role for complex interplay between SDF and TGF-β has been described. Breast CAFs have been shown to produce significantly more SDF than primary breast carcinoma cells in-vitro (Orimo, Gupta et al. 2005). This release into the tumour/tissue microenvironment has been shown to exert a paracrine pro-proliferative effect on breast tumour growth that can be increased by over-expression of SDF or decreased by the downregulation of CXCR4 expression by siRNA (Orimo, Gupta et al. 2005). Co-culture experiments have revealed that activated carcinoma-associated fibroblasts (CAFs) stimulate the proliferation of transformed (but not
normal) epithelia and, conversely, CAFs (but not normal fibroblasts) stimulate the proliferation of transformed epithelia. In prostatic cell lines, it has been demonstrated that TGF-β regulates localisation of CXCR4 to the carcinoma cell membrane and its subsequent response to CAF-derived SDF (Ao, Franco et al. 2007). Some preliminary data outlining the possible association between CXCR4 and EMT in OSCC cells in-vitro has been described and this is discussed in Section 1.6.2.4.

### 1.5.5.3 CXCR7: a second receptor for SDF

The receptor RDC1 was originally cloned in 1990 (Libert, Passage et al. 1991) but its role as a second receptor for SDF was not described until 2005 (Balabanian, Lagane et al. 2005). RDC1/CXCR7 shares many similarities with CXCR4 including a high affinity for SDF, ligand-mediated receptor internalisation, acting as a HIV-1 co-receptor and possible interaction between the two receptors (Shimizu, Haraguchi et al. 1999; Sierro, Biben et al. 2007). However, some differences have been noted, not least the inability of CXCR7 to associate with G-proteins via the intracellular domains (Balabanian, Lagane et al. 2005). CXCR7 is highly implicated in developmental processes (particularly of the haematopoietic system) although, unlike CXCR4, its double knockout is not lethal (Sierro, Biben et al. 2007). It has been proposed that the principal function of CXCR7 in organogenesis is through its role as a decoy receptor delivering ligand for lysosomal degradation (Pruenster and Rot 2006).

From these observations it might be expected that CXCR7 does not mediate functional responses in tumour cells. This is not the case. CXCR7 over-expression has been shown to promote tumour growth in nude mice (Raggo, Ruhl et al. 2005) and has been demonstrated in a number of human carcinoma cell lines (Burns, Summers et al. 2006). Investigation of prostate carcinoma specimens has revealed an association between CXCR7 expression and tumour aggression, survival pathways and the release of pro-angiogenic factors such as VEGF and CXCL8 (Wang, Shiozawa et al. 2008). Other groups have found that the interaction between CXCR4 and CXCR7 varies between cell lines (Meijer, Ogink et al. 2008). In colon carcinoma cells, proliferation was dependent on CXCR7 activity whereas in pancreatic carcinomas both CXCR7 and CXCR4 mediated proliferation in-vitro. However, either over-expression or
downregulation of CXCR7 had no effect on invasion into Matrigel or tumour growth in nude mice (Meijer, Ogink et al. 2008).

These data are part of a rapidly increasing literature investigating possible roles for CXCR7 in carcinoma progression. It is not clear at present what is the precise intracellular function for CXCR7 although it appears from these preliminary findings that it may differ between cell types. CXCR7 certainly acts as a competitor for SDF binding and may exert some of its effects on the SDF/CXCR4 pathway by targeting the ligand for lysosomal destruction. However, a more complex interplay between the receptors or their downstream signalling pathways cannot be excluded and further work is required to delineate this more clearly.

1.5.6 Summary

Chemokines receptors are G-protein linked cell surface receptors that have been shown to be associated with many important physiological roles including embryonal development, lymphocyte homing, inflammation and tissue repair. Two family members (CXCR4 and CCR7) are central to many of these processes. The wide tissue distribution of CXCR4, its fatal murine knockout and its discovery on murine stem cells lends support to the hypothesis that CXCR4 is a human stem cell marker.

There is increasing evidence that carcinomas develop the ability to subvert many of these physiological signalling pathways to promote tumour-associated characteristics including proliferation, migration, invasion, angiogenesis and EMT. For all of these processes it has been shown that the SDF/CXCR4 axis does not act alone, but rather as an (important) player within the tumour/tissue microenvironment. The prominent role of CXCR4 in the biology of both stem cells and carcinoma cells supports its possible role as a cancer stem cell marker (Kucia, Wojakowski et al. 2006).
1.6 Chemokine and chemokine receptor expression in oral squamous cell carcinoma.

The vast majority of evidence relating to roles for chemokines and their receptors in OSCC progression has focussed on CCR7 and CXCR4. This is discussed in Sections 1.6.1 and 1.6.2 respectively. With regards to chemokines, CXCL8 (Interleukin-8) has been shown to increase OSCC cell growth in-vitro (Christofakis, Miyazaki et al. 2008), and has been suggested as a possible serum biomarker for the disease (St John, Li et al. 2004). Laboratory data suggest that CXCL8 plays a role in many tumour processes and large scale clinical studies are still awaited to substantiate the sensitivity and specificity of the marker.\textsuperscript{10} Quantitative evaluation of salivary CXCL8 levels have demonstrated a considerable overlap between OSCC patients and disease-free controls suggesting its use as a screening tool would not be helpful (Arellano-Garcia, Hu et al. 2008). From these findings it would be possible to hypothesise that, due to the complexities of chemokine and chemokine receptor signalling, the manipulation of individual molecules from these families would not be associated with relevant changes in OSCC tumour behaviour. The evidence presented below shows that for (at least) CCR7 and CXCR4 this is not the case.

1.6.1 Evidence supporting a role for CCR7 in OSCC

As previously described (Section 1.2.1), clinical observation of OSCC reveals that cervical lymph node metastasis is common. Distant metastasis may be common at the microscopic level (Kotwall, Sako et al. 1987) but is uncommon at the macroscopic level (Ferlito, Shaha et al. 2001; Carlson and Ord 2002). Given the weight of evidence in the literature favouring the importance for CCR7 expression in lymphatic homing, it is reasonable to hypothesise from these clinical observations that CCR7 is more important in OSCC metastasis than CXCR4. Indeed, there is some evidence in the literature that supports a role for CCR7 expression in HNSCC. Wang et al (2004) investigated CCR7 expression in four pairs of cell lines derived from the primary lesions and lymph node metastases of squamous carcinomas of the larynx and pharynx (Wang, Xi et al. 2004). Although they claim that CCR7 mRNA expression was higher

\textsuperscript{10} In this small series (n=32) the sensitivity and specificity of the association of raised salivary CXCL8 levels with OSCC were reported as 86\% and 97\% respectively (St John, Li et al. 2004).
in the metastatic cells than in those derived from the primary lesion, their non-quantitative results show similar expression levels. Interestingly, CCR6 expression appears to reduce in the metastasis-derived cell lines. Their examination of homogenised fresh-frozen HNSCCs revealed similar levels of CCR7 expression in the primary lesion and lymph node isolates. Again CCR6 expression was reduced in the latter. One methodological criticism is that no attempt was made to exclude host stromal cells the lymph node isolates. Consequently it is impossible to determine if the CCR7 expression demonstrated was derived from host leukocytes or neoplastic cells.

The same group also investigated CCR7 mRNA expression in sub-clones of an OSCC cell line that had metastasised to the lymph nodes in three successive passages in nude mice (Wang, Xi et al. 2004). Using semi-quantitative RT-PCR they showed that CCR7 expression was increased with successive passage. However, preliminary evidence from another group does not support this finding (Uchida, Begum et al. 2003). They demonstrated that CCR7 was universally downregulated in four OSCC cell lines including two that readily metastasised to local lymph nodes in nude mice. Furthermore they showed that CXCR4 mRNA expression was increased in extracts from lymph nodes with OSCC metastatic involvement compared with primary lesions and gingival tissue from patients undergoing third molar surgery. However, their findings are subject to the same methodological criticism as before: tissue biopsies were taken whole and homogenised and no attempt was made to account for the relative contributions to the overall levels of CXCR4 mRNA from resident lymphocytes or tumour cells found in the tissue samples. Furthermore it is possible that the control tissue overlying the third molar teeth that required removal (and therefore assumed to be symptomatic) was not representative of normal mucosa. Inflammation has been shown to increase CXCR4 labelling in oral keratinocytes as outlined above (Section 1.5.3).
Evidence for the localisation of CCR7 protein in OSCC samples has been produced using immunohistochemistry by two groups (Tsuzuki, Takahashi et al. 2005; Shang, Liu et al. 2009). Both papers demonstrated CCR7 staining of OSCC cells in primary and CLN secondary deposits in 60 to 66% of clinical specimens examined. Both groups also demonstrated CCR7-positive tumours were associated with statistically significant increases in large primary disease (T3/4), lymph node metastases (pN+) and advanced disease (Stage III/IV) although no attempt was made to address the confounding effects of these variables (Tsuzuki, Takahashi et al. 2005; Shang, Liu et al. 2009). These results are suggestive that increased CCR7 production is associated with advanced disease and in one cohort this was associated with an increased risk of local recurrence and a reduced disease-free and overall survival (Tsuzuki, Takahashi et al. 2005). Some in-vitro data to support a causative role for CCR7 in metastatic disease was also reported with increased invasion through an extracellular matrix analogue (Matrigel) in response to the CCR7 ligand CCL21 (Shang, Liu et al. 2009). Knockdown of CCR7 using siRNA significantly decreased CCR7-mediated migration, invasion and proliferation of OSCC cells in-vitro (Shang, Liu et al. 2008). It has been shown that CCR7-mediated PI3K/Akt signalling protects OSCC cells from cisplatin-induced apoptosis and increases tumour cell survival. This mechanism may explain the observation of significantly less tumour growth in CCR7 ligand-deficient (plt) mice compared with normal controls (Wang, Seethala et al. 2008). In conclusion, the small amount of preliminary data available on CCR7 production by OSCC cells suggests that this chemokine receptor is a reasonable candidate for mediating tumour metastasis.

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11 Both groups used similar methodology. Briefly, CCR7 positive tumours were defined as having >=30% of OSCC cells that stained for CCR7. The threshold was determined empirically but set at an arbitrary value once assessment had been made by two independent and blinded observers (Tsuzuki, Takahashi et al. 2005; Shang, Liu et al. 2009).

12 This description is based on the abstract as the original article is written in Chinese and therefore its validity and scientific rigour cannot be tested independently of the journal’s peer review process (Shang, Liu et al. 2008).
1.6.2 Evidence supporting a role for CXCR4 in OSCC

1.6.2.1 CXCR4 protein in OSCC clinical specimens

A series of studies using immunohistochemistry to investigate CXCR4 expression in OSCC clinical specimens have been published over the last six years (Table 1.4). Two small preliminary series both demonstrated strong CXCR4 staining in cells that had metastasised to the cervical lymph nodes although, probably because of small numbers (18 and 23 respectively), no statistically significant associations with clinico-pathological features were demonstrated (Uchida, Begum et al. 2003; Delilbasi, Okura et al. 2004). In a larger series (61 patients) CXCR4 staining was demonstrated in 57% of primary lesions and 75% of lymph node metastases (Almofti, Uchida et al. 2004). Positive CXCR4 staining was significantly associated with the mode of invasion, lymphatic metastasis and reduced 5-year disease-free survival by univariate analysis. Association of the latter was no longer significant on multivariate analysis, most likely due to the confounding effect of lymph node status on prognosis (Almofti, Uchida et al. 2004). A significant relationship between CXCR4 staining in OSCC cells within the primary lesion and lymph node metastases was also demonstrated in another large series (90 patients) although only 30% of primary tumours were judged to be CXCR4 positive (Ishikawa, Nakashiro et al. 2006).

O‘Donnell et al (2005) investigated the expression profiles of a small number of primary and secondary OSCC lesions. They found no significant difference in CXCR4 mRNA expression between specimens although their methods used tissue homogenates rather than microdissection of tumour tissue (O‘Donnell, Kupferman et al. 2005). Examination of the same specimens using immunohistochemistry found no subjective difference in CXCR4 staining between primary and secondary lesions. However, the validity of these findings can be questioned as no appropriate controls or description of the assessment methods used were given.

Xu et al investigated 91 OSCC specimens and reported expression of CXCR4 in 57 (62.6%) of them (Xu, Li et al. 2006). The same findings were re-reported 3 years later (Meng, Wuyi et al. 2009). Furthermore they also described an association between CXCR4 expression and lymph node metastasis, tumour size and histological grade although it is not clear whether these are univariate or multivariate analyses. Lee
investigated 74 cases and demonstrated CXCR4-positive staining in 45 (61%) primary lesions (Lee, Jin et al. 2009). Multivariate analysis revealed that positive CXCR4 staining, recurrence and large tumour size (T3/4) were independent, significant predictors of poor overall survival. Of these, CXCR4 demonstrated the most striking effect. However, the duration of follow-up was not specified although interpretation of the Kaplan-Meier curves infers that the majority of patients were followed for less than 5 years.

In a small series of advanced OSCC (predominantly tongue and floor of mouth lesions) no significant difference was demonstrated between CXCR4 staining in primary lesions, lymph node metastases or survival (Oliveira-Neto, Silva et al. 2008). However, these results may have been skewed by the selection of cases with advanced disease and poor overall survival. In a large series of predominantly early OSCC, 20/85 (23%) of primary lesions were found to express CXCR4 (Ishikawa, Nakashiro et al. 2009). Qualitative reporting of the findings suggested that staining was strongest at the invasive front. Positive CXCR4-staining of primary lesions was associated with lymph node metastasis.

In summary, this accumulation of published literature demonstrates that CXCR4 protein staining is present in OSCC primary lesions. Collectively there is a trend for increased CXCR4 staining to be associated with more advanced disease, lymphatic metastases and reduced patient survival. However, these data have numerous shortcomings. There are a number of potential methodological inconsistencies both between studies and between observers. Selected samples vary in their site, size, stage, differentiation and overall treatment. These potentially confounding variables were often poorly controlled for both methodologically and statistically. All of the published studies used consecutive runs for immunohistochemistry (none of the specimens were run concurrently). The best method to overcome this potential bias would be to use tissue arrays with comprehensive pathological information. None of the published studies used this methodology.

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13 CXCR4 staining was graded into 4 categories by two independent blinded observers relative to the intensity of interstitial infiltrates (Lee, Jin et al. 2009).
14 Specimens were graded on a 4 point scale with 0 and 1 defined as negative and the remainder as positive. The number of observers, blinding or definition of the grading levels was not supplied (Ishikawa, Nakashiro et al. 2009).
Table 1.4: Synopsis of the published literature on the association of CXCR4 production by OSCC clinical specimens.

<table>
<thead>
<tr>
<th>Publication</th>
<th>Sample Size (n)</th>
<th>Site of Lesions</th>
<th>Number (%) of cases with CXCR4 staining in tumour cells within the Primary lesion</th>
<th>Number (%) of cases with CXCR4 staining in tumour cells within the lymph nodes</th>
<th>Statistically significant associations with positive CXCR4 staining in primary OSCC lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uchida 2003</td>
<td>18</td>
<td>SNS</td>
<td>Not stated</td>
<td>8/10 (80%)</td>
<td>None</td>
</tr>
</tbody>
</table>
| Almofti 2004         | 61              | SNS             | 35/61 (57%)                                                                     | NT                                                                              | LN metastasis (UV)  
Mode of invasion (UV)  
Reduced survival (UV) |
| Delabasi 2004        | 23              | Tongue          | 23/23 (100%)                                                                    | 9/9 (100%)                                                                     | No significant associations                                                             |
| O’Donnell 2005       | 26              | SNS             | 16/16 (100%)                                                                    | 10/10 (100%)                                                                   | No significant associations                                                             |
| Xu 2006, Meng 2009   | 91              | SNS             | 57/91 (62%)                                                                     | NT                                                                              | LN metastasis (NK)  
Tumour size (NK)  
Histolol Grade (NK) |
| Ishikawa 2006        | 90              | SNS             | 27/90 (30%)                                                                     | NT                                                                              | LN metastasis (UV)                                                                   |
| Oliveira-Neto 2008   | 41              | Tongue FOM      | Not stated                                                                      | NT                                                                              | None                                                                                   |
| Hong 2008            | 26              | SNS             | 15/26 (58%)                                                                     | NT                                                                              | PCNA labelling index (UV)                                                              |
| Lee 2009             | 74              | SNS             | 45/74 (61%)                                                                     | NT                                                                              | Reduced Survival (MV)                                                                  |
| Ishikawa 2009        | 85              | SNS             | 20/80 (23%)                                                                     | NT                                                                              | LN metastasis (UV)                                                                   |

Legend: FOM = floor of mouth, SNS = site not specified, NT = not tested, UV = Univariate analysis, MV = multivariate analysis, NK = type of statistical analysis not stated.
1.6.2.2 CXCR4 in OSCC cell migration and invasion.

A high, constitutively expressing CXCR4 cell line has been shown to invade through the ECM-analogue Matrigel in response to SDF (Yoon, Liang et al. 2007). This effect was attenuated by incubation with the pharmacological CXCR4-antagonist TN14003. Over-expression of CXCR4 was induced in an OSCC cell line by transfection including the CXCR4 insert (Uchida, Begum et al. 2004). CXCR4 over-expression was associated with an increase in migration towards SDF. However, these findings were not substantiated with blockade of the SDF/CXCR4 axis and the negative control experiments were performed with wild-type (rather than transfected cells lacking the CXCR4 insert) (Uchida, Begum et al. 2004). The migration of a constitutively CXCR4-expressing OSCC cell line (in the absence of exogenous SDF) was shown to be increased by transfection with the SDF coding sequence (Uchida, Onoue et al. 2007). This effect was attenuated by the pharmacological CXCR4-antagonist AMD3100. However, the scratch wound assays from which these findings were derived were not performed with growth-arrested cells and therefore do not differentiate between differences in cell motility or anchorage-dependent cell proliferation.

CXCR4 knock-down in OSCC cells using RNAi has been shown to reduce cell invasion through Matrigel in-vitro (Hong, Pai et al. 2009). However, no evidence has yet been published looking at the effect of CXCR4 over-expression on cell invasion through ECM analogues with appropriate controls (Uchida, Begum et al. 2004). Work focussing on possible mechanisms of bone invasion by OSCC cells in-vivo has suggested an important role for IL-6 (Tang, Chuang et al. 2008). This group has shown constitutive production of SDF by osteoblasts in-vitro. They also demonstrated that SDF increased IL-6 production by OSCC cells by a mechanism that was blocked by antibodies, antagonists or RNAi towards CXCR4. They hypothesised that, as IL-6 has been implicated in osteoclastogenesis, this mechanism may be involved in bone invasion by OSCC cells within primary lesions in-vivo (Tang, Chuang et al. 2008). What is unclear from these data is whether this may play a role in the metastatic dissemination of OSCC, although clinical observation reveals that distant, lytic bone deposits are an uncommon manifestation of the disease.
1.6.2.3 CXCR4 protein in OSCC cell proliferation

Over-expression of CXCR4 in a constitutively low-expressing OSCC cell line yielded more lymph nodes metastases following orthotopic injection in nude mice compared with untransfected controls (Uchida, Begum et al. 2004). Serial passage of metastatic OSCC sub-clones following orthotopic inoculation into nude mice has been shown to be associated with an increase in CXCR4 mRNA and protein (Yoon, Liang et al. 2007). Treatment of mice in which CXCR4-positive subclones had been implanted in the submandibular region with the CXCR4 antagonist TN14003 resulted in a significant reduction of early primary tumour growth and associated lung metastases (Yoon, Liang et al. 2007). While this evidence seemingly supports a role for CXCR4 in the growth of primary and secondary OSCC lesions, it has some flaws. Firstly, in the former experiments, the effect of transfection alone was not determined (Uchida, Begum et al. 2004). Secondly, in the latter experiments, the original sub-clones were selected out through serial animal passage (Yoon, Liang et al. 2007). Therefore, it is possible that other factors have been upregulated through this artificial process. This may limit the translation of these data to the clinical setting.

Other factors, such as the delivery of viable tumour cells to the systemic circulation, may define the pattern of metastatic dissemination of CXCR4-expressing OSCC cells. Orthotopic injection of constitutively CXCR4-expressing OSCC cells transfected with the SDF coding sequence resulted in increased lymphatic metastases without changing the volume of the primary disease or detectable distant metastases (Uchida, Onoue et al. 2007). However, when the same cells were injected intravenously the volume of lung metastases was significantly higher in clones containing the SDF insert compared with those that did not. These data suggest that differing patterns of metastasis result from cells directly entering the systemic venous system (and thus delivered directly to the pulmonary capillary bed) compared with the conventional delivery to the regional lymphatic nodes.

While the majority of the published literature on CXCR4 function in OSCC tumour growth has focussed on animal studies, there is a paucity of in-vitro data regarding its effect on proliferation. This is an important question to answer. CXCR4 knock-down in OSCC cells using RNAi has been shown to be associated with a significant reduction in real-time proliferation in-vitro (Hong, Pai et al. 2009). The effect of CXCR4 over-expression on proliferation or apoptosis has yet to be investigated in OSCC.
HIF-1α labelling was demonstrated in 51/85 (60%) of predominantly early stage OSCC primary lesions (Ishikawa, Nakashiro et al. 2009). Of the 20/85 lesions that labelled positively for CXCR4, 18 showed staining for both HIF-1α and CXCR4. HIF-1α labelling was not associated with any of the clinicopathological features recorded (Ishikawa, Nakashiro et al. 2009). However, other authors have demonstrated an association between HIF-1α staining and both prognosis and lymph node metastasis although this was in a series of predominantly late stage OSCC cases in which the prognosis was poorly defined (Uehara, Sano et al. 2009). Manipulation of OSCC cell lines in-vitro demonstrated an increase of HIF-1α and CXCR4 mRNA and protein in response to hypoxia that was abrogated by knock-down of HIF-1α expression by RNAi (Ishikawa, Nakashiro et al. 2009). While these data suggest a role for HIF-1α in the control of CXCR4 production in OSCC the exact mechanism is unclear.

1.6.2.4 CXCR4 in the epithelial-mesenchymal transition of OSCC cells.

Epithelial-mesenchymal transition (EMT) is a process that occurs during embryonic development and is typified by an increase in motility and invasiveness associated with the loss of E-cadherin expression. Over-expression of the Snail zinc-finger protein has been shown to induce EMT in OSCC cells in-vitro with an associated increase in SDF and CXCR4 expression (Taki, Higashikawa et al. 2008). Serial passage of metastatic OSCC cells through nude mice resulted in a change in morphology mirroring EMT with a concomitant reduction in E-cadherin and increase in CXCR4 expression (Yoon, Liang et al. 2007). OSCC cells showing features of EMT demonstrated an increased migratory response and upregulation of CXCR4 expression in response to SDF stimulation or TGF-β1 (Taki, Higashikawa et al. 2008). TGF-β1 production by tumour cells has been shown to induce differentiation of fibroblasts into activated myofibroblasts. These then regulate tumour growth and invasion via complex cytokine networks. Oral mucosal fibroblasts have been shown to produce SDF and hepatocyte growth factor (HpGF) in-vitro (Daly, McIlreavey et al. 2008). This was increased in response to stimulation with Interleukin-1α (IL-1α) or OSCC-conditioned media and reduced by incubation with TGF-β1. SDF and HpGF both increased OSCC cell invasion through Matrigel and in fibroblast-free organotypic

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15 Poor prognosis was defined as death (at an unspecified point) within the follow-up period of 4-16 years.
cultures, although the pattern of invasion in the latter was different compared with fibroblast-conditioned media (Daly, McIlreavey et al. 2008). In conclusion, these reports suggest that EMT in OSCC cells is associated with an aggressive tumour phenotype and increased CXCR4-mediated functional responses secondary to interactions with (activated) fibroblasts within the tumour/tissue microenvironment. What is not clear is whether CXCR4 upregulation is the cause or effect of EMT.

1.7 Summary

Chemokine receptors are mediators of physiological leukocyte recruitment to peripheral lymph nodes and distant sites. The uptake of dendritic cells to local lymph nodes is dependent upon the surface expression of the chemokine receptor CCR7. Evidence from a number of carcinoma systems supports the chemoattraction theory of metastasis. Investigation of a number of carcinomas of the aero-digestive tract suggests that tumour cell expression of CCR7 facilitates lymphatic metastasis while CXCR4 expression is most frequently associated with distant metastasis. However, it is possible that CXCR4 may mediate metastasis by mechanisms additional to its chemoattractant effect.

Although the literature on chemokine receptor expression by OSCC has accumulated over the last decade, the findings remain inconclusive. Reported results from in-vitro studies have suggested a role for CXCR4 in cell motility and tumour growth. However, the precise effect of CXCR4 over-expression remains unclear. Studies have so far failed to differentiate between effects on cell chemokinesis, chemotaxis and invasion, and can be criticised for their lack of appropriate negative controls (see above).

Different groups have reported an association between CXCR4 or CCR7 upregulation with lymph node metastasis. Clinical observation reveals that significant metastatic deposits are formed frequently in the cervical lymph nodes but rarely at distant sites. Nevertheless, micrometastases at distant sites are common at autopsy. Emerging evidence indicates that the importance of the chemokine network in tumour progression is not limited to directed cell migration. Given its clinical distribution of OSCC spread, the chemokine receptor expression profile of OSCC is intriguing.
Investigation of the modulation of the chemokines network by OSCC in vitro and in vivo may provide considerable insight into the control of metastatic dissemination.

1.8 Plan of investigation

1.8.1 Overall Study Aim
To investigate the role of chemokine receptors in the metastasis of oral squamous cell carcinoma.

1.8.2 Overall Study Hypothesis
Genetic changes in oral squamous cell carcinomas result in the over-expression of those functional wild-type chemokine receptors associated with physiological leukocyte homing to secondary lymphoid tissue. Metastatic cells delivered to the cervical lymph nodes via established lymphatic channels that bear these chemokine receptors undergo stimulation by the chemokines constitutively produced there. This chemokine receptor-mediated activation favours neoplastic growth and dissemination by increasing tumour cell migration, invasion, proliferation and survival.

1.8.3 Outline of Investigation
Initially this hypothesis will be investigated by the examination of chemokine receptor mRNA expression in cultured normal oral keratinocytes and established OSCC cell lines using microarrays. Interesting results will be verified using semi-quantitative RT-PCR and further investigated at the protein level using immunocytochemistry, flow cytometry and examination of clinical specimens using a novel technique of semi-quantitative immunohistochemistry. Chemokine receptors that demonstrate upregulation in OSCC lines (compared with normal oral keratinocytes) will be selected and stably transfected into a constitutively low-expressing OSCC cell line. These transfected cell lines (and appropriate controls) will be investigated using functional assays of chemokinesis, chemotaxis, proliferation and invasion.
Chapter 2:
Materials and Methods
2.1 Cell Culture

2.1.1 Established cell lines derived from oral squamous cell carcinomas.

1. H357
The cell line H357 was derived from a well differentiated oral squamous cell carcinoma from the tongue of a 74 year old male (Prime, Nixon et al. 1990). The original tumour was less than 4 cm in diameter with regional lymph node involvement but no distant metastases. It has been shown that orthotopic inoculation of H357 cells into the floor of the mouth of athymic mice does not result in tumourigenesis (Prime, Eveson et al. 2004).

2. TR146
The continuous cell line TR146 was derived from a human neck metastasis originating from a buccal carcinoma (Rupniak, Rowlatt et al. 1985). These cells were originally a gift to the laboratory from Cancer Research UK.

3. FaDu
The FaDu cell line (ATCC, HTB-43) was established in 1968 from a punch biopsy of a hypo-pharyngeal tumour removed from a 56 year old Hindu patient (Rangan 1972). The established line was found to contain bundles of tonofilaments in the cell cytoplasm and desmosomal regions were prominent at cell boundaries.

4. CAL27
The CAL27 cell line (ATCC, CRL-2095) was established from tissue taken prior to treatment from a 56 year old Caucasian male with a squamous cell carcinoma of the middle of the tongue (Gioanni J, Fischel JL et al. 1988). CAL27 cells are epithelial, polygonal with a highly granular cytoplasm. Immunocytochemical studies show strong positive staining with anti-keratin antibodies.

5. SCC4
The SCC4 cell line (ATCC, Number CRL-1624) was derived from a squamous cell carcinoma of the tongue of a 55 year old male (Rheinwald and Beckett 1980).
6. SCC25
The SCC25 cell line (ATCC, Number CRL-1628) was derived from a squamous cell carcinoma of the tongue of a 70 year old male (Rheinwald and Beckett 1980).

2.1.2 Established non-cancerous oral cell lines.

1. Human Gingival Fibroblasts
Primary human gingival fibroblasts (HGFs) were obtained from the departmental archive of frozen cells (Dept. of Oral Pathology, Clinical and Diagnostic Oral Sciences, Queen Mary University London). These cells were obtained from clinically healthy gingival tissues at the time third molar extraction and originally frozen in 1995 after their seventh passage.

2. Primary Normal Oral Keratinocytes
Primary normal oral keratinocytes (NOKs) were acquired from the departmental archive of frozen cells (Dept. of Oral Pathology, Clinical and Diagnostic Oral Sciences, Queen Mary University London). They were taken from normal oral mucosa at the time of third molar surgery in 1995 and frozen at the time of first passage.

2.1.3 Established non-oral cell lines and feeder cells

1. Primary Normal Skin Keratinocytes
Primary normal skin keratinocytes (NSKs) were a gift from Professor Alan Storey (Department of Cutaneous Research, Queen Mary University London). They were established from normal skin taken at the time of breast reduction surgery in 2002 and frozen at the time of first passage.

2. NIH 3T3 Swiss embryo fibroblasts
These murine fibroblasts were obtained from the European Collection of Cell Cultures (ECACC, 93061524).
2.1.4  Cell culture and passage

Established OSCC cell lines were taken and grown in 75cm² plastic tissue-culture flasks under sterile conditions in a mycoplasma-free incubator at 37°C in a humidified atmosphere with 5% CO₂. Specialised growth media (Table A2.1, Appendix 2) were supplemented with 10% Fetal Bovine Serum (FBS) where appropriate. Cells were passaged at early confluence using Trypsin/ Ethylenediaminetetraacetic acid (EDTA) solution (Invitrogen 25300-054). Cells were grown in quadruplicate to 90% confluence as determined by light microscopy. The doubling time for each cell line was calculated using proliferation assays (see Section 2.1.6).

Primary cultures of NOKs and NSKs were incubated with specialised growth media (Table A2.1, Appendix 2) under similar conditions to those outlined above with the addition of a feeder layer of growth-arrested fibroblasts. These feeder cells were prepared by the incubation of NIH-3T3 murine fibroblasts with 10 μg/ml Mitomycin C (Sigma, M4287) for 2 hours in a humidified atmosphere at 37°C with 5% CO₂ in the dark. The cells were then washed 3 times with sterile PBS, passaged, counted and seeded at a density of 2x10⁶ per T75 flask. These growth-arrested fibroblasts were allowed to adhere to the flask for 4 hours prior to the addition of the primary keratinocytes.

2.1.5  Mycoplasma testing and treatment

Samples were checked weekly for mycoplasma contamination using a combination of three complimentary techniques: immunofluorescence, polymerase chain reaction and ELISA. Cells identified as mycoplasma-positive were treated with courses of BM Cyclin (Roche, 799050) until testing negative, and then for a further two weeks (according to the manufacturer’s recommendations). After initial treatment, mycoplasma contamination was not subsequently detected in tissue culture samples. Specimen methodologies and results are given in Section A2.2 (Appendix 2).
2.1.6 **Determination of cell growth kinetics by MTT assay.**

Cell proliferation assays were performed for each cell line to determine their growth kinetics under optimal conditions. In this way the time to the end of the logarithmic growth phase was calculated for a known seeding density and culture surface area. This technique is described in detail in Section 2.8.2. Briefly, cells were passaged and mixed well to form a final cell density of $25 \times 10^3$/ml in appropriate media. Two hundred microlitres of this cell suspension were added to each of 12 wells on a 96 well plate. Seven identical plates were prepared in total and incubated at 37°C in a humidified atmosphere with 5% CO$_2$. A time course at 24-hour intervals was performed. Mean absorbance for blank wells was subtracted from each recorded value and the mean and standard deviation for each set of 12 replicates was calculated. The growth profile of each cell line was plotted against time and the exponential growth phase defined. The gradient of the exponential function was calculated across this period and the accuracy of fit determined using the Root Mean Square (RSQ). The results are summarised in Table A2.2 (Appendix 2).

2.2 **Microarrays**

2.2.1 **Overview**

Two sets of microarray experiments were performed using the Affymetrix platform. Sample handling was very similar in each case. However, by the time of the second round of experiments, the Affymetrix U133A arrays had been superseded by the U133v2.0 hardware (with associated small changes in the preparation protocol). For simplicity the protocol based on the U133A system is described with a short addendum containing the U133v2.0-based changes.
The analysis of samples using microarrays was performed using the following steps:

1. Growth of cell lines in culture
2. Extraction and purification of tRNA
3. tRNA clean-up and DNase treatment
4. Quantification of tRNA
5. Double-stranded cDNA synthesis
6. Clean-up of double-stranded cDNA
7. Synthesis of biotin-labelled cRNA
8. Clean-up of cRNA
9. cRNA fragmentation
10. Preparation for hybridisation
11. Microarray hybridisation
12. Data processing and analysis

2.2.2 Growth of cell lines in culture

In the first set of experiments six established OSCC cell lines (CAL27, FADU, H357, TR146, SCC4 and SCC25), primary normal oral and primary normal skin keratinocytes (NOK and NSK respectively) were taken and grown in cell culture as outlined previously. Cultured cells were grown in 100mm Petri dishes with 10% FBS to just before the end of the logarithmic growth phase as determined by proliferation assays. Samples from the SV cell line were prepared in an identical manner and were a kind gift from Dr Joanne Stewart (Clinical and Diagnostic Sciences, Queen Mary University London).

For the second set of experiments H357 cells transfected with and without the CXCR4 insert were stimulated with and without Stromal-cell Derived Factor (SDF). The effect of culture under conditions of high (20%) and low (2%) oxygen tension for 24 hours was also investigated using a hypoxic incubator by kind permission of Professor Ian McKenzie, Queen Mary University London.

For this experiment SDF concentrations of 1 and 100ng/ml were used compared with no stimulation.
2.2.3 Extraction, purification and quantification of total RNA

Methodologies using various agents for the extraction of RNA from cultured cells were compared. These included using Ultraspec (Biotecx, BL10050), RNAwiz (Ambion, 9736), RNAbee (AMS Biotechnology, CS-104B) and TRIzol (Invitrogen, 10296-028). On the basis of spectrophotometric and electropherographic analysis of quality and quantity the method using TRIzol and double precipitation was found to produce the best yields.

Cell culture medium was removed and the cells were washed once with sterile PBS. Care was taken to aspirate all the PBS. Five hundred microlitres of TRIzol was added for each 25cm² of cell growing surface. The TRIzol was distributed over the entire surface of each flask and incubated for 20 minutes at room temperature. The cells were mechanically disrupted using a cell scraper and the lysate collected in nuclease-free Eppendorf tubes in 750µl aliquots. One hundred and seventy five microlitres of chloroform (Sigma) was added to each aliquot, vortexed briefly and each added to heavy phase lock gel tube (Eppendorf, 0032 007.953) that had been previously centrifuged at 8,000g for 30 seconds to pellet the gel. The samples were then spun at 8,000g for 2 minutes and subsequently each colourless upper aqueous layer was carefully transferred to an Eppendorf tube.

The RNA was precipitated by the addition of 500µl of isopropanol and 1µl of glycogen (Ambion, 9510) for 20 minutes at room temperature. An RNA pellet was obtained by centrifugation at 12,000g for 10 minutes at 4°C. The supernatant was removed and the pellet was washed with 500µl of 75% ethanol followed by centrifugation at 7,500g for 5 minutes at 4°C. The supernatant was removed and the pellet was allowed to air dry for 10 minutes at room temperature. The RNA was dissolved in 200µl of RNAse free water (Ambion, 9934) for 10 minutes at 65°C. A second salt precipitation of RNA was performed by the addition of 20µl of 3M sodium acetate (Sigma, 57899), 500µl of 100% ethanol and 1µl of glycogen to each tube at -20°C overnight. An RNA pellet was subsequently produced by centrifugation at 12,000g for 20 minutes at 4°C. Washes with 500µl of 80% ethanol followed by centrifugation at 12,000g for 10 minutes at 4°C were performed twice. The supernatant was carefully removed and the pellet was allowed to air dry for 10 minutes at room temperature. The RNA was dissolved in 50µl of RNAse free water at 65°C for 10 minutes.
2.2.4 Total RNA clean-up and DNase treatment

Total RNA clean-up was performed using an RNeasy kit (Qiagen, 74104) according to the manufacturer’s instructions. One hundred micrograms of total RNA was made up to a volume of 100µl with RNase free water in an Eppendorf tube. To each tube 350µl of 1% β-mercaptoethanol (Sigma) in the supplied buffer RLT and 250µl of 100% ethanol was added and mixed by pipetting. The tube contents were then added to a spin-column placed over a 2ml collection tube. The column was centrifuged at 8,000g for 15 seconds at room temperature and the flow-through was discarded.

On column DNase digestion was performed using the RNase-free DNase kit (Qiagen, 79254). Three hundred and fifty microlitres of the supplied buffer RW1 was added to each spin column and these were centrifuged at 8,000g for 15 seconds and the flow-through discarded. Ten microlitres of DNase I stock solution, gently mixed with 70µl of buffer RDD, was added to each column and was incubated for 15 minutes at room temperature. A further spin-wash with 350µl of buffer RW1 was performed.

Each column was washed with 500µl of buffer RPE and spun at 8,000g for 15 seconds at room temperature and the flow-through discarded. This was performed twice. The columns were replaced with new collection tubes and allowed to air dry by centrifugation at 8,000g for 1 minute. The collection tubes were replaced with clean Eppendorf tubes with care being taken to avoid ethanol carryover and contamination. Thirty microlitres of RNase-free water was placed on to the column membrane, incubated for 2 minutes at room temperature and spun at 8,000g for 1 minute. This elution was performed twice.

2.2.5 Quantification of tRNA

Spectrophotometry was performed using an UVmini-1240 instrument (Shimadzu, Japan). The 260/280Å absorbance ratio was found to be critically dependent on buffer pH. Dilution in un-buffered diethyl pyrocarbonate (DEPC) treated water produced variable absorbance ratios. This problem was overcome by performing all dilutions with sodium-diphosphate (Sigma, P8135) buffer. A working solution of sodium-diphosphate spectrophotometry dilution buffer (SDSDB) in nuclease-free water was prepared fresh for each batch of samples. Baseline correction for 1x SDSDB was
performed prior to all measurements. One microlitre RNA samples were added to 99 µl of 1x SDSDB and mixed thoroughly. For each sample the values for absorbance at 260Å (A1) and 280Å (A2) were recorded. The amount of RNA contained in each sample was calculated by the formula:

\[ \text{Amount (µg/µl)} = A1 \times 4 \]

Final RNA quality was assessed using spectrophotometry and a RNA 6000 Nano Assay chip with an Agilent 2100 Bioanalyser (both Agilent Technologies). The latter was performed using the Nano LabChip kit (Agilent, 5065-4476) according to the manufacturer’s protocol: Briefly, 65 µl aliquots of Nano gel matrix were spin-filtered at 1,500 g for 10 minutes at room temperature. One microlitre of Nano dye concentrate was added to each aliquot, vortexed and spun at 13,000g for 10 minutes at room temperature. Each chip was primed with 9 µl of the gel-dye mixture using the supplied syringe pressuriser for 30 seconds. Five microlitres of the Nano marker were added to each of the wells and 1µl of heat denatured ladder (Ambion, 7152) to one control well. All samples were heat denatured in a similar way and 1µl aliquots were added to each of the sample wells. Finally, prior to reading, the loaded chip was covered with an adhesive film and vortexed on a specific shaker (IKA, MS2-S8) for 1 minute at 2,000 rpm.

Samples of suitable quality and quantity were selected by examination of the electropherograms produced by the Agilent 2100 Bioanalyser. A typical electropherogram of a suitable sample is shown in Figure 2.1. The ratio of the height of the 28S peak relative to that of the 18S peak was calculated. Evidence of good RNA quality was demonstrated by a ratio of 28S to 18S peak height of greater than 1.0.

---

17 Heat denaturation was performed by incubation at 70°C for 2 minutes
Figure 2.1: Electropherogram of a typical RNA sample. Electropherogram of a typical RNA sample produced by analysis by an Agilent 2100 Bioanalyser showing absorption peaks at 18S and 28S. This example shows RNA of suitable quality for microarray analysis demonstrated by a greater absorption (peak height) at 28S compared with 18S.

2.2.6 Double-stranded cDNA synthesis

Double-stranded cDNA was synthesised using the Superscript ds-cDNA Synthesis Kit (Invitrogen, 11917-010) according to the manufacturer’s instructions. Ten micrograms of total RNA (tRNA) of known concentration were taken and added to 100 pmol of high performance liquid chromatography (HPLC) purified T7-(dT)$_{24}$ primer (Invitrogen) on ice. The reaction volume was made up to 11 µl with RNAse free water (Ambion, 9937) and mixed by pipetting. The reaction mixture was incubated at 65°C for 10 minutes to break any RNA secondary structures present and their subsequent re-formation was inhibited by placing the tubes immediately on to ice.

Initially, single-stranded cDNA was synthesised by the following method: A master-mix comprising of 4µl of the supplied 5x First Strand Buffer, 2µl of 0.1M DTT and 1µl of dNTPs (10mM each) for each sample was prepared on ice. Seven microlitres of this master-mix were added to each tRNA sample the reaction was incubated at 42°C for 2 minutes. Subsequently four hundred units of Superscript II reverse-transcriptase were added to each sample, mixed gently and incubated at 42°C for one hour.

18 The sequence of the T7-(dT)$_{24}$ primer (Invitrogen) was GGCCAGTGAATTGTAATACGCTCACTATAGGGAGGCGGTTTTTTTTTTTTTTTTTTTT
The second strand of cDNA synthesis was then performed: A master-mix was assembled on ice by the addition of 91 µl of RNase free water, 30 µl of 5x Second Strand Buffer, 3 µl of dNTPs (10 mM each), 10 units of E. coli DNA ligase, 40 units of E. coli DNA Pol I and 2 units of E. coli RNase H for each sample. Following gentle mixing, 130 µl of the master-mix was added to each sample and the reaction was incubated at 16°C for 2 hours. Ten units of T4 DNA polymerase were then added and incubated at 16°C for a further 5 minutes. Finally 10 µl 0.5M EDTA (pH 8.0) was added.

2.2.7 Clean-up of double-stranded cDNA

To each sample of double-stranded cDNA prepared above was added 162 µl of buffer-saturated phenol. The samples were then vortexed briefly and transferred to a light phase-lock gel tube (Eppendorf, 0032 007.961) that had previously been spun at 8,000g for 30 seconds to pellet the gel. The samples were then centrifuged at 8,000g for 2 minutes at room temperature and the upper aqueous layer was transferred to a new nuclease-free Eppendorf tube.

To each tube was added 75 µl of 7.5M ammonium acetate (Sigma, A2706), 4 µl of glycogen (Ambion) and 375 µl of 100% ethanol followed by gentle mixing. The samples were centrifuged at 12,000g for 20 minutes at room temperature. The supernatant was carefully removed leaving the pellet behind; 500 µl of cold 80% ethanol was added and then spun at 12,000g for 5 minutes at 4°C. This ethanol washing was performed twice. All the ethanol supernatant was carefully removed and the pellet was allowed to air dry for 10 minutes at room temperature. The pellet was finally resuspended in 12 µl of nuclease-free water.

2.2.8 Synthesis of biotin-labelled cRNA

Synthesis of biotin-labelled complementary RNA was performed using the Bioarray high yield RNA transcript labelling kit (ENZO, Affymetrix, 900182) according to the manufacturer’s instructions. A master-mix was assembled at room temperature.

---

19 Buffer-saturated phenol was prepared as follows: 65 µl of alkalin buffer (10 mM Tris HCl, 1 mM EDTA pH 8.0) was added to 1 ml of phenol-chloroform-isoamyl alcohol in the ratio 25:24:1 (Ambion, 9732), vortexed for 2 minutes and then centrifuged briefly.
comprising of (per sample): 10µl of nuclease-free water, 4 µl of 10x HY reaction buffer, 4µl of biotin-labelled ribonucleotides, 4µl of DTT, 4µl of RNase inhibitor mix and 2µl of T7 RNA polymerase. Twenty-eight microlitres of master-mix were added to each 12µl sample prepared above and the reaction was incubated at 37°C for 5 hours with gentle mixing by tapping every hour.

2.2.9 Clean-up of cRNA

Clean up of the biotin labelled cRNA produced above was performed using the RNeasy KIT (Qiagen, 71404). Each sample was made up to 100 µl by the addition of 60 µl of nuclease-free water. Three hundred and fifty microlitres of buffer RLT and 250µl of 100% ethanol were added and mixed thoroughly by pipetting. The reaction was added to a spin-column placed over a 2 ml collection tube. The column was centrifuged at 8,000g for 15 seconds at room temperature and the flow-through was discarded. The column was washed with 500µl of buffer RPE and spun at 8,000g for 15 seconds at room temperature and the flow-through discarded. This washing stage was performed twice. The columns were replaced with new collection tubes and allowed to air dry by centrifugation at 8,000g for 1 minute. Thirty of RNase-free water was placed on to the column membrane; incubated for 2 minutes at room temperature and spun at 8,000g for 1 minute into a fresh nuclease-free Eppendorf collecting tube. This final elution step was performed twice. One microlitre of the eluted cRNA was analysed using the spectrophotometer. The minimum concentration of cRNA required was 0.6 µg/µl. Samples with concentrations below this value (but with total yields greater than 40 µg) underwent a salt and ethanol precipitation as described in Section 2.2.3 above.

A 1% agarose gel was prepared: 100 ml of 10x TAE buffer (Invitrogen, 15558-026) was diluted with 900ml DEPC-treated water. One gram of agarose (Helena Biosciences, 8201-07) was dissolved in 100 ml of 1x TAE buffer by microwave heating for 30 seconds twice followed by the addition of 60 µg of Ethidium Bromide (Sigma, E8751). The gel was poured, allowed to set and covered in 700ml of 1x TAE buffer in the gel tank.
Five hundred nanograms of each cRNA sample were denatured by heating at 70 ºC for 10 minutes in denaturing loading buffer (Sigma, S4268). Samples were mixed with 2 µl of gel loading buffer (MBI Fermentas, SM0321) and loaded on to the gel alongside a DNA ladder (Generuler 100bp Plus, MBI Fermentas, SM0321). The gel was run for at 100V for 30 minutes. Samples were checked for quality (the appearance of a smear from 100bp to 2kb with a brighter region from 500bp to 1kb) as demonstrated in Figure 2.2.

![Figure 2.2: 1% agarose gel showing typical band pattern of cRNA quality. Gel electrophoresis of six samples of fragmented cRNA (lanes A-F) run against a 1kb ladder (lane G). All samples except lane C are suitable for subsequent microarray analysis as demonstrated by a smear from 100bp to 2kb with a brighter region from 500bp to 1kb.](image)

### 2.2.10 cRNA fragmentation

RNase free, filter-sterilised 5x fragmentation buffer was prepared: 200mM Tris-acetate pH 8.1 (Sigma T1503), 500mM Potassium acetate (Sigma, P5708) and 150mM Magnesium acetate (Sigma, M2543). Thirty micrograms of cRNA and 8 µl of 5x fragmentation buffer was made up to 40 µl with nuclease-free water and incubated at 94 ºC for 35 minutes. An aliquot of 250 ng of fragmented cRNA was taken from each sample and run on an Agilent 2100 Bioanalyser using a RNA 6000 Nano Assay chip (as outlined in Section 2.2.5 above).
2.2.11 Preparation for hybridisation

Preparation of the hybridisation cocktail for each sample was performed using the Gene Chip Eukaryotic Hybridisation control kit (ENZO, Affymetrix, 900299). Vials of the 20x Eukaryotic Hybridisation Controls and Control Oligo B2 were heated at 65 ºC for 5 minutes, vortexed and spun briefly. Two buffers were prepared as outlined below: 12xMES and 2x Hybridisation Buffer. For each sample a master-mix was prepared on ice: 5 µl of Control Oligo B2 (3nM), 15 µl of 20x Eukaryotic Hybridisation Controls, 3 µl of Herring sperm DNA (10mg/ml), 3 µl Acetylated BSA (50mg/ml), 150 µl of 2x hybridisation buffer and 15 µg of fragmented cRNA. The total reaction volume was made up to 300 µl with nuclease-free water.

2.2.12 Microarray hybridisation

The hybridisation cocktail was heated to 99 ºC for 5 minutes followed by 45 ºC for a further 5 minutes, and then centrifuged at maximum speed for 5 minutes to remove any insoluble material. Initially, small aliquots of the hybridisation cocktails were run on control microarrays to confirm that the sample quality was suitable for the genome-wide arrays. Briefly, each control microarray was brought up to room temperature, filled with 200 µl of 1x hybridisation buffer, and placed in the probe array oven at 45 ºC for 10 minutes at 60 rpm. The buffer solution was removed from the array cartridge and replaced with 35 µl of the hybridisation cocktail. The array was incubated in the probe oven at 45 ºC for 16 hours at 60 rpm. Each control array was scanned and the hybridisation of sample genes across the array were analysed for signal level and consistency. All samples passed this level of quality control.

Subsequently, each genome-wide microarray (Affymetrix, U133a) was prepared in a similar fashion and incubated with 200 µl of the hybridisation cocktail under the same conditions. Duplicate samples from each cell line were hybridised separately.

20 12x MES Buffer (1.22M MES, 0.89 [Na⁺]): 64.61g MES Hydrate and 193.3g MES Sodium Salt made up to 1 litre with molecular biology grade water then 0.2µm filtered and pH checked (6.5 to 6.7).

21 2x Hybridisation Buffer (100mM MES, 1M [Na⁺], 20mM EDTA, 0.01% Tween-20): 8.3ml 12x MES Buffer, 17.7ml 5M NaCl, 4.0ml 0.5M EDTA, 0.1ml 10% Tween-20 and 19.9ml molecular biology grade water stored at 4ºC protected from light.

22 Final working concentrations of the supplied Eukaryotic Hybridisation Control preparation include bioB (1.5pM), bioC (5pM), bioD (25pm) and cre (100pM).
2.2.13 Data processing and analysis

The hybridised array was scanned using the Affymetrix image-reading system and raw data for each probe position was tabulated against the Affymetrix U133A sequence number. All data underwent a series of quality checks before any analyses were performed: Firstly, the expression levels of multiple probes for Affymetrix control genes distributed across the array were compared to determine whether the microarray had hybridised completely and evenly. Only raw expression data from arrays that had hybridised correctly were entered into the analysis programme (GeneSpring v6.0SE, Agilent Technologies). Secondly, the range of expression values for each array was compared against background to filter out absent signals. Thirdly, the values of all positive signals on each array were plotted against a normal distribution to ensure the data were parametric. Expression levels for individual genes were then normalised around a mean value of 1.0 to produce a Normalised Expression Value (NEV). This allowed comparison between samples. Finally, the software was used to calculate correlations between the samples.

In the first set of microarray experiments data from duplicate samples were available. Initially, the raw data sets were renamed using a unique, randomised and blinded identification number. The analysis software was asked to determine the pattern of similar samples and these statistical correlations were compared with the biological cell line of origin once the blinding was removed.

Values for genes with multiple addresses on the microarray were arithmetically averaged. The mean and range of each duplicate sample pair was derived from the GeneSpring software and plotted on a logarithmic scale. Individual gene expression levels in OSCC cell lines were compared with those from normal oral keratinocytes by calculation of the relative NEV by the following formula:

\[
\text{Relative NEV} = \frac{\text{NEV in OSCC cell line}}{\text{NEV in normal oral keratinocytes}}
\]
The relative NEV and range of the duplicate samples were plotted on a logarithmic scale. Fold change (plus direction) was calculated from the Relative NEV in the following way:

If Relative NEV > 1.0 → Positive Fold Change = Relative NEV
If Relative NEV < 1.0 → Negative Fold Change = 1 / Relative NEV

Statistical comparison was performed using Analysis of Variance (ANOVA). To reduce the likelihood of chance statistical correlations (false-positive results) post-hoc tests were employed. The most stringent of these was the Bonferroni correction.

2.2.14 Variations in the U133A and U133v2.0 Protocols

Some additional steps were included in the U133v2.0 protocols:

1. Eukaryotic Poly-A RNA spike-in controls were added to the sample total RNA at the beginning. These included various in-vitro synthesised polyadenylated transcripts for B.subtilis genes\textsuperscript{23} that are absent in eukaryotic samples but which have specific probe sets on the final arrays.
2. DMSO to a final concentration of 10% was added to the hybridisation cocktail
3. Data underwent normalisation using a GC-RMA pre-processor (incorporated in Genespring 6.0 SE).
4. Signal filtering was performed at a threshold calculated by multiplying the mean background reading by four (according to the manufacturer’s recommendations)

2.3 Quantitative Reverse-Transcription Polymerase Chain Reaction

2.3.1 Overview

Samples were prepared for semi-quantitative reverse-transcription polymerase chain reaction (sqRT-PCR) as follows:

\textsuperscript{23} These include lys, phe, thr and dap at final concentrations (ratio of copy numbers) of 1:100000, 1:50000, 1:25000 and 1:6667 respectively
1. Growth of cell lines in culture
2. Extraction and purification of total RNA
3. total RNA clean-up and DNase treatment
4. Quantification of total RNA
5. Total RNA reverse transcription
6. Quantitative polymerase chain reaction
7. Data processing and analysis

Sample selection and preparation was identical to Steps 1-4 of the microarray protocol outlined in Chapter 2. However, four replicate samples from each cell line were analysed by sqRT-PCR, each run in triplicate.

2.3.2 Total RNA Reverse Transcription

Two protocols were used for performing reverse transcription. Initially samples were prepared using the multi-step protocol based on the Moloney Murine Leukaemia Virus (M-MLV) reverse-transcriptase chemistry. Subsequently the one step reaction using the iScript cDNA Synthesis kit (Bio-Rad, 170-8891) was preferred due to its simplicity and cost. The iScript method had the added advantage of containing both oligo-(dT) and random hexamer primers making the cDNA produced suitable for both conventional and real-time PCR. Both methodologies are outlined below.

2.3.2.1 Moloney Murine Leukaemia Virus reverse transcriptase method.

Two micrograms of DNase-treated total RNA was taken and made up to a reaction volume of 13µl with nuclease-free water. One microgram of random hexamer primers (Promega, C1181) was added; incubated at 75°C for 5 minutes and immediately cooled on ice for 5 minutes. A master-mix was assembled on ice containing (per sample) 5µl of reaction buffer (Promega M5313), 5 µl of combined nucleotide pool and 1 unit of Moloney Murine Leukaemia Virus reverse transcriptase (MMLV-RT) enzyme (Promega, M368B). Eleven microlitres of the master-mix were added to each sample and incubated sequentially at room temperature initially for 10 minutes and then at
40°C for 50 minutes. Finally the sample volume was made up to 100 µl by the addition of 75µl of nuclease-free water. For each cell line a negative control reaction was performed in which the 1µl of MMLV-RT enzyme was replaced by an equal volume of nuclease-free water. All other reagents and reaction conditions remained the same.

2.3.2.2 iScript cDNA Synthesis method

Again, two micrograms of DNase-treated total RNA was taken and made up to a reaction volume of 15µl with nuclease-free water. A master-mix was prepared containing (per sample) 4µl of 5x iScript Reaction Mix and 1µl of iScript Reverse Transcriptase. Five microlitres of the master-mix were added to each 15µl sample. The samples were incubated at 25°C for 5 minutes, then 42°C for 30 minutes and finally 85°C for 5 minutes. If a greater volume of cDNA product was required then the reaction volume was increased with the components scaled-up proportionally. In a similar fashion to before, a no reverse-transcriptase negative control reaction was performed by the substitution of the iScript Reverse Transcriptase with 1µl of nuclease-free water.

2.3.3 Quantitative polymerase chain reaction

Quantitative RT-PCR (relative or absolute) requires reference to a normalised standard. For comparison between cell lines, relative quantitative comparison against a standard endogenous internal standard (18S RNA) was performed. The Taqman primer/probe sets used (Assays-on-Demand, Applied Biosystems) consist of a pre-optimised pair of sequence-specific PCR primers and a sequence-specific probe with a FAM reporter dye at the 5’ end and a non-fluorescent quencher at the 3’ end. In the unbound state the quencher lies in close physical proximity to the reporter negating its fluorescence. However, when bound the quencher/reporter complex separates and the reporter fluoresces when laser stimulated. Fluorescence is proportional to the concentration of amplicon in the sample. Samples in which the reverse transcription step had been omitted were used as negative-controls.
A master-mix was assembled as follows for each sample: 1.25 μl FAM-labelled sequence-specific primer/probe set, 1.25 μl VIC-labelled 18S RNA primer/probe set, 12.5 μl Taqman Universal Master-Mix (Applied Biosystems) and 8 μl PCR grade water (Ambion). Each reaction was assembled by the addition of 23 μl of master-mix to 2 μl of each cDNA sample. Four samples were analysed per cell line and each sample was run in triplicate. PCR was performed using an ABI Prism 7700 light cycler (Applied Biosystems) with the following programme: 50°C for 2 minutes, 95°C for 10 minutes, then 60 cycles of denaturing at 95°C for 15 seconds followed by annealing and extension at 60°C for 60 seconds. Initially the expression of CXCR4 and CCR7 was investigated using Assays-on Demand FAM-labelled fluoroprobes for CXCR4 (4333458, Hs 00607978) and CCR7 (4331182, Hs 00171054).

Data were interpreted using the software supplied with the Applied Biosystems 7700 light cycler. Amplification threshold (Ct) was set according to the software’s suggestion for each of the FAM and VIC dye layers. For each individual well the difference in Ct values (ΔCt) between the FAM (chemokine receptor) and VIC (18S RNA) reporters was calculated according to the following formula:

\[
\Delta C_t = C_{FAM} - C_{VIC}
\]

The mean ΔCt values for each triplicate well were calculated. Comparison of cell lines to normal oral keratinocytes was performed by calculation of the ΔΔCt value for each cell line. Finally the fold change between cell lines was calculated and plotted against a logarithmic scale.

\[
\Delta \Delta C_t (\text{CELL LINE}) = \text{mean } \Delta C_t (\text{CELL LINE}) - \text{mean } \Delta C_t (\text{NOK})
\]

\[
\text{Fold Change (CELL LINE)} = 2^{-\Delta \Delta C_t (\text{CELL LINE})}
\]
2.4 CXCR4 stable transfection

2.4.1 Overview

The aim of this procedure was to produce a transfected OSCC cell line with a high stable expression of CXCR4 mRNA. This was achieved as follows:

1. Selection of cell lines and preparation of reagents
2. Transformation of competent cells and clonal expansion
3. Clonal screening for presence of the insert
4. DNA extraction from clones
5. Plasmid ligation
6. Colony screening
7. Extraction and purification of CXCR4 plasmid DNA
8. Transfection
9. Clonal selection under antibiotic selection
10. Characterisation of transfected cell line

2.4.2 Selection and preparation of reagents.

The CXCR4 coding sequence received had been inserted into the pcDNA3 DNA plasmid (Invitrogen) using the restriction enzymes EcoR1 and Xba1. This was a kind gift of Dr Hagen Kulbe and Dr Julia Wilson (Translational Oncology Laboratory, Cancer Research UK, Queen Mary University London). The pcDNA3.1 plasmid (Invitrogen) was selected for these experiments in order to provide appropriate restriction enzyme digest sites and both DNA and protein tags for construct labelling.

Bacterial culture was performed using the LB medium. The base Lysogeny Broth (LB) was supplemented with filter-sterilised Ampicillin to a final concentration of 100µg/ml. Briefly, these were prepared by pouring 16ml of Ampicillin-supplemented molten agar into sterile Petri dishes, allowing it to solidify and then incubated, inverted, at 37°C in a dry oven for 30 minutes to remove any condensation. Agar

Lysogeny Broth was produced by adding 10g Bacto-tryptone, 5g yeast extract and 10g NaCl to 800ml of water. The pH was adjusted to 7.5 with sodium hydroxide and the volume then adjusted to 1L before autoclaving.
plates used for blue/white screening were prepared by spreading 100 µl of 0.1M dioxane-free isopropyl-β-D-thiogalactopyranoside (IPTG, Promega, V3955) and 20 µl of 50 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-thiogalactopyranoside (X-Gal, Promega, V3941) on to each plate and allowing the solutions to absorb for 30 minutes at 37°C. Stocks of bacterial clones were preserved by mixing 800µl of culture media with 200 µl of sterile 100% glycerol and freezing at -80°C.

2.4.3 Transformation of competent cells, clonal expansion and determination of transformation efficiency

Transformation was performed using JM109 competent cells (Promega). Initially, 200µl of competent cells were thawed and mixed carefully without pipetting. The bacterial suspension was divided into two equal aliquots and transferred to pre-chilled 12 ml tubes. Fifty nanograms of DNA were added to one aliquot and 0.1ng of Competent Cells control DNA (Promega) added to the other. Both tubes were immediately returned to ice for 10 minutes, then exposed to heat shock (42°C for exactly 45 seconds) in a water bath, and then returned to ice for a further 2 minutes. Nine-hundred microlitres of cold (4°C) culture media were added to each tube followed by incubation at 37°C for 1 hour on a rotational shaker at 225 revolutions per minute (rpm). Serial dilutions (x1, x10, x100 and x200) were performed for the transformed cells and spread on to sterile agar plates. The control reaction was diluted x100 and plated in-triplicate. All plates were incubated overnight at 37°C in a dry oven.

The reaction involving Competent Cells Control DNA acted as a positive control and allowed calculation of the transformation efficiency by use of the following equation:

\[
\text{Transformation efficiency (cfu/µg) = \frac{\text{mean cfu on control plate}}{\text{ng of Competent Cells Control DNA plated}}} \times 1000 \text{ng/µg}
\]

After overnight incubation, single colonies were selected from plates with an appropriate colony density and added to 9ml of Ampicillin-supplemented LB broth in sterile universals. These clonal cultures were incubated at 37°C for 24 hours on a rotational shaker at 225 rpm.
2.4.4 Clonal screening for presence of the target sequence.

Screening of single-clone cultures for a target sequence was performed using conventional polymerase chain reaction (PCR). Five microlitres of the bacterial culture were added to sterile RNase and DNase-free thin-walled Eppendorf tubes and boiled at 100°C for 10 minutes. Each tube was spun briefly and 45µl of the PCR master-mix added to each tube. The PCR master-mix comprised of (per 50µl reaction): 1µl each of sense and anti-sense primers (100pmol/µl), 1µl of dNTP (each 200 µM), 5µl of 10x reaction buffer, 0.5µl DNA polymerase, and 37.5µl of RNase and DNase-free water. PCR parameters were set at 94°C for 2 minutes followed by 40 cycles of 94°C for 1 minute, 60°C for one minute and 72°C for 1 minute, with a final incubation at 72°C for 10 minutes at the end of the reaction. The PCR products underwent electrophoresis on a 1% agarose gel at 100V until sufficient band separation was achieved. Their size was determined by comparison with a DNA ladder using the Kodak imaging detection system and software.

2.4.5 DNA extraction from clones

Bacterial clones identified as positive for the target sequence by PCR underwent DNA extraction using the Miniprep technique (Qiagen, 27104). Duplicate two millilitre samples were transferred from the clonal bacterial suspensions to Eppendorf tubes under aseptic conditions. Each column was spun on a bench centrifuge at 8,000 rpm for two minutes at room temperature to pellet the bacteria. The supernatant was discarded and the bacterial pellet resuspended completely in 250µl of Buffer P1 supplemented with RNaseA. Following the addition of 250µl of Buffer P2, each tube was carefully mixed by gentle inversion. Finally 350µl of Buffer N3 was added and each tube inverted again. The tubes were then spun at 8,000g for 10 minutes at room temperature and the supernatant carefully collected and added to spin columns. The columns were spun at 8,000g for 60 seconds, washed with 500µl of Buffer PB, spun again, washed with 750µl of Buffer PE and spun finally for 2 minutes with the flow-through discarded after each spin. Fifty microlitres of Buffer EB was added directly to the membrane of each spin column, incubated for one minute and spun as above for one minute. The DNA concentration in this final sample was measured using a UVmini-1240 spectrophotometer (Shimadzu, Japan) in a similar fashion to the RNA quantification described in Section 2.2.5. For each sample the values for absorbance at
260Å (A1) and 280Å (A2) were recorded. The amount of DNA contained in each sample was calculated by the formula:

\[
\text{DNA Amount (µg/µl)} = \text{A1} \times 5
\]

2.4.6 Restriction enzyme DNA digestion

Restriction enzyme digestion of plasmids was performed at a number of stages during the transfection procedure. For each reaction, although the DNA substrate, enzyme, buffer and possibly conditions differed, the way in which the reactions were prepared was standardised. The enzymes, buffers and conditions used are outlined in Table 2.1. A typical reaction was assembled as follows: 1 µg of DNA substrate was taken and made up to a total volume of 17.3 µl with nuclease-free water. To this was added 2 µl of Restriction Enzyme 10x buffer, 0.2 µl of acetylated bovine serum albumin (Promega, R396D) and, after mixing by pipetting, 0.5 µl of restriction enzyme. Incubations were performed at the optimum temperature for 4 hours. Five microlitre samples of the reaction products were then mixed with 1 µl of 6x loading buffer (Fermentas, R0611) run on 1% agarose electrophoresis gels at 100V against either 100bp (Fermentas, SM0421) or 1kpb (Fermentas, SM0311) DNA ladders as appropriate. On the occasions that incubation with two restriction enzymes was performed (for example EcoR1 and Xba1) a suitable combination buffer was used. For this example the MultiCore buffer was used (Promega, R999A).

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Promega Reference</th>
<th>Buffer System</th>
<th>Promega Buffer Reference</th>
<th>Optimum Incubation Temperature / °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcoR1</td>
<td>R6011</td>
<td>H</td>
<td>R008A</td>
<td>37</td>
</tr>
<tr>
<td>Xba1</td>
<td>R6181</td>
<td>D</td>
<td>R004A</td>
<td>37</td>
</tr>
<tr>
<td>BclI</td>
<td>R6651</td>
<td>C</td>
<td>R9921</td>
<td>50</td>
</tr>
<tr>
<td>SalI</td>
<td>R6051</td>
<td>D</td>
<td>R004A</td>
<td>37</td>
</tr>
<tr>
<td>Nco1</td>
<td>R6513</td>
<td>D</td>
<td>R004A</td>
<td>37</td>
</tr>
</tbody>
</table>
2.4.7 Construct isolation and extraction.

The pcDNA3-CXCR4 containing plasmid and the pcDNA3.1vB empty vector were double-digested with the restriction enzymes EcoR1 and Xba1 at 37°C for 8 hours in the appropriate buffer solution in triplicate. The clone map, PCR primers and restriction enzyme sites are shown in Figures A2.3, A2.4 and A2.5 (Appendix 2). The reaction products were run on a 1.0% agarose gel at 100V until sufficient band separation had occurred. The bands were identified under ultraviolet light, excised from the gel using a sharp scalpel, and transferred to a nuclease-free Eppendorf tube of known mass. The DNA was extracted from the gel using the QIAquick gel extraction kit (Qiagen, 28704).

The tubes containing the excised gel fragments were weighed and the mass of their contents was calculated. For each 100mg of gel, 300µl of yellow QG buffer was added to each tube. The mixture was incubated at 50°C for 10 minutes (with vortexing every couple of minutes) until the gel had dissolved. Any colour change in the QG buffer at the end of this dissolution step was corrected with the addition of 10µl aliquots of 3M sodium acetate (pH 5.0) until a yellow colour was restored. An equal volume of isopropanol was added to each tube in order to maximise yield of DNA fragments >4kbp. The contents were added to a spin column in 800µl aliquots and centrifuged for one minute. All centrifugation steps were performed at 8,000g for one minute at room temperature. The spin column membrane was washed with 500µl of buffer QG to remove all traces of agarose and spun again. Finally, the column membranes were washed with 750µl of buffer PE, spun and then spun again over an empty collecting tube. Elution of the DNA was performed using 50µl of buffer EB that was allowed to stand for one minute prior to centrifugation. Final DNA concentration was determined using a spectrophotometer as outlined above.


2.4.8 Construct ligation

The optimal molar ratio of insert (CXCR4) to vector (opened pcDNA3.1vB) was investigated. The mass of insert DNA required for each reaction was calculated according to the formula given below where kbp insert = 1.086 and kbp vector = 5.454:

\[
\text{Insert mass (ng)} = \frac{\text{mass of vector (ng) x kbp of insert}}{\text{kbp of vector}} \times \text{ratio}
\]

The ratios investigated were 1:3, 1:1 and 3:1 insert to vector respectively. Ligation was performed by adding the required amounts of CXCR4 insert DNA and pcDNA3.1vB vector DNA made up to a total volume of 17.5 µl with nuclease-free water. To this was added 2 µl of x10 T4 DNA ligase buffer and 0.5 µl of T4 DNA ligase (Promega, M1801). A negative control reaction was also prepared where 0.5 µl of nuclease-free water was substituted for the T4 DNA ligase. The reaction mixtures were then incubated at 37°C for 15 minutes. To determine the effect of the insert : vector molar ratios on ligation efficiency, the reaction products were investigated using the T7 forward and BGH reverse PCR primer pairs. These were preferable to CXCR4 primers as the latter would have been non-specific for free or incorporated inserts within the reaction mixture.

2.4.9 Preparation of constructs for transfection.

The pcDNA3.1vB-CXCR4 plasmid was prepared as detailed above. It was transformed into competent JM109 cells, as previously described, and single colonies were taken and expanded by overnight culture in liquid media. The pcDNA3.1vB empty-vector negative control plasmid was also taken and similarly expanded. All clones were screened by PCR using primers for CXCR4 and T7F/BGHR to ensure the presence of the CXCR4 insert. Clones identified as positive by PCR were then grown up, the DNA extracted by the mini-prep technique and subjected to digestion by a panel of restriction enzymes. This was to confirm the copy number and orientation of the inserts in each plasmid.

A large amount of the analysed pcDNA3.1Vb-CXCR4 plasmid DNA was then produced using the Maxi-prep plasmid kit (Qiagen, 12162). For this, a starter culture of the confirmed clones was grown in 9ml of LB media on a rotational shaker at 225
rpm at 37°C for 8 hours. Subsequently, 200 µl of the starter culture was added to a further 100ml of fresh, warmed LB media and incubated overnight at 37°C at 225 rpm. A bacterial pellet was then formed by centrifugation at 6,000g for 15 minutes at 4°C, after which the supernatant was discarded. The pellet was re-suspended in 10ml of Buffer P1 to which RNase A had been added to a final concentration of 100 µg/ml. Ten millilitres of Buffer P2 was then added to the tube, mixed by inversion, and incubated for 5 minutes at room temperature. Finally, 10 ml of Buffer P3 (pre-chilled to 4°C) was added to the tube and incubated on ice for 20 minutes. At the end of the incubation the tube was gently mixed, centrifuged at 20,000g for 30 minutes at 4°C, and then the colourless supernatant containing the plasmid DNA removed and stored on ice. The centrifugation was then repeated for a further 15 minutes and the supernatant removed and stored. Ten millilitres of Buffer QBT was applied to the filter membrane and was allowed to flow through completely under gravity. The DNA-containing supernatant was then applied to the membrane and allowed to flow through under gravity. Two, 30 ml washes of Buffer QC were then applied. The DNA was then eluted from the membrane by the addition of 15 ml of Buffer QF collected in a non-polycarbonate 50 ml tube. Ten millilitres of room-temperature isopropanol was added to the tube, mixed and centrifuged at 15,000g for 30 minutes at 4°C. The supernatant was carefully removed and discarded, taking care to leave the DNA pellet undisturbed. This was then washed with 5 ml of 70% ethanol and centrifuged again for 10 minutes. The supernatant was again removed and the pellet allowed to air dry. The DNA was dissolved in nuclease-free water and the quantity and quality were assessed using a spectrophotometer as outlined in Section 2.4.5. Finally, the DNA was made up to a concentration of 1 µg/µl and stored at -20°C.

2.4.10 Beta Galactosidase Assay and Cell Visualisation

Optimum conditions for the transfection reaction were determined using the β-Galactosidase reporter assay (E2000, Promega) and the pcDNA3.1-lacZ positive control plasmid. The assay chemistry is based on the lacZ-encoded β-Galactosidase that hydrolyses the colourless o-nitrophenyl-β-D-galactopyranoside (ONPG) to o-nitrophenol which is yellow. Standards were prepared according to the manufacturer’s instructions: a 1:10,000 dilution of 1µg/µl β-Galactosidase was
produced and used to prepare standards ranging from 1.0 to 6.0 milliunits β-
Galactosidase made up to 150µl with x1 Reporter Lysis Buffer (RLB).

For optimisation reactions, wild-type H357 cells were transfected with pcDNA3.1-lacZ DNA using the Transfast reagent (*vide infra*). Negative controls were performed using the Transfast reagent alone, without the addition of pcDNA3.1-lacZ DNA. Six wells per experimental condition were used. The cells were then incubated for a further 48 hours before the β-Galactosidase reporter assay was performed. At the end of this period, the growth medium was aspirated and the cells were gently washed twice with Mg²⁺ and Ca²⁺ free PBS. After careful aspiration of the PBS, 200µl of RLB was added to each well and incubated for 15 minutes at room temperature with gentle agitation. Following this, the wells were scraped and the lysate transferred to a 1.5ml Eppendorf tube on ice. Each tube was vortexed for 15 seconds and then centrifuged at 13,000g for 2 minutes at 4°C. One hundred microlitres of supernatant from each sample was carefully removed and used fresh. In a clean 96-well-plate, 50µl of sample, standard or negative control was added in duplicate to separate wells. A further 50µl of 2x Assay Buffer was added to each well, mixed by pipetting, covered and incubated at 37°C. After 30 minutes the reaction was stopped by the addition of 150µl of 1M sodium carbonate solution and the absorbance at 420nm read using a microplate reader. The reading for each sample duplicate was averaged, and the mean and standard deviation calculated for the six wells from each experimental condition. Statistical analysis was performed using the ANOVA test with post-hoc Bonferroni correction for multiple testing.

Visualisation of *in-situ* staining for β-Galactosidase activity was also performed. Wild-type H357 cells, transfected with the pcDNA3.1-lacZ control plasmid, were grown in sterile six well plates without antibiotic selection (*vide infra*). A pcDNA3.1vB empty-vector mock transfection was also performed to act as a negative control. Once confluence was approached, the cells were washed with PBS and fixed with 2ml per well of glutaraldehyde solution for 15 minutes. The fixative was removed, and the cells washed three times with PBS. One millilitre of 50 mg/ml X-Gal solution (Promega, V3941) was added to each well. The cells were incubated at 37°C for eight hours until visible blue staining was seen. The X-Gal solution was then removed and replaced with PBS prior to photographing.
2.4.11 Transfection Optimisation

Wild-type H357 cells were seeded on to 24-well-plates in normal growth media supplemented with 10% FCS at a sufficient density to achieve 80% confluence after 24 hours. Transfection was performed using the TransFast reagent (E2431, Promega). This reagent is comprised of the synthetic cationic lipid, N,N-[bis (2-hydroxyethyl)-N-methyl-N-[2,3-di(tetradecanoyloxy) propyl] ammonium iodide, and the neutral lipid, DOPE. On the day before the transfection procedure the TransFast reagent was thawed, 400 µl of nuclease-free water was added and the mixture was vortexed for 10 seconds. Finally the prepared reagent was stored at -20°C overnight. On the following day, the prepared reagent was warmed to room temperature and spun at 300g briefly prior to use.

The transfection procedure was performed by combining empirically-determined volumes of the TransFast reagent, plasmid DNA and serum-free media. Initially this mixture was incubated for 15 minutes at room temperature. After this time, the growth medium was aspirated from the cells and replaced with the pre-mixed TransFast/DNA/serum-free media. The cells were then incubated at 37°C and 5% CO₂ for an empirically determined time period, after which the mixture was removed and replaced with pre-warmed normal growth media supplemented with 10% FCS. Three experimental variables were investigated in order to determine the optimal conditions for transfection: 1) charge ratio of TransFast reagent to DNA, 2) amount of DNA added per well, and 3) the cell exposure time to the TransFast/DNA/serum-free media mixture. All experiments were run three times.

The influence of charge ratio on transfection efficiency was investigated. The charge ratios were manipulated by varying the ratio of TransFast reagent volume to plasmid DNA volume within the TransFast/DNA/serum-free media mixture to which the cells were exposed for one hour. Charge ratios of 1:1, 1.5:1 and 2:1 (TransFast reagent volume to pcDNA3.1-lacZ volume) over a range of starting DNA concentrations (0.2 and 0.8 µg per well) were investigated. A DNA-free negative control was also included. Six wells were used for each experimental condition and each well was analysed in duplicate for its β-Galactosidase reporter assay activity.

The effect of the starting amount of DNA on transfection efficiency was determined. The concentration of pcDNA3.1-lacZ DNA in the TransFast/DNA/serum-free media
mixture was varied between 0.2 and 2.0 µg per well, and a DNA-free negative control arm was also included. A charge ratio of 1:1 was used and the cells were exposed to the transfection mixture for a total of one hour. Again, six wells were used per experimental condition and each well was measured in duplicate for its β-Galactosidase reporter assay activity.

The relationship between the exposure time of cells to the TransFast/DNA/serum-free media mixture and transfection efficiency was investigated. A charge ratio of 1:1 was used with 1.5 µg of pcDNA3.1-lacZ DNA added per well. Exposure time was varied between 30 minutes and 2 hours. Six wells were used per time period and the β-Galactosidase reporter assay activity was measured in duplicate for each well.

### 2.4.12 Transfection procedure

A suitable wild-type OSCC cell line (H357) was selected on the basis of its low level of constitutional expression of CXCR4. Production of a stably-transfected cell line was performed in the following stages:

1. Determination of wild-type H357 killing by the antibiotic G418
2. Transfection of the pcDNA3.1vB-CXCR4 plasmid
3. Clonal selection and expansion

The effect of the antibiotic G418 sulphate ([Geneticin], Invitrogen, 10131) on wild-type H357 cells was determined using the MTT cell viability assay (as described in Section 2.8.2). The aim was to determine the G418 concentration that resulted in 90% cell death after 72 hours of exposure to the agent as per the manufacturer’s instructions. Briefly, 1 x 10⁴ cells and 200µl of fresh medium containing 10% FCS were seeded into each well of a 96-well-plate and allowed to incubate overnight at 37°C and 5%CO₂. G418 stock solutions were made up by serial dilution in growth media to yield a range of final concentrations of 100 to 1600µg/ml when was added to each of the pre-incubated wells. Twelve wells per treatment concentration were used in addition to a no-treatment control of 10µl of growth media alone. Time points of 24, 48 and 72 hours were measured and the mean and standard deviation cell survival was calculated and expressed as a percentage compared with the no-treatment control. A two-tailed
homoscedastic student t-test was used to compare the effect of increasing G418
congestion on cell survival.

Wild-type H357 cells were seeded at densities of 3, 4 and 5 x 10^5 in six well plates in
duplicate. All cells were incubated overnight with normal growth media under
optimal conditions. The following day, the wells with healthy cells at 80% confluence
(as estimated by light microscopy) were selected. The TransFast reagent was also
prepared 24 hours before use as previously described.

Optimum conditions for the transfection reaction were determined empirically using
the pcDNA3.1-lacZ control plasmid and the β-Galactosidase reporter activity assay as
described in Sections 2.4.11. The results of these experiments are outlined in Appendix
5. Three transfections were performed: experimental (pcDNA3.1vB-CXCR4), mock
(pcDNA3.1vB empty-vector) and negative control (no DNA), each with duplicate
wells. Transfection parameters were determined empirically from optimisation
experiments and scaled up to the 6 well plate format. A 1:1 molar charge ratio of
TransFast reagent for 1.5μg of DNA per well was used. Sufficient mixture was
prepared for the experimental, mock and control wells, added to 2ml of serum-free
media per well, and incubated for 15 minutes at room temperature. The growth
medium was removed from the selected wells and replaced with 2ml of the pre-
incubated TransFast/DNA/serum-free mixture per well. The plates were re-incubated
for 1 hour at 37°C and 5%CO_2 after which the transfected wells were overlayed with
normal growth media containing serum.

The transfected cells were initially grown in non-selective media for 48 hours. After
this period, the cells were trypsinised and passaged as previously described into 10cm
dishes. Preliminary experiments had determined that a concentration of 1600μg/ml of
the selective antibiotic (G418) was required to achieved 90% killing of untransfected
cells (see Appendix 5). Growth media supplemented with G418 to a final
concentration of 1600μg/ml was then introduced to transfected cells and non-
transfected negative controls. This selective growth media were changed every 48
hours to remove dead and dying cells until colony formation could be seen. When it
was possible to visualise distinct single colonies by light microscopy their positions
were marked on the surface of the dish. The cells were washed twice in PBS and sterile
paper cloning discs soaked in trypsin were applied to the marked colonies. The
positions of the cloning discs were confirmed by light microscopy. The dishes were incubated at 37°C for five minutes, after which the discs were removed and transferred into individual wells of a six well plate. The selective growth medium was replaced and the plates were incubated under optimum conditions.

In total, six pcDNA3.1vB-CXCR4 and six pcDNA3.1vB empty-vector clones were selected and expanded over a period of 3 weeks under antibiotic selection. Of the six experimental clones (labelled H357-X4.1 to X4.6), all but one (H357-X4.3) survived. All the mock-transfected clones (labelled H357-EV1 to EV6) survived. Subsequently, all transfected cells were maintained in media supplemented with G418 at a concentration of 1600 µg/ml.

2.4.13 Characterisation of the transfected cell lines.

The newly transfected cells (with and without the CXCR4 insert) were characterised at the mRNA and protein levels. Determination of CXCR4 mRNA expression by transfected cells was performed using semi-quantitative real-time RT-PCR as described in Section 2.3. Briefly, six mock-transfected clones (H357-EV1 to EV6), five CXCR4-transfected clones (H357-X4.1, 4.2, 4.4, 4.5 and 4.6) and wild-type H357 cells (H357-WT) were grown to 90% confluence (as determined by light microscopy) in appropriate media under optimal conditions. Four separate replicates of each clone were taken, the cells washed with PBS, and the total RNA harvested by the method previously outlined in Section 2.2.3. The total RNA obtained was DNase treated using the RNase-free DNase kit (Qiagen, 79254), a component of the RNeasy kit (Qiagen, 74104) as outlined in Section 2.2.4. The amount of total RNA was measured using a spectrophotometer and 2 µg of each sample was used to manufacture cDNA using the iScript cDNA synthesis kit (Bio-Rad, 170-8891) as described in Section 2.3.2.2. No reverse-transcriptase control reactions were included for each clone. Each sample was analysed in triplicate wells for CXCR4 by real-time PCR as outlined in Section 2.3.3. The relative expression of CXCR4 mRNA in each clone compared with wild-type H357 cells was calculated. Statistical comparison between clones was performed using the ANOVA test. The whole experiment was performed twice.
Determination of CXCR4 protein production by transfected cells was performed using flow cytometry and fluorescence immunocytochemistry as outlined in Sections 2.5 and 2.6 respectively.

2.4.14 Cell maintenance, FACS sorting and clonal re-selection

Stock cultures of all transfected cells (with and without the CXCR4 insert) were maintained in normal growth media supplemented with the selective antibiotic G418 to a final concentration of 1600µg/ml. After freezing, storage and subsequent thawing, the H357-X4.1 and H357-X4.2 clones underwent FACS sorting on the basis of their CXCR4 expression. Briefly, cells were prepared for FACS analysis as outlined in Section 2.4 although, in addition, this was done under strict aseptic conditions. For each clone a total of 8.5 million cells were harvested. Eight million cells were labelled with the anti-CXCR4 primary monoclonal antibody and the remaining 500,000 with an isotype control. After completion of the washing and secondary antibody stages (vide infra) the CXCR4-labelled cells were resuspended with 2mls of cold PBS in a sterile FACS tube and kept on ice in the dark. FACS analysis of CXCR4 labelling was performed using a FacsAria platform running the FacsDiva software package (both Becton Dickinson) as outlined below. Cells from the central population of CXCR4-positive cells were sorted and collected in a fresh, sterile FACS tube. Cells from each clone were resuspended in warmed growth media supplemented with the antibiotic G418 (1600 µg/ml) and plated using serial doubling dilutions into successive wells of a 24 well plate.

Cells were incubated under standard conditions of 37°C and 5%CO₂ overnight to allow attachment and then washed gently with warmed PBS and fresh selective growth media added. Wells containing cells at clonal density were identified by light microscopy, photographed and allowed to expand over a period of three weeks with passaging as required.
2.5 Flow cytometry

Established and transfected OSCC cell lines, primary oral and skin keratinocytes and human gingival fibroblasts were examined with flow cytometry. Conventional cell lines were grown in T25 flasks normal growth media containing 10% FBS and passaged at early confluence (approximately 80%) as determined by light microscopy. Early-passage primary cell lines were initially seeded with growth-arrested fibroblast feeder cells (as described in Section 2.1). Several methodological steps were performed to ensure that the feeder cells did not affect the results: Firstly, primary cells were cultured for each passage for a sufficient time to ensure that the majority of fibroblast feeder cells had died and become detached. This was confirmed by visual inspection for fibroblastoid morphologies prior to passage. Secondly, keratinocytes and fibroblasts could be differentiated on the basis of size and granularity and separate analyses of the appropriately gated populations were performed (see results). Finally, pure mono-populations of the feeder cells were examined for relevant chemokine receptor expression by flow cytometry.

Once 80% confluence had been attained, the growth media were gently aspirated and the cells washed in warmed, sterile PBS. The PBS was aspirated and the cells incubated with 1ml of non-enzymatic dissociation buffer (Sigma) at 37°C and 5% CO₂ until dissociation had occurred (typically 5 to 10 minutes). The samples were washed with 1ml of warmed PBS and centrifuged at 300g for 6 minutes. The supernatant was aspirated gently and the samples were resuspended in 200μl of cold PBS. Each sample was divided into aliquots of 100μl in clean Eppendorf tubes. For each sample, one aliquot was incubated with 50μl of 1:50 dilution primary anti-chemokine receptor mouse anti-human immunoglobulin monoclonal antibodies. Commercially available antibodies were used for these experiments: one clone raised towards CCR7 (MAB197, R&D Systems) and two separate clones raised against CXCR4 (MAB172 and MAB173, R&D Systems). To investigate CXCR4 protein production by flow cytometry two monoclonal antibodies with different binding sites were selected. Both antibodies had been validated for this technique by the commercial company and one (MAB173) has been widely used in the published literature on CXCR4. The second experimental aliquot was incubated with 50μl of isotype control monoclonal antibody diluted 1:50 in cold PBS under the same conditions to act as a negative control. All reactions were incubated for 30 minutes on ice in the dark.
Following these incubation steps, the samples were centrifuged at 1,500g for 60 seconds, the supernatant removed and resuspended with 200μl of cold PBS to wash the cells. The samples were centrifuged again and the PBS removed. A 1:300 dilution of the secondary antibody (conjugated 488 anti-mouse IgG) was prepared in cold PBS. The pellet of cells was resuspended in 50μl of secondary antibody for 15 minutes on ice in the dark. This incubation was followed with two washes with 200μl of cold PBS. The samples were finally resuspended in 200μl of cold PBS. Finally, just prior to analysis, 100μl aliquots of cold propidium iodide (Sigma, P4864) were added to each sample. Flow cytometry was performed using an FACS-Calibur instrument (BD Biosciences) and accompanying Cell Quest software.

Brief sampling of each negative control aliquot allowed the analysis parameters to be set for each cell line. Firstly a plot of forward scatter (cell size) against side scatter (granularity) was performed. Cells within the central distribution were selected (gated R1) and the remainder discounted for the purpose of the analysis. Secondly, for R1-gated cells, the propidium iodide fluorescence intensity was plotted against the chemokine receptor antibody fluorescence intensity. Cells with an intact surface membrane that excluded propidium iodide were selected (gated R2). Finally, a plot of cell count against chemokine receptor antibody fluorescence intensity for R2-gated cells was produced and overlaid against a similar plot for the matched isotype control labelled cells. Mean fluorescence values for chemokine receptor labelling and isotype controls were calculated and recorded. Analysis was based on a minimum of 10,000 counts from the R2-gated cell population.

2.6 Fluorescence immunocytochemistry and confocal microscopy

Sterile glass coverslips 13mm in diameter were taken and placed in individual wells of sterile 24 well cell-culture plates. Cells were seeded at low density in supplemented media for 48 hours as before. The cells were washed twice with PBS and fixed in cold 3.7% paraformaldehyde at pH 7.4 (15 minutes), permeabilised in 1% Triton X-100 (5 minutes) and incubated in blocking buffer (30 minutes) with three washes in PBS between each step. Primary anti-chemokine receptor monoclonal antibodies were diluted to optimal concentrations in blocking buffer (1% BSA and 10% FBS in PBS) and incubated with the cells overnight at room temperature. Negative controls with no
primary or substituted isotypes were performed for each cell line. Each sample was washed three times in PBS and then incubated with a bovine-anti-mouse secondary antibody directly conjugated to Texas Red at 1:200 dilution for an hour at room temperature. After washing three times with PBS, 1ml of 1:1000 dilution DAPI in PBS was added to each well for 5 minutes at room temperature in the dark. Each sample was washed a final three times in PBS and mounted in aqueous immuno-mount, sealed with clear resin and stored at 4°C in the dark. Non-fluorescence immunocytochemistry was performed using the same protocol with a DAB finish as outlined in Section 2.7.

Conventional microscopy was performed using a fluorescence microscope (Zeiss) and digital image capture (Nikon). Images were carefully labelled, stored on a memory card, transferred to PC and opened using the Powerpoint software package (Microsoft Corporation). A 5µm graticule was also photographed under plain light at all magnifications used in order to provide a standard reference for scale.

Confocal microscopy was performed using a laser illuminated confocal microscope (Zeiss) with accompanying image control software. Scout images were taken and the plane of focus defined. Serial scans through the focal plane were taken at increments recommended by the software package. Image folders were carefully labelled, recorded on to CD and transferred to PC. Image analysis was performed using the satellite image reformatting software package provided by the manufacturer (LSM 5 v.3.2, Zeiss). Reconstructed images were then imported into Powerpoint. In a similar way to before, a 5µm graticule was used to provide a standard reference for scale.

2.7 Immunohistochemistry

Immunohistochemistry was performed on tissue sections collected from the pathology archive of St Bartholomew’s and The London NHS Trust. Clinical specimens were used with approval of the East London and The City Research Ethics Committee (ELTC P1/04/016). Anonymous pathology reports are summarised in Table A2.1 (Appendix 2). Head and Neck Tissue Arrays (A219II, Accumax) were also investigated. The layout of the tissue array used is shown in Figure A2.1 (Appendix 2). Clinicopathological features of the specimens available on the tissue array are outlined in Table 4.1 (Chapter 4).
Specimen handling protocols basically involved the following steps:

1. Specimen selection and section cutting
2. Section rehydration and antigen retrieval
3. Blocking non-specific binding
4. Incubation with primary antibody
5. Incubation with secondary antibody
6. Colour preparation and counterstaining
7. Dehydration and mounting

In detail: The recorded minimum data set for head and neck tumour specimens were taken and salient information was entered into a spreadsheet (Excel, Microsoft Office). Specimens were selected based on their clinico-pathological parameters and the available complete diagnostic pathology reports checked for consistency. Blocks of formalin-fixed, paraffin embedded tissue were obtained from the pathology archives, orientated and cut into 5µm sections on to SuperFrost glass slides using a microtome. Positive control specimens were prepared in a similar way. Sections were stored vertically and air dried before baking in a dry oven at 65°C for 90 minutes.

The specimens were then processed in standard histological water baths and racks using solutions at room temperature (unless otherwise specified). To de-wax the specimens the slides were incubated in xylene for 15 minutes twice followed by two rinses in 100% ethanol for 10 minutes each. Endogenous peroxidise activity was blocked by incubation with 1% Hydrogen Peroxide in methanol for 30 minutes. Samples were then rehydrated by successive incubation in reducing concentrations of ethanol (100%, 75% and 50% respectively) for 5 minutes each, followed by 5 minutes in pure distilled water. Other methods of antigen retrieval were evaluated empirically using positive control specimens.25 The most effect method was determined to be microwave pressure cooking as follows: A working concentration of Antigen Unmasking Solution (H3300, Vector Laboratories) was prepared by the addition of 15ml of concentrate to 1600ml of distilled water in a microwave-proof pressure chamber. This was heated in a microwave at full power for 15 minutes until the solution was bubbling. Sections were immersed in the boiling unmasking solution, the

25 Other methods of antigen retrieval used were EDTA, citrate or protease-based.
pressure chamber sealed and returned to the microwave. The chamber and its contents were heated at full power until the pressure indicator demonstrated that maximum pressure had been achieved. Cooking was then continued for a further period. Empirically, 2 minutes was determined as optimal. After heating the pressure was released and the chamber cooled as quickly as possible with cold water without contaminating the samples. Sections were then washed quickly in distilled water followed by PBS.

A volume of PBS containing 0.1% BSA Fraction V (Sigma) was prepared fresh. Blocking buffer was prepared by adding 150µl of horse serum (Vector Laboratories) to 10ml of PBS/BSA. Samples were incubated in blocking buffer for 20 minutes at room temperature and then rinsed in PBS/BSA. Avidin and biotin blocking was achieved by incubation for 15 minutes each with Solution A and Solution B of the avidin-biotin blocking kit (Vector laboratories) respectively, with a PBS/BSA wash between stages. Sections were then rinsed with PBS/BSA followed by blocking buffer. Each slide was dried peripherally and encircled by a hydrophobic barrier (Immedge Pens).

The working concentration of each primary antibody was determined empirically. Primary antibody was diluted in blocking buffer and each section was flooded. An isotype control mouse IgG2a antibody was used as a negative control at a working concentration of 3µg/ml. All sections were sealed in a humidified chamber and incubated overnight at 4°C. After this period the slides were washed in three changes of freshly prepared wash buffer for 5 minutes each time with gentle agitation on a rotational shaker. Free liquid was then removed from around each slide and each section was flooded with a working dilution of biotinylated horse anti-mouse secondary antibody. This was incubated for 1 hour at room temperature followed by rinsing with three changes of wash buffer for 5 minutes each time. Again excess liquid was removed and Vectastain ABC solution (Vector Laboratories) was added (which had been prepared 30 minutes prior to use according to the manufacturer’s instructions) and incubated for one hour at room temperature.

26 Wash buffer: 0.15M sodium chloride and 0.05% Tween 20 in PBS
27 Working dilution of secondary antibody: 50µl in 10ml PBS/0.1% BSA
28 Vectastain ABC solution: 100µl of Solution A was mixed with 5ml of PBS and then 100µl of Solution B was added. The prepared solution was allowed to stand at room temperature for 30 minutes prior to use.
The slides were then washed with PBS for 10 minutes with gentle agitation as before and then incubated with DAB solution (Vector Laboratories) prepared according to the manufacturer’s instructions\(^{29}\) for a maximum of 5 minutes. Colour development was stopped by the removal of the DAB and rinsing with PBS. Haematoxylin counterstaining was performed by adding filtered Gill’s haematoxylin stain for 3 minutes, rinsing in tap water to produce a blue colour change then dipping briefly twice in acidified alcohol and rinsing in tap water again. Sections were then dehydrated by the sequential washing in increasing concentrations of ethanol (50%, 70%, 90% and 100% twice) and xylene twice for 5 minutes each. A small volume of xylene-based mountant (Immunomount) was added to each slide and a glass coverslip applied. For diagnostic purposes one section from each tissue block was stained with haematoxylin and eosin. This was performed by the rehydration of sections and staining with haematoxylin as described above. An additional incubation with eosin for 45 seconds was then performed. The slides were then rinsed in water, dehydrated and mounted as described above.

Tissue array specimens were prepared using an identical immunohistochemistry protocol and were then subjected to a number of post-processing steps. These are outlined in Appendix 4.

### 2.8 Proliferation Assays

#### 2.8.1 Overview

Three complementary techniques were used to perform proliferation assays:

1. 3-(4,5-dimethylthiazol-2-yl)-2,5,-diphenyl tetrazolium bromide (MTT).
2. Resazurin (Alamar Blue).
3. Bromodeoxyuridine (BrdU)

Two of the three methodologies (MTT and resazurin) are based on the biochemical transformation of compounds added to the cell supernatants by living cells. The BrdU-

\(^{29}\) DAB solution: Initially, 100µl of supplied buffer was mixed with 5ml of distilled water. Then 200µl of DAB was added, mixed and followed by 100µl of hydrogen peroxide.
based technique is based on uptake of the compound by proliferating cells which incorporate it into their DNA. In this way these methodologies selected are complementary.

2.8.2 MTT assay

This tetrazolium-based assay is dependent on the observation that the reduction potential of proliferating cells is greater than that of non-proliferating cells. The tetrazolium salt (yellow) is reduced to form formazan crystals (violet) which are insoluble in water. When the reaction product is dissolved in solvent (such as DMSO), the cells are killed. This limits the MTT assay to the measurement of one time-point only. However, data generated are representative of the proliferation activity of cells over their period of incubation with MTT, rather than cumulative as is the case with Alamar Blue (*vide infra*).

MTT assays were performed in a 96 well-plate format as follows. Cells were passaged as previously described and mixed well to form a final cell density of $2.5 \times 10^4$/ml in appropriate media. Two hundred microlitres of this cell suspension were added to each experimental or negative control well. A similar volume of media alone (no cells) was added to a final row on the plate to determine background absorption. Several identical plates were prepared and incubated overnight at 37°C in a humidified atmosphere with 5% CO$_2$ to allow the cells to adhere. After this period the wells were aspirated gently and washed twice with warmed PBS. Finally 200μl of fresh media containing the appropriate experimental conditions were added and the plates returned to the incubator.

An MTT stock solution was prepared as follows: MTT powder (Sigma, M2003) was dissolved in sterile PBS to a stock concentration of 10 mg/ml, filter sterilised and stored at 4°C protected from light. At each selected time point 20 μl of the MTT stock solution was added to each well (including no-treatment controls and media-only blanks). Care was taken to prevent contamination of the stock solution. Each well was careful mixed by pipetting. The plates were return to the incubator for four hours protected from light. At the end of this period the growth media were carefully aspirated and the plates blotted dry with paper towels. Two hundred microlitres of
DMSO (Sigma, D5879) was added to each well and incubated at room temperature for one hour protected from light. For cells grown on opaque 96-well plates (vide infra) a final pipetting step was performed to transfer 100 μl of the DMSO/cell lysate mix from each well to a fresh transparent 96-well plate suitable for absorbance readings. Absorbance at 570nm was measured using a Titertek Multiskan Plus II microplate colourimeter (Life Sciences International). Absorbance readings were entered into a Microsoft Excel spreadsheet and the mean absorbance for blank wells was subtracted from each recorded value. Using the SPSS statistical package, a P-P plot of the experimental data was prepared to compare against the normal distribution (vide infra). Subsequently, the mean and standard deviation for each set of experimental replicates were calculated and compared using the ANOVA test. Significant differences (p<0.05) between groups were further investigated with post-hoc testing using the Bonferroni correction.

2.8.3 Alamar Blue Assays

The reaction chemistry of resazurin-based assays is based on the reduction of the starting compound (blue and non-fluorescent) to resorufin (pink and highly fluorescent) in the cell supernatant (O'Brien, Wilson et al. 2000). For each experimental condition, serial fluorescence measurements may be taken without disturbing the cell culture environment. Consequently there are two significant advantages of this technique over MTT assays: Firstly, that time-series data can be generated for each well; and secondly that the cell seeding density can be checked for consistency across the plate prior to the commencement of the experimental treatments.

Assays were performed using opaque, sterile 96-well plates. The optimum number of cells per well and duration of experiments were determined empirically. Plates were set up with 5 x 10^3 cells per well in 200μl of normal growth media supplemented with 10% FCS and 5% Alamar Blue (BUF012B, Serotec) by volume. A blank row of media only (no cells) was prepared on each plate. Initially plates were incubated overnight at 37°C in a humidified atmosphere with 5% CO₂ to allow the cell populations to adhere. After this incubation period, the fluorescence emission (at a wavelength of 590nm) of each well was measured following excitation (at a wavelength of 530nm) using a
fluorescence microplate reader. Readings were tabulated using the accompanying software and exported to the SPSS statistical software package.

Each 96-well plate was analysed for consistency in cell seeding density prior to setting up any experimental conditions. This quality-control check was performed using two quick statistical tests. Firstly, the spread of the data was compared with the normal distribution by plotting the observed against expected cumulative probabilities (P-P plot). Secondly, if the data were normally distributed (P-P plot conforms to a straight line), then baseline fluorescence values for each forthcoming experimental group were compared using the ANOVA test. If no significant difference (p<0.05) could be demonstrated between groups then the plate was used for further experiments. Plates that did showed baseline differences between groups were discarded. Typical P-P plots and group comparisons are shown in Figure A2.4 (Appendix 2).

Selected plates were then washed carefully twice with warmed, sterile PBS. A stock solution of cell culture media, FCS (varied empirically) and 5% Alamar Blue was made up for all experimental wells including cell-free blanks. This was maintained at 37°C and protected from light. Experimental treatments and negative controls were prepared using aliquots of this stock solution, and 200µl were added to each well. The number of replicate wells required for each treatment condition was determined empirically. Serial fluorescence measurements were taken at two hours and at regular time points to a maximum of 72 hours as detailed above. At the end of the experimental time period it was still possible to perform MTT assays on these cells. On these occasions, the supernatants were aspirated and the cells washed twice with warmed PBS. Fresh media containing 1mg/ml MTT were added to each well and the assay was performed as described in Section 2.8.2.

2.8.4 BrdU Incorporation Assay
BrdU is a halogenated analogue of thymidine and is taken up by any cell undergoing DNA synthesis during the time of exposure. Following treatment with putative mitogens cells are pulsed with BrdU, fixed and then immunostained using antibodies raised against the BrdU label.
In these experiments, assays were performed in transparent, sterile 24-well-plates. Cells were passaged as previously described and seeded at a density of $5 \times 10^4$ per well in 500µl of basal growth media supplemented with FCS to a final concentration of 10%. Cells were incubated under standard conditions overnight, then aspirated and washed with warmed, sterile PBS. A further 500µl of basal growth media supplemented with 0.1% FCS and SDF (varying in concentration between 0 and 100ng/ml) was then added. Incubation was continued for a further 48 hours under standard conditions. The growth media were then removed and replaced with warmed basal growth media supplemented with 0.1% FCS and BrdU to a final concentration of 10µM. Incubation as before was then continued for a further empirically-determined period after which the media were aspirated, the cells washed with PBS and then fixed in 4% paraformaldehyde for 20 minutes. The wells were then washed twice with PBS and stained immediately.

To prepare the cells for staining PBS was first aspirated from each well and the cells permeabilised with 0.2% Triton for 10 minutes and then washed with PBS. To denature the DNA 4M hydrochloric acid was added for 10 minutes followed by washes with PBS pH 8.5 for 10 minutes with 2 changes then with PBS pH 7.6 for 20 minutes with 4 changes. Wells were then washed briefly with double-distilled water and then incubated with mouse anti-BrdU monoclonal antibody (Clone Bu20a, M0744, Dako) at room temperature for 2 hours. Cells were then washed with PBS for 15 minutes with 3 changes and then incubated with an anti-mouse FITC-conjugated secondary antibody at 1:80 dilution for 1 hour at room temperature protected from light. Cells were then washed with PBS for a further 15 minutes with 3 changes and then counterstained with DAPI at a final concentration of 1µg/ml in PBS for 15 minutes in the dark. After a final brief PBS wash a drop of aqueous Immunomount was added to each well and a glass coverslip applied. Plates were stored at 4ºC in the dark until visualisation.

Images were stored as photomicrographs taken with an inverted fluorescence microscope (Nikon) at x100 total magnification. Three non-overlapping positions were selected for each well and two photomicrographs taken at each position: one to record DAPI staining and the other to record FITC activity. All images were carefully

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30 Antibody final concentration of 7mg/L (1:80 of supplied solution) in PBS supplemented with 0.5% Tween 20 and 1% BSA (final concentration).
labelled, stored on a memory card, transferred to a PC and then opened in the Photoshop CS software package (Adobe Corporation). A rigorous method was employed to ensure that accurate cell counting was performed. All DAPI-stained nuclei were counted using a screen-tool that highlighted counted cells (to ensure no double counting occurred). Over the three images taken per well a total cell count of over one thousand was sought. From the images of FITC activity it was important to ensure that cells undergoing active division (concentrated bright signal) were differentiated from background (dull diffuse signal). To achieve this 10 cells clearly undergoing cell division were identified and the colour saturation profile was recorded using the on-screen tool. The mean and standard deviation of green saturation was calculated for the ten cells and the programme was used to filter the image according to the lower standard deviation less 20%. The image generated was visually inspected to ensure no artefacts were present or cells were missing. Finally a count was made of the cells identified in this way in a manner similar to that employed above.

For each well the number of cells labelled with BrdU and the total number of cells analysed were recorded. The results from replicate wells were combined mathematically. Statistical comparison for individual treatments was performed using an online Chi-Squared test generator. For data derived from dose-response experiments a Chi-Squared test for trend was performed using the MedCalc statistics software package (MedCalc Systems, Belgium). Statistical significance was accepted at the 5% level.

2.8.5 Statistical Analysis

For the resazurin-based assay the measured fluorescence values were tabulated in Microsoft Excel. The mean value for the blank wells was calculated and subtracted from the reading for each well. These time-series data were analysed according to the method outlined by Matthews (Matthews, Altman et al. 1990). The proliferation rate of cells in each individual well was calculated as follows: The value for the measurement at the two-hour time point was defined as equal to 1; subsequent measurements of the each well were then calculated relative to this value. A plot of relative fluorescence values against time revealed the duration of linear accumulation of resorufin. This was

31 Found at (http://www.georgetown.edu/faculty/ballc/webtools/web_chi.html)
determined empirically as 48 hours. Consequently, relative fluorescence values for the time points between 2 and 48 hours were selected and the gradient of the line calculated. This was defined as the relative proliferation rate. The conformity of these data to a straight line was checked by calculation of the square of the Pearson product moment correlation coefficient (R-square value). Data with R-squared values less than 0.95 were rejected. These calculations were performed for each replicate well and were grouped by experimental condition. Each group of data were then analysed for conformity to the normal distribution by P-P plots (vide supra) and, when appropriate, the mean and standard deviations calculated. Experimental groups were compared graphically by plots of the mean and standard deviation. Statistical comparison was performed using the one-way ANOVA test with determination of any significant differences between groups by post-hoc testing with the Bonferroni correction.

A statistical power calculation was also performed to determine the required number of replicate wells to be able to differentiate between the effects of various experimental conditions. This was done with an online statistics package using preliminary experimental data that had previously been analysed using SPSS.\textsuperscript{32} Firstly the conformity of the data to a normal distribution was checked. Secondly, the variances were compared for equality against the F distribution using the Levene test. Statistical conditions were selected to power the experiment to detect a two-tailed difference in the means between two experimentally-determined proliferation rates at the p<0.05 significance level on 90\% of occasions. Mean and standard deviations from preliminary data were entered into the online algorithm and the predicted number of replicate wells noted.

\textsuperscript{32} Online statistical power calculation package available at http://calculators.stat.ucla.edu/powercalc/
2.9 Assays of cell motility

Cell motility was assessed \emph{in-vitro} using three complementary techniques:

1. Gold Colloid Assays
2. Wound Scratch Assays
3. Transwell Assays

Each assay investigated a subtly different aspect of chemokinesis, chemotaxis and invasion (as discussed in the results).

2.9.1 Gold Colloid Assays

The purpose of this assay was to measure the maximal movement of single cultured keratinocytes in response to a chemokine stimulus in the absence of a directional component. The assays were performed on pre-prepared glass coverslips (13mm diameter) in a 12 well plate format. Initially all coverslips and glassware were rinsed thoroughly in distilled water to remove all traces of detergent and dry-heat sterilised. A 1\% BSA solution was prepared fresh and filter sterilised. Coverslips were held in fine, sterile forceps, immersed in the 1\% BSA solution, drained completely by blotting, immersed briefly in 100\% ethanol and drained again. They were then placed singly in individual tissue culture wells. The coverslips were then coated with gold colloid as follows: Firstly solutions of 0.387g of anhydrous sodium carbonate (451614, Sigma) in 100ml of sterile distilled water and gold(III) chloride (318645000, Acros Organics) to a final concentration of 6.85mg/ml in sterile distilled water were prepared. In a conical flask 15 ml of the sodium carbonate solution, 4.5 ml of the gold chloride solution and 27.5 ml of sterile distilled water were added and mixed while heating to boiling point. To this 4.5 ml of a 0.1\% formaldehyde solution was added, mixed and a colour change from red to purple observed. One millilitre aliquots of this final mixture were added to each well containing a BSA coated coverslip. The culture plate was covered and incubated overnight at 4\°C.

Finally a wash buffer was prepared comprising of 500ml Hanks’ Balanced Salts Solution (HBSS) supplemented with 5ml penicillin/streptomycin, 10ml sodium
pyruvate and 10ml 1M Hepes buffer. The gold colloid solution was aspirated from each well and the coverslips were rinsed twice with wash buffer. One millilitre of wash buffer supplemented with Type 1 rat-tail collagen (354236, Becton Dickinson) to a final concentration of 15µg/ml was added to each well and incubated at 37°C for two hours. The wells were aspirated again and the coverslips rinsed with wash buffer once. A total of 4,000 cells suspended in 1ml of growth media supplemented with 0.1% FCS and SDF to varying final concentrations were added to each well. The culture plates were covered and incubated at 37°C with 5% CO₂ for 20 hours. After this period the wells were aspirated, rinsed once with wash buffer and the cells fixed by the addition of 2ml of 0.1% formaldehyde in PBS to each well for 15 minutes at room temperature. Cells were then stained for surface receptors using the protocol for fluorescence immunocytochemistry outlined in Section 2.6.

Analysis was performed using photomicrographs of individual cells taken at x1000 magnification under oil immersion. Images were opened using the Image-J software package (NIH) and the area cleared of gold colloid particles around the cell was marked out (Figure 2.3). The total area covered was recorded and converted into square micrometres using a formula derived from photomicrographs of a graticule at the same magnification. Twelve individual cells from each treatment condition were measured and the mean and standard deviation calculated.

**Figure 2.3: Method of determining area covered in gold colloid assays.** Cells were incubated on sterile coverslips coated with gold colloid for 20 hours as outlined above. Photomicrographs were taken of single cells under oil immersion (a), scale bar represents 25µm. The area cleared of gold colloid particles was drawn out (b), and the area calculated using NIH Image J software. Twelve cells per coverslip were analysed, tabulated and the mean +/- standard deviation calculated.
2.9.2  **Scratch Wound Assays**

The purpose of this assay was to measure the maximal movement of confluent cultured keratinocytes in response to a chemokine stimulus (without a directional vector). The assays were all performed in conventional, sterile 24-well plates. Cells were trypsinised and passaged as described previously. Varying concentrations of each cell type (typically 100x10^3, 200x10^3 and 300x10^3 per millilitre) were prepared in normal growth media supplemented with 10% FCS. Different plates were prepared using the different concentrations with 500µl added to each well. The plates were then incubated for 16 hours under standard conditions of 37ºC and 5% CO₂.

The following day, the plate with the most even cell distribution corresponding to 90-100% confluence was selected and the others discarded. This plate was then washed twice with sterile PBS and then incubated with serum-free media containing 10µg/ml mitomycin C and 0.5% BSA for 2 hours under standard conditions. Two vertical and two horizontal scratches were created in each well with a sterile 200µl pipette tip using reasonable downward pressure. This created eight areas for analysis in each well (as outlined in Figure 2.4).

![Figure 2.4: Schematic representation of an individual well prepared for a scratch wound assay. Briefly, a confluent culture was prepared in each well and then scratched twice in both the horizontal and vertical directions with a 200µl micropipette tip. In this way eight individual areas for analysis were created within a single chamber. For details see text.](image-url)
Figure 2.5: Schematic representation of the method employed to measure scratch wound closure. Confluent cultures were prepared and scratched as previously outlined (Figure 2.4). Wells were incubated and photomicrographs were taken at various time points: for example 28 hours (a), 24 hours (b) and 4 hours (c). Comparison was made with baseline measurements taken at $T_{\text{zero}}$ (d). Residual wound distances ($X$) were measured at the same distance ($m$) above the intersection in all images and were calculated as a percentage of ($T_{\text{zero}}$).

The wells were then washed three times with sterile, warmed PBS and aspirated carefully. Serum-free growth media supplemented with 0.5% BSA with or without treatments was added to each well to a total volume of 500µl. Positive and negative controls were included on each plate and each experiment was repeated a minimum of three times. A photomicrograph was recorded from each of the eight areas for analysis (shown in Figure 2.4) using an inverted microscope (Nikon) at x20 magnification. Each area in each well was photographed sequentially at the outset ($T_{\text{zero}}$) and at predetermined intervals over a 28 hour time period. From each experiment, four or five images were obtained for each area. These images were analysed together at the end of the experiment using the Image-J (NIH) software package. The final image was analysed first and the minimum distance (in pixels) between the two sides of the wound was measured (Figure 2.5). The distance ($M$) this occurred above the
perpendicular line of the second scratch was also recorded. For images taken of this area taken at previous time points the distance between the sides (X) was measured at point (M) above the intersection (Figure 2.5). In this way distances between the closest points of the wound edges were recorded over various time periods including $T_{\text{zero}}$. These were expressed as a percentage of the value for $T_{\text{zero}}$. For each experimental condition the mean and standard deviation for the eight areas were plotted against time. If a wound was found to have completely closed then the value for that time point was defined as 0%.

2.9.3 Transwell Assays

2.9.3.1 Overview

Assays of cell migration and invasion were performed using the Transwell assay system. These were performed in a sterile 24-well plate format using polycarbonate membranes with 8 µm pore sizes (Falcon 353097, Becton Dickinson). A diagram of the assay system is shown in Figure 2.6. Essentially, cells under investigation were added to the upper chamber at a known density. Cells adhered to the upper surface of the basal polycarbonate membrane that contained pores of known diameter. Here they were exposed to chemotactic stimuli contained within the growth media added to the lower chamber. Over a period of time a proportion of the cells migrated through the membrane pores to reach the lower surface of the membrane. At the end of the experiment the number of cells that had reached the lower surface was determined. Cell migration was studied in this way or, alternatively, cell invasion was investigated by the addition of material (such as Matrigel) to the upper surface of the membrane.
2.9.3.2 Membrane coating and blocking

The effect of coating the polycarbonate membrane with various matrix proteins was investigated. Solutions of 50µg/ml rat tail collagen type 1 (BD Biosciences, 354236) and 10µg/ml fibronectin (Sigma, F2006) were prepared. To coat the lower surface of the polycarbonate membranes, 250µl of the working solutions were added to the lower chambers and the inserts introduced (with care not to trap air below the membrane). The upper surfaces were coated by the further addition of 50µl of the working solutions to the upper chamber, again with care not to introduce any air. The plates were then incubated at 37°C in a humidified atmosphere with 5% CO₂ for two hours. Finally, the working solutions were removed and the membranes were washed gently with sterile PBS.

Figure 2.6. Diagram of the Transwell assay system. Briefly, polycarbonate membranes with a defined pore size were prepared either as supplied (cell migration) or supplemented with an extracellular analogue (such as Matrigel). Media containing a known concentration of cells were added to the upper chamber while cell-free media containing a known concentration of the chemoattractant were added to the lower chamber. Cells were allowed to adhere and the apparatus was incubated for a defined period under optimal conditions. The upper surface of the membrane was then cleaned mechanically and the cells adherent to the lower surface were fixed, stained and counted. For further description see text.

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33 Rat tail collagen type 1 solution (BD Biosciences, 354236) was prepared: the stock solution (3.12 mg/ml) was diluted in 0.02N acetic acid to a final concentration of 50µg/ml then filter sterilised and stored at 4°C. Fibronectin solution(Sigma, F2006) was prepared: as follows the stock solution (1 mg/ml) was diluted in sterile PBS to give a final concentration of 10µg/ml.
Migration buffer was prepared fresh as follows: Dulbecco’s Modified Eagle Medium (Cambrex, BE12-604F) and HAM nutrient mixture F12 (Cambrex, BE12-615F) were mixed in a 3:1 ratio, supplemented with 0.5% bovine serum albumin (0.25g/50ml) and filter sterilised. Both sides of the polycarbonate membranes were then blocked with migration buffer. Six hundred microlitres of migration buffer were added to the lower chamber and 100µl to the upper (again ensuring no air bubbles). The membranes were returned to the incubator for a further hour.

2.9.3.3 Preparation of cells and wells.

Cells were grown in 25cm² flasks to 80% confluence as determined by light microscopy. The growth media were aspirated and the cells washed in warmed, sterile PBS. One millilitre of non-enzymatic cell dissociation solution in PBS (Sigma, C5914) was added to each flask and the cells returned to the incubator until they were no longer adherent. Passage was performed as previously described and the cells finally resuspended at a density of 1 million per millilitre in freshly prepared, warmed migration buffer. The contents of the upper chamber were aspirated and 100µl of the cell solution (containing 1 x 10⁵ cells) was added to the upper chamber. The chemoattractant to be tested was diluted in migration buffer to the required concentrations with the appropriate negative controls. Three hundred microlitre aliquots were added to the lower chambers and the inserts were gently lowered into place, again ensuring no air bubbles were trapped. The plates were then returned to the incubator. Three replicates were included for each experimental condition including negative controls. When included, pharmacological antagonists and bioneutralising antibodies (and isotype controls) were added to the upper chamber in the concentrations stated. Positive controls were performed with the addition of 10% FCS to the lower chamber.

At the end of the incubation period the plates were transferred to the fume cupboard. The migration buffer from the upper and lower chambers was removed and the cells fixed in 10% formalin for 10 minutes at room temperature. The formalin was then aspirated and the cells stained with a solution of 0.5% crystal violet in 10% ethanol for 10 minutes. After staining the cells were immersed in water for 10 seconds. The upper surface only was then dried firmly with first a dental roll and then a cotton bud
to remove all remaining adherent cells in the upper chamber. The lower surface was carefully preserved untouched. After rigorous cleaning of the upper surface the polycarbonate membrane (with stained adherent cells on the lower surface only) was excised using a fresh scalpel and fine forceps. Each membrane was briefly air dried, immersed in xylene and then mounted under a glass coverslip in non-aqueous resin on a clean, labelled, standard microscope slide. Gentle downward pressure was applied with the forceps on the coverslip to flatten the membrane and exclude any air bubbles. The mounting resin was allowed to harden overnight.

2.9.3.4 Cell counting and data analysis.

Stringent methods were used to reduce observer bias during data collection: Firstly, all experimental wells were allocated a random identification number by an independent observer. A record of these numbers and their associated experimental conditions was kept separately and securely. The matching microscope slides were re-labelled with this number in a way that retained the blinding of the person doing the counting. The blinding was only removed once all data recording and analysis had been performed. Secondly, all membranes were photographed using a digital camera and a photomicroscope. Images were taken from randomly selected, non-overlapping fields at x200 original magnification. The edges of the membrane were excluded because mechanical cleaning of the upper surface always left a small zone of adherent cells around the peripheries. Each membrane yielded approximately 20 images which were carefully labelled, downloaded on to computer and printed out.

Image printouts were checked for quality and clarity. Intact cells were marked and counted. Cellular material remaining only in a membrane pore was not included in the count. The total number of cells per image was recorded on a Microsoft Excel spreadsheet. The total number of images counted per membrane was determined by means of cumulative mean plots produced in Microsoft Excel in real-time. Essentially this method calculates the cumulative mean and standard deviation of the data as they are recorded. Further images were counted until the changes in the calculated mean were less than 5% for at least two iterations. A simple computer model of the process was generated to test the methodology (Figure 2.7). It showed that the cumulative mean varied by less than 5% of their value after 10 data had been included. This
occurred for the six datasets generated with varying means. This model was supported by experimentally-derived data which, in the majority of cases, involved counting cells on approximately ten images. The cumulative mean from the count of each membrane was taken and averaged for the three replicate wells. Although from multiple images, the cumulative mean for each well represents only a single data point.

![Figure 2.7](image)

**Figure 2.7: Computer modelling of cumulative mean plots.** Twenty integer values were randomly generated by computer to conform to a normal distribution of mean ($\mu$) and standard deviation $0.25\sigma$. Six datasets were produced where $\mu=10, 20, 40, 80, 160$ and $320$. Cumulative means were calculated and the percentage change plotted against the iteration number. Results show that in all cases the cumulative mean varies by less than 5% of its value before the tenth iteration.
Chapter 3:
Characterisation of established OSCC cell lines
3.1 Introduction

The role of chemokine receptors in leukocyte homing has been well established. The specific chemokine receptors CXCR4 and CCR7 have been specifically implicated in the mechanism of homeostatic selective re-circulation of T-lymphocytes. Their role in tumour metastasis has also been supported by evidence in breast, prostate, gastric and oesophageal carcinomas. More recently some preliminary work has been published on the role of CXCR4 and CCR7 in the dissemination of OSCC. The limitations of the methodologies of these studies have previously been discussed (Chapter 1) and, therefore, the role of any chemokine receptors in the metastatic pathways of OSCC has yet to be fully elucidated. Established OSCC cell lines provide a useful tool to investigate chemokine receptor mRNA expression and protein production in-vitro. They provide a population of tumour cells distinct from the adjacent tissue stroma found in clinical biopsies. Consequently the mRNA and protein levels of a whole range of chemokine receptors can be determined with a view to manipulation of gene expression and investigation with functional assays as required. The aim of the work presented in this chapter is to determine the expression of chemokine receptor mRNA in established OSCC cell lines and to investigate associated protein levels where appropriate.

3.2 Chapter Hypotheses

1. The mRNA expression of chemokine receptors associated with the lymph node homing dendritic cells (CCR7 and CXCR4) varies between OSCC cell lines and primary keratinocytes.

2. CXCR4 surface protein production reflects mRNA levels in established OSCC cell lines.
3.3 Chapter Aims & Objectives

3.3.1 Overall Aim
To determine the pattern of chemokine receptor mRNA expression and protein production in primary keratinocytes and selected, established OSCC cell lines.

3.3.2 Specific Objectives
1. To perform a quantitative screening of chemokine and chemokine receptor mRNA expression in primary keratinocytes and OSCC cell lines using microarrays.
2. To compare the mRNA expression of selected chemokine receptors in OSCC cell lines using the second technique of semi-quantitative, real-time RT-PCR.
3. To determine an OSCC cell line that constitutively expresses CXCR4 mRNA at low levels.
4. To determine the surface expression of CXCR4 protein on normal oral keratinocytes in culture and in-vivo.
5. To compare the surface expression of CXCR4 and CCR7 protein on established OSCC cell lines.

3.4 Results

3.4.1 Microarrays
3.4.1.1 Experimental overview
Microarray analysis was performed on established OSCC cell lines and primary normal oral and skin keratinocytes grown to early confluence under optimum conditions (including media supplemented with 10% FCS). To ensure good quality data were generated, the efficiency of microarray hybridisation was determined prior to analysis. Where available, data from duplicate samples were compared and combined. The expression of chemokines and chemokine receptors in primary keratinocytes and in established OSCC cell lines was determined and compared.
Eighteen arrays from 9 cell lines were processed. Sample data were analysed for quality according to the four criteria outlined in Section 2.2.13. Signals from internal controls identified two arrays that had failed to hybridise correctly (one each from SCC25 and CAL27). Consequently data from these arrays were excluded from the analysis and the SCC25 and CAL27 cell lines were analysed on the basis of one array each. Data quality and replicate matching was performed as outlined in Section 2.2.13 and the results are presented in Appendix 3.

3.4.1.2 Chemokine Receptor mRNA expression by Primary Cultures of Normal Oral and Skin Keratinocytes

A comprehensive microarray screening of the expression of all known chemokine receptors (CCR1-10, CXCR1-6, XCR1 and CX3CR1) was performed on seven established oral cell lines (H357, TR146, CAL27, FADU, SCC4, SCC25 and SV) and primary cultures of normal oral and skin keratinocytes as outlined above. Normalised expression values (NEV) were calculated for each gene in each sample as described in Section 2.2.13. The NEV for the chemokine receptors in normal oral and skin keratinocytes are represented in Figure 3.1.

For normal oral keratinocytes, these data show that there was only a small degree of variability (0.31 to 1.70) in expression levels for the majority of chemokine receptors. CXCR1 displayed a greater than three-fold reduction in expression compared with the normalised mean. Taking a two-fold change in expression as significant, this was the only chemokine receptor in normal oral keratinocytes to show a significant change in expression away from the normalised mean. For normal skin keratinocytes, again only a small degree of variability in the expression of chemokine receptor mRNA was demonstrated. However, the NEVs of four chemokine receptors were found to lie outside a two-fold change from the normalised mean: CCR3, CCR6 and CXCR5 were expressed at relatively low levels (NEV 0.37 0.22 and 0.39 respectively). CXCR1 (NEV = 2.04) was the only chemokine receptor expressed at relatively high levels.

In summary, chemokine receptor mRNA expression in NOK demonstrated no striking variation from the norm. Expression in NSK cells showed more variability but no consistent pattern was observed when compared with NOK. These results

34 A reduction in expression expressed as fold-change was calculated as the reciprocal of the NEV. In the case of CXCR1 this was 1.0/0.314 = 3.18 or just over a three-fold change.
demonstrated the expression of chemokine receptor mRNA in laboratory cultures of normal keratinocytes for comparison with similar data derived from established OSCC cell lines.

Figure 3.1: Normalised expression of chemokine receptor mRNA by normal oral and skin keratinocytes. Normal oral keratinocytes (NOK) from third molar surgery (Section 2.1.2) and normal skin keratinocytes (NSK) taken during breast reduction surgery (Section 2.1.3) were taken and cultured in four parallel flasks. Normalised chemokine receptor mRNA expression was determined by microarray analyses run in duplicate. Bars represent the mean (range) of the duplicate samples. For NOK only one mean chemokine receptor NEV (CXCR1) lies more than two-fold from the normalised mean. For NSK, this occurs with four chemokine receptors (CCR3, CCR6, CXCR1 and CXCR5). The raw data are given in Table A3.1 (Appendix 3).
3.4.1.3 Chemokine Receptor mRNA expression by Established OSCC cell lines.

Seven established oral cell lines (H357, TR146, CAL27, FADU, SCC4, SCC25 and SV) were analysed by microarray as outlined above. The mean NEVs for all known chemokine receptors in all the cell lines were calculated as outlined in Section 2.2.13. These values are represented in Figure 3.2 with the raw data given in Table A3.2 (Appendix 3). Compared with the normalised mean, CXCR4 demonstrated the highest chemokine receptor mRNA expression in the oral cell lines tested. This occurred in two OSCC cell lines, SCC4 and SCC25 (NEV 8.3 and 10.1 respectively). The normalised expression of CXCR4 was essentially unchanged in the remaining five cell lines (NEV range 0.76 to 0.85) which was very close to the values obtained for NOK and NSK (NEV of 0.96 and 0.80 respectively). The expression of the chemokine receptor CCR7 was significantly decreased in the SCC25 cell line (NEV 0.27). In the remaining cell lines relative CCR7 expression did not significantly differ from that of normal oral keratinocytes.

Apart from the high expression of CXCR4 mentioned above, only two other chemokine receptors were expressed more than two-fold above the normalised mean: CCR5 in the SCC4 cell line and CXCR2 in FADU cells (NEV 3.7 and 2.5 respectively). All other changes in chemokine receptor expression in oral cell lines were reductions compared with the normalised mean. TR146 cells expressed CCR1 and CCR3 at low levels (NEV 0.45 and 0.47 respectively) while the expression of CCR3, CCR6 and CCR8 was similarly low in FADU cells (NEV 0.35, 0.43 and 0.27 respectively). The CAL27 cell line also expressed CCR8 at low levels, but also CXCR1 and CXCR5 (NEV 0.24, 0.26 and 0.36 respectively). CXCR5 was also expressed at low levels by H357 cells (NEV 0.48). The SV40 transformed cell line [SV] demonstrated a unique profile of chemokine receptor expression with low levels of CCR4, CXCR1, CXCR2 and CX3CR1 detected (NEV 0.29, 0.19, 0.29 and 0.34 respectively). CX3CR1 was also expressed at low levels by SCC4 cells, in addition to CXCR3 (NEV 0.48 and 0.49 respectively). Finally, XCR1 was expressed at lower levels in all OSCC cell lines, reaching significance in SCC4, SCC25 and TR146 (NEV 0.26, 0.30 and 0.43 respectively). A representation of the relative expression of all chemokine receptors in all oral cell lines compared with NOK is given in Table 3.1.
Figure 3.2: Chemokine Receptor mRNA Expression by Established OSCC Cell Lines. Solid bars represent the chemokine receptor NEV (averaged for duplicate samples where applicable). For description of results see text. The raw data are given in Table A3.2 (Appendix 3).
In summary, these data indicate that CXCR4 upregulation was the most striking change in chemokine receptor expression in some, but not all, OSCC cell lines. This was not demonstrated in the normal oral or skin keratinocytes tested. Most other changes that occurred in chemokine receptor expression involved reduction in mRNA levels compared to normalised means. Interestingly CCR7, one of the chemokine receptors strongly associated with physiological leukocyte homing to lymph nodes, was not upregulated in oral cell lines or normal keratinocytes. The expression of chemokine mRNA by NOK, NSK and established OSCC cell lines was also determined and the results are presented in Section A3.3 (Appendix 3).

Table 3.1: Significant Changes of Chemokine Receptor Normalised Expression Values in Established OSCC Cell Lines.

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This table is derived from data presented in Table A3.4 (Appendix 3). Legend: ▲ = upregulation 2-5 fold, ▲▲ = upregulation greater than five-fold, ▼ = downregulation 2-5 fold, ▼▼ = downregulation greater than five-fold.
3.4.2 Chemokine receptor mRNA expression levels determined by semi-quantitative Reverse Transcription Polymerase Chain Reaction.

Semi-quantitative RT-PCR was used to determine the relative expression of chemokine receptor mRNA by cultured cells using an alternative methodology. Complimentary-DNA synthesised from the same RNA used in the microarray analyses was investigated with additional replicates included. Data normalisation against an overall expression level for multiple genes was not possible for sqRT-PCR results. Consequently the expression levels of each mRNA species in NOK was defined as equal to one and expression in all other cell lines was calculated relative to this to yield semi-quantitative results. Compared with NOK, CXCR4 mRNA expression was significantly higher in all cell lines examined with the exception of NSK, TR146 and H357 (Figure 3.3). Expression in skin and oral keratinocytes showed no significant difference. Two cell lines (SCC4 and SCC25) demonstrated CXCR4 upregulation of more than 1,000-fold compared with NOK (p<0.001). Three further cell lines (SV, FADU and CAL27) showed significant upregulation of CXCR4 (compared with NOK) although the magnitude of this increase varied considerably (93, 25 and 5 times respectively). The mRNA expression of other chemokine receptors by OSCC cell lines was also measured by sqRT-PCR for comparative purposes. The relative expression of CCR7 by NSK and OSCC cells lines demonstrated no significant differences from NOK (Figure 3.4a). NOK and NSK demonstrated very similar levels of CCR7 mRNA expression. Although CCR7 mRNA expression was less in the SCC4 and SCC25 cell lines and increased in the remaining OSCC cell lines, none of these differences were of a sufficient magnitude to achieve statistical significance. CCR4 mRNA expression showed some variation between cell lines (Figure 3.4b).

Expression by primary skin keratinocytes was nearly ten-fold greater than by primary oral keratinocytes (p<0.001). The SCC4 and SV cell lines were the only established OSCC cell lines in which CCR4 mRNA expression was significantly altered, compared with NOK (p<0.001). In SCC4 cells, CCR4 expression showed a three-fold increase, while in SV cells a five-fold decrease in expression was demonstrated, compared with NOK. The remaining cell lines investigated (SCC25, FADU, CAL27 and untransfected H357 cells) showed no significant alteration in CCR4 mRNA expression compared
with NOK. Expression of XCR1 mRNA showed significant differences in most cell lines compared with NOK (Figure 3.4c). Expression in NSK was ten-fold less than in NOK (p<0.001). In most of the established OSCC cell lines, XCR1 mRNA was also significantly reduced compared with NOK: in the TR146, SCC4 and SCC25 cell lines expression was reduced 20-, 30- and 100-fold respectively (p<0.001), while in the FADU and H357 cell lines expression was reduced two- and three-fold respectively (p<0.05). XCR1 mRNA expression was increased in the CAL27 and SV cell lines two- and five-fold respectively (p<0.05), compared with NOK.

Figure 3.3: Expression of CXCR4 mRNA by normal keratinocytes and OSCC Cell Lines.
Relative CXCR4 mRNA expression in normal keratinocytes and OSCC cell lines cultured under optimal conditions to the end of their logarithmic growth phase. Results show two high-expressing OSCC cell lines (SCC4 and SCC25), three expressing at intermediate levels (SV, FADU and CAL27) and two (H357 and TR146) expressing similar levels to normal oral and skin keratinocytes. All OSCC cell lines except H357 and TR146 demonstrated significantly more CXCR4 mRNA expression than normal oral and skin keratinocytes (ANOVA with Bonferroni correction for multiple testing). Mean (solid bars) and range (lines) of four replicates (each measured in triplicate) are shown expressed as relative fold change compared with CXCR4 in normal oral keratinocytes. Graph shows the cumulative results of two experiments performed. Legend: *** = p<0.001, * p<0.05, ns = not significant.
Expression of the chemokine XCL1 was also determined by sqRT-PCR (Figure 3.4d). XCL1 was selected for investigation as it is the XCR1 ligand and thus made a receptor/ligand pair, as well as being a representative of the chemokines also investigated by microarray (Appendix 3). Interestingly, XCL1 expression was less than NOK in only NSKs (30-fold, p<0.001). XCL1 expression in OSCC cell lines was increased compared with NOK: in five cell lines (SCC4, SCC25, SV, CAL27 and TR146) this increase was relatively small (two-to three-fold, p<0.05). However, in the H357 and FADU lines the increase was seven- and 21-fold respectively (p<0.001).

To investigate the relationship between the microarray results and the sqRT-PCR data obtained from similar samples, a simple graphical representation of the raw expression data was produced. From this it was determined that the range of magnitude of the sqRT-PCR results was much greater than that demonstrated by the microarrays. To investigate this further the expression values for all the genes examined by both methods (CXCR4, CCR7, CCR4, XCR1 and XCL1) were pooled. Following a log(10) transformation of both data sets, a clear linear relationship of gradient 0.32 between the results was demonstrated (Figure 3.5). This relationship was maintained over the entire range of expression levels exhibited. Using linear regression the Root Mean Square (R²) value was calculated as equal to 0.645 suggesting a probability value of less than 0.001.

In summary, there was a high concordance between the log-transformed mRNA expression values derived by the two independent methods (sqRT-PCR and microarrays). CXCR4 was the chemokine receptor expressed at the highest level by some, but not all, the established OSCC cell lines, compared with NOK. There was no significant variation in CCR7 mRNA expression.
Figure 3.4: Messenger RNA expression by Normal Keratinocytes and OSCC Cell Lines. All cells were cultured under optimal conditions to the end of their logarithmic growth phase. Panels represent CCR7 (a), CCR4 (b), XCR1 (c) and XCL1 (d) mRNA expression in cell lines relative to NOK. For a detailed description of the results see text. Mean (solid bars) and range (lines) of four replicates (each measured in triplicate) are demonstrated for each cell line. Statistical significance was determined using ANOVA with the Bonferroni correction for multiple testing. Legend: NOK = normal oral keratinocytes; NSK = normal skin keratinocytes; * = p<0.05, ** = p<0.01, *** = p<0.001. Panels represent the cumulative results of three experiments performed.
Figure 3.5: The relationship between RT-PCR and microarray values for chemokine and chemokine receptor relative expression values. Relative expression values for the chemokine receptors CXCR4, CCR7, CCR4 and XCR1, and the chemokine XCL1 were determined by both microarrays and qRT-PCR. A scatterplot of the Log_{10} values yielded a linear relationship of best fit (Root mean square = 0.645, linear regression p<0.001) with gradient of 0.32.
3.4.3 CXCR4 and CCR7 protein production by primary keratinocytes and gingival fibroblasts.

Following initial investigation at the mRNA level, CXCR4 and CCR7 surface protein production by primary oral and skin keratinocytes was determined initially using flow cytometry. Cells were passaged using growth-arrested fibroblast feeder cells as previously described (Section 2.1.4) and grown until 80% confluence as determined by light microscopy. The data plots for CXCR4 and CCR7 labelling of primary normal oral and skin keratinocytes, and untransformed human gingival fibroblasts (HGFs) are given in Figure 3.6. No significant CXCR4 or CCR7 labelling was demonstrated for either normal oral or skin keratinocytes. In HGF cells, CXCR4 labelling was marginally higher compared with isotype antibody controls (Mean Fluorescence Values [MFV] 7.26 and 4.17 respectively). No surface CCR7 was detected on HGFs by flow cytometry. Although very few cells with a fibroblast-like morphology were seen in the cultures prior to dissociation, the presence of contaminating feeder cells could not be excluded. Therefore, further analyses were performed to compare CXCR4 expression in a mono-population of 3T3 feeder cells and combined culture with primary oral and skin keratinocytes (Figure 3.7). Results showed no CXCR4 labelling of the 3T3 population grown in isolation or in combination with the primary keratinocytes. Furthermore, when differentiated by relative size, neither component of the combined keratinocytes/feeder cell culture demonstrated significant CXCR4 labelling.

To investigate further these flow cytometry findings of CXCR4 labelling in NOK, a second, complementary technique was employed. Two anti-CXCR4 monoclonal antibodies (MAB172 and MAB173, R&D Systems) were used for flow cytometry while the latter was used for immunocytochemistry. Primary oral and skin keratinocytes (together with control mono-populations of fibroblast feeder layers) were examined using immunocytochemistry. No significant staining for either CXCR4 or CCR7 was demonstrated compared with isotype antibody controls. However, HGFs revealed very weak staining for CCR7 and a slightly stronger signal with CXCR4, compared with isotype and no primary antibody controls (Figure 3.8). This finding is in concordance with the low level of CXCR4 labelling of HGFs demonstrated by flow cytometry (Figure 3.6, panel E). However, the very low level of CCR7 immunostaining in HGFs (Figure 3.8, panel B) was not replicated using flow cytometry (Figure 3.6, panel F).
Figure 3.6: Flow cytometry of CXCR4 and CCR7 in primary keratinocytes and human gingival fibroblasts.

Cells were grown to 80% confluence, passaged and incubated with anti-CXCR4 (A, C and E) or anti-CCR7 (B, D and F) monoclonal antibodies or isotype controls. Data plots of cell counts against fluorescence intensity for NOK (A and B), NSK (C and D) and HGF (E and F). Table shows Mean Fluorescence Values (MFV). No significant CXCR4 or CCR7 labelling was demonstrated by either oral or skin keratinocytes. HGF cells demonstrated a very low density of CXCR4, but not CCR7. One representative experiment shown of two performed.

Legend: NOK = primary normal oral keratinocytes; NSK = primary normal skin keratinocytes; HGF = untransformed human gingival fibroblasts.

<table>
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</tr>
<tr>
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Figure 3.7: CXCR4 labelling of primary cultured keratinocytes and their feeder fibroblasts.

Primary oral and skin keratinocytes were cultured with growth-arrested 3T3 fibroblasts under optimum conditions. At 80% confluence and maximal fibroblast detachment the cells were dissociated and labelled with anti-CXCR4 monoclonal antibodies (clone 173) prior to flow cytometry. Monocultures of 3T3 cells were used for comparison. The normal size distribution of 3T3 cells was determined from pure cultures (G) and only a population of cells outside of this (population P1, panel G) was defined. The P1 population was selected from primary cultures of oral and skin keratinocytes (A and D respectively). Comparisons between labelled cells (C and F respectively) and unlabelled controls (B and E respectively) demonstrated that very low levels of oral and skin keratinocytes labelled positively for CXCR4 (4.3% and 3.1% respectively, panel I). 3T3 cells did not demonstrate CXCR4 labelling (H). One representative experiment shown of two performed. Legend: NOK = normal oral keratinocytes; NSK = normal skin keratinocytes.

<table>
<thead>
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</table>
Figure 3.8: Human gingival fibroblast expression of CXCR4 and CCR7. Human gingival fibroblasts were cultured in optimal growth media supplemented with 10% FCS to approximately 80% confluence. Cells were fixed and labelled using standard immunocytochemical methods outlined in Section 2.6. Primary antibodies used were CXCR4 (A); CCR7 (B); isotype control (C); and no primary antibody (D). HGFs stained positively for CXCR4 and weakly for CCR7 with no significant non-specific binding demonstrated in negative controls. Scale bar represents 100µm. Representative images are shown from three experiments performed.

In summary, no CXCR4 or CCR7 labelling for NOK or NSK was demonstrated by either flow cytometry or immunocytochemistry. These findings concur with the low levels of CXCR4 and CCR7 mRNA expression displayed by these cells in culture. Again, these findings provide a baseline for comparison with established OSCC cell lines.
To determine their surface expression of CXCR4 protein, established OSCC cell lines were grown under optimal conditions to 80% confluence, dissociated and analysed with flow cytometry (Figure 3.9). All OSCC cell lines tested demonstrated a homogenous distribution of size and granularity conforming to the normal distribution. Less than 15% of the total number of cells stained strongly with propidium iodide and were gated out. CXCR4 labelling was greatest on the SCC4 cell line (panel A) with a mean fluorescence value of 24.89 (compared with 9.64 for the isotype antibody control). CXCR4 signal above background was also detected on SCC25 and CAL27 cells (panels B & C) with mean fluorescence values of 17.97 and 14.48 respectively. No significant CXCR4 labelling was detected on either TR146 or FADU cells (panels E & F). The H357 cell line was also examined and the results are presented in Section 3.4.5.

As before, immunocytochemistry was performed to investigate CXCR4 protein staining expression by these established OSCC cell lines (Figure 3.10). The most significant CXCR4 staining was seen with SCC4 cells (Figure 3.10 (a)) compared with isotype controls (Figure 3.10 (f)). SCC25 (Figure 3.10 (b)) and CAL27 (Figure 3.10 (c)) cells were seen to stain positively for CXCR4 but to a lesser extent than SCC4 cells. The staining pattern in these three cell lines was diffuse and homogenous. CXCR4 staining in TR146 (Figure 3.10 (d)) and FaDu (Figure 3.10 (e)) cells was very weak, focal and heterogeneous in comparison. Consequently it was demonstrated that cell lines positive for CXCR4 labelling by flow cytometry (SCC4, SCC25 and CAL27) also showed positive staining by immunocytochemistry. Cell lines negative for CXCR4 by flow cytometry (TR146 and FaDu) showed only weak and heterogeneous staining. Two CXCR4 positive cell lines (SCC4 and CAL27) were cultured as described above and investigated with further immunocytochemistry experiments to try and visualise more clearly the distribution of CXCR4 protein within the cells. Initially conventional fluorescent immunocytochemistry was performed on SCC4 and CAL27 cells grown at a low density on glass coverslips under optimum conditions (Figure 3.11 panels a-d). Results revealed that for both SCC4 and CAL27 cells the highest CXCR4 staining intensity was concentrated around the nuclei extending into the cytoplasmic compartment (panels a and b respectively). Isotype antibody control experiments revealed no positive staining (panels h and i). Compared with the outline of the
nuclear structures (highlighted by the DAPI counterstain - panels c and d respectively)
the results were suggestive that CXCR4 protein expression was outside the nucleus.
To determine the location of the staining within the cell more accurately confocal
fluorescence microscopy was performed. For both SCC4 and CAL27 cells this revealed
diffuse staining for CXCR4 that appears to be associated with the cell membrane rather
than the nucleus (panels e and f respectively).  

35 Computer reformatting and three-dimensional (3-D) reconstruction of the confocal data
allows perspectives on the pattern of cell staining not possible with conventional microscopy.
However, 3-D reconstruction images yield most information when manipulated on the
computer screen and are only poorly represented by 2-D printouts and therefore have been
omitted from this thesis.
Figure 3.9: CXCR4 surface protein production by OSCC cell lines. Cells were grown to 80% confluence under optimum conditions. Prior to analysis cells were incubated with anti-CXCR4 antibodies (or isotype controls) and propidium iodide. Cell count was plotted against fluorescence intensity for: SCC4 (A); SCC25 (B); CAL27 (C); TRI46 (D); and FADU cells (E). Table shows Mean Fluorescence Values (MFV) for anti-CXCR4 (clone 173) and isotype control antibodies. SCC4, SCC25 and CAL27 cells demonstrated CXCR4 labelling in excess of isotype controls. CXCR4 protein was not detected on the surface of TRI46 and FADU cells. One representative experiment shown of two performed. Legend: anti-CXCR4 antibodies (green line); isotype controls (purple shading).

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Established OSCC cell lines were cultured under optimum conditions on sterile glass coverslips until 80% confluent. Cells were fixed, permeabilised and stained using monoclonal antibodies against CXCR4 (clone 173) conjugated to Texas Red. Conventional light microscopy revealed strong staining for the SCC4 cell line (a) and, to a lesser degree, SCC25 (b) and CAL27 (c) cells. Only very weak staining was seen for TR146 (d) and FaDu (e) cells compared with isotype antibody controls (f). Representative images shown from three replicates performed. Scale bar represents 100µm.
Figure 3.11: CXCR4 staining of cultured SCC4 and CAL27 cells. Representative images for SCC4 (panels a, c, e & h) and CAL27 (panels b, d, f and i) are shown using conventional (a-d, h & i) and confocal (e & f) microscopy. Staining was performed using anti-CXCR4 monoclonal antibodies (a, b, e & f) with nuclear counterstain (c - i). Isotype antibody negative controls were also performed (h & i). Scale bar 20µm. For results see text.
3.4.5 CXCR4 protein production by untransfected H357 cells

To determine the surface expression of CXCR4 protein in the established H357 cell line, these cells were cultured under optimal conditions (including 10% FCS) until 80% confluence, then dissociated and prepared for flow cytometry. Labelling with two anti-CXCR4 monoclonal antibodies was compared. Both antibodies detected low levels of CXCR4 on the surface of untransfected H357 cells (Figure 3.12): MFV for clones 172 and 173 were 9.2 and 14.7 respectively compared with isotype controls (MFV = 4.4). A similar pattern of results was obtained with a second, replicate experiment (panel H). These data suggest that the CXCR4 receptor was produced on the surface of these cells at very low levels and further experiments were performed to investigate this. Immunocytochemistry on permeabilised cells was used to visualise the pattern and location of CXCR4 protein (Figure 3.13). Conventional peroxidase-based staining for CXCR4 demonstrated clear staining of H357 cells (Figure 3.13 panel a) compared with isotype antibody controls (Figure 3.13 panel b). At higher magnification, using fluorescent labels, staining could again be demonstrated (Figure 3.13 panel c) although, following comparison with the nuclear counterstain (Figure 3.13 panel d), the CXCR4 protein appeared to be associated with the nuclear structures rather than the cytoplasmic membrane. Further examination of these experimental preparations with confocal microscopy revealed that CXCR4 labelling overlaid the nuclear membrane and nucleolar structures with a low intensity of cytoplasmic staining (Figure 3.13 panels e & f).

Analysis of these data suggested that CXCR4 protein was produced at very low levels on the cytoplasmic membrane of untransfected H357 cells. However, previous results had demonstrated that CXCR4 mRNA was expressed by these cells, and immunocytochemistry for the protein showed some staining of predominantly perinuclear structures. From these results it was hypothesised that overexpression of CXCR4 mRNA would result in an increase in protein localising to the cytoplasmic membrane. Experiments were designed to test this hypothesis and are described in Chapter 5.
Figure 3.12: CXCR4 surface production by untransfected H357 cells. Untransfected H357 cells were cultured under optimum conditions until approximately 80% confluent, dissociated and labelled for analysis with propidium iodide and anti-CXCR4 monoclonal antibodies clone 172 (A & B), clone 173 (C & D) or isotype controls (E & F). Only live cells were gated (B, D & F). Low levels of CXCR4 labelling were demonstrated by both antibody clones (G & H). Data from two replicate experiments are given (H) although only one is shown (A-G).
Figure 3.13: CXCR4 staining in untransfected H357 cells. Cells from the established H357 cell line were cultured under optimum conditions, fixed, permeabilised using detergent and stained for immunocytochemistry using anti-CXCR4 monoclonal antibodies (MAB173, R&D Systems). Peroxidase-based methods revealed diffuse staining (a) compared with isotype antibody negative controls (b). Using conventional microscopy fluorescence-based methods demonstrated that staining was less densely associated with the cytoplasmic membrane than with structures associated with the nucleus (c) as highlighted by the nuclear counterstain (d). Perinuclear staining may have been artificially increased by the use of detergents in preparation of the cells prior to labelling. Confocal microscopy at 0.5µm intervals further delineated this predominantly peri-nuclear relationship of CXCR4 staining (e) with occasional expression within the cytoplasmic space (f). Scale bar represents 100µm (a & b), 20µm (c-e) and 10µm (f).
3.4.6 CCR7 protein production by OSCC cell lines

Parallel experiments to those outlined previously were performed to investigate the production of CCR7 by established OSCC cell lines. Both flow cytometry and immunocytochemical techniques were carried out using commercially available monoclonal antibodies to the CCR7 receptor validated in the literature. Three repeat experiments failed to demonstrate detectable CCR7 labelling on the surface of any of the cell lines tested by flow cytometry (Figure 3.14).

Immunocytochemistry was performed to investigate these findings further. These experiments showed barely detectable perinuclear CCR7 staining in permeabilised cells with fluorescence- or peroxidase-based methods (Figure 3.15). Isotype control antibodies demonstrated no labelling.

Immunofluorescence experiments are represented by results for untransfected H357 and CAL27 cells. No CCR7 labelling was detected on any of the six established OSCC cell lines investigated.
Figure 3.14: CCR7 surface labelling in established OSCC cell lines by flow cytometry. Cells were grown to 80% confluence under optimum conditions. Prior to analysis cells were incubated with anti-CCR7 antibodies or isotype controls followed by propidium iodide. Cell count was plotted against fluorescence intensity for: SCC4 (A); SCC25 (B); CAL27 (C); TRI46 (D); and FADU cells (E). Mean fluorescence values were tabulated for anti-CCR7 and isotype controls. Labelling in excess of isotype controls was not detected on the surface of any of the cells tested. One representative experiment shown of three performed. Legend: anti-CCR7 antibodies (green line); isotype controls (purple shading).
Figure 3.15: CCR7 labelling of H357 and CAL27 cells by immunofluorescence. Cells were cultured under optimum conditions, fixed, permeabilised and stained with anti-CCR7 monoclonal antibodies using fluorescence-based reporters. Untransfected H357 cells (a & b) and CAL27 cells (c-f) are shown. Barely detectable perinuclear staining was demonstrated with anti-CCR7 antibodies (a & c) but not isotype controls (e). Comparisons can be made with the DAPI nuclear counterstains of the corresponding areas (b, d & f). Scale bar represents 20µm.
3.4.7 Summary of experimental results

- Microarray samples that had been blinded and randomised were paired according to their biological cell line of origin by cluster analysis.
- Microarray and sqRT-PCR measurements of relative mRNA expression demonstrated a linear association when their logarithmically-transformed values were plotted.
- Little variability was shown in the levels of chemokine receptor mRNA expression by normal oral keratinocytes except for CXCR1 which is expressed at a lower level than other chemokine receptor family members.
- The highest level of mRNA expression of all the chemokine receptors was demonstrated by CXCR4 in two of the OSCC cell lines tested.
- CCR7 mRNA was expressed at similar or relatively lower levels in all the OSCC cell lines compared with normal oral keratinocytes.
- H357 cells demonstrated similar levels of CXCR4 mRNA expression compared with normal oral keratinocytes.
- CXCR4 or CCR7 protein production was not demonstrated by normal oral or skin keratinocytes.
- Human Gingival Fibroblasts demonstrated some CXCR4 expression in vitro.
- Three established OSCC cell lines (SCC4, SCC25 & CAL27) that showed CXCR4 mRNA expression also demonstrated CXCR4 protein production associated with the cytoplasmic compartment.
- CCR7 protein production was not demonstrated in any of the established OSCC cell lines examined.
3.5 Discussion

A significant variation in chemokine and chemokine receptor mRNA expression and protein production was demonstrated between primary cultures of oral keratinocytes and established OSCC cell lines. The quantitative and qualitative data produced were derived by several complimentary techniques. Before the significance of the findings was examined it was important to review the strengths and weaknesses in each of these techniques particularly with respect to comparing the data generated for particular chemokine receptor mRNA species. This is discussed in Appendix 3.

3.5.1 CXCR4 mRNA expression and protein production by normal keratinocytes

The results presented here have demonstrated that CXCR4 mRNA was expressed at low levels in NOK and NSK. This is mirrored in the literature although similar experiments are often either small numbers of normal controls or part of studies focusing on HIV infection. CXCR4 mRNA expression has been described in oral and gingival keratinocytes (Uchida, Begum et al. 2003; Chen, Zha et al. 2004). These findings are substantiated by gene array studies on laser-capture specimens from normal oral mucosa (Moutsopoulos, Nares et al. 2007). CXCR4 mRNA (and protein) have been demonstrated in cultures of normal colonic (Jordan, Kolios et al. 1999) but not salivary gland epithelial cells. (Moore, Rahemtulla et al. 2003) In my experiments, both microarrays and sqRT-PCR were used semi-quantitatively with the expression levels in other cell lines calculated relative to NOK. No absolute level of CXCR4 mRNA expression was determined and this could be addressed by further work using carefully calibrated RNA standards.

My results demonstrated that cultured normal keratinocytes did not label positively for CXCR4 protein with either flow cytometry or immunocytochemistry. Liu et al demonstrated CXCR4 surface protein labelling in a small subset of normal oral keratinocytes derived from 3rd molar surgery (Liu, Zha et al. 2003). Moore et al showed a greater proportion of positive cells in samples taken from periodontal flap surgery (Moore, Rahemtulla et al. 2003). It can be assumed that there was significant inflammation present in the latter group of patients undergoing periodontal surgery.
and that low grade inflammation was common in the 3rd molar region. It was from a similar source that the NOKs used in these experiments were derived (Section 2.1.2). The effect of localized inflammation on CXCR4 expression and production in oral keratinocytes is not known. My results demonstrated no upregulation of inflammation-induced chemokine receptor mRNA expression although no distinct pattern was seen. This could be investigated further by pathway analysis of the entire microarray dataset focusing on pro-inflammatory mechanisms. Exposure of NOK to ethanol at low concentrations *in-vitro* increases CXCR4 surface protein (but not mRNA expression), although it is not known if this is secondary to a pro-inflammatory mechanism (Liu, Zha et al. 2003; Chen, Zha et al. 2004).

My observational data on CXCR4 expression in NOKs did not address the question of whether these receptors were functional. To do this would have required either careful passage without feeder cells or growth in a feeder-free environment. The ability of CXCR4-tropic HIV-1 viruses to infect NOKs in culture (Liu, Zha et al. 2003; Moore, Rahemtulla et al. 2003) and the abrogating effect on this infection by co-culture with SDF (Chow, Yu et al. 2002) provides indirect evidence that a small number of normal keratinocytes express CXCR4 surface receptors at least capable of ligand binding and receptor internalisation. Data on CXCR4-mediated downstream cell signalling in these instances were not documented.

While CXCR4 surface protein was not detected on NOK or NSK in my experiments it is possible that functional protein is produced by these keratinocytes *in-vivo*. If this is increased at sites of inflammation, together with the potential role of pro-inflammatory mediators in tumour progression, this would be an interesting field for further investigation (Balkwill and Mantovani 2001). An initial step would be to investigate CXCR4 staining in clinical samples of normal and inflamed oral mucosa.

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37 The normalised mRNA expression of inflammation-induced chemokine receptors CXCR1, CXCR2, CCR1, CCR2 and CCR3 were not significantly upregulated in NOKs (Section 3.4.1.2).
3.5.2 CXCR4 mRNA expression in established OSCC and other head and neck carcinoma cell lines

CXCR4 mRNA was expressed at low levels in NOK and surface protein was not detected. Considering the hypothesis that CXCR4 plays a role in OSCC tumour progression, it might be expected that the mRNA would be upregulated in OSCC cell lines. My results show that CXCR4 mRNA was the only chemokine receptor to be expressed at significantly higher levels (ten-fold increase) in two out of six OSCC cell lines. This upregulation in some, but not all, cell lines suggests that CXCR4 overexpression is not ubiquitous in tumour progression. Indeed a similar pattern of CXCR4 mRNA expression in established OSCC cell lines is described in the majority of the published literature: high expression of CXCR4 mRNA was demonstrated in two out of three (Uchida, Begum et al. 2003), three out of six (Katayama, Ogino et al. 2005), three out of eight (Ishikawa, Nakashiro et al. 2006), but none out of 13 (Muller, Sonkoly et al. 2006) OSCC cell lines tested. CXCR4 gene expression is a prerequisite of protein production and its absence in OSCC cells would not support (although not refute) the overall hypothesis of this thesis. My findings, together with those from the literature, suggest that CXCR4 mRNA expression is not-ubiquitous in OSCC cells in-vitro. This may be a reflection of the genome of the individual cell line or, alternatively, a change in the transcriptome in response to cell culture conditions. It has been suggested that CXCR4 surface production is induced and maintained by the tissue/tumour microenvironment from observations of CT-26 colon carcinoma cells where CXCR4 mRNA was strongly upregulated in-vivo but lost again following a few days of ex-vivo culture. (Zeelenberg, Ruuis-Van Stalle et al. 2003) The effect of individual cell culture conditions on CXCR4 mRNA expression could be investigated empirically. One variable of clinico-pathophysiological interest would be the effect of hypoxia particularly on CXCR4 mRNA expression (Uchida, Onoue et al. 2007).

CXCR4 mRNA upregulation (compared with normal epithelia) has been demonstrated in other head and neck carcinomas cell lines including salivary adenoid cystic carcinoma (two out of four), thyroid (seven out of ten) and nasopharyngeal carcinoma (Moore, Rahemtulla et al. 2003 ; Wang, Xi et al. 2005; Muller, Sonkoly et al. 2006; De Falco, Guarino et al. 2007). Interestingly, clinical observations demonstrate that adenoid cystic carcinoma is one of the few salivary tumours to demonstrate distant metastasis although it is not clear from these data alone what role CXCR4 plays in this
process.\textsuperscript{38} The finding of increased CXCR4 mRNA expression in a BCC-derived cell line (clinically a tumour that is locally invasive but very rarely metastasises) further clouds the picture of what role CXCR4 plays in epithelial tumour progression. What is well established is that tumourigenesis is dependent on multiple factors within the tumour/tissue microenvironment (Balkwill and Mantovani 2001).

Semi-quantitative analysis revealed a ten-fold increase in CXCR4 expression in the SCC4 and SCC25 cell lines, compared with NOK. Upregulation of CXCR4 mRNA in primary thyroid carcinomas ranged from five to 65 times greater than in cultures of normal thyroid cells (De Falco, Guarino et al. 2007). Examination of other epithelial carcinomas has demonstrated CXCR4 upregulation one magnitude greater than found in OSCC. For example, the BCC-derived cell line BCC-1/KMC, CXCR4 was expressed 72-fold more than all other chemokine receptors (Chen, Yu et al. 2006). My data demonstrate that only CXCR4 mRNA was constitutively expressed at high levels in OSCC cell lines. This pattern has also been described in the literature (Uchida, Begum et al. 2003). However, Muller et al investigated a comprehensive panel of chemokine receptor mRNAs and found no discernable pattern amongst the OSCC cell lines examined (Muller, Sonkoly et al. 2006). The exact mechanisms underpinning the differences in the magnitude of upregulation remain unclear. What remains to be determined is whether this large range in upregulation observed \textit{in-vitro} is representative of the \textit{in-vivo} situation. This could be addressed by laser-capture microdissection of small islands of tumour cells from clinical specimens and analysis with multiplexed semi-quantitative RT-PCR. This was one of the original objectives of my experimental work as discussed earlier.

In summary, low constitutive CXCR4 mRNA expression in NOK, with significant upregulation in some, but not all, established OSCC cell lines is consistent with the hypothesis that the SDF-CXCR4 axis is, in some way, involved with the malignant process. However, at a mechanistic level, it is likely that CXCR4 mRNA expression levels would be poorly predictive of the behaviour of these cells \textit{in-vivo}. To test this hypothesis further, examination of CXCR4 protein production was required.

\textsuperscript{38} The observed clinical behaviour of adenoid cystic carcinomas is that they metastasise haematogenously (not via the lymphatics) to distant sites as a late event. Here tumour deposits may enter a latent state and not produce clinically relevant lesions until quite some time after initial presentation. The role of CXCR4 in this pattern of tumour behaviour is not clear (Muller, Sonkoly et al. 2006).
3.5.3 CXCR4 protein production by established OSCC and other carcinoma cell lines

At first inspection, the relationship between CXCR4 mRNA expression and surface protein production appears simple: high levels of CXCR4 mRNA expression demonstrated by SCC4 and SCC25 cells were associated with positive labelling with flow cytometry and surface staining with immunocytochemistry. Similar patterns of protein production (as determined by flow cytometry) have been reported in other OSCC cell lines expressing high levels of CXCR4 mRNA (Uchida, Begum et al. 2003; Katayama, Ogino et al. 2005; Ishikawa, Nakashiro et al. 2006); and in other high CXCR4-expressing carcinoma cell lines including salivary adenoid cystic (Muller, Sonkoly et al. 2006), thyroid (De Falco, Guarino et al. 2007), basal cell (Chen, Yu et al. 2006), renal (Schrader, Lechner et al. 2002) and non-small cell lung carcinoma (Su, Zhang et al. 2005).

Some cell lines (SV and FaDu) demonstrated CXCR4 mRNA expression at levels ten- to 20-fold higher than NOK with sqRT-PCR but only very low levels of CXCR4 surface protein levels were detected with flow cytometry. One explanation is that FaDu cells produce functional CXCR4 protein but this is only associated with the cell membrane at low levels. The presence of low (but detectable) cytoplasmic staining of CXCR4 protein in FaDu cells by immunocytochemistry supports this hypothesis and further investigation using confocal microscopy and co-staining with localising antibodies would provide more evidence. Samara et al have demonstrated functional responses to SDF stimulation (calcium flux and cell adhesion) by FaDu cells in-vitro despite the expression of low amounts of CXCR4 mRNA (Samara, Lawrence et al. 2004). This provides further evidence that mRNA expression levels are poorly predictive of cell behaviour. Functional assays involving the FaDu cell line analysed in my experiments would determine whether these findings could be replicated.

Muller et al showed small but detectable levels of CXCR4 protein with flow cytometry in OSCC cell lines with extremely low levels of mRNA expression (Muller, Sonkoly et al. 2006). Interestingly, despite surface protein labelling, these cells demonstrated no migratory response to SDF in Transwell chemotactic assays. There are several
mechanisms that add to the complexity between mRNA transcription and functional protein production. Firstly, abundant mRNA transcripts may not be translated; while another possibility is that CXCR4 undergoes a significant degree of post-translational control. Immunocytochemistry revealed that the OSCC cell lines expressing high amounts of CXCR4 mRNA also labelled positively for CXCR4 protein. Although this technique is not quantitative, there was a suggestion that low expressing cell lines stained less strongly for CXCR4 protein. However, care must be taken in extrapolating these results as the transcriptomes of different cell lines may differ significantly (as demonstrated by microarray analysis) and it is this difference, rather than that of CXCR4 mRNA abundance, that may determine CXCR4 protein production. A CXCR4-transfected cell line would permit the investigation of this further by reducing possible confounding (genomic) factors.

With regards to differences in post-translational control: this may result in CXCR4 protein not reaching the cell membrane, not reaching it in a functional form, reaching it in a functional form but dimerising into a configuration not amenable to antibody binding, or reaching it and immediately being endocytosed by either ligand-dependent or independent mechanisms. The staining of both the nuclear membrane and the cytoplasmic components of cells by anti-CXCR4 antibodies in permeabilised OSCC cells suggest that the protein is manufactured but not always associated with the cell membrane. Again, its location within cell compartments could be determined by confocal microscopy and co-labelling with localising antibodies.

While cells with little CXCR4 surface labelling may be responsive to SDF stimulation, the converse may also be demonstrated. CXCR4 protein associated with the cell membrane may be available for antibody binding but demonstrate no functional capacity. The hepatocellular carcinoma cell line Hep G2 stains positively for surface CXCR4 but fails to undergo phosphorylation and internalisation upon ligand binding (Mitra, De et al. 2001). Transfection of HepG2 with wild-type CXCR4 restored receptor function. Even among other hepatocellular carcinoma cell lines that label positively for surface CXCR4 with rapid protein translocation from the membrane and cytoplasm to the perinuclear region following stimulation with SDF, not all cell lines demonstrated changes in migration and proliferation (Schimanski, Bahre et al. 2006). Consequently, to determine the effect of CXCR4 mRNA expression on cellular responses to SDF
gradients, an experimental model was developed in which the overexpression of wild-type CXCR4 mRNA was achieved by transfection (Chapter 5).

In summary, established OSCC cell lines with high constitutive expression of CXCR4 mRNA also demonstrated protein labelling with antibodies. This localised to both the cell membrane and the perinuclear/cytoplasmic compartments. Conversely, cell lines with low CXCR4 mRNA expression demonstrated significantly less protein labelling, little of which was associated with the cell membrane. However, as demonstrated in the literature, surface protein labelling with monoclonal antibodies is poorly predictive of ligand-induced functional responses and these must be determined empirically (Chapters 6 & 7).

### 3.5.4 CCR7 mRNA expression and protein production by normal keratinocytes, OSCC and other carcinoma cell lines

The roles of the chemokine receptors CXCR4 and CCR7 in models of homeostatic leukocyte homing have been clearly defined. Consequently pioneering investigations of similar mechanisms in carcinoma metastasis focused on both these receptors. Results from my experiments demonstrated low levels of CCR7 mRNA by microarray and sqRT-PCR with no significant variation between normal and neoplastic-derived oral cell lines. Similar findings have been reported in other established OSCC cell lines (Uchida, Begum et al. 2003). While Muller et al demonstrated a wide variation in absolute levels of CCR7 mRNA expression in ten primary OSCC tumours, her group reported no surface CCR7 labeling revealed by flow cytometry but a positive signal from the intracellular compartment and positive cytoplasmic staining with immunocytochemistry (Muller, Sonkoly et al. 2006). I identified no CCR7 protein labeling on flow cytometry but some barely detectable perinuclear staining with immunocytochemistry. While it is possible that CCR7 mRNA upregulation and increases in surface protein levels could be induced in-vivo, it has been shown in the literature that stimulation with either growth factors or pro-inflammatory cytokines did not induce surface CCR7 in OSCC cell lines (Muller, Sonkoly et al. 2006).

Results from my experiments demonstrated low levels of CCR7 mRNA expression and no labeling of CCR7 protein. One methodological criticism of these experiments is the
lack of a positive control cell line. While an internal positive control was available for
sqRT-PCR investigation of CCR7, there was no external positive control for either these
or the protein assays. A Hodgkin lymphoma-derived cell line, in which CCR7
expression and surface protein are consistent features, has been described and would
be suitable if available (Hopken, Foss et al. 2002). Alternatively, CCR7-transfection of
an established OSCC cell line might demonstrate CCR7 protein production, although
this would be subject to the possible variation in post-translational control as discussed
above. However, CCR7 transduction of murine mammary carcinoma cells did result in
detectable protein production (Croci, Nicoletti et al. 2007).

The established OSCC cell lines investigated in these experiments did not demonstrate
significant CCR7 mRNA expression or protein labeling. However, CCR7 mRNA
expression in other epithelial tumours of the aero-digestive tract varies widely with
occasional cell lines demonstrating functional responses (Mashino, Sadanaga et al.
2002; Ding, Shimada et al. 2003). Interestingly, similar experiments have demonstrated
substantial increases in CCR7 mRNA expression in lymph node metastasis-derived cell
lines compared with those derived from their matched primary lesions (Muller,
Sonkoly et al. 2006). Indeed CCR7 expression has been shown to increase through
successive passages of cell lines through nude mice (Wang, Xi et al. 2005; Wang, Zhang
et al. 2005). These findings suggest that, while constitutive levels of CCR7 expression
may be low, they are upregulated during the process of lymph node metastasis.
However, what is not clear from these data is whether CCR7 upregulation facilitates
the progression of tumour cells to the lymph node, or whether CCR7 is induced by
factors within the lymphoreticular microenvironment. Data from leukocyte homing
support the former although the extrapolation of these to carcinoma metastasis must
be done with caution and further empirical evidence is desirable.

XCR1 was expressed at lower levels in all OSCC cell lines compared with normal oral
keratinocytes. These findings are consistent with observations in the literature which
suggest that XCR1 is rarely seen in tumour cells, predominantly due to its very limited
physiological expression profile (Zlotnik 2004). A more mechanistic hypothesis would
be that tumours downregulate XCR1 in order to remain unresponsive to its ligand
XCL1 (Lymphotactin). XCL1 is constitutively produced by gamma-delta T-
lymphocytes that reside in the epithelial compartment and bear a dendritic phenotype
(Boismenu, Feng et al. 1996). In this way, downregulation of XCR1 would reduce
tumour cell responses to lymphocyte-derived XCL1 and facilitate evasion of the host lymphocytic response. In reality, it is likely that this hypothesis is over-simplistic. XCL1 production by intraepithelial lymphocytes attracts neutrophils and natural-killer cells that express the XCR1 receptor (Huang, Li et al. 2001). Consequently, it may be a reduction in XCL1 production (rather than XCR1) that is important in evading the anti-tumour host response. This is supported by evidence in solid tumours including myeloma where XCL1 transfection of tumour cells induced a dense neutrophil infiltration and lymphocyte-mediated tumour regression (Cairns, Gordon et al. 2001). Interestingly, my results showed that XCL1 was expressed at high levels by two OSCC cell lines (CAL27 and FADU) and at low levels by another two (SCC4 and SCC25). It is possible to hypothesise that the former cell lines may have attracted a denser neutrophil infiltrate and greater lymphocyte-mediated tumour regression in-vivo than the latter pair.

Together these results suggest that, even in cells with high expression of XCL1, there is low receptor expression and an autocrine pathway is not supported at an mRNA level. The role of the Lymphotactin-XCR1 axis has not been investigated in squamous cell carcinoma, particularly of the head and neck. While this line of study would indeed be interesting, it lies outside the scope of this project.

### 3.6 Conclusions

The results presented in this chapter show that some (but not all) established OSCC cell lines constitutively produce CXCR4 (but not CCR7) mRNA and surface protein. It was also observed that surface protein levels were higher in cell lines expressing greater amounts of CXCR4 mRNA. Following these preliminary findings in observational studies it was decided to focus investigation on the chemokine receptor CXCR4. It was hypothesised that the abundance of CXCR4 mRNA was a key determinant of surface protein levels and function. Consequently, CXCR4 transfection of a constitutively low expressing established OSCC cell line was developed to test this (Chapter 5). Functional assays were also performed to determine the response of constitutively high-expressing and CXCR4-transfected cells to SDF gradients (Chapters 6 and 7). However, the biological relevance of these in-vitro assays would only be confirmed if CXCR4 protein was observed in OSCC tumourigenesis in-vivo. The
strongest mechanistic evidence for this would come from animal models (although this could not be justified at this stage without further preliminary data). Consequently it was decided to do further observational investigation of CXCR4 in OSCC clinical specimens (Chapter 4).
Chapter 4:

CXCR4 protein production in OSCC clinical specimens
4.1 Introduction

Evidence from the literature has demonstrated a role for CXCR4 and CCR7 in the selective homing of leukocytes. Animal studies have also shown that these chemokine receptors may play a part in the metastatic mechanisms of certain carcinomas. Results presented in the previous chapter have demonstrated that CXCR4 (but not CCR7) protein is constitutively produced on the surface of cultured OSCC cells. However, the distribution of CXCR4 surface protein in-vitro was found to be heterogeneous with only a minority of cells staining positively despite the relative upregulation of CXCR4 mRNA expression compared with NOK. Although derived from clinical specimens, it is not clear from in-vitro data alone whether the behaviour of these cell lines reflects the in-vivo situation, particularly as the tissue/tumour microenvironment is impossible to replicate under culture conditions. Consequently, before undertaking assays of cell function, it was important to determine if CXCR4 staining was demonstrated in clinical specimens. This could potentially be achieved using a number of complimentary techniques including immunohistochemistry, in-situ hybridisation and laser-capture microdissection with (multiplexed) sqRT-PCR. By far the simplest of these was immunohistochemistry and, once published experimental protocols could be validated empirically, this was a useful technique to produce qualitative data on the distribution of CXCR4 protein within clinical specimens derived from archived histopathological specimens in which the lymph node status was known.

To achieve this, samples were taken from archived, formalin-fixed, primary resection specimens and investigated with immunohistochemistry. Lymph node status was defined by histological examination of the accompanying modified radical neck dissection using conventional, light microscopy based techniques. However, the limitation of comparing results between specimens using this technique was quickly identified and a novel, quantitative assessment of staining using tissue arrays was developed, validated and used.
4.2 Chapter hypothesis

Increased CXCR4 protein production in primary OSCC clinical samples is positively associated with histopathological observation of cervical lymph node metastases.

4.3 Chapter Aims and Objectives

4.3.1 Overall Aim

To determine the pattern of CXCR4 production in clinical patient samples.

4.3.2 Specific Objectives

1. To develop a quantitative technique for the assessment of CXCR4 immunohistochemistry staining of clinical patient samples.
2. To investigate the relationship between clinico-pathological factors and CXCR4 staining of the nucleus and/or cytoplasm.
3. To explore the association between CXCR4 protein expression and lymph node metastasis in head and neck squamous cell carcinomas.

4.4 Results: CXCR4 protein staining in clinical specimens.

4.4.1 Overview

Initial experiments were performed to establish the validity and reproducibility of the CXCR4 immunohistochemistry protocol. Following this, representative sections from primary OSCC lesions and their associated lymphatic metastases were then compared. Finally, a novel quantitative method of analysing staining intensity was developed, validated and used to investigate numerous specimens from squamous carcinomas arising in the head and neck (predominantly originating in the oral cavity and oropharynx).
4.4.2 Preliminary investigations

Initially a series of CXCR4 positive control sections of human tonsil were prepared and various methods of antigen retrieval were examined empirically. This included enzyme digestion, heating in a variety of buffer solutions and microwaving. A tightly defined experimental protocol was derived and used in all subsequent experiments. Preliminary experiments were carried out with tissue blocks selected from the clinical resection specimens of ten patients. Each specimen comprised of matched primary tumour and lymph node metastases excised from the same patient in continuity. Clinicopathological features of the specimens examined are outlined in Table A2.3 (Appendix 2). Where available, an examination of matched, adjacent, histologically normal mucosa was also performed. Representative results and a discussion of these preliminary findings are presented in Section A4.1 (Appendix 4).

The collective results from these preliminary investigations suggested that more CXCR4 protein was found in the cytoplasmic compartment of lymph node metastases compared with tumour cells from the matched primary lesions. Where available, comparison with adjacent, histologically normal oral mucosa suggested that CXCR4 staining was more intense in OSCC cells and that CXCR4 staining occurred in both primary OSCC lesions and their associated lymph node metastases. The limitations of qualitative, immunohistochemical data are that results are not easily collated and batch sizes are often small. Therefore, to address these issues, a method by which immunohistochemical slides could be prepared in batches and analysed quantitatively was devised.
4.4.3 Quantitative determination of CXCR4 expression in OSCC primary tumours

4.4.3.1 Overview

The aim of these experiments was to compare the CXCR4 protein distribution in a series of OSCC clinical specimens derived from a number of different patients. In order to achieve a meaningful comparison between specimens two criteria had to be fulfilled. Firstly, specimen preparation had to be identical in order to standardise antigen retrieval and antibody binding. This was addressed using tissue arrays. Secondly, a quantitative method of assessing staining intensity was developed, validated and used to reduce inter-observer error in the assessment of staining patterns.

4.4.3.2 Validation of the technique

The method of immunohistochemistry analysis (as outlined in Section 2.7 and used in the preliminary experiments described in Section A4.1, Appendix 4) was optimised empirically. Commercially available head and neck SCC tissue arrays were then taken and stained for CXCR4 protein with post-hoc processing as outlined in Section A4.2 (Appendix 4). Briefly, saturation measurements at nine standardised areas across the field of view were taken and the mean (standard error) values calculated. Yellow saturation values were found to be the most sensitive marker of peroxidase activity. Results from samples with two replicates per array were compared, demonstrating a high level of concordance for both the nuclear and cytoplasmic compartments. A random subset of 20 images were analysed on two separate occasions with a high concordance between results.

4.4.3.3 Results of quantitative immunohistochemical analysis

Only tissues from primary lesions were represented on the tissue arrays. Pathological lymph node status was determined for each sample from the accompanying detailed
histology reports. A summary of clinicopathological variables for each specimen represented on the array is given in Table 4.1. Their distribution on the array is shown in Figure A2.1 (Appendix 2). Values for nuclear and cytoplasmic staining by tissue type were obtained and plotted (Figure 4.1). Each scatterplot point (panels a)i), b)i) and c)i)) represents the mean (95% confidence interval) for the measured cytoplasmic and nuclear staining intensity (horizontal and vertical axes respectively). The bar charts (panels a)ii), b)ii) and c)ii)) represent the mean (standard deviation) cytoplasmic and nuclear staining intensity (light and dark bars respectively) for each specimen according to the tissue and nodal status. All statistical comparisons were performed using the Student t-test. Relationships that failed to reach statistical significance were not highlighted.

For the seven specimens of lip tissue represented on the array (Figure 4.1 panels a)i) and a)ii)) the CXCR4 staining pattern was consistently different in primary lesions that had pathological evidence of lymph node metastasis (n=2) compared with tumours that had not spread (n=4). These clusters were also distinct from the one specimen of normal lip mucosa represented on the array. Primary lip lesions with pathological evidence of CLN metastases (pN+) demonstrated significantly more cytoplasmic CXCR4 staining (p<0.05), but significantly less nuclear staining (p<0.001) compared with primary lip lesions without pathological evidence of CLN spread (pN0). However, statistical comparisons in this small subset of lesions must be interpreted with caution and validation of these changes was investigated with a larger sample of OSCC lesions.

In total 26 specimens of oral tissue were represented on the array (Figure 4.1 panels b)i) and b)ii)). A clustering of normal (n=6), metastatic (n=11) and non-metastatic (n=9) tissue samples was demonstrated. Normal oral tissues generally had the lowest intensity of both nuclear and cytoplasmic staining. Compared with normal mucosa, primary OSCC lesions without lymphatic spread (pN0) demonstrated significantly more nuclear CXCR4 staining (p<0.01). Primary OSCC lesions with pathological evidence of CLN metastases (pN+) demonstrated significantly more nuclear and

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39 In 23/31 (75%) tumour specimens represented on the array the staging information was derived from histological reports following neck dissection. On the remainder the staging was from a combination of clinical and radiological findings (Table 4.1).
40 The methodologies used are outlined in Section 2.7 (Chapter 2) & Section A4.2 (Appendix 4).
41 According to the ICD-10 definitions outlined in Appendix 1.
cytoplasmic staining of CXCR4 compared with normal controls (p<0.01 & p<0.05 respectively). In a similar pattern to the lip lesions described above, primary pN+ OSCC lesions showed significantly more cytoplasmic CXCR4 staining (p<0.05) compared with primary pN0 lesions. Only one pN0 tumour clustered in the middle of the lymph node positive group and this was a moderately differentiated pT2 lesion of the floor of the mouth.

Thirty one SCCs from all sites in the head and neck (with eight matched normal controls) were represented on the array (Figure 4.1 panels c)i) and c)ii)). A similar clustering pattern was observed to that described above for OSCC lesions. Interestingly, three of the four outlying specimens from the pN0 cluster comprised laryngeal, oesophageal and lip carcinomas. Overall the pattern of staining intensities for normal, pN0 and pN+ HNSCCs was identical to that of OSCC described above. Particularly the intensity of cytoplasmic staining in primary pN+ HNSCC lesions was significantly greater (p<0.01) than for primary pN0 HNSCC lesions.

CXCR4 staining intensity data was reformatted according to disease stage (Figure 4.2, panels a)i) and a)ii)) and tumour size (Figure 4.2, panels b)i) and b)ii)). Clusters were tighter based on disease stage (panel a)i)) compared with size of the primary tumour (panel b)i)). Significantly more nuclear staining was observed for all disease stages (1-4) compared with normal controls (p<0.05). No significant differences in nuclear staining were demonstrated between disease stages. Cytoplasmic staining levels were similar in Stage 1 and Stage 2 disease (compared with normal controls). However, the intensity of cytoplasmic staining was significantly greater in Stage 3 and Stage 4 disease compared with the other groups (p<0.05). Nuclear staining was significantly greater for all primary tumour sizes (T1-T3) compared with normal controls (p<0.05). Only T3 lesions showed significantly increased cytoplasmic CXCR4 staining compared with normal, T1 and T2 lesions. Although it was tempting to analyse trends of staining intensity associated with primary tumour site and grade of histological differentiation, there were insufficient replicates on the array to control for the important confounding variables of tumour size and nodal status.

42 Including all OSCCs and SCCs of the upper oesophagus, larynx and lip
43 For disease stage and tumour size statistical comparisons were performed using ANOVA with post-hoc correction for multiple testing.
44 No statistical comparison was possible for T4 tumours as only one T4 oral cavity SCC was represented on the tissue array.
A qualitative assessment of the CXCR4 staining patterns of OSCC lesions on the tissue arrays was also made (Figure 4.3). This revealed that a homogeneous pattern was seen most often (panels b and d). The distribution was also occasionally heterogeneous (panels a and c). This was consistent for both the cytoplasmic and nuclear compartments. Subjective assessment of laryngeal lesions (Figure 4.4) only revealed heterogeneous staining (panels a and b). However, variability in both nuclear and cytoplasmic staining strength was seen (panels c and d) with the tendency that lesions with evidence of lymphatic metastases demonstrating a greater intensity of CXCR4 staining (panels b and d).

4.4.4 Summary of experimental results

1. Qualitative assessment appeared to demonstrate more CXCR4 protein labeling in the cytoplasmic compartment of lymphatic metastases compared with tumour cells from matched primary lesions (Section A4.1, Appendix 4).

2. Quantitative assessment of CXCR4 staining in tissue sections was reproducible and concordant between matched samples.

3. An increased intensity of CXCR4 staining (particularly cytoplasmic) in OSCC primary lesions was associated with pathological confirmation of lymph node metastases.
Table 4.1: Summary of clinicopathological features for each specimen represented on the tissue arrays

<table>
<thead>
<tr>
<th>Site</th>
<th>Age (years)</th>
<th>Sex</th>
<th>T Status</th>
<th>N Status</th>
<th>Method of Staging</th>
<th>Disease Stage</th>
<th>Normal Control Available</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buccal</td>
<td>50</td>
<td>M</td>
<td>1</td>
<td>0</td>
<td>CL</td>
<td>1</td>
<td>Y</td>
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Legend: M = male, F = female, ND = neck dissection, CL = clinical
Figure 4.1: CXCR4 staining intensity in SCC tissue array samples by tissue type and lymph node status. Lip SCC (a), oral SCC (b) and all HNSCCs (c). For details see text. Legend N0 = CLN negative, N+ = CLN positive, *= p<0.05, **= p<0.01, ***= p<0.001.
Figure 4.2: CXCR4 staining intensity in oral SCC tissue array samples by disease stage and by primary tumour size. SCC lesions from the oral cavity and matched normal controls were stained for CXCR4 and colour saturation data were collected blind to tissue of origin as described in Section 2.7. Mean values (points) and 95% confidence intervals (error bars) were plotted by disease stage (a)i) and tumour size (b)i). Mean (standard deviation) were calculated and plotted for cytoplasmic (light bars) and nuclear (dark bars) for each group of replicates represented. Statistical comparisons were performed using ANOVA. Results reaching significance (compared with normal controls) are highlighted. Legend: * = p<0.05.
Figure 4.3: CXCR4 immunohistochemistry of OSCC tissue array specimens. Tissue arrays were taken and prepared as outlined in Section 2.7. Immunohistochemistry was performed with monoclonal antibodies to CXCR4 (a-d) and isotype controls (e & f). Sections of a pT1N0M0 SCC from the molar alveolus (a, c & e) and a pT1N0M0 buccal SCC (b, d & f) are shown. Staining patterns in OSCC lesions were variably heterogeneous (a & c) or homogeneous (b & d). Variation in the intensity of nuclear (c) and cytoplasmic (d) staining was also demonstrated. Isotype antibody controls demonstrated no non-specific binding (e & f). Scale bar represents 250µm (a, b & e) or 50µm (c, d & f).
Figure 4.4: CXCR4 immunohistochemistry of laryngeal SCC tissue array specimens. Tissue arrays were taken and prepared as outlined in Section 2.7. Immunohistochemistry was performed with monoclonal antibodies to CXCR4 (a-d) and isotype controls (e & f). Sections of a pT4aN0M0 SCC (a, c & e) and a pT4N2cM0 SCC (b, d & f) from the larynx are shown. CXCR4 staining patterns in laryngeal SCC lesions varied in intensity (a-d) in both the cytoplasmic (c) and nuclear (d) compartments. Isotype antibody controls demonstrated no non-specific binding (e & f). Scale bar represents 250µm (a & b) or 50µm (c-f).
4.5 Discussion

Before interpreting these results it is important to analyse the strengths (and weaknesses) of the experimental methodologies used. These are discussed in Appendix 4. Due to numerous methodological inconsistencies within the literature, comparison of my results with those described in the literature must be performed with caution. For completeness (and where possible) the relevant methodologies are summarised as footnotes.

My results support the hypothesis that CXCR4 surface protein is produced at very low levels by occasional basal keratinocytes. Matched normal controls represented on the tissue arrays demonstrated weak nuclear and cytoplasmic staining for CXCR4. Similar findings have been reported for oral mucosa (Delilbasi, Okura et al. 2004; Moutsopoulos, Nares et al. 2007), prostatic epithelia (Sun, Wang et al. 2003) and in a proportion of normal breast epithelia by some (Salvucci, Bouchard et al. 2006) but not other authors (Kato, Kitayama et al. 2003). However, other groups have reported no CXCR4 staining in normal oral mucosa (Katayama, Ogino et al. 2005). The reasons for these apparent discrepancies are not clear. Artefactual explanations include variations in experimental technique, particularly antigen retrieval and staining protocols. One criticism of numerous published series is that they fail to include the appropriate positive and negative controls in order to exclude these confounding factors. Extravasated CXCR4-positive leukocytes within the epithelial compartment may be mistaken for keratinocyte labelling. This could be addressed by the careful observation of cell morphology by experienced pathologists or the use of co-labelling for leucocyte-specific surface markers.

My examination of normal oral mucosa revealed focal staining of intra-epithelial cells with leukocyte morphology, as well as endothelial cells and occasional basal keratinocytes. Similar findings have been described by other groups (Delilbasi, Okura et al. 2004; Moutsopoulos, Nares et al. 2007) although not universally so (Katayama, Ogino et al. 2005). CXCR4 staining has been demonstrated in all layers of normal oral epithelium except for the lamina propria (Jotwani, Muthukuru et al. 2004). In patients with chronic periodontitis, CXCR4-positive cells were also observed in the lamina propria although no consistent relationship between CXCR4 staining and inflammation was described (Jotwani, Muthukuru et al. 2004). However, this
association does not explain the finding of CXCR4-labelled basal keratinocytes. One possibility is that cells in different niches within the epithelial structure demonstrate different phylogenetic properties including some with stem cell characteristics (Mackenzie 2005). In oral mucosa, epithelial stem cell populations localise to the rete ridges and are thought to be more autonomous than other epithelial stem cells which are more dependent on cell-to-cell interactions with non-epithelial cells (Tudor, Locke et al. 2004). While definitive oral epithelial stem cell markers remain elusive, the SDF-CXCR4 axis has been implicated in many pathways associated with stem cell activity. (Kucia, Reca et al. 2005; Mackenzie 2005). The transmission of the X4 strain of the HIV-1 virus across intact epithelial surfaces facilitated by the CXCR4 surface protein coreceptor provides further (indirect) evidence for CXCR4 production in oral mucosa. Real variation in CXCR4 expression within normal epithelia may be due to true differences in expression in anatomically distinct sites, or potentially secondary to responses to the local tissue microenvironment (such as antigenic or pro-inflammatory stimuli). The lack of consistent data in the literature points towards the need for further research.

My results demonstrate surface CXCR4 staining in primary OSCC lesions. However, the hypothesis that CXCR4 protein production is associated with malignant transformation is not supported by the observation of similar levels of cytoplasmic staining in normal and pTxpN0 lesions. Staining of primary tumour cells was heterogeneous and findings from cultured OSCC cell lines also demonstrated that CXCR4 surface labelling was not ubiquitous. It is possible that differences between cells within the same primary tumour or established cell line are attributable to genetic instability within individual cells. Alternatively CXCR4 production may be induced by factors within the extracellular microenvironment. While staining appeared unrelated to the proximity to the invasive front there was an appreciable difference in staining intensity in lymph node metastases (compared with their matched primary tumours). It is not clear from these observational data at which point along the metastatic pathway is CXCR4 upregulated. While some authors have commented on increased CXCR4 labelling at the invasive front, this area was not clearly represented in sufficient numbers of my specimens to be able to make a valid judgement (Ishikawa, Nakashiro et al. 2006; Muller, Sonkoly et al. 2006). Recent evidence from pancreatic adenocarcinoma reveals a subpopulation of CXCR4-positive cancer stem cells at the invasive front (Hermann, Huber et al. 2007). Abrogation of this subpopulation
significantly reduced metastases without affecting tumourigenicity, suggesting that CXCR4-mediated signalling is an early event in the metastatic pathway.

CXCR4 staining was observed in all malignant specimens represented on the tissue arrays. However, the strength of cytoplasmic staining in pN0 oral lesions was statistically indistinguishable from that of normal matched controls. Therefore, when comparing to similar reports in the literature, the investigation of matched controls is of paramount importance. However, for the reasons outlined in Appendix 4, direct comparison between different batches is subject to numerous potential methodological inconsistencies.

The frequency of positive CXCR4 labelling of primary OSCC lesions reported in the literature varies from 29 to 100% (for full references see Table 1.4, Chapter 1). One explanation could be the different methodologies employed. However, it is interesting to note that groups reporting weak staining of normal tissues demonstrated the highest frequencies (57 to 100%) of positive CXCR4-labelling of primary OSCC lesions (Almofti, Uchida et al. 2004; Delilbasi, Okura et al. 2004). Conversely, authors that described no CXCR4 staining of normal mucosa reported less frequently stained primary OSCC specimens (29 to 34%) (Katayama, Ogino et al. 2005; Ishikawa, Nakashiro et al. 2006; Muller, Sonkoly et al. 2006). This observation is independent of the experimental method and assessment criteria used by each author and suggests that the robust comparison between these series is extremely difficult.

Cytoplasmic CXCR4 staining in the primary lesions of pN+ specimens was significantly greater compared with pN0 samples. This statistically significant relationship was observed independently in groups of lip, oral cavity and all head and neck SCCs on the array. The relationship between cytoplasmic staining intensity in

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45 Sections were defined as positive if more than 25% of tumour cells stained for CXCR4 or SDF respectively. Sections with absent or less than 25% staining were classified as negative (Almofti, Uchida et al. 2004).
46 The method by which staining was assessed and graded was not described in the paper (Delilbasi, Okura et al. 2004).
47 Positive CXCR4 staining was defined as more than 10% of tumour cells demonstrating membrane and cytoplasmic staining as determined by one observer (Ishikawa, Nakashiro et al. 2006).
48 CXCR4 staining was categorised as strong, moderate, weak or absent as compared with staining in interstitial infiltrates by three independent, blinded observers (Katayama, Ogino et al. 2005).
early (Stage 1 and 2) and late (Stage 3 and 4) disease was significantly different. Considering early stage disease is (by definition) lymph node negative, this finding adds further weight to the hypothesis that lymph node status is the clinicopathological variable most closely associated with cytoplasmic CXCR4 production. There were insufficient samples on the array to investigate the relationship between tumour size (T stage) and CXCR4 staining while controlling for the confounding variable of nodal status. This may explain the increased nuclear and cytoplasmic staining demonstrated with increasing T stage. Considering that metastatic cells are ultimately derived from the primary focus, this association provides evidence (albeit indirect) supporting the hypothesis that CXCR4 mediates lymphatic metastasis in OSCC. This is in agreement with the consensus of the published literature on OSCC (Almofti, Uchida et al. 2004; Katayama, Ogino et al. 2005; Ishikawa, Nakashiro et al. 2006). Only one group found no consistent relationship between CXCR4 or CCR7 staining in primary lesions and lymph node metastases (Muller, Sonkoly et al. 2006). It is important to note that the statistical comparison performed in my results differs considerably from other authors. My findings demonstrate a higher intensity of CXCR4 staining within the primary lesions with lymph node metastases compared to those without. The staining intensity was measured quantitatively and compared statistically using tests based on the normal distribution. In this way bias was reduced and the data generated was more reliable and robust. Other authors have forced observations into a dichotomy and performed statistical comparisons on these ordinal outcomes. Together, these data suggest that lymph node metastasis is more common from primary OSCC lesions that demonstrate higher levels of cytoplasmic CXCR4 staining, although this association is not ubiquitous. What these simple analyses do not account for are the (biologically relevant) factors such as receptor density, functionality and heterogeneity amongst cells. A more mechanistic approach in-vitro is required to address these questions and this is presented in Chapters 6 and 7.

In a very large series of breast carcinomas, no clear association between either nuclear or cytoplasmic positivity for CXCR4 and lymph node metastases was demonstrated (Salvucci, Bouchard et al. 2006). Statistically significant association has only been shown in cervical adenocarcinoma and colorectal carcinomas (Yang, Lee et al. 2007;
Yoshitake, Fukui et al. 2008). This may reflect differences in tumour biology between these epithelial carcinomas particularly given the not infrequent clinical observation of widespread breast cancer metastasis without lymphatic involvement (vide infra).

Overall, CXCR4 cytoplasmic staining was greater in primary lesions that demonstrated lymph node metastases compared with those that did not. From the preliminary examination of matched primary and secondary lesions, a tendency towards denser staining in the metastatic cells within the lymph nodes was observed, although this could not be examined statistically. The lymphoreticular stroma constitutively produces SDF and this may induce, as well as bind with, tumour CXCR4 thereby initiating a paracrine stimulation pathway. Co-localisation of SDF and CXCR4 staining has been demonstrated in up to 47% of cases conferring a spindle-shaped morphology to the tumour cells (Uchida, Onoue et al. 2007). Further evidence has suggested that this autocrine SDF/CXCR4 pathway mediates the proliferation and motility of OSCC lymphatic metastases in-vivo. Co-localisation of SDF and CXCR4 has also been associated with lymph node metastasis in oesophageal and colorectal carcinomas (Sasaki, Natsugoe et al. 2008; Yoshitake, Fukui et al. 2008). This may be important in primary lesions where SDF is not constitutively expressed, but in the lymph node parenchyma, SDF is widely produced and may form a chemotactic gradient towards the periphery of developing lesions.

As discussed in the Chapter 3, the cellular location of CXCR4 staining may be significant. Only protein associated with the cell membrane will be exposed to extracellular ligand. The process of ligand-mediated receptor internalisation may sequester functional protein within the intracellular compartments and CXCR4 has also been shown to undergo oligomerisation and internalisation without the presence of SDF (Babcock, Farzan et al. 2003). Furthermore it has been shown that endogenous SDF binds to newly synthesised CXCR4, inhibiting its translocation to the cell surface (Zeelenberg, Ruuis-Van Stalle et al. 2003). Conversely, CXCR4 protein within the cell may not be functional. This may be due to intrinsic deficiencies preventing function or the inability to access the cytoplasmic membrane. In arrestin-deficient cells, chemokine

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49 Multivariate analysis included age (>50 years), primary tumour size, depth of stromal invasion, lymphovascular invasion and histological grade (Yang, Lee et al. 2007).
50 Positive SDF staining was defined as over 10% of >1,000 tumour cells in 10 high-power fields as determined by two independent, blinded observers (Sasaki, Natsugoe et al. 2008).
receptors are unable to recycle to the cell surface and are held within the perinuclear recycling compartments (Vines, Revankar et al. 2003). Consequently, one criticism of the literature is the inference that cellular responses to SDF are defined by the density of CXCR4 staining of the cell membrane. Peri-nuclear staining may reflect receptor internalisation and, therefore, may be a better indicator of CXCR4 activation than cell membrane staining. Strategies to address this include the analysis of CXCR4 staining in light of proxy markers of tumour behaviour. For example, in a small series of randomly selected OSCC cases, positive CXCR4 staining was demonstrated on the cell membrane or in the cytoplasm in 15 out of 26 (58%) cases (Hong, Pai et al. 2009). The PCNA labelling index was significantly higher in the CXCR4-positive group.

What is the mechanism of CXCR4 nuclear staining? If it is related to endogenous SDF release and CXCR4 internalisation then one might expect to see a difference between tumour cells that do and do not produce SDF. This is not the case. Primary colorectal carcinomas in which nuclear CXCR4 staining was observed were associated with metastatic lymph node deposits demonstrating cytoplasmic staining (Yoshitake, Fukui et al. 2008). This could reflect a shift between tumour/tissue microenvironments, possibly mediated by hypoxia-related or pro-inflammatory factors. My data on OSCC matched primary/lymph node secondary specimens were insufficient to produce a valid assessment. This would need to come from investigation of a tissue array containing an appropriate number of suitably matched specimens.

A number of authors have demonstrated a statistically significant association between increased CXCR4 staining of primary OSCC lesions and poor clinical outcome (Almofti, Uchida et al. 2004; Katayama, Ogino et al. 2005; Ishikawa, Nakashiro et al. 2006; Lee, Jin et al. 2009). However, multivariate analysis was employed in only two of these series to attempt to control for confounding variables (Katayama, Ogino et al. 2005; Lee, Jin et al. 2009).51 In these cases, positive CXCR4 staining of the primary lesion was associated with significantly reduced disease-specific survival. Nevertheless, when calculated from the raw data, the Positive Predictive Values (PPV) for CXCR4 staining the clinical endpoints of lymph node metastasis, tumour

51 Multivariate analysis by one group included age (>60yrs), male gender, primary site (floor of mouth), poor tumour differentiation, advanced clinical stage (III & IV) (Katayama, Ogino et al. 2005). The other group included the variables of primary size, lymph node metastasis, recurrence and high MMP-9 expression (Lee, Jin et al. 2009).
recurrence, Stage IV disease and five-year survival were 66%, 66%, 66% and 57% respectively (Almofti, Uchida et al. 2004). In short, based on these data, CXCR4 status was only marginally better than chance at defining clinically important outcomes.

The meaningful association of SDF/CXCR4 staining patterns with clinico-pathological variables is fraught with methodological difficulties. The majority of studies in the literature have not been powered sufficiently to determine any associations, particularly accounting for confounding variables associated with survival such as tumour stage (Scotton, Wilson et al. 2002; Burger, Glodek et al. 2003; Kato, Kitayama et al. 2003; Sun, Wang et al. 2003; Yasumoto, Koizumi et al. 2006; De Falco, Guarino et al. 2007; Wen, Zhu et al. 2007; Yang, Lee et al. 2007; Zhang, Lu et al. 2007; Kajiyama, Shibata et al. 2008; Sasaki, Natsugoe et al. 2008; Yoshitake, Fukui et al. 2008). These studies fail at two separate levels: firstly, inherent variability in the experimental and assessment methodologies used precludes meaningful interpretation of the data. Secondly, the arbitrary numerical data produced in this way do not demonstrate a likelihood of a particular finding occurring at a frequency that was significantly different to chance alone.

Interestingly, in a large series of breast carcinomas, cancers showing positive cytoplasmic CXCR4 staining were more likely to be high grade, hormone receptor negative and to overexpress HER2 (Salvucci, Bouchard et al. 2006). A mechanism by which HER2-mediated breast carcinoma metastasis is dependent on CXCR4 upregulation has been postulated (Li, Pan et al. 2004). High nuclear and high cytoplasmic CXCR4 staining were independently associated with significant reductions in tumour-specific patient survival of 46 and 70% respectively. These associations were independent of other predictors of prognosis. The relationship between increased cytoplasmic staining and decreased survival was strongest in the groups of patients who were lymph node negative or had not undergone chemotherapy. It can be inferred from these observations that, in primary breast carcinoma, surface expression of CXCR4 may be more important in mediating systemic

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52 Positive Predictive Values were calculated from the raw data by the formula (True Positives)/(True Positives + False Positives).
53 Multivariate analysis included age, pT stage, BRE grade, number of positive lymph nodes, oestrogen receptor (ER) and progesterone receptor (PR) status (Salvucci, Bouchard et al. 2006).
metastases (and consequently patient demise) compared with loco-regional spread. This is particularly seen in patients who do not undergo systemic chemotherapy. These clinical observations are in concordance with *in-vivo* studies that demonstrate the role of CXCR4-mediated metastatic mechanisms in murine models of breast carcinoma (Muller, Homey et al. 2001).

One criticism of my preliminary work was the lack of pN0 specimens examined. Although this was addressed with the tissue array experiments, it should be noted that even detailed histological examination cannot guarantee the absence of micrometastases. In the clinical situation, these tiny foci of metastatic cells have debatable prognostic significance (Woolgar 1999). However, for the evaluation of metastatic mechanisms, the ability of cells to break away and form new colonies is hugely important. While the staging of the tissue arrays was done on histological examination of functional neck dissections, it is not known how assiduous the pathologists had been when examining for micrometastases. In a study on oesophageal squamous carcinoma, eight out of 46 (17%) specimens assessed as lymph node-negative by H&E staining had detectable micrometastases by cytokeratin staining and all of these were CXCR4 positive (Sasaki, Natsugoe et al. 2008). Not only does this highlight the limitations of current pathological investigations (developed to inform clinical decision making), but it also raises the intriguing observation of CXCR4-positive lymphatic micrometastases within SDF-rich lymph node parenchyma. It is not known from this “snapshot” view whether these were lesions that were actively growing, or if they had entered a quiescent state. This could be tested by a number of complex experimental methodologies including serial animal studies or intra-vital microscopy. Overall, these data are consistent with the hypothesis that CXCR4 is not the sole mediator of metastatic progression, but rather one implement in the molecular “toolbox” that mediates tumour dissemination *in-vivo*. 

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4.6 Conclusions

Investigation of clinical specimens offers a unique insight into the location of CXCR4-producing cells in-vivo. These findings presented in this chapter were consistent with the overall hypothesis that the SDF/CXCR4 axis plays a role in OSCC metastasis. However, data derived from observational studies of clinical specimens serve predominantly to inform in-vitro and, particularly, in-vivo experiments. The localisation of a single protein belies the complexity of the tumour/tissue microenvironment. Nevertheless, if CXCR4 had not been detected on primary or secondary tumour deposits in clinical specimens then its role in OSCC metastasis in-vivo would have been questionable regardless of the findings from in-vitro studies. These data did demonstrate CXCR4 in OSCC clinical samples and the next aim was to investigate the role of the molecule in functional assays of cell activity associated with tumourigenesis. In order to achieve this, an appropriate experimental model was created by overexpressing CXCR4 mRNA in an established OSCC cell line in-vitro.
Chapter 5:
Establishing a stable, CXCR4-transfected OSCC cell line
5.1 Introduction

Higher levels of CXCR4 mRNA have been shown in some, but not all, established OSCC cell lines (Chapter 3). In cell lines with the highest expression levels, associated CXCR4 surface protein was also demonstrated. This was in contrast to NOKs in which both CXCR4 mRNA and surface protein was barely detectable. CXCR4 protein was demonstrated in archived clinical OSCC samples (Chapter 4). The pattern of staining intensity appeared to correlate with histologically-confirmed lymphatic metastases. Together these data support the hypothesis that CXCR4 is associated with OSCC metastasis.

Observational data can only provide evidence of association rather than causation. The ability to over-express CXCR4 mRNA in cells that constitutively produce the protein at very low levels would provide a useful experimental model in which the effect of CXCR4 gene upregulation on cell phenotype could be tested. In this way, one element of the observed differential gene expression resulting from carcinogenesis can be replicated in-vitro. Following preliminary in-vitro studies this may then be translated into animal models to determine the effects of single gene over-expression in-vivo.

Genetic manipulation of cell lines can be achieved in a number of ways. Insertion of a single coding sequence immediately downstream of a known promoter provides the most reliable mechanism of stable changes in mRNA upregulation. Other strategies exist for the downregulation of genes, predominantly through the use of small interfering RNA (siRNA) species. These are not applicable to gene overexpression. Despite the association of CXCR4 expression with a number of solid and haematological malignancies, there have been remarkably few reports on CXCR4-transfection of carcinoma cell lines. This chapter documents the development and investigation of the transfection of a constitutively low-expressing established OSCC cell line with CXCR4.
5.2 Chapter Hypotheses

Stable over-expression of CXCR4 mRNA in a constitutively low-expressing established OSCC cell line results in significantly increased surface CXCR4 protein production.

5.3 Chapter Aims and Objectives

5.3.1 Overall Aim

To produce an established OSCC cell line that produces functional surface CXCR4 protein at high levels.

5.3.2 Specific Objectives

1. To select an established OSCC cell line that constitutively expressed CXCR4 mRNA at very low levels.
2. To transform an expression vector with the wild-type CXCR4 insert in the correct alignment.
3. To transfected the H357 cell line with the CXCR4-containing vector along with appropriate controls.
4. To isolate and expand positive clones using antibiotic selection.
5. To establish the CXCR4 mRNA and protein levels produced by clones and their controls.

5.4 Results

5.4.1 Overview

The aim of these experiments was to perform a stable CXCR4 transfection of an OSCC cell line that constitutively expressed the gene at low levels. The H357 cell line was selected. Data presented in Chapter 3 demonstrated that untransfected H357 cells expressed low levels of both CXCR4 mRNA (as determined by microarray and sqRT-
PCR) and CXCR4 surface protein (as determined by flow cytometry analysis and immunocytochemistry).

The methodology employed is described in Section 2.4. A brief outline of the steps performed is given below:

1. Isolation of the CXCR4 coding sequence
2. Transformation of competent cells and clonal expansion
3. Clonal screening for presence of the CXCR4 insert
4. DNA extraction from clones
5. Plasmid ligation
6. Colony screening
7. Extraction and purification of CXCR4 plasmid DNA
8. Transfection
9. Clonal expansion under antibiotic selection
10. Characterisation of the transfected cell line

5.4.2 Extraction of the CXCR4 coding sequence

Firstly, the presence of the CXCR4 sequence in the donated pcDNA3 plasmid was investigated. This was performed by transforming JM109 competent cells with the donated plasmid thought to contain the CXCR4 coding sequence cassette. The calculated transformation efficiency of the control reaction was $6.6 \times 10^6$ cfu/μg of DNA added.\(^{54}\)

Single colonies were incubated overnight and then screened for the CXCR4 sequence by PCR. Three strongly positive clones were identified. To confirm the presence of the CXCR4 sequence, DNA was extracted from the positive clones and incubated with a panel of restriction enzymes (Figure 5.1). The BamH1 restriction enzyme was predicted to cut the entire CXCR4 construct containing pcDNA3 plasmid twice: once in the pcDNA3 multiple cloning site and once in the CXCR4 cassette yielding fragments

\(^{54}\) Transformation efficiency calculated as outlined in Section 2.4.3.
of 6100 and 480 base pairs in length.\textsuperscript{55,56} It was also predicted that the pcDNA3 plasmid without the CXCR4 construct would be cut only once. Furthermore pcDNA3 plasmids containing multiple copies of the CXCR4 construct would be cut more than twice. Results showed that the BamH1 restriction enzyme digestion of the pcDNA3-CXCR4 plasmid yields two fragments, 6100 and 480 base pairs in length, confirming the presence of one CXCR4 insert per plasmid. Incubation of the pcDNA3-CXCR4 plasmid with the restriction enzymes Xba1 and EcoR1 confirmed that they only made a single cut in the plasmid.

![Figure 5.1: Restriction enzyme digestion of the pcDNA3-CXCR4 plasmid.](image)

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Eco</th>
<th>Xba</th>
<th>Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Observed</td>
<td>5502</td>
<td>7200</td>
<td>6008</td>
</tr>
<tr>
<td>1025</td>
<td>7400</td>
<td>524</td>
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</tr>
<tr>
<td>Expected</td>
<td>5446</td>
<td>6532</td>
<td>6100</td>
</tr>
<tr>
<td>1086</td>
<td>6532</td>
<td>480</td>
<td></td>
</tr>
</tbody>
</table>

Legend: LD = 1kbp ladder, Eco = EcoR1, Xba = Xba1, Bam = BamH1.

\textsuperscript{55} pcDNA3 plasmid sequence available at (http://www.invitrogen.com/content/sfs/vectors/pcdna3_rest.htm)

\textsuperscript{56} Restriction enzyme digests predicted using the WebCutter 2.0 software package available at http://rna.lundberg.gu.se/cutter2/
To isolate the CXCR4 sequence, double-digestion of the pcDNA3-CXCR4 plasmid with the EcoR1 and Xba1 restriction enzymes was performed. The pcDNA3.1vB plasmid was digested in the same way to open the multiple cloning site at the appropriate locations. Both reaction products were separated on an agarose gel, excised and the DNA extracted (Figure 5.2).

Following DNA extraction, the CXCR4 insert and linear pcDNA3.1vB plasmid were ligated. The optimum conditions for ligation were determined using a range of insert to vector ratios (1:3, 1:1 and 3:1). Ligation efficiency was determined by the density of the PCR band for the T7forward and BGH reverse primers. Results showed that there was no significant difference between the molar ratios of 1:3, 1:1 and 3:1 of insert to vector respectively (Figure 5.3).

Figure 5.2: Restriction enzyme digests of the pcDNA3-CXCR4 and pcDNA3.1vB plasmids. Restriction enzyme digestion products prior to gel extraction were run on a 1% agarose gel. The pcDNA3-CXCR4 and pcDNA3.1vB plasmids were double-digested with the restriction enzymes EcoR1 and Xba1 to yield the CXCR4 coding sequence (1086 base pairs) and open plasmid (5446 base pairs). Both linear sequences were excised and extracted from this gel. Legend: LD = GeneRuler 1kbp DNA ladder.
Figure 5.3: The effect of Insert / Vector Molar Ratio on the Efficiency of CXCR4 Ligation.

PCR products generated by the T7forward and BGH reverse primers following ligation of the CXCR4 insert into the open pcDNA3.1vB plasmid: the effect of variation of the insert / vector ratio. Each reaction was sampled in-triplicate. There was no obvious difference in band density for the three molar ratios tested. Representative results are shown from one experiment of three performed. Legend: PC = pcDNA3-CXCR4 positive control, NC = negative control (ligation mixture without addition of T4 ligase), LD = GeneRuler 100bp DNA ladder, EV = pcDNA3.1vB empty-vector negative control, ratios = insert : vector molar ratios.

5.4.3 Transformation of competent cells, clonal expansion and screening

JM109 competent cells were transformed with the pcDNA3.1vB-CXCR4 plasmid with an efficiency of $4.3 \times 10^6$ cfu/μg of DNA added. Twenty clones were expanded from single colonies and underwent PCR with CXCR4 primers. Eleven clones demonstrated PCR products 232 base pairs in size, consistent with the presence of CXCR4 (Figure 5.4). PCR for CXCR4 was repeated on one clone which was also investigated using the T7 forward and BGH reverse primer pair (Figure 5.5). Results showed that for the pcDNA3.1vB-CXCR4 plasmid and the pcDNA3-CXCR4 positive control, the T7/BGH PCR products were consistent with the 1298bp band expected. With CXCR4 primers, an expected band corresponding to 232bp was demonstrated. In the pcDNA3.1vB empty-vector plasmid, no CXCR4 band was seen and the T7/BGH amplicon was 257bp, demonstrating there was no interposed CXCR4 coding sequence.
Figure 5.4: Screening of the transformed bacterial colonies for the pcDNA3.1vB-CXCR4 construct by PCR. CXCR4 PCR products run by gel electrophoresis against a 100bp DNA ladder showing bands consistent with the predicted amplicon size of 232 base pairs. Representative results are shown from one experiment of three performed. Negative control (DNA-free water) showed no band (data not shown). Legend: LD = 100 base pair ladder, 1-11 = bacterial clones, PC = pcDNA3-CXCR4 positive control DNA.

Figure 5.5: Screening of a selected transformed bacterial colonies for the CXCR4 and the T7/BGH primer pairs. PCR products for CXCR4 (left) and T7-BGH (right) run by gel electrophoresis against a 100 bp DNA ladder. Plasmids screened: 1) pcDNA3.1vB-CXCR4 construct (X4) showing a positive band for CXCR4 (232 bp) and the T7-BGH band at 1298 bp enclosing the CXCR4 coding sequence; 2) pcDNA3.1vB empty vector negative control (EV) showing no band for CXCR4 and the T7-NGH band and 257 bp (no interposed CXCR4 coding sequence); and 3) pcDNA3-CXCR4 positive control (PC) with similar results to (1). Representative results are shown from one experiment of three performed. Legend: LD = 100 base pair ladder.
The selected pcDNA3.1vB-CXCR4 and empty-vector clones underwent restriction enzyme digestion to determine the number and orientation of the CXCR4 coding sequences inserted (Table 5.1). The following pattern of pcDNA3.1vB-CXCR4 plasmid digestion was demonstrated: the SalI restriction enzyme yielded a fragment of 4500 and 2100 base pairs (predicted 4356 and 2188 base pairs). The EcoR1 and Xba1 enzymes gave single fragments of 6500 and 6400 base pairs respectively (both predicted at 6544 base pairs). Combined digestion with both EcoR1 and Xba1 resulted in two fragments of 5600 and 1153 base pairs (predicted 5458 and 1086 base pairs). The NcoI restriction enzyme was shown to cut four times giving fragments of 3500, 2200, 842 and 360 base pairs (predicted 3345, 2069, 735 and 338 base pairs). For the pcDNA3.1vB empty vector the following pattern of restriction enzyme digestion was shown: the NcoI enzyme cut three times giving fragments of 3600, 1583 and 815 base pairs (predicted 3347, 1415 and 735 base pairs). SalI cut twice with bands at 3500 and 2300 base pairs (predicted 3309 and 2188). The EcoR1 and Xba1 enzymes yielded single fragments measuring 5600 and 5400 base pairs respectively (both predicted at 5497 base pairs). In combination, EcoR1 and Xba1 yielded only one fragment of 5400 base pairs although two were predicted (5438 and 39 base pairs). It is likely that the tiny 39 base pair fragment was not resolved sufficiently at the end of the gel.

Finally the empty pcDNA3.1vB plasmid was transformed into competent cells as described above. Single clones were incubated and screened by PCR using primers for the T7 promoter (forward) and BGH reverse priming site (reverse). In an empty plasmid this yielded an amplicon 257 base-pairs in length.

5.4.4 CXCR4 Transfection of H357 cells
The optimum conditions for the transfection reaction were determined empirically using the pcDNA3.1-lacZ control plasmid and the β-Galactosidase reporter activity assay. The effect of the three variables investigated (charge ratio, starting DNA concentration and exposure time) are described in Appendix 5. The killing effect of the antibiotic G418 on untransfected H357 cells was determined empirically and is also reported in Appendix 5.
Table 5.1: Tabulation of observed and expected band sizes following restriction enzyme digestion of the pcDNA3.1vB-CXCR4 plasmid and pcDNA3.1vB empty-vector control.

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>Observed (base pairs)</th>
<th>Expected (base pairs)</th>
<th>plDNA3.1vB-CXCR4</th>
<th>pcDNA3.1vB empty vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>NcoI</td>
<td>3500</td>
<td>3345</td>
<td>3500</td>
<td>3600</td>
</tr>
<tr>
<td></td>
<td>2200</td>
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<td>3500</td>
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<tr>
<td></td>
<td>842</td>
<td>6544</td>
<td>1583</td>
<td>3309</td>
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<tr>
<td></td>
<td>360</td>
<td>6544</td>
<td>815</td>
<td>5497</td>
</tr>
<tr>
<td>SalI</td>
<td>4500</td>
<td>2069</td>
<td>4500</td>
<td>3309</td>
</tr>
<tr>
<td></td>
<td>2100</td>
<td>2188</td>
<td>3600</td>
<td>1415</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3309</td>
<td>1415</td>
</tr>
<tr>
<td>EcoR1</td>
<td>6500</td>
<td>735</td>
<td>5600</td>
<td>5497</td>
</tr>
<tr>
<td></td>
<td>6400</td>
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</tr>
<tr>
<td>EcoR1 Xba1</td>
<td>5600</td>
<td></td>
<td>1153</td>
<td>39</td>
</tr>
</tbody>
</table>

Restriction enzyme digestion products from the pcDNA3.1vB-CXCR4 plasmid and pcDNA3.1vB empty-vector control run on 0.75% agarose gel against a 1kbp DNA ladder. Incubation of the pcDNA3.1vB-CXCR4 plasmid reveals excellent correlation with predicted fragment number and size: NcoI yields four bands (3500, 2200, 842 and 360 base pairs), and SalI two bands (4500 and 2100 base pairs). EcoR1 and Xba1 alone yield one band (6500 and 6400 base pairs respectively) and in combination two bands (5600 and 1153 base pairs). Restriction enzyme digestion of the pcDNA3.1vB empty-vector control also demonstrates good agreement with fragment predictions: NcoI yields three bands (3600, 1583 and 815 base pairs), and SalI two bands (3500 and 2300 base pairs). Single incubation with EcoR1 and Xba reveal one band each (5600 and 5400 base pairs), while in combination one band is seen (5400 base pairs) when two are predicted. All experiments were performed three times and the mean observed values are given.

In summary, H357 cells were transfected with 1.5µg of the pcDNA3.1vB-CXCR4 plasmid (or the pcDNA3.1vB empty vector control) per well, at a charge ratio of 1:1, with an exposure time of one hour as described in Section 2.4.14. Transfected cells were grown in non-selective media for 48 hours and then passaged, clones selected and expanded using selective media containing G418 at a concentration of 1600µg/ml (as outlined in Section 2.4.15). Five CXCR4-containing and six empty-vector clones were expanded into populations large enough to permit characterisation and further investigation.
5.4.5 Characterisation of CXCR4-transfected H357 Clones

5.4.5.1 CXCR4 mRNA expression

To determine the effect of transfection on CXCR4 mRNA expression transfected H357 clones (with and without the CXCR4 construct), cells were cultured under optimal conditions under antibiotic selection and investigated with sqRT-PCR (Figure 5.6). Compared with untransfected cells, CXCR4 mRNA expression was essentially unchanged in transfected cells lacking the CXCR4 insert. In cells containing the CXCR4 insert, mRNA expression was significantly increased: three clones (H357-X4.4, H357-X4.5 and H357–X4.6) demonstrated increases in CXCR4 expression of 42, 800 and 2,000 times respectively (compared with transfected controls lacking the CXCR4 insert). However, in two clones (H357-X4.1 and H357–X4.2), CXCR4 mRNA species were over half a million times more abundant than in similarly matched controls.

In a separate experiment, transfected cells with (H357-X4.1) and without (H357-EV1) the CXCR4 insert were cultured under varying conditions in serum-reduced media (0.1% FCS) for 24 hours. Total RNA was harvested and subjected to microarray analysis. CXCR4 mRNA expression was 45 to 90-fold higher (p<0.001, Student t-test) in the cells containing the CXCR4 insert (NEV range 4.88 – 7.34), compared with those that did not (NEV range 0.08 – 0.11) (Figure 5.7). Despite variations in the experimental conditions (including SDF stimulation and a reduced oxygen tension) CXCR4 mRNA expression levels remained relatively stable in cells transfected with and without the CXCR4 construct.
Figure 5.6: Relative expression of CXCR4 mRNA by untransfected H357 cells and transfected cells with and without the CXCR4 insert. Untransfected and transfected H357 cells (five clones with and six clones without the CXCR4 insert) were grown under optimum conditions to 90% confluence as determined by light microscopy. Messenger RNA expression was determined by qRT-PCR and represented as fold change relative to untransfected H357 cells. Bars represent the mean (range) fold change of four replicates each measured in triplicate. All CXCR4 transfected clones expressed significantly more CXCR4 mRNA than the untransfected or empty-vector mock transfected cells (p<0.001). There was no significant difference in CXCR4 mRNA expression between wild-type and any of the empty-vector clones (p>0.05). The CXCR4 transfected clones 4.1 and 4.2 expressed significantly more (p<0.001) CXCR4 mRNA than the other transfected clones (4.4, 4.5 and 4.6). No Template Controls and samples incubated without Reverse Transcriptase produced no signal for CXCR4 after 40 cycles. Statistical comparison with expression levels in untransfected H357 cells was performed with ANOVA incorporating the Bonferroni correction for multiple testing. The figure represents the cumulative results of two experiments performed. Legend: UT = Untransfected H357; EV1-EV6 = H357 empty vector clones; X4.1-X4.6 = CXCR4-transfected H357 clones; ns = not significant (compared with untransfected); *** = p<0.001 (compared with wild-type).
Figure 5.7: CXCR4 mRNA expression in transfected H357 cells with and without the CXCR4 insert subject to a range of experimental conditions. H357 cells with and without the CXCR4 insert were seeded at known densities and grown for 24 hours in normal growth media supplemented with 0.1%FCS. Cells were stimulated with varying concentrations of SDF (0, 1 and 100 ng/ml) and varying oxygen tensions (2% and 20%). Total RNA was harvested and examined with microarrays. Figure represents the mean normalised expression value for both microarray CXCR4 mRNA probes for each experimental condition. Results show that CXCR4 expression is 45 to 90 fold higher in those clones containing the CXCR4 insert, compared with those that do not (p<0.001, Student t-test). CXCR4 expression levels were independent of other experimental variables such as SDF and oxygen concentrations. Legend: hypox = low (2%) oxygen tension; normox = high (20%) oxygen tension.
5.4.5.2 CXCR4 surface protein production

To determine the production of CXCR4 surface protein by transfected cells (with and without the CXCR4 insert), clones were grown under optimum conditions to 90% confluence in selective media, dissociated and investigated with flow cytometry. Labelling with two unrelated anti-CXCR4 monoclonal antibodies (clones 172 and 173) yielded similar results (Figure 5.8). Two of the five clones containing the CXCR4 insert (H357-X4.1 and H357-X4.2) demonstrated very high CXCR4 surface expression (MFV 93.6 and 120.8 respectively). Some surface labelling was shown by the H357-X4.5 clone (MFV 25.4). Signal levels for the H357-X4.4 and H357-X4.6 clones (MFV 11.2 and 10.1 respectively) were similar to those demonstrated by the mock-transfected empty-vector controls (MFV 9.45). Background signal for the isotype antibody controls for all clones investigated was universally low (MFV <6). Interestingly the two high CXCR4-expressing clones (H357-X4.1 and H357-X4.2) demonstrated a biphasic distribution of fluorescence intensity. The density plot of cell size against granularity for all the clones investigated showed two distinct populations of cells.

Further experiments were performed to test the working hypothesis that the biphasic levels of CXCR4 surface protein demonstrated in the high-expressing clones (H357-X4.1 and H357-X4.2) were a reflection of the size of different cell populations represented in the size scatterplot (Figure 5.9). Live cells were gated into two populations based on size. For all clones investigated, small live cells demonstrated the same percentage labelling for CXCR4 as large live cells. Consequently these data suggested that CXCR4 surface expression was unrelated to cell size. Furthermore, both the H357-X4.1 and H357-X4.2 clones demonstrated a similar overall percentage of positively labelled cells (74.8% and 80.1% respectively) and mean fluorescence intensity within the positive population (120.4 and 124.1 respectively). These findings demonstrated that a small proportion (20 to 25%) of the H357-X4.1 and H357-X4.2 transfected clones were not expressing CXCR4 on the cell surface. Consequently it was decided to select out CXCR4-labelled cells from these “mixed” populations by fluorescence-assisted cell sorting (FACS). This was performed according to the methods outlined in Section 2.4.17. Selected CXCR4-positive cells were cultured and passaged in selective growth media for use in further experiments.

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57 Data from both antibodies (clones 172 and 173) are tabulated in Figure 5.8. For simplicity mean fluorescence (MF) values quoted in the text are for the 173 clone.
Figure 5.8: CXCR4 surface expression in transfected H357 cells. Cells were cultured under optimum conditions. Prior to analysis anti-CXCR4 monoclonal antibodies or isotype controls and propidium iodide were added. Plots of cell count against fluorescence intensity are given for: A) H357-X4.1; B) H357-X4.2; C) H357-X4.4; D) H357-X4.5; E) H357-X4.6 and F) empty-vector mock transfected clones. Mean fluorescence measurements for each monoclonal antibody in live cells were tabulated (G). H357-X4.1 and H357-X4.2 cells demonstrated high levels of CXCR4 surface expression with both anti-CXCR4 monoclonal antibody clones. CXCR4 surface labelling was not demonstrated on the remaining CXCR4-transfected cells and empty-vector mock-transfected controls. Legend: Antibody labelling of cell populations: anti-CXCR4 clone 172 (red line); anti-CXCR4 clone 173 (green line); and isotype control (purple shading).
Figure 5.9: CXCR4 protein labelling in transfected H357 cells sorted by size. Cells were cultured in growth media with 10% FCS until 80% confluent. Prior to analysis cells were incubated with anti-CXCR4 monoclonal antibodies (MAB172 & MAB173) or isotype controls and propidium iodide. For each clone two populations were gated (R1 & R2) dependent on size (left column) and live cells selected (R4, middle column). Plots of cell count against fluorescence intensity (right column) are given for: untransfected (a); empty-vector mock transfected (b); H357-X4.1 (c); and H357-X4.2 clones (d). Results show that similar CXCR4-labelling densities are represented in the two cell populations gated by size. Blue line = MAB172, Red line = MAB173.
Qualitative investigation was performed using immunocytochemistry. H357 cells transfected with the vector including the CXCR4 insert demonstrated far greater staining for the protein than transfected cells lacking the CXCR4 insert (Figure 5.10). Using a peroxidase-based technique a strong homogeneous cytoplasmic staining was shown in CXCR4-transfected clones with occasional cells (approximately 5% by cell count) showing even denser cytoplasmic labelling. In contrast empty-vector mock-transfected cells demonstrated only a very weak cytoplasmic staining that was difficult to distinguish from background. However, in these control clones, occasional stronger staining of the nucleus was also seen, suggesting that some CXCR4 protein was being produced but that this was not associated with the cytoplasmic membrane. In order to investigate this further, confocal fluorescent immunocytochemistry on the same clones was performed (Figure 5.11). These images revealed that in clones containing the CXCR4 insert, staining for the protein was throughout the cytoplasmic component of the cell (panels a and b). In contrast, clones lacking the CXCR4 insert demonstrated CXCR4 protein labelling but this was predominantly associated with the perinuclear structures (panel c). A similar pattern of staining was shown with untransfected cells from the same H357 cell line (panel d).

5.4.6 Summary of Results

- The CXCR4-coding sequence was excised from the donated plasmid, confirmed and ligated into a new vector.
- Competent cells were transformed with the new CXCR4-containing vector (with appropriate controls) and amplified with clonal selection.
- The validity, copy number and orientation of the CXCR4 sequence in the new vector were confirmed.
- H357 cells were transfected with the vector (with and without the CXCR4 insert) and clonally expanded.
- CXCR4 mRNA was significantly upregulated in all transfected clones containing the CXCR4 insert.
- Two of these clones (H357-X4.1 and H357-X4.2) demonstrated significantly more cytoplasmic and surface CXCR4 protein than transfected controls lacking the CXCR4 insert.
Figure 5.10: Peroxidase-based immunocytochemistry of transfected H357 cells. The clone H357-X4.1 stably transfected with CXCR4 (a, c & d) and empty-vector mock transfected controls (b) were cultured on glass coverslips to early confluence, fixed, permeabilised and stained for CXCR4 using monoclonal antibodies (clone 173) with peroxidase-based techniques. Dense CXCR4 staining was revealed for transfected cells (a) compared with mock-transfected controls (b). Isotype antibody (c) and no primary antibody (d) negative controls revealed no staining. Scale bar represents 100µm.
Figure 5.11. CXCR4 staining of transfected H357 cells. Transfected H357 cell clones with (a&b) and without (c) the CXCR4 insert were grown at low density on glass coverslips along with untransfected controls (d). After fixation cells were incubated with anti-CXCR4 monoclonal antibodies (a-d) or isotype controls (e). Transfected cells containing the CXCR4 insert showed significant cytoplasmic labelling (a&b). The majority of transfected cells without the insert (c) showed CXCR4 staining associated with the nuclear membrane (dashed arrow) with only occasional cytoplasmic staining (solid arrow). Scale bar represents 20µm.
5.5 Discussion

Prior to its manipulation, the expression of CXCR4 in untransfected H357 cells was carefully characterised at the mRNA and protein levels using a number of complimentary techniques (Chapter 3). It was demonstrated to have the lowest CXCR4 mRNA expression of all the OSCC cell lines investigated with levels comparable to NOKs. In a similar study, Uchida et al transfected an OSCC cell line that expressed CXCR4 mRNA at a constitutively low level (Uchida, Begum et al. 2004). Subsequently it was confirmed that mock-transfected H357 cells lacking the CXCR4 construct had similar very low levels of expression. These were important observations as it confirmed that the transfection process alone did not appear to influence the expression of CXCR4 in the empty-vector mock-transfected H357 clones. Characterisation of untransfected H357 cells suggested that these cells contained the CXCR4 coding sequence although this was not determined directly by sequencing. Consequently, it was theoretically possible that CXCR4 expression could be increased in mock-transfected cells by incorporation of the CMV promoter contained within the empty vector directly upstream of the CXCR4 coding sequence. Empirical evidence showed that this was not the case and confirmed the suitability of the empty-vector mock-transfected cells as suitable negative controls. However, these findings are not universal: results from the transfection of BCC cells using a retroviral vector without the CXCR4 insert increased CXCR4 mRNA expression three fold (Chen, Yu et al. 2006). The reason for this was not clear although it was possible that the incorporation of the vector may have occurred upstream of the constitutive CXCR4 coding sequence thereby altering its basal expression compared with untransfected controls.

Excising the CXCR4 construct out the donated plasmid and splicing it into a different vector involved a number of additional experimental steps. However, to maximise the usability of the transfected cell line in subsequent investigations it was decided to produce a construct which could be tagged by alternative methods. The ability to label the cells with other monoclonal antibodies gave the facility to mark transfected cells without interfering with ligand binding sites directly. However, in retrospect, a vector including a green fluorescent protein (GFP) motif might have been preferable as this would have allowed real-time monitoring rather than the multiple fixing and labelling steps required for antibody-based techniques (Kawamata, Uchida et al. 1999; Uchida, Begum et al. 2004).
Untransfected H357 cells were surprisingly resistant to G418. A concentration of 1600µg/ml was required to achieve 90% cytotoxicity after 72 hours. This is significantly higher than the concentrations used on other oral-derived cell lines reported in the literature which ranged from 400 to 1000µg/ml (Shintani, Mihara et al. 2002; Uchida, Begum et al. 2004). Consequently there was a concern that G418-mediated cell toxicity could confound the results of subsequent protein-based and functional experiments. This was addressed by maintaining transfected cells in G418-supplemented media but then transferring them to defined, G418-free media for 24 hours prior to exposure to experimental conditions.

The validity of the sequence, its orientation and copy number could have been performed following transformation of competent cells without the prior excision of the CXCR4 insert from the donated plasmid. Confirming the validity of the CXCR4 sequence was performed using PCR and restriction enzyme digestion with band matching. While these techniques provide evidence supporting the identity of the CXCR4 sequence, the data they provide are not conclusive. One methodological weakness of this work is that the CXCR4 sequence was never confirmed by direct sequencing and this could be performed with further experiments. Numerous CXCR4-specific sqRT-PCR reactions and antibody binding experiments on the transfected clones all yielded positive results for transfected clones containing the CXCR4 insert. However, unexpected results – such as those from the three clones (H357-X4.4, H357-X4.5 and H357-X4.6) in which upregulated CXCR4 mRNA was detected but produced no detected surface protein by flow cytometry – might be usefully analysed in this way, as one of the possible explanations of this finding is that the CXCR4 insert had been altered in a way that prevented protein translation but produced a mRNA sequence detectable by PCR. Work by Zhang et al has shown that point mutations in the CXCR4 sequence can result in changes in constitutive activity but, as yet, no studies have demonstrated a difference in antibody binding resulting from point mutations (Zhang, Navenot et al. 2002).

The magnitude of CXCR4 mRNA upregulation following transfection with the CXCR4 insert was surprising. By sqRT-PCR this varied from 40 to 500,000-fold. Unfortunately comparative, quantitative data from the literature are not available for OSCC or NSCLC cells (Uchida, Begum et al. 2004; Su, Zhang et al. 2005). However, the basal cell carcinoma-derived cell line BCC-1/KMC was transduced with CXCR4 which increased
CXCR4 mRNA expression 60-fold (Chen, Yu et al. 2006). This may be a manifestation of a different experimental methodology or related to the observation that CXCR4 is constitutively expressed 72-fold more in BCC-1/KMC compared with other chemokine receptors. Microarray analysis of one of the highest CXCR4-expressing clones (H357-X4.1) revealed CXCR4 upregulation 45 to 90-fold higher than in transfectants lacking the CXCR4 insert. Data presented in Chapter 3 demonstrate that the relative magnitudes of sqRT-PCR and microarray expression data are linearly related after logarithmic transformation. Applying the same formula to these results, the expected change in CXCR4 expression, as determined by microarray, would be approximately 65. This correlates well with the observed values. These findings suggest that CXCR4 is very highly expressed in all five surviving H357 clones transfected with the CXCR4 insert. However, flow cytometry detected significantly increased levels (75%) of CXCR4 surface protein in the two highest expressing clones (H357-X4.1 and H357-X4.2). Similar experiments in NSCLC cell lines increased CXCR4 surface protein from 20 to 50% (Su, Zhang et al. 2005).

Clones in which CXCR4 mRNA expression was more moderately increased (42-2,116-fold) failed to produce surface protein detectable with flow cytometry but did demonstrate some staining of perinuclear structures with immunocytochemistry. From these data alone, it is not clear why these clones did not produce surface protein. One possible cause could be mutation in the CXCR4 coding sequence preventing the translated protein reaching the cell membrane. This could be investigated by the sequencing of the coding sequence of all the clones looking for discrepancies from the wild-type. An alternative explanation would be that significant overexpression of CXCR4 mRNA favours surface protein production in a similar way to that observed in untransfected cells. However, evidence that the expression / translation process is under complex control comes from the observation that established OSCC cell lines which constitutively produce CXCR4 surface protein (SCC4 and SCC25) express the mRNA species at lower levels than several CXCR4-transfected H357 cells that do not demonstrate surface protein. At what level(s) the control of surface protein production

58 The BCC-1/KMC basal cell carcinoma-derived cell line was transduced with CXCR4 using the retroviral vector pLNCX2 (Chen, Yu et al 2006).
59 Log10 gradient was measured as 0.32 (Chapter 3). PCR measured mean H357-X4.1 CXCR4 upregulation as 506,370-fold. $10^{(\log_{10}(506,370)\times0.32)}=66.9$. Therefore, by this transformation, the expected upregulation of CXCR4 as measured by microarray would be 66.9. The observed values were 45-90.
is determined is not clear from these results, although its further delineation might be useful in the clinico-pathological context as it is this mechanism that may define the cell’s responsiveness to endogenous SDF and therefore the influence of CXCR4 over-expression on cell behaviour. However, this hypothesis assumes that the OSCC cell has the constitutive ability to respond to CXCR4 activation by SDF. In order to test this it was necessary to derive an experimental model in which cells differed only by the amount of CXCR4 expressed. The results in this chapter demonstrate that this has been achieved. In the hepatoma cell line HepG2 it has been shown that although constitutively CXCR4 mRNA and surface protein was detectable, the receptor did not undergo phosphorylation or internalisation upon ligand binding (Mitra, De et al. 2001). However, using a pcDNA3 vector, insertion of wild-type CXCR4 restored receptor function suggesting that the downstream signalling machinery was already available within the cell (Mitra, De et al. 2001). My flow cytometry data have demonstrated that CXCR4 surface protein was increased in the H357 clones containing the CXCR4 insert. Further functional studies were required to determine if the cells were able to respond to SDF gradients in-vitro without further manipulation of the cell-signalling pathways.

Further transfections reducing the constitutive high CXCR4-expressing cell lines SCC4 and SCC25 might have produced interesting experimental models. Varying methodologies to decrease CXCR4 translation have been described in the literature including anchoring CXCR4 via KDEL-receptors in the endoplasmic reticulum (Zeelenberg, Ruuis-Van Stalle et al. 2003). Stable transfection with CXCR4 antisense RNA has been described for NSCLC (Su, Zhang et al. 2005) and nasopharyngeal carcinoma (Hu, Deng et al. 2005) with an associated decrease in cell surface expression (compared with untransfected controls).

5.6 Conclusions

These results have demonstrated that the stable overexpression of wild-type CXCR4 mRNA caused a significant increase in surface protein levels. This provided a useful experimental model in which to investigate the effect of SDF stimulation on OSCC cells which differ only by the inclusion of the CXCR4 construct. However, constitutive expression of CXCR4 mRNA (as detected by sqRT-PCR) and associated protein production (as labeled by monoclonal antibodies) is not always indicative of functional
responses to SDF (Mitra, De et al. 2001). These findings can be extrapolated to CXCR4-transfected cells thereby confirming the importance of functional assays of cell behaviour. The role of CXCR4 in OSCC tumour biology can be investigated using a number of *in-vitro* assays relevant to the metastatic process (Chapters 6 and 7). The inclusion of a transfected cell line with high CXCR4 protein production (together with its negative control) provides a useful system with which to investigate this.
Chapter 6: The effects of CXCR4 function on cell proliferation and apoptosis in established OSCC cell lines and CXCR4 transfectants
6.1 Introduction

My results so far have demonstrated that normal oral and skin keratinocytes (NOK and NSK) constitutively express very low levels of CXCR4 mRNA and protein (Chapter 3). However, while some established OSCC cell lines demonstrate very low constitutive levels of CXCR4 mRNA expression and protein production, others (predominantly SCC4 and SCC25) show high levels of CXCR4 mRNA expression and positive protein labelling in both the nuclear and cytoplasmic compartments. Furthermore it has been shown that the established OSCC cell line H357 can be induced to express high levels of CXCR4 surface protein by stable transfection with the coding sequence (Chapter 5). Investigation of tissue sections has revealed an association between CXCR4 protein staining and lymphatic metastasis (Chapter 4).

Consequently, two hypotheses might be proposed: firstly that CXCR4 upregulation may occur during the process of malignant transformation, although not ubiquitously so. Secondly that alterations in cell phenotype produced in this way are associated with beneficial changes in tumour cell survival within the host/tumour microenvironment. From the perspective of Paget’s “Seed and Soil” theory of tumour metastasis (Paget 1889; Fidler 2003), does the upregulation of functional CXCR4 receptors convey an alteration of the tumour “Seed” so that it increases proliferation when in the environment of certain host “Soil”?

One significant criticism of the published work investigating CXCR4 expression and metastasis in other carcinoma systems is that it has focussed on the directional (chemotactic) mechanisms of the process. The potential role of CXCR4 in other processes within the multi-step metastatic pathway has often been overlooked. Consequently, observed associations between CXCR4 expression and the presence of metastases may really reflect a selective advantage for CXCR4-positive primary tumour cells before the advent of metastases.

To test these hypotheses, direct human clinical evidence would be preferable. This could be derived from sequential biopsies of oral mucosa that was undergoing transformation in-vivo from a normal phenotype through dysplasia to frank malignancy. However, such material is hard to obtain, not least for the ethical considerations of knowingly allowing lesions to progress to carcinomas. Furthermore,
it would not give information on the direct effect of CXCR4 upregulation on tumour cell behaviour. To overcome these difficulties a more pragmatic empirical approach was employed: data on the impact of CXCR4 expression on cell function would be collected using *in-vitro* studies (Chapters 6 and 7) to compliment the investigation of *ex-vivo* tissue sections harvested at varying stages of the disease process (Chapter 4).

This chapter and the next aim to investigate the effect of the SDF-CXCR4 axis on molecular and cellular functions of cultured OSCC cells *in-vitro*. This starts with an assessment of ligand-mediated receptor internalisation and continues to investigate the effect of SDF stimulation on proliferation and apoptosis. The effects of CXCR4 stimulation on cell motility (chemokinesis, chemotaxis and directed invasion) will be addressed in Chapter 7.

### 6.2 Chapter Hypothesis

SDF stimulation of CXCR4-expressing OSCC cell lines affects cell proliferation and apoptotic activity *in-vitro*.

### 6.3 Chapter Aims and Objectives

#### 6.3.1 Overall Aim

To investigate the effect of SDF stimulation on CXCR4 receptor-mediated proliferation and apoptosis.
6.3.2 Specific Objectives

1. To determine the effect of SDF stimulation on CXCR4 receptor internalisation.
2. To investigate the impact of SDF stimulation on OSCC cell proliferation.
3. To compare the proliferation rates of untransfected and CXCR4 transfected H357 cells.
4. To determine the effect of CXCR4 receptor blockade on cell proliferation rates.
5. To deduce the influence of CXCR4 stimulation on apoptosis in OSCC cells.

6.4 Results

6.4.1 Receptor Internalisation

6.4.1.1 Overview

CXCR4 undergoes rapid clathrin-mediated internalisation following ligand binding. This results in a transient reduction in cell surface receptor levels after exposure to SDF. The majority of internalised CXCR4 undergoes lysosomal degradation although some is recycled. Although some ligand-activated CXCR4 signalling pathways are thought to be independent of receptor internalisation, the activation of CXCR4 by SDF triggers Gα- and β-arrestin-2-mediated signalling cascades which initiate internalisation (Zhang, Wang et al. 2001; Roland, Murphy et al. 2003). It can be hypothesised that for CXCR4 to be fully functional in response to ligand binding it must be mobile within the lipid bilayer and free to internalise upon activation. Therefore the effect of SDF activation on CXCR4 receptor surface density was investigated.
6.4.2.1 The effect of serum-starvation and SDF stimulation on CXCR4 surface labelling.

To minimise the potentially confounding effect of media supplementation with serum, all experiments involving stimulation with SDF were done under serum-reduced conditions. Consequently, to determine the effect of serum-reduction on the surface expression of CXCR4 in the high expressing transfected H357 cell clones, a series of preliminary experiments were performed.

Briefly, cells were grown in culture, dissociated using enzyme-free methods, labelled with monoclonal antibodies (with appropriate isotype controls), incubated with propidium iodide and analysed with flow cytometry. Cells that failed to exclude propidium iodide were gated out. Results demonstrated that overnight incubation in a serum concentration reduced from 10 to 0.1% was associated with a reduction in CXCR4 receptor surface density (Figure 6.1). In the H357-X4.1 clone, serum-starvation reduced the mean fluorescence of live cells by 21%. In H357-X4.2 cells the change was approximately double this figure. Both differences were statistically significant (p<0.001, Student t-test). In both untransfected and empty-vector mock-transfected cells the mean fluorescence of the CXCR4-labelled cells did not differ significantly from that of the isotype monoclonal antibody control.

In the next series of experiments the effect of SDF stimulation on the surface expression of CXCR4 was determined in CXCR4-transfected H357 cell clones under conditions of serum-starvation (Figure 6.2). Compared with unstimulated controls, CXCR4-transfected cells incubated with SDF (100ng/ml) for one hour demonstrated a significant reduction in CXCR4 surface expression (p<0.001, Student t-test). The magnitude of this reduction was 48% and 23% for H357-X4.1 and H357-X4.2 cells respectively. Exposure of these cells to SDF (100ng/ml) for a 16 hour period resulted in a further significant decrease in surface CXCR4 labelling as detected by flow cytometry analysis (p<0.001, Student t-test). Compared with unstimulated controls, the size of this reduction was 63% and 24% for H357-X4.1 and H357-X4.2 cells respectively. Untransfected and mock-transfected H357 cells lacking the CXCR4 insert demonstrated no significant change in CXCR4 surface expression when incubated with SDF.
Figure 6.1: The effect of serum starvation on CXCR4 surface expression. Half a million cells were cultured overnight in growth media supplemented with either 10% or 0.1% FCS. Prior to FACS analysis cells were incubated with anti-CXCR4 monoclonal antibodies or isotype controls and propidium iodide. Plots of cell count against fluorescence intensity are given for: A) untransfected; B) empty-vector mock transfected; C) H357-X4.1; and D) H357-X4.2 clones. Mean fluorescence measurements for populations of over 50,000 live cells from three experimental replicates were tabulated. Overnight serum-starvation resulted in a statistically significant reduction of mean fluorescence of approximately 21 and 42% for H357-X4.1 and H357-X4.2 cells respectively. CXCR4 expression on untransfected and empty-vector mock-transfected cells was undetectable for all incubation conditions. Figures are representative plots from one of three experiments performed; quantitative data are pooled from all three replicates. Legend: Antibody labelling of cell populations: CXCR4 and 10% FCS (green line); CXCR4 and 0.1% FCS (red line); and isotype control and 10% FCS (black line); (***) denotes p<0.001 (Student t-test).
Figure 6.2: The effect of SDF stimulation on CXCR4 surface expression. Half a million cells were cultured in growth media supplemented with 0.1% FCS and 100ng/ml of SDF for either one hour or overnight. Prior to FACS analysis cells were incubated with anti-CXCR4 monoclonal antibodies or isotype controls and propidium iodide. Plots of cell count against fluorescence intensity are given for: A) untransfected; B) empty-vector mock transfected; C) H357-X4.1; and D) H357-X4.2 clones. Mean fluorescence measurements for populations of over 50,000 live cells from three experimental replicates were tabulated. Stimulation of H357-X4.1 and H357-X4.2 cells with SDF for 1 hour significantly reduced CXCR4 surface expression by approximately one half and one quarter respectively (p<0.001, student t-test). Incubation with SDF overnight significantly reduced CXCR4 surface expression by approximately two-thirds and one quarter respectively, compared with unstimulated controls (p<0.001, student t-test). CXCR4 expression on untransfected and empty-vector mock-transfected cells was undetectable for all incubation conditions. Figures are representative plots from one of three experiments performed; quantitative data are pooled from all three replicates. Legend: Antibody labelling of cell populations: CXCR4 and no SDF (red); CXCR4 and SDF (100ng/ml) for 1 hour (orange); CXCR4 and SDF (100ng/ml) overnight (blue); and isotype control (black); (***)) denotes p<0.001 with Student t-test.
In conclusion the results of these simple experiments demonstrate that exposure to SDF reduces CXCR4 surface receptor density in high expressing transfected cell lines under experimental conditions including serum starvation.

6.4.2 The effect of SDF on cell proliferation

6.4.2.1 Overview

The overall aim of this set of experiments was to investigate the effect of SDF stimulation on the proliferation rate of a range of CXCR4-positive OSCC cell lines and transfected clones. A number of experimental factors that might potentially confound the effect of SDF stimulation on cell proliferation were identified:

1. Cell seeding density
2. Duration of incubation
3. Cell culture supplementation with FCS
4. Media supplementation with EGF

These included the effect of basic culture conditions as well as growth media components (for example mitogenic factors such as EGF). A statistical power calculation was also performed to determine the number of replicate wells required to detect likely differences in cell proliferation rates. These factors were tested empirically and the results are given in Section A6.2 (Appendix 6).

6.4.2.2 The effect of SDF stimulation on cell proliferation

Once the optimum cell-culture parameters had been empirically defined, a series of experiments were performed to investigate the effect of SDF stimulation on cell proliferation. Initially the effects of SDF stimulation (100ng/ml) on proliferation were determined for a number of established OSCC cell lines and transfected H357 cells with and without the CXCR4 insert (Figure 6.3). Pooled data were derived from three identical experiments performed on the established cell lines H357, CAL27 and SCC4. Only SCC4 cells demonstrated a statistically significant increase in proliferation of 26% (p<0.001). Of the transfected H357 cells containing the CXCR4 insert (X4.1, X4.2 and
X4.4), only the X4.1 and X4.2 clones demonstrated a significant increase in proliferation of 22% and 23% respectively in response to SDF (p<0.01). No effect of SDF on the proliferation of untransfected and empty-vector mock-transfected H357 cells was shown. When the magnitude of the proliferation response to SDF was plotted against the availability of surface CXCR4 protein (as determined by mean fluorescence measurements using flow cytometry) an approximately linear relationship was demonstrated (Figure 6.3 panel b). The gradient of the line of approximate best fit was greater for established cells than for transfected clones. In summary, these results demonstrated that SDF stimulation only significantly increased proliferation in established cell lines or transfected H357 cells with demonstrable CXCR4 surface protein.
Three established OSCC cell lines (CAL27, SCC4 and H357), three CXCR4-transfected H357 clones (X4.1, X4.2 and X4.4) and mock-transfected empty-vector cells were incubated for 72 hours under optimum conditions (10% FCS) and in serum-reduced conditions (0.1% FCS) with or without 100ng/ml of SDF. The effect of serum reduction on proliferation is shown in Figure A6.7 in Appendix 6. The effect of SDF treatment (compared with unstimulated controls) under reduced serum conditions is shown in panel (a). Bars represent the mean (standard deviation) of combined data from three identical experiments each containing twelve replicate wells per cell treatment. Statistical analysis was performed using the Student t-test. All comparisons were not statistically significant unless indicated. Of the established cell lines investigated only SCC4 demonstrated a significant increase in proliferation in response to SDF. A significant pro-proliferative effect of SDF was demonstrated in the CXCR4-transfected clones H357-X4.1 and H357-X4.2 but not in H357-X4.4, empty-vector
mock transfected or untransfected H357 cells. There appeared to be a roughly linear relationship between surface CXCR4 levels (as determined by flow cytometry) and the magnitude of the proliferative effect of SDF – panel (b). However, the gradient of the suggested line of best fit differed between untransfected (solid line) and transfected (dashed line) cells. Legend: ** = p<0.01; *** = p<0.001. The figure represents pooled data from three identical experiments.

6.4.2.3 The effect of SDF concentration on cell proliferation

The response of untransfected and transfected H357 cells (with and without the CXCR4 insert) to SDF concentrations ranging from 0.1 to 100ng/ml under serum-reduced conditions was investigated (Figure 6.4). H357-X4.1 cells demonstrated a small (5%) but significant (p<0.05) increase in proliferation following stimulation with SDF at 1 ng/ml, compared with unstimulated controls. Increasing SDF concentrations to 100 ng/ml produced a near-linear increase in the proliferation of this clone to a maximal value of 17% (p<0.001). SDF stimulation resulted in similar, significant increases in H357-X4.2 cell proliferation with an identical pattern of dose-response to a maximal value of 14% at 100 ng/ml of SDF (p<0.001). Compared with unstimulated controls, untransfected and empty-vector mock-transfected H357 cells demonstrated no significant differences in proliferation in response to SDF stimulation (Figure 6.4 panels a & b).

The results from these two sets of experiments (represented in Figures 6.3 and 6.4) both showed proliferation was increased in cells that produced CXCR4 surface protein, either constitutively (SCC4) or following transfection (H357-X4.1 and H3457-X4.2), in response to SDF stimulation. Furthermore, cells that produced little or no CXCR4 surface protein (CAL27, H357, mock-transfected H357 and H357-X4.4) demonstrated no change in proliferation in response to SDF. These data therefore support the hypothesis that SDF exerts a pro-proliferative effect on CXCR4-producing cells. The aim of the next series of experiments was to test this hypothesis using a second and complimentary methodology.
Figure 6.4: The effect of SDF on H357 cell clone proliferation: Dose-response. Four H357 clones – untransfected (H357-UT), empty-vector (H357-EV) and two CXCR4-transfected (H357-X4.1 and H357-X4.2) were seeded at equal densities and incubated in optimum (10% FCS) and serum-reduced (0.1% FCS) with supplementation of SDF to final concentrations ranging from 0 to 100ng/ml for 72 hours. The effect of serum-reduction on proliferation in the absence of SDF is given in Figure A6.1 (Appendix 6). Proliferation rates were calculated as a percentage of untreated (SDF 0), serum-reduced controls. Plots represent the mean (standard error) of pooled data from three identical experiments each containing 12 replicate wells and five timepoints per treatment. Statistical analysis was performed using the ANOVA test with post-hoc Bonferroni correction. H357-X4.1 and H357-X4.2 cells demonstrated statistically significant increases in proliferation in response to SDF concentrations of 1 to 100 ng/ml. No significant change in proliferation in response to SDF stimulation was demonstrated for either untransfected or empty-vector mock-transfected cells. Legend: * = p<0.05; *** = p<0.001. The figure represents pooled data from three identical experiments.
The effect of SDF stimulation on BrdU incorporation

The effect of SDF stimulation on established OSCC cell lines and transfected cells has been demonstrated in Sections 6.4.2.2 and 6.4.2.3 above. However, as discussed in Section A6.3 (Appendix 6), resazurin-based assays measure cell viability rather than proliferation directly. Consequently a series of experiments was performed to determine the effect of SDF stimulation on more direct measures of cell proliferation (BrdU incorporation). To complement the data derived from the previous experiments (Section 6.4.2.2) this was firstly performed on established cell lines (H357, CAL27 and SCC4) and subsequently on H357 cells transfected with and without the CXCR4 insert.

The effect of SDF stimulation (range 0 to 100ng/ml) on BrdU incorporation by established cell lines was determined and data from three identical experiments was combined (Figure 6.5). In SCC4 cells, SDF concentrations of 0.1 ng/ml and 1 ng/ml increased BrdU uptake by 6% and 9% respectively (compared with unstimulated controls). This achieved statistical significance (p<0.05, χ² test) for the latter but not the former. Increasing the SDF concentration to 10 ng/ml further increased BrdU uptake to 14% (p<0.01). However, further increments of SDF concentration were not associated with further increases in proliferation suggesting that a maximal response was achieved at 10 ng/ml of SDF. CAL27 cells and untransfected H357 cells demonstrated no significant effect of SDF stimulation on proliferation.

In a separate series of similar experiments, the effect of SDF stimulation on BrdU incorporation by transfected H357 cells with and without the CXCR4 insert was determined (Figure 6.6). BrdU incorporation by both the CXCR4-transfected clones investigated (H357-X4.1 and H357-X4.2) was increased by 5% and 8% respectively in response to 0.1 ng/ml of SDF, although this difference failed to achieve statistical significance when compared with unstimulated controls. Increasing the SDF concentrations to 1 ng/ml resulted in a greater increase in proliferation in H357-X4.1 and H357-X4.2 cells by 14% and 18% respectively. This difference was statistically significant (p<0.01, χ² test). Further increases in SDF concentration to 10 ng/ml produced smaller but significant increases in BrdU incorporation compared with unstimulated controls. However, stimulation with SDF at 100 ng/ml resulted in lower proliferation rates than observed with SDF at 10 ng/ml. Both untransfected (H357-UT) and empty-vector mock-transfected (H357-EV) cells showed no significant change in proliferation in response to SDF stimulation.
In summary these experiments showed that only cells containing the CXCR4 sequence and expressing the surface protein either constitutively (SCC4) or following transfection (H357-X4.1 and H357-X4.2) demonstrated an increase in BrdU incorporation in response to SDF stimulation. Maximal increases of between 14 and 18% (compared with unstimulated controls) were seen at SDF concentrations between 1 and 10 ng/ml. Higher SDF concentrations did not yield further increases in proliferation. Cells not expressing CXCR4 surface protein demonstrated no significant change in BrdU incorporation in response to SDF stimulation. These results concur closely with those derived from the resazurin-based assays described in Section 6.4.2.3 and, in combination, provide significant evidence supporting the hypothesis that the SDF mediates increases in proliferation in OSCC cells that demonstrate CXCR4 surface protein. To test this hypothesis further a series of experiments was performed to determine the effect of blockade of the SDF-CXCR4 axis on cell proliferation.
Figure 6.5: The effect of SDF stimulation on cell proliferation in established cell lines. CAL27, SCC4 and untransfected H357 cells were seeded at known densities in 24 well plates and allowed to adhere overnight. Cells were then stimulated in reduced-serum (0.1% FCS) media supplemented with varying concentrations of SDF (0, 0.1, 1, 10 and 100ng/ml) for 48 hours. Cells were then pulsed with SDF-free media supplemented with 10% FCS and 10µM BrdU for two hours then fixed, stained for BrdU incorporation and counterstained with DAPI. Each well was photographed in three positions. The total cell count and number of BrdU labelled nuclei were recorded (a minimum of 1,000 cells were counted per experimental condition per replicate). The dose-response of each cell type to SDF (expressed as a percentage of unstimulated controls) was plotted. BrdU incorporation by SCC4 cells was significantly increased (p<0.05, χ² test) by stimulation with SDF at concentrations ranging between 1 and 100ng/ml. CAL27 and untransfected H357 cell proliferation did not change significantly in response to SDF stimulation. Legend: * = p<0.05 ; ** = p<0.01. The figure represents pooled data from three identical experiments.
Figure 6.6: The effect of SDF stimulation on CXCR4-transfected H357 cell proliferation. Untransfected, empty-vector mock-transfected and two clones containing the CXCR4 insert and producing surface protein (H357-X4.1 and H357-X4.2) were seeded at known densities in 24 well plates and allowed to adhere overnight. Cells were then stimulated in reduced-serum (0.1% FCS) media supplemented with varying concentrations of SDF (0, 0.1, 1, 10 and 100ng/ml) for 48 hours. Cells were then pulsed with SDF-free media supplemented with 10% FCS and 10µM BrdU for two hours then fixed, stained for BrdU incorporation and counterstained with DAPI. Each well was photographed in three positions for both BrdU labelling and DAPI. No signal was detected when an isotype primary antibody was compared with the DAPI control. Representative raw data images are presented in Figure A6.2 (Appendix 6). The total and number of BrdU labelled nuclei were recorded (a minimum of 1,000 cells were counted per treatment per replicate). The panel represents the dose-response of each cell type to SDF (expressed as a percentage of unstimulated controls) derived from cumulative data from three identical experiments. Untransfected and transfected cells lacking the CXCR4 insert showed no significant response to SDF stimulation. Clones containing the CXCR4 insert demonstrated a clear response to SDF with maximum proliferation at SDF concentrations between 1 and 10ng/ml of SDF (p<0.05, χ² test). Further increases in SDF concentration did not increase BrdU incorporation rates. Overall Chi-squared testing for trend revealed no significant relationships. Legend: * = p<0.05; ** = p<0.01. Panel represents cumulative data from three identical experiments.
6.4.2.5 The effect of CXCR4 blockade on cell proliferation

The effect of AMD3100 (a pharmacological CXCR4 antagonist) on the SDF-induced proliferation of transfected H357 cells (with and without the CXCR4 insert) was determined by a fluorescence-based proliferation assay (Figure 6.7). Results showed that incubation with AMD3100 (1µg/ml) alone had no statistically significant effect on the proliferation of transfected or untransfected cells (compared with untreated controls). Stimulation of cells with SDF (10ng/ml) alone resulted in statistically significant (p<0.001) increases in proliferation for H357-X4.1 and H357-X4.2 cells of 13% and 15% respectively (compared with untreated controls). Proliferation of H357-X4.1 and H357-X4.2 was significantly reduced (p<0.001) by the combined incubation of cells with SDF (10 ng/ml) and AMD3100 (1µg/ml). Co-culture of these CXCR4-transfected clones with both SDF and AMD3100 abrogated proliferation to levels statistically indistinguishable from those of untreated controls and cells cultured with AMD3100 alone. Untransfected (H357-UT) and empty-vector mock-transfected (H357-EV) cells demonstrated no statistically significant change in proliferation in response to SDF alone, AMD3100 alone or combined treatment with SDF and AMD3100 (compared with untreated controls). In summary, these data demonstrate that the pro-proliferative effect attributed to SDF stimulation in previous experiments was abrogated by blockade of the SDF-CXCR4 axis using the receptor antagonist AMD3100.

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60 The SDF concentration of 10 ng/ml was used as it had been previously shown to exert the maximal effect on cell proliferation in transfected H357 cells containing the CXCR4 insert.
Figure 6.7: The effect of AMD3100 treatment on H357 cell proliferation. Four H357 clones (untransfected, empty-vector and two CXCR4 transfectants – H357-X4.1 and H357-X4.2) were incubated with 10ng/ml with and without additional treatment with 1µg/ml AMD3100 for 72 hours in conditions of 0.1% FCS. Serial fluorescence measurements were taken and the proliferation rate in each experimental condition was calculated. Bars represent mean (standard deviation) percentage change in proliferation compared with untreated controls (0.1%FCS only) from three identical experiments each with 12 replicate wells per treatment. Statistical analysis using the Student t-test was performed. Treatment with AMD3100 alone resulted in no change in cell proliferation. Stimulation with SDF alone resulted in a significant increase in proliferation in H357-X4.1 and H357-X4.2 cells but not mock- or un-transfected cells. Combined treatment with both SDF and AMD3100 reduced proliferation to levels statistically indistinguishable from treatment with AMD3100 alone. Legend: AMD = AMD3100 at 1µg/ml; SDF = SDF at 10 ng/ml; UT = untransfected; EV = empty-vector; *** = p< 0.001. The figure shows cumulative data from three identical experiments performed.

6.4.2.6 Summary of proliferation assay data

These results have clearly demonstrated that SDF stimulation increases proliferation in cells that produce CXCR4 surface protein either constitutively (SCC4) or following transfection (H357-X4.1 and H357-X4.2). The magnitude of the response appears to be dependent on both the availability of CXCR4 surface protein and the concentration of SDF in the culture media. Further supportive evidence was derived from blockade of the SDF-CXCR4 axis which resulted in abrogation of the SDF-mediated pro-proliferative effects in normally responsive cells.

Similar findings were demonstrated using techniques based on BrdU incorporation (a direct measure of DNA synthesis) and fluorescence-based assays of cell viability. While data derived from BrdU incorporation were suggestive that the SDF-CXCR4 axis mediated increases in cell proliferation, it was possible that the results of the cell viability assays were a combination of cell growth (proliferation) and cell death (apoptosis and necrosis). To investigate this further the effect of CXCR4 stimulation on cell death was determined.
6.4.3 The effect of SDF stimulation on cell death

Data demonstrating the pro-proliferative effect of SDF on cells bearing the CXCR4 receptor have been outlined in Section 6.4.2. What is not clear from cell viability assays alone is the mechanism by which SDF exerts this effect. One possibility was that cell apoptosis was reduced by SDF stimulation resulting in a net increase in the accumulation of cellular material detected by cell viability assays. Data derived from direct measurements of cell proliferation (BrdU incorporation) have suggested that this effect is small. However, several techniques exist by which apoptotic activity can be evaluated. An assay of caspase 3/7 activity was selected as this measures the “executioner arm” of the apoptotic pathway. Initially the caspase assay was validated and optimised using H357 cells incubated with varying concentrations of staurosporine, a known inducer of apoptosis (Figure A6.3, Appendix 6).

Once the caspase 3/7 assay had been validated it was necessary to determine the effect of serum-reduction on H357 cell apoptosis. Preliminary experiments were performed to determine the effect of serum reduction on apoptosis in untransfected H357 cells (Figure A6.4, Appendix 6). Further experiments were then performed to determine the effect of SDF stimulation on the caspase 3/7 activity in transfected H357 clones cultured under reduced-serum conditions (0.1% serum (Figure 6.8)). Stimulation with SDF (100ng/ml) had no statistically significant effect on caspase 3/7 activity in untransfected H357 cells compared with untreated controls. Treatment with AMD3100 (1μg/ml), either alone or in combination with SDF (100ng/ml), also produced no statistically significant difference in caspase 3/7 activity in untransfected H357 cells compared with untreated controls. A similar pattern of results was obtained for transfected H357 cells both with and without the CXCR4 insert. In summary, AMD3100 and SDF (either alone or in combination) had no significant effect on apoptotic activity in transfected or untransfected H357 cells.
Figure 6.8: The effect of SDF stimulation on H357 cell apoptosis. Four H357 cell clones (untransfected, empty-vector mock-transfected and two CXCR4-transfected – H357-X4.1 and H357-X4.2) were incubated in 0.1% FCS for 24 hours. Cells were either left untreated in 0.1% FCS (controls) or stimulated with AMD3100 (1µg/ml) or SDF 100ng/ml either alone or in combination. Caspase 3/7 activity was measured after 24 hours. Bars represent mean (standard deviation) of cumulative data derived from two identical experiments each with 12 replicate wells. Data are expressed as a percentage of untreated controls (0.1% FCS alone). Statistical comparison between groups was performed with the ANOVA test. There was no statistically significant difference between the treatment groups (SDF or AMD either alone or in combination) compared with controls. Legend: AMD = AMD3100 1µg/ml; UT = untransfected H357 cells; EV = empty-vector mock-transfected H357 cells; X4.1 and X4.2 = CXCR4-transfected H357 clones. Figure represents cumulative data derived from two identical experiments.
6.4.4 Summary of experimental results

6.4.4.1 Receptor internalisation

- CXCR4 surface expression in CXCR4-transfected H357 cells was reduced by 20 to 40% following incubation in low-serum (0.1%) media (Figure 6.1).
- Exposure of CXCR4-transfected H357 cells to SDF (100ng/ml) for one hour decreased CXCR4 surface expression by 25 to 50% with no further change after 16 hours (Figure 6.2).

6.4.4.2 Proliferation and Apoptosis

- SDF stimulation (100ng/ml) produced a significant 26% increase in the proliferation of the constitutively CXCR4-expressing cell line SCC4 (Figure 6.3).
- SDF stimulation (100ng/ml) produced significant increases (22-23%) in the proliferation of transfected H357 with (but not without) the CXCR4 insert (Figure 6.3).
- A clear dose-response between SDF concentration and increases in proliferation was demonstrated for H357 cells transfected with (but not without) the CXCR4 insert (Figure 6.4).
- Increasing concentrations of SDF produced significantly increased levels of BrdU incorporation for cells that did constitutively express CXCR4 (SCC4) but not for those that did not (Figure 6.5).
- Increasing concentrations of SDF produced increased levels of BrdU incorporation in CXCR4-transfected H357 cells with a maximum response at 10ng/ml (Figure 6.6).
- Co-stimulation with the CXCR4 antagonist AMD3100 significantly abrogated the SDF-mediated increases in cell proliferation of CXCR4-transfected H357 cells (Figure 6.7).
- SDF and AMD3100 stimulation (alone or in combination) had no significant effect on the apoptotic activity of untransfected H357 cells or those transfected with or without the CXCR4 insert (Figure 6.8).
6.5 Discussion

Before interpreting the results of the experiments presented in this Chapter it is useful to reflect upon the relative strengths and weakness of the methodologies employed and the statistical analysis performed on the data generated. This can be found in Section A6.3 (Appendix 6). Ligand-induced receptor internalisation is a key feature of chemokine (and cytokine) signalling mechanisms. Response to SDF gradients by CXCR4-expressing haematopoietic stem cells has been shown to be associated with the incorporation of the receptor and the GTPase Rac-1 into membrane lipid rafts (Wysoczynski, Reca et al. 2005). Consequently it would be possible to hypothesise that the response to SDF gradients is dependent on factors controlling intracellular lipid biochemistry. Direct evidence to support this notion comes from in-vitro observations that depletion of membrane cholesterol reduces the response to SDF by CXCR4 positive tumour cells (Kucia, Reca et al. 2005). Evidence that indirectly supports this hypothesis may be drawn from the in-vivo observations that coincident treatment with the “statin” family of compounds (that inhibit the intracellular synthesis of cholesterol) appears to be associated with an anti-tumour effect. Furthermore, some authors have suggested that other drugs (such as polyene antibiotics) that alter the formation of intracellular lipid rafts may also have an anti-cancer action (Kucia, Reca et al. 2005). Membrane-expressed haematopoietic phosphatase (CD45) associates with CXCR4 within lipid rafts and this can be blocked by β-cyclodextrin (which inhibits lipid raft formation) (Fernandis, Cherla et al. 2003).

The activated SDF-CXCR4 complex undergoes rapid internalisation through a mechanism involving G-protein-coupled receptor kinases. This is followed by the binding of β-arrestin (Cheng, Zhao et al. 2000). Although internalised CXCR4 may be re-expressed on the surface (blocking de novo synthesis), this process may be blocked in haematopoietic stem-cells by heparin (Kucia, Reca et al. 2005) or by cross-linking of L-selectin (Ding, Issekutz et al. 2003). CXCR4 in the plasma membrane can undergo internalisation in both a constitutive and a ligand-dependent fashion. It has recently been demonstrated that these two internalisation pathways are mediated by separate mechanisms (Futahashi, Komano et al. 2007). Evidence in the literature is inconclusive with regards to the hypothesis that CXCR4 internalisation is a requirement for all its signalling activity. It has been shown that SDF-induced phosphorylation of MAPKp42/44 still occurs using a mutant receptor that does not undergo
internalisation (Zhang, Wang et al. 2001). Furthermore, MAPKp42/44 phosphorylation is mediated by the third intracellular domain of the receptor which is not involved in receptor internalisation (Roland, Murphy et al. 2003).

Studies have been performed using mutant receptors to identify which part of the CXCR4 molecule is responsible for signalling (Roland, Murphy et al. 2003). These have shown that only the third intracellular loop is involved in the binding of Gαi proteins, calcium mobilisation and MAPKp42/44 activation but not receptor internalisation. SDF-mediated chemotaxis was dependent on the second, third and C-terminal intracellular domains, suggesting that migration is dependent on several complementary signalling events rather than one particular pathway. WHIM syndrome (warts, hypogammaglobulinaemia, immunodeficiency and myelokathexis) is associated with an activating mutation of CXCR4 characterised by truncations in the C-terminus region (Hernandez, Gorlin et al. 2003; Kawai, Choi et al. 2005). Following stimulation with SDF (100nM for 30 minutes) a reduction in surface CXCR4 levels of approximately 60% in wild-type and 30% in mutant receptor was observed. Receptor recovery after 120 minutes was twice as great in the mutant group compared to wild-type. Lymphoblastoid cells from these donors demonstrate greater calcium flux in response to SDF compared with normal controls (Kawai, Choi et al. 2005).

In cells constitutively producing detectable CXCR4 surface protein (SCC4), stimulation with SDF (100ng/ml) resulted in significantly increased proliferation. CAL27 cells (which demonstrated small amounts of CXCR4 surface protein by flow cytometry) showed a small increase in proliferation in response to SDF but this failed to reach statistical significance. Transfected H357 cells containing the CXCR4 insert demonstrated significant increases in proliferation in response to SDF (up to 100ng/ml) in a dose dependent manner. This effect was abrogated by co-incubation with the pharmacological CXCR4 antagonist AMD3100. The magnitude of the response to maximal stimulation with SDF (100ng/ml) was positively associated with the abundance of CXCR4 surface protein (as measured by the MFV determined by flow cytometry). The gradient of this association differed between transfected and non-transfected cells. This may be explained by differences in the binding capacity, downstream signalling or phenotypic responses of the different cell lines. Further experiments could be aimed at investigating these possible confounding variables.
From the observations that SDF-mediated increased proliferation, further experiments were performed to determine if the magnitude of this effect was dependent on factors associated with the SDF-CXCR4 axis (such as surface levels of CXCR4 or SDF concentrations) or just reflected differences between cell lines. It might be expected that untransfected cell lines producing surface CXCR4 protein at higher levels might show greater increases in proliferation in response to SDF compared with H357 cells. However, this is not the case. SCC4 cells demonstrated increased SDF-mediated proliferation by both fluorescence assay and BrdU incorporation, but CAL27 cells did not (despite demonstrating CXCR4 surface protein with flow cytometry). This may be an inherent feature of the different cell lines. However, it must be remembered that the statistical power of the BrdU incorporation studies was relatively low and further work may demonstrate a small, but statistically significant effect. Dose-response for SCC4 cells was maximal at 10 to 100ng/ml of SDF. Further work could be performed to investigate the effect of bio-neutralising antibodies or pharmacological antagonists on the SDF-mediated increase in SCC4 proliferation. These findings were consistent with the only other published results which demonstrated no further increase in proliferation with SDF concentration in excess of 10 to 25ng/ml in untransfected OSCC (Katayama, Ogino et al. 2005), hepatoma (Sutton, Friand et al. 2007), cervical (Zhang, Lu et al. 2007) and gastric carcinoma (Yasumoto, Koizumi et al. 2006) cell lines; and abrogation of the effect with anti-CXCR4 antibodies.

As mentioned earlier, the magnitude of the pro-proliferative effect of SDF in untransfected cells was associated with the availability of surface CXCR4 protein as measured by flow cytometry. The maximal response ranged from 5-8% for CAL27 cells and 15-26% for SCC4 cells. Other authors have demonstrated a range of changes in maximal response to SDF in untransfected gastric (10%) (Yasumoto, Koizumi et al. 2006), hepatoma (10%) (Sutton, Friand et al. 2007), breast (25%) (Wendt, Cooper et al. 2008), ovarian (40%) (Scotton, Wilson et al. 2002; Kajiyama, Shibata et al. 2008), thyroid (40%) (Castellone, Guarino et al. 2004; De Falco, Guarino et al. 2007), pancreatic (40%) (Marchesi, Monti et al. 2004), cervical (50-100%) (Yang, Lee et al. 2007; Zhang, Lu et al. 2007), oral (50%) (Katayama, Ogino et al. 2005) and basal cell (125%) (Chen, Yu et al. 2006) carcinomas. Does this represent changes in CXCR4-mediated signalling (surface density, ligand concentration or down-stream signalling), variation between carcinoma cell lines or differences in assay systems? It is difficult to make substantive conclusions when comparing data between cell lines or between tumour types. The most robust
data come from studies using cell lines in which single-gene transfections have been made. Nevertheless, in my experiments on untransfected cells there was good correlation between fluorescence-based and BrdU incorporation assays. In the literature the proportional changes in measurements of DNA synthesis were much greater than the variation in cell counts (Scotton, Wilson et al. 2002; De Falco, Guarino et al. 2007). This is due to methodological differences: changes in cell count over time are calculated relative to a baseline value whereas measures of DNA synthesis are “snapshot” views. MTT assays offer similar snapshot views and, indeed, studies using this technique tended to report greater differences in proliferation rates in response to SDF. In my experiments it would have been possible to perform MTT assays once the final fluorescence-based measurements had been made. This would provide a dataset complimentary to the first and a more accurate reflection of what was happening at the end of the incubation period. These combined techniques might be useful in further work.

The pro-proliferative effect of SDF stimulation on the transfected H357 cells containing the CXCR4 insert was significant (20-44%). Mock-transfected H357-EV cells lacking the CXCR4 insert did not demonstrate significant proliferative responses to SDF stimulation. Interestingly, both CXCR4-containing clones (H357-X4.1 and H357-X4.2) demonstrated similar levels of surface CXCR4 by flow-cytometry (Chapter 5) and proliferative responses to SDF stimulation that were statistically indistinguishable from each other (compared with untreated controls). However, transfection (with or without the CXCR4 insert) increased proliferation rates in unstimulated cells under serum-starved conditions compared with untransfected controls (Figure A6.1, Appendix 6). The influence of transfection on proliferation is discussed below. The shape and magnitude of the SDF dose-response curves for H357-X4.1 and H357-X4.2 proliferation were similar for each technique used. However, with fluorescence-based assays, 100ng/ml of SDF achieved maximal responses (approximately 14-17%) in H357-X4.1 and H357-X4.2 cells respectively. However, dose-response curves based on BrdU incorporation revealed maximal responses (approximately 15-18%) at 1-10ng/ml of SDF for both clones. Other experiments investigating the effect of SDF on the proliferation of OSCC-derived cells transfected with and without the CXCR4 insert are not described and comparison with the literature is not possible.
The inconsistency between the different assay systems regarding the SDF dose producing the maximal proliferative effect may be real or artefact. As discussed above, methods measuring DNA synthesis offer a snapshot view at the end of the incubation period while techniques comparing growth rates over time may be unduly influenced by early readings. To exclude methodological artefact, comparison with MTT measurements may be more useful. Transfected cells containing the CXCR4 insert demonstrated higher levels of surface CXCR4 protein (Chapter 5) and consequently may be more sensitive to exogenous SDF ligand. This may explain the greater response of transfected cells to low SDF concentrations compared with untransfected cells (Figure 6.6). Furthermore, it might be possible that these cells produce SDF in-vitro thereby exerting a direct proliferative effect via autocrine stimulation. Consequently, it might be expected that incubation with AMD3100 alone would reduce cell proliferation by blockade of the autocrine SDF/CXCR4 signalling pathway. Data from these experiments demonstrated that AMD3100 alone did not produce a statistically significant change in cell proliferation (Figure 6.8). Furthermore, the hypothesis of autocrine SDF production was not supported by evidence from assays of soluble protein level (data not shown). However, it is possible that ligand-mediated receptor internalisation may reduce soluble SDF protein levels below the detection threshold of commercial ELISA kits. Further studies using probes for SDF mRNA and Western blotting techniques would reveal insights into SDF gene transcription and levels of post-translational control of protein production by cells and could be included in further work. Investigation of prostate carcinoma cell lines demonstrated a reduction in proliferation following treatment with anti-CXCR4 antibodies (Sun, Wang et al. 2003). CXCR4 silencing in SDF-secreting breast and Mc3 salivary mucoepidermoid carcinoma cells resulted in reduced growth compared with controls (Lapteva, Yang et al. 2005; Wen, Zhu et al. 2007). In combination, these findings suggest that stimulation of the SDF-CXCR4 axis by either autocrine or paracrine ligand results in increased proliferation of some, but not all, tumour systems.

As discussed above, the effect of stimulation of the SDF-CXCR4 axis on tumour cell proliferation is the subject of much debate in the literature. There remains a paucity of published evidence regarding its effects on OSCC-derived cell lines and the data presented here would make a substantial contribution to this. The majority of recent reports that include proliferation studies (using a range of complimentary methodologies) suggest that SDF stimulation increases growth in cells producing
functional CXCR4. However, this conclusion is not ubiquitous. SDF (up to 100ng/ml) had no effect on the proliferation of SAS-H1 cells derived from an OSCC primary lesion (K Muraoka, poster communication, IADR annual scientific congress [Brisbane] 2006). No effect of SDF stimulation on proliferation was demonstrated in non-small cell lung carcinoma (NSCLC) (Phillips, Burdick et al. 2003), glioma (Zhou, Larsen et al. 2002), renal (Pan, Mestas et al. 2006), hepatocellular (Schimanski, Bahre et al. 2006) and laryngeal SCC cells (Tan, Chu et al. 2008). Consequently it seems likely that the magnitude of the effect of SDF on cell proliferation is mediated by the availability of surface CXCR4 receptors, ligand concentration and intrinsic factors within the cell lines. What is not completely clear from the literature is whether these are idiosyncrasies between cell lines or features of different tumour tissues. An answer to the question might be derived by a better understanding of the signalling pathways involved in modulating CXCR4-mediated changes in proliferation rate.

While there is increasing empirical evidence that SDF stimulation augments cell proliferation there is little data available to delineate the mechanisms involved. Indirect effects of SDF on cell proliferation will be discussed below. However, it has been proposed that SDF binding exerts a direct proliferative effect by altering the intracellular balance between the activated p38 stress kinase negative regulator and the PI-3K/AKT axis positive regulator (Vlahakis, Villasis-Keever et al. 2002). It has been well documented in oral and other carcinomas that binding of SDF to the CXCR4 axis activates a G-protein mediated cascade (Bonavia, Bajetto et al. 2003) resulting in rapid phosphorylation of the PI-3K/Akt and ERK1/2 pathways (Zhou, Larsen et al. 2002; Katayama, Ogino et al. 2005; Muller, Sonkoly et al. 2006; Yasumoto, Koizumi et al. 2006). However, although there is a significant volume of literature that demonstrates the effect of both of these pathways on cell proliferation, the only direct evidence pertaining to CXCR4 is from Yang, Lee et al, 2007. They showed that the ERK1/2 inhibitor PD98059 and the PI3K/Akt inhibitor LY294002 independently reduced CXCR4-mediated proliferation in BCC cells in culture (Yang, Lee et al. 2007). Interestingly, deletion of the –COOH terminal from the CXCR4 protein resulted in constitutional activation of ERK (Ueda, Neel et al. 2006). However, it is not clear whether this increased proliferation observed in these cells was due to ERK activation or a reduction in cell-adhesion molecule mediated signalling (vide infra).
Reduction of serum concentration was associated with a decrease in cell viability and an increase in apoptotic activity. Serum deprivation at 0.1% FCS doubled caspase-3/7 activity in untransfected and transfected H357 cells equally (Figure A6.4, Appendix 6). The magnitude of this effect was slightly greater than that described in the literature where increases in apoptotic activity were approximately 20% and 30% in ovarian and pancreatic carcinoma cells respectively (Marchesi, Monti et al. 2004; Kryczek, Lange et al. 2005). These differences may be due to variation in the absolute concentrations of serum used, characteristics of the carcinoma cells or features of the assay systems used. Variations in serum concentration appear an unlikely explanation as final FCS levels varied between 3% and 0% (Marchesi, Monti et al. 2004; Kryczek, Lange et al. 2005). Consequently it seems most likely that the differences may be due to intrinsic properties of the cells. Increased caspase-3/7 activity can be induced in OSCC-derived cell lines by incubation with cisplatin (R Damerau, poster communication, IADR annual scientific congress [New Orleans] 2007) and further work could investigate the effects of chemotherapy agents and radiation on apoptosis in these cells.

No reports on the effect of SDF stimulation on apoptosis in OSCC-derived cells are available in the literature. However, a reporting bias may be present as authors may choose not to present negative findings. In other carcinoma systems opinion is divided. No effect of SDF stimulation on low-serum induced apoptosis was demonstrated in breast (Wendt, Cooper et al. 2008), small-cell (Hartmann, Burger et al. 2005), or non small-cell lung carcinomas (Phillips, Burdick et al. 2003). However, chemical or radiation-induced apoptosis was significantly reduced by SDF, albeit at high concentrations (100-500ng/ml), in hepatoma (Sutton, Friand et al. 2007), cervical (Yang, Lee et al. 2007), adenoid cystic (Muller, Sonkoly et al. 2006), pancreatic (Marchesi, Monti et al. 2004), papillary thyroid (Castellone, Guarino et al. 2004) and basal cell carcinomas (Chen, Yu et al. 2006). In ovarian carcinoma cell lines VEGF has been shown to potentiate the SDF-mediated reduction in low-serum induced apoptosis (Kryczek, Lange et al. 2005).

Considering the stages of metastasis within the “Seed and Soil” hypothesis, proliferation is important for neoplastic progression at both the primary and secondary sites. Increased tumour cell growth confers a selective advantage to clones of neoplastic cells. On the one hand, it is possible that the SDF-CXCR4 axis provides an
additional mechanism by which cancer cells may respond in the tumour-tissue microenvironment. On the other, as some of these and other published results have suggested, there is indirect evidence for autocrine pathways that influence SDF-CXCR4 signalling. The differing effects of endogenous and exogenous sources of SDF on CXCR4-positive OSCC cells has been recently postulated (Uchida, Onoue et al. 2007). They argue that exogenous SDF within the lymph nodes mediates regional OSCC metastasis whereas endogenous production of SDF by OSCC cells facilitates haematogenous spread. Although distant metastasis is an uncommon, late occurrence in OSCC, there is increasing evidence that OSCC cells enter the systemic circulation (either directly or via lymphatic efferents) at an early stage in disease progression. If this is the case then clinical observations show that the vast majority of tumour emboli fail to establish themselves as distant metastases of a clinically detectable size. This failure of tumour proliferation at distant sites may be due to a reduced efficiency at a number of stages of the metastatic process including extravasation and angiogenesis (Fidler 2003). However, early work with OSCC-derived cell lines in animal models suggests that the SDF-CXCR4 axis may play a role (Uchida, Onoue et al. 2007) and the data presented here suggest that stimulation of this pathway exerts a pro-proliferative effect, at least in-vitro. However, rapid tumour growth without associated angiogenesis results in a hypoxic microenvironment. The interrelation between hypoxia and CXCR4 signalling is beginning to be unravelled, with particular focus on Hypoxia-inducible Factor-1α (HIF-1α). It has been demonstrated that HIF-1α is a potent regulator of both SDF (Ceradini and Gurtner 2005) and CXCR4 (Staller, Sulitkova et al. 2003; Pan, Mestas et al. 2006) expression under hypoxic conditions. Tissue hypoxia also induces VEGF production which has been shown to potentiate the effects of SDF on increased proliferation and reduced apoptosis in ovarian carcinoma cells (Kryczek, Lange et al. 2005). The effect of VEGF and SDF co-stimulation on the proliferation and apoptotic activity of OSCC-derived cells has yet to be investigated but may form a mechanism co-ordinating tumour growth and neo-angiogenesis at primary and secondary sites.

The effect of SDF stimulation on cell proliferation may not be (entirely) a direct consequence of CXCR4-mediated signalling. Evidence comes from haematopoietic progenitor cells that SDF may induce other autocrine growth factors directly (GraftFaure, Leveque et al. 2000) (Hodohara, Fuji et al. 2000) or, through increased cell motility, expose cells to paracrine factors secreted nearby (Kucia, Reca et al. 2005).
Finally, the apparent pro-survival effect of SDF may act through CXCR4-mediated changes in cell-cell adhesion. Ueda et al demonstrated that deletion of the -COOH terminal domain from CXCR4 resulted in a substantial increase in cell proliferation (Ueda, Neel et al. 2006). This increase occurred in the absence of SDF, suggesting that this was not a direct SDF-mediated effect on proliferation but, perhaps, a manifestation of a down-regulation of cell-to-cell contact and anchorage-dependence as evidenced by associated changes in the cell phenotype. In separate work on OSCC-derived cell lines producing wild-type CXCR4 surface protein, endogenous SDF production (approximately 1ng/ml) significantly increased anchorage-independent cell growth (soft agar assay) but not anchorage-dependent cell growth compared with controls (Uchida, Onoue et al. 2007). The effect of CXCR4 signalling on cell adhesion and its indirect effect on cell proliferation remains intriguing and would be a potential direction for further work. Possible strategies include the use of selective signalling pathway inhibitors, CXCR4-transfection of adhesion molecule deficient OSCC cells lines (such as E-cadherin negative H376 cells) or selective adhesion molecule gene silencing in the CXCR4-transfected H357 cells.

6.6 Conclusions

The results presented in previous chapters have demonstrated that transfection including the CXCR4-insert significantly increases production of the protein on the cell surface in-vitro. CXCR4 staining has also been demonstrated on clinically-derived specimens of OSCC metastasis. The data presented in this chapter demonstrated the functional response of CXCR4-producing cells to SDF stimulation. Stable transfection with the CXCR4 coding sequence significantly increases cell proliferation in response to SDF suggesting that the downstream signalling cascade mediating these effects is already present. Similarly, CXCR4 blockade was shown to attenuate these responses. As discussed in Chapter 1, metastatic mechanisms in-vivo encompass a number of additional cell processes including survival, angiogenesis, migration and invasion. Data presented here have demonstrated that CXCR4 production has no significant effect on cell apoptosis in-vitro. However, much of the early work on homeostatic chemokine receptors focussed on their role in selective leukocyte homing and a role for CXCR4 in cell migration and invasion is suggested. This will be investigated in Chapter 7.
Chapter 7:

The effects of CXCR4 function on cell motility in established OSCC cell lines and CXCR4 transfectants
7.1 Introduction

The characterisation of established OSCC-derived cell lines and CXCR4 transfectants has provided the background material to investigate the function of CXCR4 in these cells. Data presented in the previous chapter have demonstrated that SDF stimulation of CXCR4-expressing cells results in increased cell proliferation without a significant effect on apoptotic activity.

From its role in developmental biology it is clear that CXCR4 has an important effect on stem cell positioning (cell migration) and organogenesis (proliferation and survival). The role of the SDF/CXCR4 axis in lymphocyte homeostasis is also reliant on similar pathophysiological processes. Metastasis is a multistep process incorporating not only cell growth and survival, but also angiogenesis, migration and invasion. The association of CXCR4 expression with directed cell movement within the developing embryo and the immune system has stimulated a number of studies into its expression in carcinoma systems. Functional CXCR4 has been demonstrated in a number of tumours (reviewed in Section 1.5, Chapter 1). Furthermore, constitutive production of the ligand has been shown in a number of organs that are the common metastatic destinations for these cancers.

Clinically relevant OSCC metastases are often present in the cervical lymph nodes and less frequently at distant sites. However, the micrometastatic burden of this disease may be greater than the clinical picture suggests. What is not clear from the emerging literature on CXCR4 expression by OSCC-derived cell lines is the effect of SDF stimulation on directed and undirected cell movement. This chapter presents conclusive evidence that the SDF/CXCR4 axis mediates chemokinesis, chemotaxis and invasion in OSCC cell in-vitro.

7.2 Chapter Hypothesis

SDF stimulation of CXCR4-expressing OSCC cells mediates increased chemokinesis, chemotaxis and invasion in-vitro.
7.3 Chapter Aims and Objectives

7.3.1 Overall Aim
To investigate the effect of SDF stimulation on CXCR4 receptor-mediated OSCC cell motility.

7.3.2 Specific Objectives
6. To optimise laboratory assays for the determination of chemokinesis, chemotaxis and directed cell invasion.
7. To investigate SDF-induced chemokinesis in CXCR4-expressing OSCC cells.
8. To determine SDF-induced chemotaxis in CXCR4-expressing OSCC cells.
9. To analyse the role of the SDF-CXCR4 axis in directed OSCC cell invasion in vitro.

7.4 Results

7.4.1 The effect of SDF on cell movement.
The aim of these experiments was to investigate the effect of SDF on cell movement. This was to test the hypothesis that:

a) Motility in CXCR4-positive cells would be increased by stimulation with SDF in a dose-dependent manner.

b) Maximal cell movement would be achieved when a SDF gradient was present.

In order to test these hypotheses complimentary methodologies were employed to differentiate between cell motility in the presence or absence of a ligand gradient (chemotaxis and chemokinesis respectively).
7.4.1.1 The effect of SDF on chemokinesis (Gold colloid assays)

Gold colloid assays were performed to investigate the effect of direct SDF stimulation on undirected cell movement (chemokinesis) in the absence of a chemotactic gradient. To overcome the possible confounding effect of intercellular interactions on cell movement, the motion of single cells was analysed using this methodology.

Preliminary experiments were performed to establish the validity of the technique. The hypothesis that, within a particular time frame, the area cleared of gold colloid represents the perimeter of the excursion of the cells was tested. The relationship between the surface area covered by the cytoplasm and the area of gold colloid cleared from around the cells at the end of the incubation period was determined using H357-X4.1 cells exposed to 100ng/ml of SDF for 20 hours (Figure 7.1). The results demonstrated an area of gold colloid clearance over four-fold larger than the area defined by the cytoplasmic membrane at the time of fixation (Figure 7.1 panel d). From this finding it was concluded that the cells had undergone movement over the surface of the coverslip during the incubation period and that the boundary seen did not represent the cytoplasmic membrane of a single cell. Similar data were derived from H357-X4.2 cells (data not shown). Consequently it was assumed that a measure of gold colloid clearance could be used as an indicator of cellular chemokinesis.

A second series of experiments (10 replicates per condition) investigated the effect of SDF concentration on the chemokinesis of solitary transfected H357 cells with and without the CXCR4 insert (Figure 7.2). Without SDF stimulation both H357-X4.1 and H357-X4.2 cells demonstrated more motility (23% and 44% respectively, p<0.01) than H357-EV cells lacking the CXCR4 insert. Upon stimulation with SDF at concentrations of 1, 10 and 100ng/ml H357-X4.1 cells showed an increase in chemokinesis of 23%, 25% and 42% respectively (p<0.01) compared with unstimulated (SDF 0) controls. A similar pattern of response to SDF stimulation was seen with H357-X4.2 cells although the magnitude of the increased chemokinesis was less (8%, 7% and 20% more chemokinesis in response to 1, 10 and 100ng/ml SDF respectively). In H357-X4.2 cells only the highest concentration of SDF (100ng/ml) produced an increase in chemokinesis that achieved statistical significance (p<0.05). Furthermore, H357-EV cells demonstrated an increase in chemokinesis in response to SDF

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61 All statistical comparisons were performed using a two sample, homoscedastic Student t-test.
stimulation in a pattern similar to H357-X4.1 cells. Compared with unstimulated (SDF 0) controls, H357-EV motility was increased by 35%, 29% and 44% in response to 1, 10 and 100ng/ml SDF respectively (p<0.05). However, in absolute terms, the motility of H357-EV cells exposed to 100ng/ml SDF was the same as unstimulated H357-X4.2 cells; while the chemokinesis of H357-X4.1 and H357-X4.2 cells stimulated with 100ng/ml SDF was also identical. Finally, all three cell types demonstrated a similar degree of motility in response to 1ng/ml SDF as with 10ng/ml.

In summary, this small set of experiments investigated the effect of SDF stimulation on the chemokinesis of transfected H357 cells with and without the CXCR4 insert. Results suggest that increasing concentrations of SDF do increase chemokinesis. However, from the response of H357-EV cells it appears that overexpression of CXCR4 is not required to modulate SDF-mediated chemokinesis in this assay system. What is not clear from these experiments is the effect of directional vectors on cell movement. These “vectors” can either be defined by cell-cell interactions or extracellular gradients and further experiments were performed to investigate these respectively.
Figure 7.1: Determination of area covered in gold colloid assays. H357-X4.1 cells were incubated for 20 hours on sterile coverslips coated with gold colloid at a density of 4,000 cells per well. Cells were labelled with an anti-CXCR4 monoclonal antibody, a Texas Red-conjugated secondary antibody and counterstained with DAPI. Confocal fluorescence and brightfield microscopy was performed at x400 magnification and analysed using Zeiss LSM image software. Panels show images from the blue channel (a), red channel (b), brightfield (c) and overlaid red channel plus brightfield (d). Results demonstrate that the area of gold colloid cleared by the cells (panel c) is greater than the area occupied by the cytoplasmic membrane (panel b) which is, in turn, greater than the area of the cell nuclei (panel a). Scale bar represents 50µm.
Figure 7.2: The effect of SDF on cell chemokinesis. Transfected cells with (H357-X4.1 & H357-X4.2) and without (H357-EV) the CXCR4 insert were incubated for 20 hours on sterile coverslips coated with gold colloid at an initial density of 4,000 cells per well. Growth media was supplemented with SDF to yield final concentrations varying between 0 and 100ng/ml. Cells were washed, fixed, mounted and photographed under oil immersion. Raw data are given in Figure A7.1 (Appendix 7). Areas were recorded for 10 cells per experimental condition and the mean and standard deviations represented in a histogram (a). The same mean values were plotted against SDF concentration on a logarithmic scale (b), standard deviations were omitted for clarity. Statistical analysis was performed using the Student t-test. Legend: EV=H357 Empty Vector, X4.1= H357-X4.1, *=p<0.05, **=p<0.01 and ***=p<0.001. Scale bar represents 50µm.
7.4.1.2 The effect of SDF on directed chemokinesis (Scratch assays)

The results from the gold colloid assays demonstrate that single transfected cells with or without the CXCR4 insert increase their chemokinesis in response to SDF. However, this methodology does not investigate cells in contact with each other as is found in-vivo. An in-vitro model of this (the scratch assay) was used to analyse further the effect of SDF on chemokinesis.

Preliminary experiments were performed to optimise the cell seeding density, photographic intervals and incubation times (data not shown). Photomicrographs were taken at defined intervals (Figure 7.3, panels a, c & e) and a robust and reproducible method for measuring the distance between cells on either side of the scratch was tested empirically (Figure 7.3, panels b, d & f). For each experimental condition eight replicates were measured.

The effect of SDF concentration on scratch wound closure against time was determined for untransfected as well as transfected H357 cells with and without the CXCR4 insert (Figure 7.4). In the absence of SDF, all cell lines tested demonstrated no significant differences in the percentage of scratch wound closure over the 28 hour period investigated (range 41% to 51%). For all experiments positive controls using growth media supplemented with 10% FCS showed complete or near complete closure of wounds at 28 hours (mean remaining wound distance ranging from 0% to 18%). When pre-incubation with mitomycin C was omitted, all cell lines demonstrated complete or near complete wound closure at 20 hours. Statistical comparison of all cell lines tested under positive and negative control conditions demonstrated no significant differences.62

Following supplementation of the growth media with SDF, H357-X4.1 cells demonstrated significantly faster closure of scratch wounds: the mean percentage wound distance remaining at 28 hours was 51%, 23%, 27% and 6% for SDF concentrations of 0, 1, 10 and 100ng/ml respectively. A similar pattern of response was exhibited by H357-4.2 cells at 28 hours although with a reduced magnitude: mean percentage wound distances were 43%, 37%, 37% and 25% for SDF concentrations of 0, 1, 10 and 100ng/ml respectively. Interestingly, both untransfected (H357-UT) and

62 p>0.05, ANOVA
mock transfected (H357-EV) cells which lacked CXCR4 overexpression showed no response to SDF supplementation regardless of concentration. These findings suggest that SDF mediates faster scratch wound closure in growth-arrested H357 cells which have been modulated to overexpress CXCR4.

To test this hypothesis further a series of experiments was performed on responsive cell lines (H357-X4.1 and H357-X4.2) in which the SDF-CXCR4 axis was blocked by either pharmacological antagonists (AMD3100) or bioneutralising monoclonal antibodies (Figure 7.5). As before, unstimulated (SDF 0), growth-arrested cells from both cell lines demonstrated similar rates of scratch wound closure (range 45% to 48% at 28 hours). Supplementation with 100ng/ml SDF resulted in complete H357-X4.1 cell wound closure and reduced the remaining distance to 28% for H357-X4.2 cells at 28 hours. For both H357-X4.1 and H357-X4.2 cells incubation with either AMD3100 or anti-CXCR4 monoclonal antibodies completely abrogated the effect of SDF stimulation (100ng/ml) to the level of unstimulated (SDF 0) negative controls. However, the addition of isotype control monoclonal antibodies had no detrimental effect on the response to SDF supplementation.

In summary, these data provide compelling evidence that CXCR4 overexpression confers a response to SDF that mediates faster wound closure in the scratch assay system. The confounding effects of proliferation were excluded by initiating growth arrest prior to SDF exposure. Consequently, in assays in which the vector of cell movement was defined by an asymmetry in intercellular contacts, it can be hypothesised that the changes in wound closure rate observed were due to SDF-mediated chemokinesis. What is not clear from these results is whether the SDF-CXCR4 axis can mediate directed cell movement along chemotactic gradients. Further experiments using an alternative assay system were required to determine this.
Figure 7.3: The effect of SDF on scratch wound closure: determination of experimental procedure. Confluent wells of mitomycin-treated, growth-arrested cells were scratched to produce 8 replicates per well and subsequently incubated with varying concentrations of SDF as outlined in Section 2.9.2 (Chapter 2). In this representative experiment an SDF concentration of 1ng/ml was used. Photomicrographs (a, c & e) were taken at varying time and the distance between cell edges measured at a set distance from the intersection (as represented by “x” and “m” respectively in panels b, d & f). The mean percentage decrease in each distance (compared with baseline T0) and standard deviation was then calculated for each experimental condition. Scale bar represents 400µm.
Figure 7.4: The effect of SDF on scratch wound closure. Confluent wells of growth-arrested untransfected (H357-UT) and transfected cells with (H357-X4.1 and H357-X4.2) and without (H357-EV) the CXCR4 insert were prepared and scratched as previously described to produce 8 replicates per experimental condition. Growth media were supplemented with varying concentrations of SDF (0, 1, 10 and 100ng/ml). Photomicrographs were taken at varying time points (0, 5, 20 & 28 hours) and the distance between cell edges measured and expressed as a percentage of baseline (T0). Data from three identical experiments were combined and the mean distances were plotted against time (error bars were omitted for clarity). Statistical comparisons were performed using ANOVA. Legend: SDF concentration (0-100ng/ml), FCS M+ = Fetal Calf Serum with mitomycin, FCS M- = Fetal Calf Serum without mitomycin, *=p<0.05 and **=p<0.01.
Figure 7.5: The effect of SDF-CXCR4 blockade on scratch wound closure in responsive cell lines. Confluent wells of growth-arrested CXCR4-transfected cells (H357-X4.1 and H357-X4.2) were prepared and scratched as previously described to produce 8 replicates per condition. Growth media were supplemented without or with 100ng/ml SDF. Wells containing SDF were further supplemented with AMD3100, bioneutralising anti-CXCR4 monoclonal antibodies or isotype controls. Photomicrographs were taken at varying time points (0, 5, 20 & 28 hours) and the distance between cell edges measured and expressed as a percentage of baseline (T0). Data from three identical experiments was combined and the mean distances were plotted against time (error bars were omitted for clarity). Statistical comparisons were performed using ANOVA. Legend: AMD=AMD3100, mAb=anti-CXCR4 monoclonal antibodies, iso Ab=isotype control monoclonal antibodies, *=p<0.05 and **=p<0.01.
Gold colloid and scratch assays were previously performed to determine the effect of the SDF-CXCR4 axis in undirected and directed chemokinesis respectively. To investigate cell movement in response to SDF gradients the Transwell assays system was optimised and used. Preliminary empirical data (in conjunction with reports from the literature) revealed that keratinocytes adhered poorly to untreated polycarbonate membranes (data not shown). Therefore initial experiments were performed to investigate the effect of membrane coating on cell migration. The effects of pre-coating the polycarbonate membranes with type 1 collagen or fibronectin were compared against no pre-treatment. Cells were prepared in low-serum media (0.1% FCS) with and without the addition of a positive chemotactic stimulus (10% FCS) added to the lower chamber (Figure 7.6). Results demonstrated that without pre-coating there was a limited migratory response to 10% FCS but this was potentiated threefold by pre-treating with collagen. The difference between collagen coating on both surfaces and only the lower was negligible for both 10% FCS and negative controls. Interestingly, a significantly increased (p<0.001, Student t-test) migratory response was demonstrated when the lower surface was coated with fibronectin, even with FCS absent from the lower chamber. In the absence of FCS, coating of both surfaces with fibronectin decreased the number of migrating cells by approximately 60% (p<0.001). These results are suggestive that fibronectin alone acts as a significant haptotactic stimulus for these cells. Type 1 collagen facilitates adhesion (compared with no treatment) but does not act as an independent chemoattractant. All further experiments were performed with Type 1 collagen coating of the lower polycarbonate surface only.

The effect of transfection (with and without the CXCR4 insert) and SDF stimulation on the adhesion of cells was investigated using Transwell membranes pre-incubated with Type 1 collagen on the lower surface only (Figure 7.7). Results revealed that transfection had no effect on adhesion compared with untransfected controls. Addition of SDF (100ng/ml) to both the upper and lower chambers increased the numbers of migrating cells between two and threefold. While this increase was greatest in cells containing the CXCR4 insert (H357-X4.1 and H357-X4.2) the numbers of migrated cells was small and difference was not statistically significant. Addition of FCS to the lower chamber only was used as a positive control.
Figure 7.6: The effect of membrane coating on cell migration. CXCR4-transfected H357 cells were plated at known density in the upper chamber of a Transwell assay. Type 1 collagen or fibronectin had been used to pre-coat the polycarbonate membranes on either on either the upper and lower, or lower surfaces only. Media containing either 10% FCS or migration buffer (negative control) were added to the lower chambers. Three replicate wells per experimental condition were included. Cells were incubated overnight, fixed, stained and counted. Data from three identical experiments were combined and the mean (standard deviation) cell count per high power image was plotted against treatment type. Statistical analysis was performed with the Student t-test. In membranes with no pre-treatment, cells only migrated towards 10% FCS. Collagen coating potentiated this effect approximately threefold. However, it stimulated no significant migration when migration buffer was added to the lower chamber. Fibronectin coating resulted in the most cell migration towards 10% FCS. However, when there was no chemotactic stimulus in the lower chamber, fibronectin stimulated the migration of cells when applied to the lower surface alone. This effect was attenuated when both surfaces were pre-coated with fibronectin. Legend: MB = migration buffer, *** = p<0.001.
Figure 7.7: The effect of CXCR4 transfection and SDF stimulation on cell chemokinesis. Untransfected H357 cells (H357-UT) and H357 cells transfected with (H357-X4.1 & H357-X4.2) and without (H357-EV) the CXCR4 insert were prepared in migration buffer and known numbers were seeded in upper Transwell assay chambers which had been pre-incubated with Type 1 collagen on the lower surface. SDF (to a final concentration of 100ng/ml) was added to both upper and lower chambers of some wells. FCS (to a final concentration of 10%) was added to the lower chamber only of some wells to act as a positive control. Three replicate wells per experimental condition were prepared. Plates were incubated for 3 hours under optimal conditions. The numbers of cells on the lower surface were counted per high power image. Data from three identical experiments were pooled and the cumulative means (standard deviations) were calculated and plotted. Statistical comparison was performed using the Student t-test. SDF stimulation but not transfection (with or without CXCR4) demonstrated a significant increase on cell adhesion. Legend: MB = migration buffer, ** = p<0.01.
Figure 7.8: The chemotactic and chemokinetic effect of SDF. CXCR4-transfected H357 cells were seeded at a known density in the upper chamber of a Transwell assay. Migration buffer was added to the lower chamber and supplemented with either recombinant SDF (to a final concentration of 100ng/ml), 10% FCS (positive control) or nothing (negative control). An equal concentration of SDF was added to the upper chamber of one group of wells. Three replicate wells per experimental condition were prepared. Cells were incubated for 3 and 12 hours. Data from three identical experiments were pooled and the mean (standard deviation) cell count per high power image were plotted against treatment type and incubation period. Statistical analysis was performed using ANOVA with the Bonferroni post-hoc correction for multiple testing. There was some movement (chemokinesis) of cells in the negative control wells and this was increased approximately twofold by the addition of SDF to both upper and lower chambers. A chemotactic gradient of SDF dramatically increased the number of cells migrating to the lower chamber. This was about 65% of the number migrating towards 10% FCS. The pattern of results was similar for the 3 and 12 hour time points although values were approximately 50% greater after the longer incubation. Legend: MB = migration buffer, ** = p<0.01.
Figure 7.9: CXCR4-transfected H357 cell migration towards SDF: Time course. CXCR4-transfected cells were seeded at known densities in the upper chamber of Transwell assays. Migration buffer with or without SDF at 100ng/ml was added to the lower chambers and inserts were incubated for a variety of durations from 1 to 8 hours. Three replicate wells per experimental condition were performed. Data were pooled from three identical experiments. Mean (SD) cell number per high-powered image was plotted against time. Addition of migration buffer (MB) alone to the lower chamber resulted in a slow increase in cell migration to the lower surface of the membrane (red line). This relationship was linear (R-squared = 0.924, dotted line). Addition of SDF to the lower chamber significantly increased cell migration (blue line). This increase was greatest over the earlier time points and plateaued later. This relationship was best modelled with a logarithmic function (R-squared = 0.988, solid black line). Legend: MB = migration buffer, *** p<0.001, Student t-test.
Initially, the effects of recombinant SDF on chemokinesis and chemotaxis were determined (Figure 7.8). Results demonstrated that CXCR4-transfected H357 cells do demonstrate random chemokinesis when cultured in migration buffer alone for 3 hours. When 100ng/ml of SDF was added to both upper and lower chambers (obviating the effect of any chemotactic gradient), chemokinesis was approximately doubled. Creation of a chemotactic gradient across the membrane by the addition of 100ng/ml of SDF to only the lower chamber increased cell migration by approximately 100% percent (compared with the chemokinetic effect previously described). Addition of 10% FCS to the lower chamber resulted in the greatest migratory response (approximately 50% greater than towards 100ng/ml SDF). Incubation of cells under similar conditions for 12 hours yielded a similar pattern of results, although the numbers of migrating cells were approximately 50% greater than for the 3 hour time point. This is suggestive that some, but not all, cell migration occurs within the first 3 hours of incubation. Consequently, a time-course experiment was performed to determine the effect of incubation duration on migration.

Results demonstrated that when no chemotactic gradient was present there was a gradual increase in cell migration to the lower surface with time (Figure 7.9). This relationship was best modelled with a linear function (R-squared = 0.924). When cells were exposed to a chemotactic gradient of 100ng/ml of SDF across the membrane, there was a statistically significant increase in cell migration at all time points (p<0.001, Student t-test). At one hour, three times more cells had moved in response to SDF than with migration buffer alone. The rate of change of SDF-induced cell migration was greatest between the early time points and plateaued after approximately 5 hours. The relationship with time was best modelled with a logarithmic function (R-squared = 0.988).

The dose-response of CXCR4-transfected H357-X4.2) to varying chemotactic gradients of SDF was determined (Figure 7.10). Consistent with earlier findings, H357-X4.2 cells demonstrated some migration to the lower surface of the Transwell assay even in the absence of SDF (negative control). The greatest number of migrated cells was demonstrated towards 10% FCS (positive control). However, with an increase in SDF concentration in the lower chamber up to 50ng/ml, there was a concomitant 150% increase in the number of cell migrating through (p<0.001, Student t-test). The number of cells that migrated towards 50ng/ml of SDF was approximately 70% of the size of
the response to the 10% FCS positive control. Interestingly, increasing the SDF concentration in the lower chamber from 50 to 100ng/ml decreased the number of migrating cells by 30% but this change failed to achieve statistical significance. The control wells also yielded interesting results: Firstly, transfected H357 cells lacking the CXCR4 insert (H357-EV) demonstrated a similar number of migrating cells to the negative controls. The migratory response of H357-X4.2 cells to 100ng/ml of SDF was also significantly reduced (p<0.01) to baseline levels by the addition of the pharmacological CXCR4 antagonist AMD3100 at concentrations of 1µg/ml to the upper chamber. Similar attenuation was demonstrated by the addition of bioneutralising anti-CXCR4 monoclonal antibodies (but not isotype controls) to the upper chamber. Finally, the addition of 50ng/ml of SDF to the upper chamber also significantly reduced (p<0.01) the migratory response towards 100ng/ml of SDF (in the lower chamber) back to baseline levels.

A similar experiment was performed to investigate the effect of SDF gradient on SCC4 cell migration (Figure 7.11). A small number of SCC4 cells migrated to the lower surface of the membrane over the 8 hour time period. This was significantly increased (p<0.001, Student t-test) by the addition of SDF to the lower chamber in a dose-dependent fashion over the concentration range tested (25-100ng/ml). Increasing the SDF concentration from zero to 100ng/ml increased the number of migrating SCC4 cells fivefold. The maximum elicited response to SDF was approximately 45% of the size of the response to the positive control (10% FCS). Addition of SDF to the upper chambers reduced the chemokine gradient across the membrane and significantly reduced (p<0.05) the number of migrating cells: For low concentrations (12.5ng/ml) the reduction in the migratory response was proportional to the reduction in the chemotactic gradient across the membrane. However for greater concentrations added to the upper chamber (50ng/ml) there was a greater decrease in migratory response than would be predicted by a linear proportion. Addition of AMD3100 (1µg/ml) to the upper chamber abrogated the response to SDF back down to baseline (unstimulated) levels (p<0.001). A similar significant reduction was demonstrated with the incubation of cells with bioneutralising anti-CXCR4 monoclonal antibodies, but no significant change was demonstrated with their isotype controls.
Figure 7.10: CXCR4-transfected H357 cell migration towards SDF: Dose-response. Known densities of CXCR4-transfected H357 cells (clone X4.2) were seeded into the upper chambers of Transwell inserts. SDF at given concentrations were added to the lower chambers and the wells were incubated under optimum conditions for 8 hours. Three replicate wells per experimental condition were performed and data from three identical experiments were pooled. Bars represent mean (SD) cell counts per high-power field expressed as a percentage of positive control (FCS only). Statistical comparisons were performed using the Student t-test. Significantly more (p<0.001) cells migrated towards the SDF stimulus in a dose-dependent fashion up to concentrations of 50ng/ml. Further increases in SDF concentration did not yield significant further increases in migration. The chemotactic response was significantly attenuated (p<0.01) by the addition to the upper chamber of SDF (50ng/ml), AMD3100 (1µg/ml), bioneutralising anti-CXCR4 monoclonal antibodies (25µg/ml), but not by an isotype-control monoclonal antibody. Compared with unstimulated controls, empty-vector mock-transfected H357-EV cells showed no significant migratory response to 100ng/ml SDF. Legend: all SDF concentrations given in ng/ml; EV = empty vector mock-transfected H357 cells; AMD = AMD3100 at 1µg/ml; Ab = bioneutralising anti-CXCR4 monoclonal antibodies at 25µg/ml; iso = isotype control monoclonal antibodies at 25µg/ml; FCS = 10% FCS; *** = p<0.001, ** = p<0.01, ns= not significant (Student t-test).
Figure 7.11: SCC4 cell migration towards SDF: Dose-response. Wild-type SCC4 cells were seeded at known densities into the upper chambers of Transwell inserts. SDF at varying concentrations was added to the lower chambers and the wells were incubated for 8 hours under optimum conditions. Three replicate wells per experimental condition were performed and data from three identical experiments were pooled. Bars represent mean (SD) cell counts per high-power field expressed as a percentage of positive control (FCS only). Statistical comparisons were made using the Student t-test. Some cells migrated to the lower surface in the absence of SDF (negative control). The greatest migratory response was to 10% FCS (positive control). Significantly more (p<0.05) cells migrated in response to increasing SDF gradients across the membrane to the maximum value tested (100ng/ml). Further addition of SDF to the upper chambers significantly reduced (p<0.05) the migratory response. Incubation of cells with AMD3100 (1µg/ml) or bioneutralising anti-CXCR4 monoclonal antibodies (25 µg/ml) significantly reduced migration (p<0.001) to levels similar to unstimulated (negative) controls. Addition of isotype control antibodies to the upper chamber yielded no significant change in migration pattern. Legend: All SDF concentrations in ng/ml; AMD = AMD3100 at 1µg/ml; Ab = bioneutralising anti-CXCR4 monoclonal antibodies at 25µg/ml; iso = isotype control monoclonal antibodies at 25µg/ml; FCS = 10% FCS; *** = p<0.001, * = p<0.05, ns= not significant (Student t-test).
7.4.2 The effect of SDF on cell invasion

Preliminary experiments were performed to determine the optimum duration for the incubation of invasion assays (Figure 7.12). Results showed that very few cells invaded through the Matrigel to reach the lower surface of the membrane in the absence of a chemotactic gradient. With SDF, however, the invasive response of H357-X4.2 cells showed three phases: Initially, there was a latent phase in which no cells were seen on the lower membrane until 8 hours had elapsed. Secondly, between 8 and 16 hours, there was a linear phase in which the numbers of invading cells rose proportionally with time. Finally, after 24 hours, there was an exponential phase where the number of cells counted on the lower membrane exceeded what would have been predicted from a continuation of the linear phase. Interestingly, the morphology of the cells on the membrane surface after 24 hours was different to that seen at previous time points: Small clusters of dividing cells could be seen in the former but not in the latter (data not shown). This is suggestive that, after 24 hours, early invading cells were proliferating. Consequently, in order to negate this confounding effect, invasion assays were run for 16 hours overnight.

The invasive response of CXCR4-transfected H357 cells to varying concentrations of SDF was determined (Figure 7.13). Some cells invaded through the Matrigel to reach the lower surface of the membrane in the absence of SDF (negative control), although when 10% FCS was added to the lower chamber (positive control) the number of invading cells increased approximately sixfold. H357-X4.2 cells (a high CXCR4-expressing clone) demonstrated a linear dose-response to SDF in concentrations from zero to 100ng/ml with significantly more (p<0.001, Student t-test) cells invading with each increment SDF gradient tested (25, 50 and 100ng/ml). The number of invading cells was increased nearly sixfold over this range of concentrations. The numbers of invading cells towards 100ng/ml of SDF was statistically indistinguishable to that towards 10% FCS. Interestingly, addition of 50ng/ml of SDF to the upper chamber significantly reduced H357-X4.2 invasion (towards 100ng/ml of SDF) to levels similar to negative controls (p<0.001, Student t-test). Invasion of transfected H357 cells lacking the CXCR4 insert (H357-EV) was statistically indistinguishable from unstimulated (negative) H357-X4.2 controls despite the presence or absence of SDF. Incubation of cells with AMD3100 (1µg/ml) or anti-CXCR4 monoclonal antibodies (25µg/ml) significantly attenuated the invasive response towards 100ng/ml of SDF (p<0.001, Student t-test). This attenuation was not complete; residual invasion greater than
negative controls by about 25 and 35 percent respectively (although this failed to achieve statistical significance). The addition of isotype control monoclonal antibodies to the upper chambers had no effect on invasion.

Figure 7.12: CXCR4-transfected cell invasion towards SDF: Time course. Known densities of H357-X4.2 cells were added to the upper chamber of Transwell assays supplemented with Matrigel. Migration buffer with or without 100ng/ml SDF was added to the lower chambers. Wells were incubated for between 2 and 24 hours. Three replicate wells per experimental condition were performed and data were pooled from three identical experiments. Mean (SD) cell number per high power field was plotted against time. No cells were seen on the lower membrane until the 8 hour time point. Very few cells demonstrated invasion in the absence of a chemotactic gradient. In response to SDF significantly more cells demonstrated invasion compared with migration buffer alone. A linear increase in invasion with time over the 8 to 16 hour time period (R-squared = 0.962, dotted line) was demonstrated. The most cells were seen on the lower membrane after 24 hours. Legend: MB = migration buffer, *** = p<0.001, Student t-test.
Finally, the invasive response of SCC4 cells towards SDF was determined (Figure 7.14). Very few SCC4 cells invaded through the Matrigel in the absence of SDF. The creation of a chemokine gradient by the addition of SDF to the lower chambers resulted in a significant increase \( (p<0.001, \text{ Student t-test}) \) in invasion in an approximately linear dose-response up to a concentration of SDF of 50ng/ml. Further increasing the SDF gradient to 100ng/ml produced no statistically significant increase in invasion. The invasive response towards 100ng/ml of SDF was approximately fifteen times greater than that demonstrated in the absence of SDF \( (p<0.001, \text{ Student t-test}) \) but about two-thirds of the response to the 10% FCS positive control \( (p<0.01, \text{ Student t-test}) \).

Interestingly, addition of SDF to the upper chambers almost completely attenuated \( (p<0.001, \text{ Student t-test}) \) the invasion of SCC4 cells towards 100ng/ml of SDF. This effect was similar for the addition of 12.5 and 50ng/ml of SDF to the upper chamber. Incubation of cells with the pharmacological CXCR4 antagonist AMD3100 (1µg/ml) or anti-CXCR4 monoclonal antibodies (25µg/ml) also significantly reduced \( (p<0.001, \text{ Student t-test}) \) SCC4 cell invasion. For a SDF gradient of 100ng/ml across the Matrigel-coated membrane, the addition of isotype control antibodies demonstrated no significant effect on invasion.

In summary, these results demonstrate that both the constitutively CXCR4-producing cell line SCC4 and the transfected H357 cells containing the CXCR4 insert both migrate and invade across the Matrigel extracellular matrix analogue in response to SDF. The magnitude of this response appears to be related to the concentration gradient of SDF and the CXCR4 surface receptor density (as determined in Chapters 3 and 4). Furthermore these chemotactic responses were significantly attenuated by the addition of either SDF to the upper chamber (reducing the chemokine gradient) or by incubation with pharmacological (AMD3100) or bio-neutralising anti-CXCR4 monoclonal antibodies.
Figure 7.13: CXCR4-transfected H357 cell invasion towards SDF: Dose-response. Known densities of CXCR4-transfected H357 cells (clone X4.2) were seeded into the upper chambers of Transwell inserts supplemented with Matrigel. SDF at given concentrations were added to the lower chambers and the wells were incubated for 16 hours under optimum conditions. Three wells per experimental condition were prepared and the data from three identical experiments were pooled. Bars represent mean (SD) number of cells per high-powered field expressed as a percentage of positive control (10% FCS). Statistical comparisons were performed using the Student t-test. Significantly more (p<0.001) cells invaded through Matrigel towards the SDF stimulus in a dose-dependent fashion across the range of concentrations tested. The chemotactic response was significantly abrogated (p<0.001) by the addition to the upper chamber of SDF (50ng/ml). The addition of AMD3100 (1µg/ml) or bioneutralising anti-CXCR4 monoclonal antibodies (25µg/ml) demonstrated a significant attenuation (p<0.001) of the invasive response. Isotype-control monoclonal antibodies had no significant effect on invasion. Transfected H357 cells lacking the CXCR4 insert (H357-EV) showed a similar invasive response with and without 100ng/ml SDF which was statistically indistinguishable from unstimulated (negative) controls. Legend: All SDF concentrations given in ng/ml; EV = empty vector mock-transfected H357 cells; AMD = AMD3100 at 1µg/ml; Ab = bioneutralising anti-CXCR4 monoclonal antibodies at 25µg/ml; iso = isotype control monoclonal antibodies at 25µg/ml; FCS = 10% FCS; *** = p<0.001, ns = not significant (Student t-test).
Figure 7.14: SCC4 cell invasion towards SDF: Dose-response. SCC4 cells were seeded at known densities into the upper chambers of Transwell inserts supplemented with Matrigel. SDF at varying concentrations was added to the lower chambers and the assays incubated for 16 hours under optimum conditions. Three wells per experimental condition were prepared and the data from three identical experiments were pooled. Bars represent mean (SD) number of cells per high-powered field expressed as a percentage of positive control (10% FCS). Statistical comparisons were performed using the Student t-test. Very few cells invaded through the Matrigel in the absence of SDF (negative control). Significantly more (p<0.001) SCC4 cells invaded towards SDF added to the lower chamber in a dose dependent fashion up to a SDF concentration of 50ng/ml. Further increasing the SDF gradient to 100ng/ml did not produce a statistically significant increase in invasion. Cell invasion was significantly reduced (p<0.001) to levels of the negative control by the addition to the upper chamber of SDF (12.5 or 50ng/ml), AMD3100 (1µg/ml) or anti-CXCR4 monoclonal antibodies (25µg/ml). The addition of isotype control monoclonal antibodies to the upper chamber did not reduce cell invasion significantly. The greatest number of invading cells was demonstrated towards 10% FCS (positive control). Legend: SDF concentrations in ng/ml; AMD = AMD3100 at 1µg/ml; Ab = bioneutralising anti-CXCR4 monoclonal antibodies at 25µg/ml; iso = isotype control monoclonal antibodies at 25µg/ml; FCS = 10% FCS; *** = p<0.001, ** = p<0.01, ns = not significant (Student t-test).
7.4.3 Summary of experimental results

- Solitary, transfected H357 cells containing the CXCR4 insert demonstrated the most chemokinesis in response to SDF stimulation (Figures 7.1 & 7.2).
- CXCR4-transfected H357 cells in confluent culture demonstrated the quickest healing of scratch wounds in response to SDF gradients (Figures 7.3 & 7.4). This relationship was attenuated by CXCR4 receptor blockers (Figure 7.5).
- FCS and fibronectin gradients potentiated the migration of H357 cells across Transwell membranes (Figures 7.6 & 7.7).
- CXCR4-transfected H357 cells demonstrated chemotaxis in response to SDF gradients (Figure 7.8) that was maximal at 8 hours (Figure 7.9). Cell invasion increased over a period of 24 hours (Figure 7.12).
- SCC4 cells that constitutively produce CXCR4 protein migrated (Figure 7.11) and invaded (Figure 7.14) towards SDF in a dose-dependent manner.
- Similar responses were demonstrated by CXCR4-transfected H357 cells for both migration (Figure 7.10) and invasion (Figure 7.13).
- SDF-mediated responses in migration and invasion were attenuated by blockade of CXCR4 receptors.

7.5 Discussion

Before considering the implications of these results it is prudent to review the strengths and weaknesses of the experimental methodologies employed. These are discussed in Appendix 7. Proliferation and migration in the OSCC cell lines studied were anchorage dependent. In the absence of Type 1 collagen, cells adhered poorly to the polycarbonate Transwell membranes. It has been reported that SDF stimulation mediates increased adhesion extracellular matrix (ECM) proteins by CXCR4-producing head and neck squamous cell carcinomas (HNSCC) (Samara, Lawrence et al. 2004), small-cell lung (Burger, Glodek et al. 2003; Hartmann, Burger et al. 2005), ovarian (Kajiyama, Shibata et al. 2008), prostate (Taichman, Cooper et al. 2002) but not salivary mucoepidermoid carcinoma (Wen, Zhu et al. 2007) cell lines. There is little consensus in the literature with regards to the precise signalling mechanisms that modulate adhesion although in all cases blockade of the CXCR4 receptor abolishes the effect.
Downstream, it is likely that multiple integrin subunits are involved, mediated by Rho GTPases or ERK but not receptor-associated G-proteins (Taichman, Cooper et al. 2002; Burger, Glodek et al. 2003; Samara, Lawrence et al. 2004; Hartmann, Burger et al. 2005). While my results suggested that fibronectin also promoted CXCR4-transfected H357 cell adhesion, the effect of SDF stimulation on this was not examined.

The influence of cell adhesion on the metastatic process is complex. However, at a very simplistic level, it might be thought that increased adhesion may favour tumour proliferation and extravasation from the circulation, while reduced attachment may promote cell migration and invasion. The expression of adhesion molecules during different phases of the neoplastic process is likely to vary significantly. Preliminary findings suggest that CXCR4 may play a role in the increase or decrease of cell adhesion in response to endogenous or exogenous sources of SDF. It has been shown that SDF stimulation reduces ECM adherence by confluent colorectal carcinoma cells undergoing anoikis (Wendt, Cooper et al. 2008). While my preliminary experiments (performed to optimise the Transwell assays) demonstrated that cells were attracted to fibronectin, their design did not allow further comment on the influence of the SDF/CXCR4 axis on OSCC cell adhesion. This could be studied using simple ECM attachment assays (with blockade of various cell signalling pathways) or more complex assays of cell-cell adhesion (for example with endothelial cells). These methodologies would be potential avenues of further work.

The hypothesis that the SDF-CXCR4 axis mediates increased motility in OSCC cells in-vitro is supported by the data presented in this chapter. As discussed in the previous chapter, the magnitude of the effect may be due to a number of different factors including ligand concentration, ligand source, surface protein density, cell line motility and assay type. In three different assay systems, exposure of CXCR4-overexpressing cells to media supplemented with SDF produced an increase in both chemokinesis and chemotaxis. However, a simple relationship between dose and response was not observed. In the gold colloid and scratch wound assays, H357-X4.1 and H357-X4.2 cells showed significant increases in motility when the SDF concentration was increased from 0 to 1ng/ml, and from 10 to 100ng/ml, but no difference between SDF concentrations of 1 and 10ng/ml. In high expressing OSCC cell lines a similar dose-response pattern was observed in some (Almofti, Uchida et al. 2004) but not all (Uchida, Begum et al. 2003; Katayama, Ogino et al. 2005) Transwell invasion assays.
However, comparative data from invasion assays should be interpreted with caution (*vide infra*). Similar data from gold colloid and scratch wound assays are not available in the literature.

Chemokinetic (gold colloid) assays demonstrated that cell motility was increased by 20-40% with SDF stimulation (100ng/ml) in transfected H357 cells with and without the CXCR4 insert. This suggests that SDF increases random cell movement in cells with high and low levels of CXCR4 surface protein even in the absence of a directional stimulus. One similar published study demonstrated that one cell line (out of two tested) demonstrated a doubling of random cell movement in response to SDF despite both producing CXCR4 mRNA and functional protein (Ishikawa, Nakashiro et al. 2006). However, protein production in these cell lines was investigated with Western Blotting and not flow cytometry (hence its location inside or outside the cell membrane could not be determined). Recent work on human mesenchymal stem cells (MSCs) has demonstrated that SDF-1 stimulation activates Jak2/STAT3 as well as MEK/ERK1/2 signalling, which promotes migration of MSCs toward tumour cells. Focal adhesion kinases and paxillin were significantly activated which correlated with reorganization of F-actin filaments in MSCs (Gao, Priebe et al. 2009).

In the Transwell migration assays, the dose-response relationship for both H357-X4.2 and SCC4 cells demonstrated a similar pattern but a differing magnitude. SCC4 cells demonstrated an increase in chemotaxis proportional to SDF concentration across the range investigated (0 to 100ng/ml). It is possible that, at SDF concentrations below 100ng/ml, the magnitude of response was determined by ligand availability rather than downstream signalling. Further evidence supporting ligand concentration as a determinant of migratory response is available in the literature: Similar peak chemotactic responses at 100ng/ml SDF were reported by groups investigating cell lines constitutively expressing CXCR4 derived from head and neck SCC (Katayama, Ogino et al. 2005), nasopharyngeal (Hu, Deng et al. 2005), breast (Muller, Homey et al. 2001; Helbig, Christopherson et al. 2003) and non-small cell lung (Phillips, Mestas et al. 2005) carcinomas. Breast carcinoma cells transfected with wild-type CXCR4 demonstrated a peak migratory response to 12.5ng/ml SDF with migration decreasing with further increases in SDF concentration up to 250ng/ml (Ueda, Neel et al. 2006). SDF-induced invasion was abrogated by co-culture with AMD3100 or neutralising anti-CXCR4 monoclonal antibodies. Similar antibody concentrations produced
identical results in 3 other OSCC cell lines (Katayama, Ogino et al. 2005). Only in adenoid cystic carcinoma cells has it been shown that increasing SDF concentrations to supra-physiological levels (up to 1500ng/ml) produces any further increase in chemotaxis in the Transwell system (Muller, Sonkoly et al. 2006).

SDF concentrations measured *in-vitro* range from 1 to 10ng/ml for SDF-transfected OSCC cell lines and gingival fibroblasts respectively (Almofti, Uchida et al. 2004; Uchida, Onoue et al. 2007). In breast carcinoma cells transfected with modulated CXCR4, SDF concentrations of 0.1ng/ml were demonstrated in complete serum but not serum-free media (Ueda, Neel et al. 2006). Measurements *in-vivo* have found SDF concentrations of 25ng/ml have been found in malignant ascites (Kryczek, Lange et al. 2005). Significantly faster rates of scratch wound closure have been demonstrated by CXCR4-producing OSCC cells endogenously expressing SDF (approximately 1ng/ml) compared with controls (Uchida, Onoue et al. 2007). However, these experiments ran for 48 hours without the induction of growth arrest and therefore the confounding effect of cell proliferation cannot be determined. In contradistinction, increased endogenous production of SDF in breast carcinoma cells was associated with reduced cell chemotaxis towards exogenous SDF stimuli and *vice versa* (Wendt, Cooper et al. 2008). The quantitative effects of mechanisms such as ligand-mediated chemokine receptor internalisation and the tumour-tissue microenvironment on SDF concentration gradients *in-vivo* are difficult to determine.

In assays of scratch wounded, growth-arrested monolayers, the effects of SDF stimulation on the motility of CXCR4-transfected H357 cells were clear. The rate of wound closure differed significantly between clones and was reproducible across experiments. This was due to variation in cell motility rather than cell proliferation, although the reasons for the former are not clear. CXCR4 blockade abrogated the SDF-mediated response suggesting that the receptor remains functional in both clones. However, the more motile clone (H357-X4.1) constitutively produced higher levels of surface protein and enhanced ligand-mediated receptor-internalisation compared with H357-X4.2 cells. If this explanation is valid then it might be expected that the efficacy of the SDF/CXCR4 signalling mechanisms would vary between clones. The hypothesis of altered receptor sensitivity could be investigated by measurements of calcium flux and the effect of signalling pathway blockade on cell function. The effects of altered CXCR4 receptor cycling on cell motility have been demonstrated in breast
carcinoma cells (Ueda, Neel et al. 2006). Cells transfected with COOH-truncated CXCR4 closed scratch wounds quicker than wild-type controls. However, demonstrated changes in proliferation rate were not controlled for and this truncation has wide ranging effects on cell adhesion and epithelial-to-mesenchymal transition. Furthermore, while COOH-truncated CXCR4 cells demonstrated faster basal migration levels there was no clear dose response to SDF. While the two clones I investigated demonstrated different receptor sensitivities it seems unlikely that this was due to similar terminal deletions. It is also possible that the transfection process had resulted in other differences in the transcriptomes between the clones (for example an effect of the plasmid gene promoter upon genes modulating cell motility). This would require a more in-depth analysis to determine the exact insertion site although its effect on gene expression could be investigated by microarray analysis of stimulated cells.

Does the evidence support the hypothesis that SDF-mediated cell migration and invasion is proportional to CXCR4 receptor surface density independent of cell line? Compared with SCC4 cells, increased CXCR4 surface receptor density (as demonstrated by mean fluorescence intensity measurements by flow cytometry), a maximal chemotactic response to lower concentrations SDF (25-50 ng/ml) and a threefold increase in the numbers of migrating cells at this concentration were all demonstrated empirically by H357-X4.2 cells. Furthermore, pre-incubation of cells in the upper Transwell chamber with SDF significantly reduces chemotaxis possibly due to a diminution of available CXCR4 secondary to ligand-induced receptor internalisation. Invasive response has been shown to vary in association with levels of CXCR4 mRNA and protein in different constitutively expressing OSCC cell lines (Uchida, Begum et al. 2003; Almofti, Uchida et al. 2004; Ishikawa, Nakashiro et al. 2006).

Surface CXCR4 protein (as detected by flow cytometry) may not mediate chemotaxis in response to SDF in all cell lines. Hu et al demonstrated that only two out of seven CXCR4-producing nasopharyngeal carcinoma cell lines migrated towards an SDF gradient in-vitro (Hu, Deng et al. 2005). This suggests that CXCR4 receptor function and signalling is not ubiquitous. However, it is not clear whether this is due to defects in the CXCR4 protein, deficits in the SDF/CXCR4 signalling pathways or due to variations in other associated characteristics of the cells (such as invasiveness, cell adhesion or proliferation). Transfected H357 cells lacking the CXCR4 insert (H357-EV)
were responsive to increasing concentrations of SDF as determined by the gold colloid assay. The pattern of response was similar to transfected cells that did contain the CXCR4 insert. H357-EV cells do demonstrate very low levels of constitutive CXCR4 surface labelling and it is tempting to attribute this observed behaviour to this. An alternative hypothesis is that it is the incorporation of the transfection plasmid that confers enhanced chemokinesis in these cells irrespective of the inclusion (or not) of the CXCR4 insert. However, in both scratch wound and Transwell assays no chemotactic response of H357-EV to SDF was observed. One possibility is that the data generated for H357-EV cells in the gold colloid assay is not truly representative although the results are averaged from 10 replicate cells and the experiment was repeated twice. Further work to investigate this effect could compare transfected with untransfected cells and also focus on the blockade of the CXCR4-SDF axis with pharmacological antagonists, monoclonal antibodies or inhibitors of the signalling pathways discussed above. In this way the validity of the observed data could be tested.

Despite their frequency in the literature, comparisons of invasive potential between cell lines are fundamentally flawed (Almofti, Uchida et al. 2004; Uchida, Begum et al. 2004; Katayama, Ogino et al. 2005). Matrix degradation is a key mechanism in neoplastic invasion. OSCC cells have been shown to produce matrix metalloproteinases (MMPs) and urokinase plasminogen activators (uPAs) constitutively in culture (Kawamata, Nakashiro et al. 1997). While some authors have established an association between SDF stimulation and production of activated MMP-9 by HNSCC cells (Samara, Lawrence et al. 2004), the majority of the published literature disagrees for OSCC (Onoue, Uchida et al. 2006) and other carcinomas including ovarian (Scotton, Wilson et al. 2002). It has been suggested that matrix degradation is an independent feature of tumour cell behaviour and chemokine stimulation merely defines the directional component (Scotton, Wilson et al. 2002). My data suggest that SDF stimulation mediates more than just directed chemotaxis alone due to the demonstrable effect on chemokinesis. This is supported in the OSCC literature (Ishikawa, Nakashiro et al. 2006). My results clearly demonstrate the ability of CXCR4-transfected H357 cells to invade through Matrigel. A clear dose-response relationship between SDF concentration and invasiveness was demonstrated in CXCR4 expressing cells in concordance with other published studies on OSCC-derived cell lines (Uchida, Begum et al. 2003; Rehman and Wang 2008).
While invasion may be greatly increased in highly expressing (transfected) cell lines it should be remembered that the functional endpoint measured is a manifestation of many mechanisms within the cell line under investigation. The signalling pathways that modulate these functions have been investigated by some authors. Both the ERK1/2 and Akt/PKB pathways are thought to be involved in migration mediated by the SDF-CXCR4 axis (Uchida, Begum et al. 2003; Uchida, Begum et al. 2004; Katayama, Ogino et al. 2005). More recently Rehman et al have suggested that NF-κB signalling has a role in SDF-mediated Matrigel invasion via a TNF-α-independent mechanism (Rehman and Wang 2008). This finding is replicated in breast carcinoma cells and raises the intriguing prospect of an autocrine loop as NF-κB has been shown to induce CXCR4 expression in these cells (Helbig, Christopherson et al. 2003). Further experimental investigation might have focussed on the selective blockade of the MEK, P13K, SFK and NF-κB pathways with the synthetic inhibitors PD098059, LY294002, PP1 and IKKβVI respectively. The disadvantage of using such inhibitors is that they have a far ranging effect on cell function which is not restricted to the migratory endpoint of the pathway under investigation. Careful control experiments must be performed to ensure that, for example, changes in migration or invasion are not secondary to changes in proliferation (Rehman and Wang 2008). Consequently, the observed changes in cell phenotype may not always be attributable to the effect on single pathways.

Perhaps the most informative line of further work would be to investigate differences between a single cell line transfected with and without the CXCR4 insert. This would allow direct comparison of the effects of CXCR4 overexpression in different aspects of chemokinesis, chemotaxis and invasion while controlling for other characteristics of the cell line. My data have demonstrated functional differences between clones containing the CXCR4 insert suggesting that insertion site may influence phenotype. It is also possible that certain cell lines may not be appropriate to use in particular assay systems. My preliminary findings using H357 cells on dermal collagen in organotypic culture showed no significant invasion. This may be due to a reduced MMP/uPAs activity produced by the cell line which has been shown to be poorly tumourigenic in vivo (Prime, Eveson et al. 2004). Consequently, an alternative cell line with greater constitutive MMP activity but low CXCR4 expression may be suitable for transfection.
Transfection with or without the CXCR4 insert made no significant impact on the response of H357 cells to wounding in the absence of SDF. However, H357-X4.1 cells were considerable more responsive to SDF to stimulation than H357-X4.2. As the CXCR4 mRNA expression and surface receptor labelling were similar for both clones, this suggests that where the gene was incorporated into the host cell line genome may have a significant effect on the change in phenotype arising. Nevertheless such changes are not universally demonstrated as the response of both clones to SDF stimulation in the gold colloid assays was almost identical. It is likely that the overexpression of the CXCR4 sequence does not ubiquitously lead to increases in cell chemotaxis but the functional response measured is dependent on other factors within the cellular microenvironment. These might include intracellular binding, adhesion to extracellular matrix molecules (such as fibronectin and heparin sulphated proteoglycans) and possibly the effect of these on SDF ligand binding efficiency (Mbemba, Gluckman et al. 2000; Sbaa-Ketata, Vasse et al. 2001).

Hu, Deng et al (2005) demonstrated that, of two CXCR4-producing nasopharyngeal carcinoma cell line subclones produced, only one of which demonstrated SDF-mediated chemotaxis *in-vitro*. While both subclones formed primary lesions following orthotopic injection into nude mice but only the SDF-responsive one metastasised to local lymph nodes and the lungs. Anti-sense CXCR4 abrogated the in-vitro chemotaxis and reduced lymphatic and lung metastases *in-vivo* (Hu, Deng et al. 2005). These data demonstrate that blockade of SDF/CXCR4 signalling decreases, but does not abolish, metastasis *in-vivo*, suggesting that the metastatic process is dependent on more pathways than CXCR4 alone.

### 7.6 Conclusions

The results presented in previous chapters have demonstrated that SDF stimulation results in increased proliferation of OSCC cells bearing the CXCR4 receptor *in-vitro*. Data presented in this chapter supports the hypothesis that CXCR4 mediates directed cell movement *in-vitro*. This was demonstrated in response to SDF stimulation both with and without a directional gradient. Transfected cells containing the CXCR4 insert demonstrated a greater response than those without, suggesting that the CXCR4 downstream signalling cascade that mediates cell motility in these cells was present
and functional prior to transfection. Similarly, blockade of the SDF-CXCR4 axis attenuated these responses \textit{in-vitro}. In combination with the results presented in previous chapters, these data support the hypothesis that CXCR4-mediated responses play a role in OSCC cell metastasis through effects on proliferation, chemotaxis and invasion.
Chapter 8:

Conclusions
8.1 Clinical context

Oral squamous cell carcinoma is a dismal disease. Overall, approximately one third of patients diagnosed with the condition in the United Kingdom will die as a result of it. Recent publications have shown that disease-specific 5-year survival has changed little in the last decade (Woolgar, Rogers et al. 1999; Rogers, Brown et al. 2009). Modern therapeutic regimes are still associated with significant morbidity and mortality despite the advent of new strategies including microvascular reconstruction and Intensity-Modulated Radiotherapy (IMRT). Independent clinicopathological features associated with poor prognosis are positive primary tumour resection margins, the presence of lymph node metastases, extracapsular spread from lymph node deposits and metastatic dissemination (Woolgar 2007). All of these features represent the ability of the tumour to invade locally and spread to distant sites. Clinicians planning individual treatment algorithms face the difficulty of estimating the likelihood of disseminated disease based on clinical, radiological and histological investigation. The sensitivity and specificity of these combined assessments still fall far short of perfect and, at best, treatment recommendations are based on informed estimates (Weiss, Harrison et al. 1994; Okura, Aikawa et al. 2009). Consequently, the ability to predict accurately the likelihood of metastatic dissemination from analysis of the primary OSCC lesion would be extremely beneficial to clinicians and their patients.

With the advent of modern laboratory techniques the focus of predicting metastases has fallen on the characterisation of molecular biomarkers. Numerous single markers have been statistically associated with OSCC lymph node metastases. However, sensitivity and specificity data have been universally disappointing with the conclusion that these molecular analyses yield little clinical benefit at present. Gene arrays have offered the ability to profile multiple markers from single tumours with the associated benefits in statistical prediction (O’Donnell, Kupferman et al. 2005; Hunter, Thurlow et al. 2006). However, again their findings are based on retrospective analyses of association and are limited by the methods of mRNA extraction from tissues. As previously discussed, tumour/tissue homogenates offer little insight into the specific gene signatures of tumours (compared with the surrounding stroma). For molecules such as CXCR4 this is a significant handicap due to its physiological expression in the peri-tumour infiltrate. One strategy to overcome this would be to use laser-capture microdissection with multiplexed semi-quantitative RT-PCR. This was
attempted in this thesis but found to be technically very difficult with confounding influences (such as tissue preparation and preservation) impossible to control for without the prospective collection of specimens. Further work using LCMD/sqRT-PCR with fresh clinical material would provide an interesting insight into the expression of CXCR4 and other markers in OSCC ex-vivo.

8.2 A novel, quantitative method for using immunohistochemistry

In this thesis, immunohistochemistry (IHC) was used to investigate the pattern of CXCR4 staining in archived clinical OSCC specimens. Traditional IHC is subject to the limitations of methodological reproducibility and inter-observer variation discussed in Chapter 4. The most common faults in the reported literature are the failure to include appropriate positive and negative controls, and the (often arbitrary) grading systems used to convert qualitative images into quantitative results. The lack of consistency in the latter precludes comparison between published series. To address these issues I developed a novel method by which the intensity of IHC cell staining could be examined. It also produced robust and reproducible, quantitative data that could be examined with appropriate statistical tests. In this way the inherent biases associated with the conventional assessment of IHC specimens were addressed. However, this method alone was not able to control for the possible confounding effects of variable staining intensities caused by the processing of individual samples in batches. To overcome this I used tissue arrays of suitable clinical material. In this way, all specimens were exposed to identical incubation conditions and any variability between batches was eliminated. For this reason the use of tissue arrays has significant advantages. One limitation of my work was that there were insufficient samples represented on the array to permit the independent examination of the association of CXCR4 staining intensity with possibly interrelated variables such as tumour site, tumour size/thickness, histological differentiation and nodal metastasis. This could be overcome by creating larger arrays containing more specimens (Salvucci, Bouchard et al. 2006). As far as I am aware this method of IHC, which combines the use of tissue arrays and an independent, quantitative assessment of staining intensity, has not been previously described in the literature.
My results demonstrated a statistically significant increase in CXCR4 staining of the cytoplasm in primary OSCC lesions that demonstrated lymph nodes metastases (pN+) compared with primary lesions in which no lymph node metastases were detected (pN0). No difference in cytoplasmic staining intensity between normal tissue and pN0 primary OSCC lesions was shown. These data suggest that CXCR4 staining of the cytoplasm is positively associated with lymph node metastasis but not with neoplastic transformation. However, nuclear CXCR4 staining was significantly increased in primary oral and other head and neck SCCs compared to case-matched normal controls. This suggests that CXCR4 staining in the nucleus is positively associated with neoplastic transformation (but not lymph node metastasis).

These IHC results highlight the critical importance of including matched normal controls in IHC analyses as, without these, no comment can be made on the level of staining intensity observed in the primary lesions. The prevalence of positive CXCR4 staining in normal oral mucosa varies widely in the literature (reviewed in Section 4.5, Chapter 4). This is probably a reflection of the variety of IHC methodologies employed. My preliminary investigations suggested a positive but lower level of staining in case-matched normal mucosa (Figure 4.1, Appendix 4). However, using the quantitative methodology accounting for background staining, a similar intensity of specific CXCR4 staining in normal mucosa and pN0 primary OSCC was demonstrated. This has not been previously described. This may be due to the methodological inadequacies in other published studies as previously discussed. Alternatively it may be due to sampling error resulting from the small numbers of case-matched normal controls available on the tissue arrays. Further work using this novel, quantitative methodology to investigate CXCR4 staining in normal mucosa would be recommended. Prospectively-harvested, non-inflamed, normal oral mucosa from young individuals without significant exposure to environment risk factors for OSCC (heavy alcohol and tobacco use) would be of particular interest. It is questionable whether pericoronal tissue taken at the time of the removal of symptomatic 3rd molar teeth provides a suitable normal control.

As discussed above, there are severe limitations to the combined techniques of LCMD/sqRT-PCR. My results have shown that novel analysis of conventional IHC can yield significant insights into the distribution of CXCR4 staining in primary OSCC.
lesions. These data serve to inform *in-vitro* experiments where CXCR4 (and other chemokine receptors) production and function can be investigated.

### 8.3 Insights from *in-vitro* investigations.

In this study, microarrays were used to perform a wide ranging screen of chemokine receptor mRNA expression in six established OSCC cell lines. CXCR4 was found to be the most highly expressed chemokine receptor. Data derived from *in-vitro* experiments using microarrays and sqRT-PCR demonstrated a significant increase in CXCR4 mRNA expression in some (but not all) OSCC cell lines compared with normal oral keratinocytes. CXCR4 mRNA was expressed in normal oral keratinocytes, but at very low levels. Examination of CXCR4 protein production revealed very low levels in normal oral keratinocytes (Figure 3.9, Chapter 3). In OSCC cells with low levels of CXCR4 mRNA expression, the protein staining was weak (Figures 3.11 & 3.12, Chapter 3) and limited predominantly to the perinuclear structures (Figure 3.15, Chapter 3). Established cell lines with high constitutive levels of CXCR4 mRNA expression and cells transfected with the CXCR4 insert demonstrated stronger surface protein staining (Figure 3.13, Chapter 3 and Figure 5.11, Chapter 5 respectively).

Observational data from established OSCC cell lines (Chapter 3), clinical specimens (Chapter 4) and transfected OSCC cells containing the CXCR4 insert (Chapter 5) were consistent in demonstrating low levels of CXCR4 in normal cells, increased levels in the perinuclear structures in low-expressing OSCC cells and significant cytoplasmic staining in high-expressing OSCC cells. IHC data suggested that cytoplasmic staining was associated with lymph node metastasis. What was unclear from these observations was the role of CXCR4 in OSCC cell function. This marked the second part of the thesis in which attention was focussed on CXCR4 through the production of a stably-transfected cell line and the assessment of the SDF/CXCR4 axis on cell proliferation, apoptosis, migration and invasion.

The microarray data presented here have demonstrated a significant diversity in the transcriptomes of the established OSCC cell lines investigated. This underpins the key limitation of comparing the results of functional assays in a variety of cell lines: there are a number of genetic factors that may explain any significant differences that are
detected. Consequently a more robust method of investigating the effect of the expression of one particular gene is to manipulate its transcription. The method chosen in this thesis was over-expression in a constitutively low-expressing established OSCC cell line by stable transfection with a vector including the CXCR4 insert (together with appropriate controls). An alternative method would have been to select a high-expressing cell line and knock-down CXCR4 using small, interfering RNA (siRNA). From the literature, CXCR4 appears to confer a positive effect on certain cell functions. Therefore the advantage of the former technique compared with the latter is that experiments can be designed looking for an increase in effect, rather than a decrease or absence. However, the two techniques are complimentary and further work targeting CXCR4 for gene silencing in the SCC4 cell line could produce another model for investigation with functional assays.

One criticism of functional assays performed *in-vitro* is that they do not represent the complexity of the *in-vivo* tumour/tissue microenvironment. This can be universally applied to all cell culture-based experiments. However, these techniques offer the unique opportunity to analyse specific characteristics and to record any changes in response to the manipulation of environmental conditions. In the planning of this study, observational data derived from clinical specimens and cell culture experiments were used to focus the aim of functional assays of OSCC tumour behaviour *in-vitro*. The ability of tumours to metastasise is dependent on a number of cellular processes (summarised in Table 1.1, Chapter 1) (Fidler 2003). However, changes in cell behaviour required to establish lymph node metastases are likely to be different to those required for distant dissemination. This may explain the pattern of OSCC disease observed clinically where spread to the cervical lymph nodes is seen commonly, but sizeable systemic deposits are only rarely encountered.

Previous studies in the literature have focussed on the ability of CXCR4 and other chemokine receptors to mediate cell migration in OSCC and other carcinomas (reviewed in Sections 1.6.2.2 and 1.5.4, Chapter 1 respectively). My results demonstrated that transfected OSCC cells containing the CXCR4 insert showed significant increases in chemokinesis, chemotaxis, migration and invasion, compared with transfected cells lacking the CXCR4 insert (Chapter 7). Established OSCC cell lines constitutively expressing relatively high amounts of CXCR4 also migrated and invaded towards SDF gradients through pathways that could be attenuated by
blockade of the SDF/CXCR4 axis. The methodologies of previously published studies have been inadequate, predominantly due to lack of appropriate controls (reviewed in Section 1.6.2.2, Chapter 1). Therefore the data presented here are the first to provide conclusive evidence that CXCR4 over-expression causes an increase in OSCC cell migration and invasion in-vitro. It was also demonstrated (using scratch wound assays) that a concentration gradient of SDF was not essential for CXCR4-mediated chemotaxis in growth-arrested cells. While it has been shown that SDF can bind ECM moieties such as heparin sulphate, clear chemokine gradients are difficult to demonstrate within the tissue/tumour microenvironment. Cell-cell interactions, including the expression of cell-adhesion molecules and the release of MMPs, may play a significant role in defining the direction of cell movement while SDF promotes tumour cell chemokinesis. Further investigation of these mechanisms could be performed using organotypic culture models. In this way the delivery of exogenous SDF stimulation could be controlled through either supplementation of the growth media or pre-incubation of the collagen/ECM matrix. One particular facet of CXCR4 function that could be explored using this method is the role of fibroblasts (or fibroblast-conditioned media) to induce EMT. However, one limitation of the organotypic model is its inability to recreate the effect of cell-cell interactions. This is only achievable in co-culture experiments or animal models, although the latter preclude the precise manipulation of single constituents of the cell microenvironment.

The parallels between the mechanisms of tumour dissemination and physiological lymphocyte homing are probably over-simplistic. The release of activated lymphocytes via lymphatic channels to the venous circulation facilitates their systemic delivery. However, these cells can pass freely through the lung beds and the post-capillary venules of tissue in which the signals for selective re-circulation are not present. This is not the same for the much larger carcinoma cells that may undergo mechanical arrest in the microcirculation and are targeted for destruction by the host immune system if they are not equipped to evade it. However, the role of chemokine receptors in lymph node metastasis may mirror more closely the multi-step mechanism of dendritic cell recruitment to lymph nodes (Table 1.3, Chapter 1). Defined anatomical pathways deliver cells down afferent lymphatic channels to the first echelon of draining lymph nodes. In this way tumour cells are able to disseminate to the lymph node parenchyma that is rich in chemokines. Functional chemokine receptors on the carcinoma cell surface may then mediate changes in cell behaviour in
response to this new microenvironment. Metastatic deposits may then develop within the lymph node if the tumour is able to proliferate while avoiding destruction by the host immune system. It is possible that immune-mediated mechanisms that promote tumour growth may confer a particular survival advantage to metastatic cells.

The results presented here provide clear evidence of a role for CXCR4 in OSCC cell proliferation but not apoptosis (Chapter 6). SDF concentration and CXCR4 availability were found to be independent factors that determined the magnitude of the pro-proliferative response. Consequently, cells bearing functional, surface CXCR4 might be expected to access lymph nodes where they would be stimulated to proliferate, migrate and invade. Despite the positive relationship between CXCR4 staining and poor clinical outcome described in the literature, the possible association between CXCR4 and extracapsular spread outside the lymph node has not been tested. This might yield further evidence regarding the role of the SDF/CXCR4 axis in the progression of lymph node disease, one of the major determinants of patient outcome.

8.4 Limitations and suggestions for further work

One of the features of CXCR4 is its ability to signal through a variety of different cellular pathways. The results presented here have focussed on the functional responses of high-expressing CXCR4 cells to SDF. No examination of the signalling pathways mediating each response was made. This could be achieved using a number of different, complimentary techniques including Western Blotting to determine the activated, phosphorylated form of signalling kinases, or co-incubation with pharmacological antagonists against selected pathways. The elucidation of the phases of kinase phosphorylation is critically dependent on the duration between stimulation and measurement. This may vary for each of the pathways involved which may be multiple for the function observed. The strength of data generated following single gene transfection (compared with appropriate controls) is that the native, potential signalling pathways within the recipient cells are left unaffected. Therefore, while pathway analysis may yield useful information on CXCR4-mediated mechanisms, the translational benefit is limited to defining which pharmacological pathway antagonists may be of use clinically.
The *in-vitro* evidence presented here strongly suggests that CXCR4 over-expression mediates a number of cell functions required for carcinoma cell metastasis. However, as mentioned above, one of the limitations of culture-based experimental data is that it may poorly represent conditions *in-vivo*. Therefore, orthotopic inoculation of the transfected OSCC cell line containing the CXCR4 insert could be performed in nude mice along with appropriate mock-transfected controls. The mouse model would then reveal data on the effect of CXCR4 upregulation on the development of primary and metastatic lesions both in the cervical lymph nodes and distant sites. However, the H357 cell line has been shown to be poorly tumourigenic in nude mice (Prime, Eveson et al. 2004). If tumour growth could not be demonstrated (with or without the CXCR4 insert) then it is likely that this would be a representation of features of the cell line rather than the over-expression of CXCR4. One strategy would be to select and transfet an alternative established OSCC cell line, or alternatively select a constitutively high expressing cell line and transfet them with a construct that produced anti-CXCR4 siRNA. One limitation of the transfection vector selected in this study was the reliance on antibody labelling to determine surface protein levels. Other methods exist to make quicker, real-time assessments of construct expression. These include the incorporation of a green fluorescent protein (GFP) marker which would allow investigation of tumour dissemination without immunofluorescence. This could be performed on sacrificed tissue in the conventional way, or in live animals using intra-vital microscopy. An alternative technique to determine the pattern and volume of tumour burden without sacrificing the animal would be to use PET/CT scanning, potentially with a co-transfected marker.

One of the most surprising findings from the characterisation of established OSCC cell lines was that significant levels of CCR7 mRNA or protein were not demonstrated. CCR7 appears to be the predominant chemokine receptor involved with the uptake of activated dendritic cells to peripheral lymph nodes. The CCR7 -/- knockout mouse lacks this ability, although comparison with CXCR4 -/- knockouts is not possible as the latter is fatal. There is some evidence in the literature that some OSCC cell lines produce functional CCR7 protein *in-vitro* and that CCR7 staining in clinical specimens is associated with advanced disease and lymph node metastasis (reviewed in Section 1.6.1, Chapter 1). One limitation of the work presented in this thesis is that investigation of CCR7 was restricted to established OSCC cells. With more time it would be necessary to optimise the IHC staining protocol for CCR7 and investigate the
same preliminary specimens and tissue arrays using the novel, quantitative IHC method described. In this way correlation between in-vitro and clinical findings could have been made. If positive CCR7 staining was not demonstrated in clinical samples then this would not preclude investigation of its functional role in cell culture. However, this work would be limited to the study of a transfected cell line in which CCR7 mRNA was over-expressed. Additionally any functional activity demonstrated could not be readily translated to the in-vivo situation where the protein had not been shown to exist.

CXCR7 (RDC1) was only recently discovered as a second receptor for SDF (Balabanian, Lagane et al. 2005). While it was initially thought to play a role predominantly in organogenesis, a number of groups have described CXCR7-mediated changes in tumour cell proliferation in-vitro (reviewed in Section 1.5.5, Chapter 1). The function of CXCR7 appears to vary between carcinomas and, unfortunately, retrospective examination of the microarray data presented here shows that RDC1 was not represented on the array. Consequently it would be interesting to investigate archived cDNA for CXCR7 expression levels and to determine protein levels using flow cytometry and IHC. From the literature, CXCR4 production is poorly predictive of CXCR7 levels and their co-expression and interaction would be important to delineate. Further manipulation of this could be performed by performing transfection of a constitutively low-expressing cell line with a single insert or both in combination.

8.5 Translational considerations

The ability of carcinoma cells to invade locally, disseminate widely and then proliferate at distant anatomical sites is unlikely to be due to a change of expression of a single gene. This fact explains why individual biomarkers are universally poor at predicting metastases. However, physiological processes (such as the uptake of activated dendritic cells by lymph nodes) are attractive targets for subversion by neoplastic cells. Over-expression of the molecules that mediate such mechanisms expose the carcinoma cell to pathophysiological processes (such as the inflammatory response) that may further promote tumour progression (Balkwill and Mantovani 2001). From the tumour’s perspective, a further, indirect benefit of subverting physiological pathways is that therapeutic strategies aimed at blocking these mechanisms may have substantial
and debilitating side effects that preclude their use in patients. This phenomenon has been demonstrated in patients with HIV who have been administered pharmacological antagonists to CXCR4.

If the role of individual biomarkers is clinically limited and the development of anti-CXCR4 therapies handicapped by systemic toxicity, is there any translational benefit in studying the role of CXCR4 in OSCC metastasis? The answer is clearly yes. Current treatment strategies for early stage disease (in which no involvement of the CLN can be felt or imaged) focus on an assessment of risk of occult, subclinical tumour deposits in the neck. While the results of prospective, randomised, clinical trials are still a decade away the accepted yardstick remains an arbitrary threshold (Weiss, Harrison et al. 1994; Okura, Aikawa et al. 2009). Any method of assessing the diagnostic biopsy taken from the primary tumour that would inform the decision-making process with regards to the prospective treatment of the N0 neck would be clinically beneficial. In this way a panel of validated biomarkers with defined sensitivity and specificity could help clinicians and their patients decide on the best course of treatment in situations where the decision is not clear-cut. Data presented here demonstrate an association between cytoplasmic CXCR4 protein and lymph node metastasis. This assessment could be performed in the clinical situation by conventional IHC of the diagnostic biopsy taken from the primary lesion.

Other ways in which anti-CXCR4 therapies may be helpful without the burden of systemic toxicity include the use of radio-sensitising agents. These compounds do not cause side effects themselves but potentiate the anti-tumour effect of external beam radiotherapy on cells that have taken them up. Limitations of this technique include the effects on the physiological upregulation of surface CXCR4 within the parenchyma of the cervical lymph nodes targeted in the radiation field. Involvement of the SDF/CXCR4 axis in induction of apoptosis was not demonstrated by my results. This, combined with their systemic side-effects, suggests that the role of CXCR4 antagonists in adjuvant chemotherapy is likely to be extremely limited.

Finally there are distinct benefits in studying the mechanisms by which CXCR4 may mediate lymph node and distant metastasis. Although sequential involvement of the CLN is the most common pattern of OSCC tumour spread, the prevalence of lymph node skipping has been reported by a number of authors (reviewed in Section 1.2.1,
Chapter 1). This is particularly important when more minimally invasive techniques of CLN assessment and treatment are considered. These include sentinel node biopsy (SNB) in which the first echelon of draining lymph nodes are identified and sampled. The rationale of this treatment is that only patients with a positive sentinel node progress to formal neck dissection. The danger with this treatment algorithm is the false negative SNB in which tumour has spread to the CLN but the screening investigation (SNB) fails to predict it.

The metastatic process is highly inefficient, suggesting that, of the huge number of genetic variants created, only a few cells develop the ability to access and grow at distant sites. Detailed autopsies of patients with OSCC have demonstrated that small, subclinical distant metastases may be relatively common, but they rarely progress to form clinically significant lesions. This failure to enlarge may be due to properties inherent within the tumour metastases, or due to the confounding effect of rapid progression of loco-regional disease precipitating early demise of the patient. Clinical observation supports (at least in part) the former hypothesis. Therefore investigation of the mechanisms controlling tumour growth arrest (despite CXCR4 bearing cells disseminating to tissue microenvironments with abundant SDF) may yield interesting insights into the control of metastatic pathways.

In conclusion, the results presented in this thesis demonstrate that CXCR4 protein levels on the cell membrane of primary OSCC lesions are associated with CLN metastases. This may be mediated by the positive effects of CXCR4 activation on proliferation, migration and invasion. While individual biomarkers may lack significant statistical power in predicting clinical metastases, these data support the inclusion of CXCR4 protein in a prognostic panel. This would be amenable to investigation of diagnostic biopsies by IHC. However, the complexity of multiple IHC testing precludes its current use in the clinical setting. Further prospective work focussing on the specific removal of tumour cells (for example by LCMD) followed by multiplexed sqRT-PCR might automate this procedure, albeit at an mRNA level. While this work would provide significant insights into the metastatic mechanisms of OSCC, its clinical application remains limited (at least in the short-term).
Appendix 1:
Supplement to Chapter 1-
Introduction and
Literature Review
Table A1.1: International Statistical Classification of Diseases and Related Health Problems (10th Revision 2007): Malignant neoplasms of lip, oral cavity and pharynx (C00-C14)

<table>
<thead>
<tr>
<th>Site &amp; Code</th>
<th>Sub-Code</th>
<th>Sub-site</th>
<th>Definition</th>
<th>Included</th>
<th>Excluded</th>
</tr>
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<tbody>
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<td>C00 Lip</td>
<td>C00.0 to C00.9</td>
<td>Dorsal surface of base of tongue</td>
<td></td>
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<tr>
<td>C01 Malignant neoplasm of base of tongue</td>
<td>C02.0</td>
<td>Dorsal surface of tongue</td>
<td>Anterior two-thirds of tongue, dorsal surface</td>
<td></td>
<td>X</td>
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<tr>
<td></td>
<td>C02.1</td>
<td>Border of tongue</td>
<td>Tip of tongue</td>
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<td></td>
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<td></td>
<td>C02.2</td>
<td>Ventral surface of tongue</td>
<td>Anterior two-thirds of tongue, ventral surface</td>
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<td></td>
<td>C02.3</td>
<td>Anterior two-thirds of tongue, part unspecified</td>
<td>Frenulum linguae</td>
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<td>Middle third of tongue NOS</td>
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<tr>
<td></td>
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<td>Mobile part of tongue NOS</td>
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<td>Upper gum alveolar (ridge) mucosa gingiva</td>
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<td>Lower gum alveolar (ridge) mucosa gingiva</td>
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<td>Gum, unspecified</td>
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<td>Anterior floor of mouth</td>
<td>Anterior to the premolar-canine junction</td>
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<td>C04.9</td>
<td>Floor of mouth, unspecified</td>
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<td>C05 Malignant neoplasm of palate</td>
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<td>Sub-site</td>
<td>Definition</td>
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<td>Buccal mucosa NOS</td>
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<td>Vestibule of mouth</td>
<td>Buccal sulcus (upper)(lower)</td>
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<td>Retromolar area</td>
<td>Labial sulcus (upper)(lower)</td>
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<td>Minor salivary gland, unspecified site / Oral cavity NOS</td>
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<tr>
<td><strong>C07</strong> Malignant neoplasm of parotid gland</td>
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<td><strong>C08</strong> Malignant neoplasm of other &amp; unspecified major salivary glands</td>
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<td>Tonsillar fossa</td>
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<td>Branchial cyst [site of neoplasm]</td>
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<td>Junctional region of oropharynx</td>
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<td>Oropharynx, unspecified</td>
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<tr>
<td>Site &amp; Code</td>
<td>Sub-Code</td>
<td>Sub-site</td>
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<td>C11</td>
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<td>Floor of nasopharynx</td>
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<tr>
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<td>Nasopharyngeal surface of soft palate (anterior)(posterior) Posterior margin of choana / septum</td>
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<td>Aryepiglottic fold: NOS marginal zone</td>
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<td>Posterior wall of hypopharynx</td>
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<td>x</td>
<td></td>
</tr>
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<td>C13.8</td>
<td>Overlapping lesion of hypopharynx</td>
<td></td>
<td></td>
<td>x</td>
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<td>Hypopharynx, unspecified</td>
<td>Hypopharyngeal wall NOS</td>
<td></td>
<td>x</td>
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</tr>
<tr>
<td>C14</td>
<td>C14.0</td>
<td>Pharynx, unspecified</td>
<td></td>
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<td>C14.2</td>
<td>Waldeyer's ring</td>
<td>Malignant neoplasm of lip, oral cavity and pharynx whose point of origin cannot be classified to any one of the categories C00-C14.2</td>
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<td></td>
<td></td>
<td>x</td>
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<td>C15</td>
<td>Oesophagus</td>
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<td>x</td>
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<td>C32</td>
<td>Larynx</td>
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<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>C33</td>
<td>Trachea</td>
<td></td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td>C43 &amp; C44</td>
<td>C43.0</td>
<td>Upper</td>
<td></td>
<td></td>
<td>x</td>
</tr>
<tr>
<td></td>
<td>C44.0</td>
<td>Lower</td>
<td></td>
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Appendix 2:

Supplement to Chapter 2

-Materials & Methods
## A2.1 Supplementary Tables & Figures

### Table A2.1: Components of Specialised Growth Media for Cell Culture

<table>
<thead>
<tr>
<th>Cell Line(s)</th>
<th>Base Media</th>
<th>Supplements (final concentrations)</th>
</tr>
</thead>
</table>
| 357          | 3:1 Dulbecco’s Modified Eagle Medium (DMEM) with sodium pyruvate and 1000mg/ml glucose (31885-023, Invitrogen) to Ham F-12 (N-4888, Sigma), (Formanek, Millesi et al 1996) | Hydrocortisone 0.4μg/ml, (H0135, Sigma)  
Transferrin 5μg/ml, (T5391, Sigma)  
Insulin 5μg/ml, (I-1882, Sigma)  
Cholera Toxin 1 x 10^{-10}M, (C-3012, Sigma)  
Epidermal cell Growth Factor (EGF) 10ng/ml, (E1257, Sigma)  
Adenine 1.8 x 10^{-4} M, (A3159, Sigma)  
Penicillin/Streptomycin 50u/ml & 50μg/ml  
Fungizone 2.5μg/ml  
10% FCS |
| FADU CAL27   | RPMI 1640 with 25mM Hepes & L-Glutamine (BE12-115F) | Penicillin/Streptomycin 50u/ml & 50μg/ml  
Fungizone 2.5μg/ml  
10% FCS |
| TR146 HGF 3T3 | (DMEM) (BE12-604F) | Penicillin/Streptomycin 50u/ml & 50μg/ml  
Fungizone 2.5μg/ml  
10% FCS |
| SCC4 SCC25   | 1:1 DMEM : Ham F-12 with 15mM Hepes & L-Glutamine (BE12-719F) | Penicillin/Streptomycin 50u/ml & 50μg/ml  
Fungizone 2.5μg/ml  
Hydrocortisone 400ng/ml  
10% FCS |
| SVpgC2a      | Defined Keratinocyte SFM medium (Gibco) | Penicillin/Streptomycin 50u/ml & 50μg/ml  
Insulin 4μg/ml  
Hydrocortisone 0.5μg/ml  
EGF 25ng/ml |
Table A2.2: Determination of growth kinetics of OSCC & 3T3 cell lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Doubling Time (Hours)</th>
<th>Time to end of log growth phase / hr *</th>
<th>No. of Cell divisions to end of Log Growth Phase</th>
<th>Accuracy of the modelling of the Exponential Phase (RSQ)</th>
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<td>H357</td>
<td>19.9</td>
<td>112</td>
<td>5.6</td>
<td>0.972</td>
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<tr>
<td>TR146</td>
<td>20.6</td>
<td>88</td>
<td>4.3</td>
<td>0.979</td>
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<tr>
<td>CAL27</td>
<td>43.3</td>
<td>136</td>
<td>4.9</td>
<td>0.914</td>
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<tr>
<td>FaDu</td>
<td>24.3</td>
<td>112</td>
<td>3.6</td>
<td>0.971</td>
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<tr>
<td>SCC4</td>
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<td>3.8</td>
<td>0.979</td>
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<tr>
<td>SCC25</td>
<td>20.3</td>
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<td>3T3</td>
<td>20.9</td>
<td>112</td>
<td>5.3</td>
<td>0.999</td>
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Legend: * = Based on seeding density of 5x10^3 per well
Table A2.3:  Pathology Report Summary of ten preliminary cases of tongue OSCC selected for CXCR4 immunohistochemistry.

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<td>F</td>
<td>M</td>
<td>M</td>
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<td>Pathological Staging</td>
<td>T2N1M0</td>
<td>T2N2bM0</td>
<td>T4N2b</td>
<td>T2N0M0</td>
<td>T2N0M0</td>
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<tr>
<td>Primary tumour site</td>
<td>Tongue</td>
<td>Tongue</td>
<td>Tongue &amp; FoM</td>
<td>Tongue</td>
<td>Tongue</td>
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<tr>
<td>Primary tumour size (cm)*</td>
<td>2.1</td>
<td>3.4</td>
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<td>Maximum depth of invasion (cm)</td>
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<td>Differentiation</td>
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<td>Moderate Cohesive</td>
<td>Moderate Cohesive</td>
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<td>Invasive front</td>
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<td>Total number of lymph nodes excised</td>
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<td>18</td>
<td>N/A</td>
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<td>Number of positive neck lymph nodes</td>
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<td>invasion (cm)**</td>
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<td>1</td>
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<td>Anatomical level of positive lymph nodes</td>
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<td>2</td>
</tr>
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<td>Lymph node metastasis size (cm)*</td>
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<td>Yes</td>
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<td>Yes</td>
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</tbody>
</table>

Legend:  FFPE = formalin-fixed paraffin-embedded;  FoM = Floor of Mouth;  * = maximum dimension as measured in the plane of section;  ** = maximum depth of invasion as measured from estimated epithelial surface.
Figure A2.1: Diagram of the layout of the Head & Neck Tissue Array (A219II, Accumax)
Figure A2.2: Restriction Enzyme digest of pcDNA3-CXCR4 with XbaI, EcoRI and BamHI. A schematic representation of the restriction enzyme binding sites for XbaI, EcoRI and BamHI in the pcDNA3 plasmid containing the CXCR4 insert. Fragment sizes are given in base pairs (bp).
Figure A2.3: PCR primers for T7F/BGH-R and CXCR4 on pcDNA3-CXCR4 and pcDNA3. Schematic representation of the binding sites for the PCR primer pairs for T7F/BGH-R and CXCR4 on the pcDNA3 plasmids with (panel a) and without (panel b) the CXCR4 insert. Fragment sizes are given in base pairs (bp).
Figure A2.4: pcDNA3.1 plasmid map. A diagram of the pcDNA3.1 plasmid (a) and detail of the binding site (b) showing the CXCR4 insertion site following restriction enzyme digestion with EcoR1 and Xba1.
Figure A2.5. Tests for the consistency of cell seeding density prior to Alamar Blue assays. 96 well plates were seeded with cells at a known density and incubated overnight with 5% Alamar Blue. Fluorescence measurements were taken and an observed against expected cumulative probability (P-P) plot was produced (A). Subsequently, the baseline fluorescence measurements of future experimental groups were compared (B). Bars represent mean (standard deviation) of twelve replicate wells. ANOVA reveals no significant difference between groups (p>0.05).
A2.2 Protocol for Mycoplasma testing

Cells were taken and grown in parallel with flask cultures on sterile glass coverslips in a 24-well plate at an initial density of 1x10^4 cells/well under standard conditions for 72 hours. They were washed twice with sterile PBS and then incubated with 50μl of 10μg/ml 4',6-Diamidino-2-phenylindole dimethylsulfoxide (DAPI) nuclear fluorescent stain for ten minutes in the dark. The coverslips were mounted in aqueous Immunomount (ThermoShandon, 9990402), allowed to dry and viewed under the fluorescence microscope. Speckled staining of the cytoplasm was taken as evidence of mycoplasma infection. Mycoplasma positive cell lines were treated as outlined in Section 2.1.5 (Chapter 2) and were rechecked at the end of the treatment regimen.

An additional ELISA-based method was employed to screen the transfected H357 cells prior to their use in observational or functional experiments. Briefly, aliquots from cell culture supernatants were taken at confluence, spun at 8,000g for 1 minute and incubated according to the manufacturer’s instructions (Roche). Colour was allowed to develop for a standardised period (30 minutes) and the plates were then read in a standard microplate reader. Transfected cells with and without the CXCR4 insert were tested with antibodies raised against four common mycoplasma species. Results show levels generated by the cell lines were equivalent to negative control levels and well below the response levels of internal positive controls (Figure A2.6).
Figure A2.6: ELISA-based mycoplasma screening of transfected H357 cells. Transfected H357 cells with and without the CXCR4 insert were cultured and the supernatant was sampled. Analysis using an ELISA-based protocol was performed according to the manufacturer’s instructions. Samples were incubated with antibodies raised against four common mycoplasma species. Absorbance recordings for cell supernatants were similar to those recorded for internal negative controls. Readings from internal positive control samples were substantially higher.
Appendix 3:
Supplement to Chapter 3
- Characterisation of established OSCC cell lines
A3.1  Statistical processing of array data

For each array, histogram plots of the log_{10} transformation of each normalised gene expression values for all represented genes were produced. These plots were shown to be similar in shape between arrays and graphically followed a normal distribution (Figure A3.1). A normal distribution of each log_{10} transformed (but not the untransformed) normalised expression values was confirmed using P-P plots.

![Histogram plots of the log_{10} transformation of all genes represented on the microarrays.](image)

**Figure A3.1:** Histogram plots of the log_{10} transformation of all genes represented on the microarrays. Microarrays (U133A, Affymetrix) were hybridised against cDNA manufactured from NOK, NSK and established OSCC cell lines (as outlined in Section 2.2, Chapter 2). Internal quality-control genes demonstrated that 17 arrays had hybridised correctly. The normalised expression values (NEV) of all genes represented on the microarrays were plotted against frequency for the 17 arrays (each represented by an individual line). Each curve conformed to the normal distribution centred on the value 1.00.
A phylogenetic tree of all the samples mapping all genes on the array demonstrated that each sample duplicate most closely matched to its reciprocal pair (Figure A3.2). The concordance values between sample replicates from each cell line ranged from 0.92 to 0.97 compared with a maximum value of 0.798 for the concordance between samples derived from different cell lines (SCC4 and SCC25). These results confirmed that there was a high reproducibility of results between sample replicates. Consequently, the validity of the mathematical amalgamation of data for the cell line replicates is supported by the biological correlation of cell line type with these phylogenetic and concordance analyses.

Figure A3.2: Phylogenetic gene tree of all microarray samples. The gene expression profile of all genes represented on the microarrays were compared for samples that had hybridised correctly (n=17). Where appropriate the results from duplicate samples were combined.
A3.2 Raw data tables for chemokine receptor mRNA expression as determined by microarrays

Table A3.1: Chemokine Receptor mRNA expression raw data for NOK and NSK microarrays.

<table>
<thead>
<tr>
<th></th>
<th>NEV mean (min, max)</th>
<th></th>
<th>NEV mean (min, max)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NOK</td>
<td>NSK</td>
<td>NOK</td>
</tr>
<tr>
<td>CCR1</td>
<td>0.88 (0.54, 1.88)</td>
<td>0.83 (0.14, 1.45)</td>
<td></td>
</tr>
<tr>
<td>CCR2</td>
<td>0.65 (0.28, 1.93)</td>
<td>0.89 (0.43, 2.02)</td>
<td></td>
</tr>
<tr>
<td>CCR3</td>
<td>0.84 (0.34, 2.11)</td>
<td>0.37 (0.36, 0.37)</td>
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</tr>
<tr>
<td>CCR4</td>
<td>0.60 (0.23, 1.57)</td>
<td>1.46 (1.29, 1.66)</td>
<td></td>
</tr>
<tr>
<td>CCR5</td>
<td>1.45 (1.01, 2.08)</td>
<td>0.76 (0.64, 0.91)</td>
<td></td>
</tr>
<tr>
<td>CCR6</td>
<td>0.76 (0.74, 0.78)</td>
<td>0.22 (0.19, 0.26)</td>
<td></td>
</tr>
<tr>
<td>CCR7</td>
<td>1.20 (1.02, 1.41)</td>
<td>0.79 (0.63, 0.98)</td>
<td></td>
</tr>
<tr>
<td>CCR8</td>
<td>0.60 (0.43, 0.84)</td>
<td>1.93 (1.55, 2.41)</td>
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</tr>
<tr>
<td>CCR9</td>
<td>1.19 (1.09, 1.29)</td>
<td>1.68 (1.66, 1.70)</td>
<td></td>
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<tr>
<td>CCR10</td>
<td>0.81 (0.80, 1.20)</td>
<td>0.73 (0.71, 0.90)</td>
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<tr>
<td>CXCR1</td>
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<td>2.04 (1.14, 2.95)</td>
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<tr>
<td>CXCR2</td>
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<td>1.37 (0.83, 1.52)</td>
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<tr>
<td>CXCR3</td>
<td>0.85 (0.34, 2.69)</td>
<td>1.19 (0.50, 1.79)</td>
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<tr>
<td>CXCR4</td>
<td>0.96 (0.53, 1.22)</td>
<td>0.80 (0.56, 1.15)</td>
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<tr>
<td>CXCR5</td>
<td>0.70 (0.32, 1.53)</td>
<td>0.39 (0.23, 0.53)</td>
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</tr>
<tr>
<td>CXCR6</td>
<td>1.34 (0.76, 2.09)</td>
<td>1.05 (0.49, 2.50)</td>
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<tr>
<td>XCR1</td>
<td>1.70 (0.95, 3.05)</td>
<td>0.98 (0.56, 1.72)</td>
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<tr>
<td>CX3CR1</td>
<td>0.96 (0.55, 1.69)</td>
<td>0.85 (0.66, 1.08)</td>
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Raw data for Figure 3.1 (Chapter 3)
Table A3.2: Chemokine Receptor mRNA expression raw data for established OSCC Cell Lines.

<table>
<thead>
<tr>
<th></th>
<th>SCC4</th>
<th>SCC25</th>
<th>CAL27</th>
<th>H357</th>
<th>FADU</th>
<th>TR146</th>
<th>SV</th>
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<tbody>
<tr>
<td>CCR1</td>
<td>0.926</td>
<td>0.764</td>
<td>0.661</td>
<td>0.646</td>
<td>1.777</td>
<td>0.450</td>
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<td>CCR2</td>
<td>0.760</td>
<td>0.847</td>
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<td>1.171</td>
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<td>CCR3</td>
<td>0.882</td>
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<td>0.716</td>
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<td>0.469</td>
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<td>CCR4</td>
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<td>0.893</td>
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<td>0.790</td>
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<td>1.370</td>
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<tr>
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<td>CCR6</td>
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<td>0.686</td>
<td>0.427</td>
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<td>CCR7</td>
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<td>0.940</td>
<td>1.953</td>
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<td>1.206</td>
<td>0.274</td>
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<td>CCR9</td>
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<td>1.409</td>
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<td>CXCR5</td>
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<td>0.482</td>
<td>0.712</td>
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<td>0.743</td>
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<td>0.727</td>
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Raw data for Figure 3.2 (Chapter 3) averaged for duplicate samples were appropriate.
A3.3 Chemokine mRNA expression by Primary Cultures of NOK, NSK and Established OSCC cell lines.

The Affymetrix U133A microarray contains probes for all known chemokines (CCL1-27, CXCL1-14, XCL1 and CX3CL1) with the exception of CCL6, CCL9, CCL10, CCL12 and CCL26. The normalised expression values (NEV) of chemokines for normal oral and skin keratinocytes were calculated (as outlined in Section 2.2.13) and plotted (Figure A3.3).

These results show a considerable degree of variability in the expression of chemokine mRNA (compared with the normalised mean) for normal oral keratinocytes. In the majority of cases the duplicate values were highly concordant. Out of the C-X-C family of chemokines, CXCL1, CXCL2, CXCL3, CXCL5, CXCL6 and CXCL8 were expressed at high levels (NEV 5.6, 3.9, 18.5, 37.3, 19.1 and 6.5 respectively). CXCL13 was the only C-X-C chemokine expressed at low levels in NOK (NEV 0.20). Only three members of the C-C chemokine family were highly expressed by NOK: CCL14, CCL20 and CCL22 (NEV 2.9, 8.9 and 2.7 respectively). However, seven C-C chemokines were expressed at low levels by these cells: CCL1, CCL2, CCL3, CCL5, CCL23, CCL25 and CCL27 (NEV 0.17, 0.29, 0.26, 0.49, 0.25, 0.43 and 0.29 respectively). Finally, the chemokine CX3CL1 was highly expressed in NOK (NEV 5.1).

There were a number of similarities between the chemokine expression profiles in keratinocytes from the skin and the mouth: Normal skin keratinocytes showed high expression of CXCL5, CXCL6 and CX3CL1 (NEV 8.2, 6.1 and 5.3 respectively) and low expression of CXCL13 (NEV 0.14). However, some differences in the expression of C-X-C chemokines between NSK and NOK were also demonstrated: In NSK the chemokines CXCL1, CXCL3 and CXCL8 were not highly expressed and CXCL2 was only expressed at low levels (NEV 0.46). Finally, CXCL7 was highly expressed in NSK (NEV 20.2) but at normal levels in NOK.
Figure A3.3: Normalised expression of chemokine mRNA by normal oral and skin keratinocytes. Solid bars represent the NEV of chemokine mRNA for normal oral (NOK) and skin (NSK) keratinocytes (averaged for duplicate samples). For description of results see text.
Figure A3.4a: C-C Chemokine mRNA Expression by Established OSCC Cell Lines. Solid bars represent the chemokine NEV (averaged for duplicate samples where applicable). For description of results see text.
Figure A3.4b: C-X-C, X-C and C-X3-C Chemokine mRNA Expression by Established OSCC Cell Lines. Solid bars represent the chemokine NEV (averaged for duplicate samples where applicable). For description of results see text.
The C-C and X-C chemokines were less variably expressed by NSK and demonstrated fewer similarities with the pattern of expression in NOK. The chemokines CCL2, CCL8, CCL17, CCL18, CCL21 and XCL1 were expressed at low levels in NSK (NEV 0.23, 0.31, 0.42, 0.45, 0.47 and 0.33 respectively). Interestingly, no chemokines of the C-C family were expressed at high levels in NSK. The mean normalised expression values for all chemokines represented on the microarrays were calculated for the cell lines tested (as outlined in section 2.4.2.13). These NEVs are represented for C-C chemokines (Figure A3.4a) and for C-X-C, X-C and C-X3-C chemokines (Figure A3.4b).

The pattern of chemokine mRNA expression by oral cell lines was complex and highly variable. However, several trends can be identified from analysis of significant differences in expression of greater than twofold from the normalised mean: Firstly, the majority of changes in chemokine expression were shown to be associated with their downregulation. However, only CCL1 was ubiquitously downregulated in all the oral cell lines tested (NEV range 0.12 to 0.37). Secondly, when chemokine expression in individual cell lines was compared some patterns emerged. Both SCC4 and SCC25 demonstrated low expression of CCL1, CCL2, XCL1 and particularly CXCL1, while showing upregulation of CXCL2 and CXCL3. In addition, SCC25 demonstrated downregulation of CCL3, CCL4, CCL7, CCL8, CCL11, CCL16, CCL18, CCL21, CXCL5 and CXCL7; while SCC4 showed low expression of CCL17, CCL23, CXCL11, CXCL12 and CXCL13. The CAL27 cell line demonstrated some similarities with SCC25 cells, for example the low expression of CCL1, CCL2, CCL4, CCL7, CCL8, CCL21 and CXCL7. However, CAL27 cells showed the greatest upregulation of chemokines including CCL3, CCL11, CCL16, CCL17, CCL18, CXCL10, XCL1, and particularly CXCL11 and CX3CL1. In contrast, H357 cells showed few changes in chemokine expression but was the only cell line in which CCL21 was highly expressed (NEV 2.3). The expression pattern in FADU cells showed some similarities with CAL27, notably downregulation of CCL1, CCL4, CCL8, CCL14, CXCL13, CXCL14 and upregulation of CCL18 and XCL1. TRI46 cells also showed some similarities to CAL27 cells particularly in their C-X-C chemokine profile: both cell lines showed low expression of CXCL7 and CXCL13, with high expression of CXCL10 and CXCL11. Again the SV cell line demonstrated a unique pattern of chemokine expression: CCL1, CCL4, CCL7, CCL17, CCL18, CCL22, CCL24, CXCL7, CXCL8, CXCL13, XCL1 and particularly CCL20, CXCL1, CXCL2 and CXCL3 were all downregulated. SV was the
only cell line to show high expression of CCL2. A summary of these changes is given in Table A3.3.

### Table A3.3: Summary of Significant Changes of Chemokine Normalised Expression Values in Oral Cell Lines

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>SCC4</th>
<th>SCC25</th>
<th>CAL27</th>
<th>H357</th>
<th>FADU</th>
<th>TR146</th>
<th>SV</th>
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Representation of changes in NEV in oral cell lines. This table is derived from data presented in Figures A3.4a&amp;b (Appendix 3). Legend: ▲ = upregulation 2-5 fold, ▲▲ = upregulation greater than fivefold, ▼ = downregulation 2-5 fold, ▼▼ = downregulation greater than fivefold.
A3.4 Discussion of experimental techniques

A3.4.1 Selection of established cell lines

The derivation of the established OSCC cell lines used in these experiments is reasonably well documented in the literature (details given in Chapter 2.1.1). It is tempting to hypothesise that cell lines derived from primary tumours and metastases show changes in gene expression levels principally attributable to either these phenotypic characteristics or their anatomical location of origin (or both). However, this supposition is flawed for a number of reasons: Firstly, the accuracy of representation of the original tumour by the cell line is unclear. While some authors maintain that cell lines are well representative of (at least specific fractions of) the tumours they were derived from (Mackenzie 2005), not all literature is in agreement (Prime, Nixon et al. 1990). Secondly, an investigation of a large number of tumours would be required to determine any consistent patterns of gene expression. While this experiment investigated six established OSCC cell lines other authors have been more ambitious using lines derived from OSCC clinical specimens (Hunter, Thurlow et al. 2006).

It is tempting to attribute changes in gene expression to clinico-pathological parameters relating to the index patient for each cell line. However, this reasoning is flawed for the reasons given above. Consequently, now that the in-vitro gene expression profile for these established OSCC cell lines has been determined, the logical progression would be to investigate their associated in-vivo phenotype using a suitable animal model. Interestingly some data are available in the literature: Prime et al inoculated athymic mice (Balb/C, nu/nu) with the H357 cell line and found it to be non-tumorigenic (possibly due to Smad3-mediated TGFβ1-induced growth inhibition) (Prime, Eveson et al. 2004; Pring, Prime et al. 2006). However, as the H357 cell line demonstrates, cells may grow well in culture but not in-vivo. This is most likely due to changes in their transcriptome (and consequently in their phenotype) induced by factors in the tumour/tissue microenvironment such as cytokines and hypoxia. These variables are easier to control in-vitro than in-vivo but investigation by the former may allow better planning of the latter. An interesting experiment to perform using the H357 cell line would be observation of the invasive/metastatic patterns of clones with and without the CXCR4 construct. This would allow a more direct measure of the
influence of CXCR4 on the multistep process of metastasis (Fidler 2003). Studies with similar methodologies have recently been performed on other carcinomas including oral squamous (Uchida, Begum et al. 2004), pancreatic (Saur, Seidler et al. 2005), non-small cell lung (Phillips, Burdick et al. 2003; Su, Zhang et al. 2005) and prostate (Sun, Schneider et al. 2005) as well as melanoma (Murakami, Maki et al. 2002).

An alternative approach has also been described in which nude mice have been orthopically inoculated with OSCC cell lines and then tumour cells harvested from resultant metastatic deposits (Zhang, Liu et al. 2002; Chen, Zhang et al. 2003; Wang, Xi et al. 2004). This process was repeated several times thereby creating a form of \textit{in-vivo} “passaging” of OSCC cells. The authors argue that subsequent “passages” should increase the expression of genes associated with metastasis. However, this methodology is flawed because it cannot distinguish between association and causation. What would be more informative is insertion of candidate genes identified in these experiments into cell lines and a comparison made between their metastatic behaviours.

\textbf{A3.4.2 Cell culture methodologies}

The aim of these experiments was to determine the expression of chemokines and their receptors in established OSCC cell lines and to compare them with primary cultures derived from normal keratinocytes. The validity of this comparison is dependent on two biological assumptions: Firstly that the NOK sample was really representative of normal oral mucosal tissue. Secondly that the total RNA extracted from the primary cultures was representative of the keratinocyte population (rather than the fibroblast feeder layer).

The primary NOK culture used was derived from gingival tissue excised during the routine removal of human lower third molar teeth. Clinical information regarding the indications for dental extraction was not available. However, it is possible that either: i) pathological processes (for example inflammatory conditions such as recurrent pericoronitis) were present in the soft tissues around these teeth at the time of tissue
harvesting; or ii) that the gene expression profile of the NOK sample might have been changed from its in-vivo state by the selection and expansion in-vitro over a couple of passages. If these were the case then it might be hypothesised that inflammatory changes present in the donor tissues would be represented in the transcriptomes of the primary keratinocyte cultures derived from them. However, a couple of levels of empirical microarray data do not support this: Firstly, expression of pro-inflammatory chemokines was low in the NOK sample. Secondly, a similar pattern of gene expression was shown for NOK and NSK (harvested from normal skin during breast reduction) both overall (Figure A3.2, Appendix 3) and particularly for chemokines (Figure A3.3, Appendix 3) and their receptors (Figure 3.1, Chapter 3). A third method would be to use pathways analysis to investigate expression levels of mediators of pro-inflammatory signalling pathways in the NOK (or NSK) samples. The latter method is limited by the fact that gene transcription (measured by the microarray) does not always accurately represent protein translation or kinase activation, both of which require carefully timed analysis with Western blotting to determine their precise activity.

Two complimentary methods were used to minimise 3T3 fibroblast genetic contamination: Firstly microscopic examination was performed to determine the impact of mitomycin treatment on cell attachment with time. Secondly as the feeder layer was originally derived from an albino Swiss mouse embryo its reactivity with the human cDNA array was likely to be reduced. One limitation was that this cross-reactivity could not be determined empirically and no data were available from the manufacturer. FACS methodologies (used elsewhere in this thesis) were considered as adjuncts in the process of cell separation. Basic FACS analysis could be used to sort cell populations based on differences in cell size / granularity. However, complete separation based on these parameters alone requires distinct populations of cells or otherwise the intermediate group will be discarded. This may be overcome by labelling with monoclonal antibodies against fibroblast-specific cell surface markers. However, this technique is dependent on a number of incubation and wash steps during which gene expression profiles may alter in an unpredictable fashion. Consequently the methodology used was a compromise between cell selectivity and

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63 According to the 2001 NICE guidelines for the removal of third molar teeth the only indication for the removal of asymptomatic wisdom teeth in the absence of pathology is prior to orthognathic surgery (http://guidance.nice.org.uk/TA1/guidance/pdf/English)
maintaining the cells in optimal growing conditions until the total RNA was harvested. The use (and ultimate concordance) of the duplicate samples provided evidence that this strategy was reasonable and reproducible.

Factors such as serum supplementation can have a significant effect on global gene expression profiles (Iyer, Eisen et al. 1999). Consequently, in order to minimise the effect of potentially confounding variables, the culture conditions used were standardised.

A3.4.3 Comparison of microarray and sqRT-PCR data

When a comparison is made between the relative expression values of mRNA species generated by sqRT-PCR and microarray it is important to consider that each data set have been normalised in different ways. Semi-quantitative RT-PCR data is calculated relative to expression in a defined species (in this case normal oral keratinocytes) and the data transformation is simple. However, expression levels for individual genes derived from microarray data are calculated relative to a normalised mean for each array. This value is determined from the expression levels of all the genes represented on the array. Microarray expression data for individual genes can be viewed as quantitative (rather than semi-quantitative). This is based on one of the key assumptions of microarray data processing: that global expression profiles for each sample are both similar and normally distributed. Analysis of these data shows that both these conditions are met (Figure A3.1). Therefore it is assumed that variations in signal strength between samples are due to variations in hybridisation efficiency. Internal controls within the microarray methodology lend validity to this assumption by measuring hybridisation efficiency across the entire surface of the array. Therefore comparing quantitative microarray data with sqRT-PCR results for single genes can be misleading if mRNA expression in the normalising sample for PCR (in this case NOK) does not equal 1 as determined by microarray. This was demonstrated with all genes compared although the greatest difference was found with XCR1 where the microarray NEV in NOK was 1.70. This problem can be addressed by redefining the NEV in NOK is as equal to 1.00 and performing the simple mathematical transformation to the other expression data to generate relative NEVs. When this is performed there is a significant linear correlation between the data obtained by microarray and sqRT-PCR,
but only when the log of each value is plotted. Why this is dependent on a logarithmic function is not obviously clear. It seems most likely that, due to technical constraints and the post-hoc mathematical transformations involved, the dynamic range available to microarray data does not match that of the sqRT-PCR system. What is irrefutable is that the pattern of gene expression derived from each technique is similar. Furthermore this occurs across a variety of genes tested and across a wide range of values (Figure 3.7, Chapter 3).

One criticism of the methodologies used to determine mRNA expression is the reliance on commercial products. It was assumed that the primer/probe sets for both the microarrays and sqRT-PCR reactions were appropriate and correct. This could have been tested using conventional RT-PCR reactions using self-designed primers and subsequent sequencing of the reaction product. In this way the results could have been confirmed by comparison to known sequences, although relative quantification of the results would have been more difficult.

A3.4.4 Flow cytometry and FACS

Flow cytometry was used with the specific aim to study chemokine receptor protein located on the surface of cells. Specific methodologies were used to maximise the validity of the results: Firstly, non-enzymatic dissociation was performed to ensure surface proteins were minimally disturbed. Secondly, all incubation steps were performed on ice to maintain cellular integrity and minimise chemokine receptor internalisation. And finally, a DNA fluorochrome (propidium iodide) was used to enable the gating out of cells that did not have an intact cell membrane.

The results from the flow cytometry experiments were represented as histograms of cumulative frequency comparing labelled and unlabelled (isotype control antibody) populations. There is debate in the literature regarding the presentation of data in this format. Certainly these histograms do not represent variations in size, granularity and variations in propidium iodide staining. However, as the cell populations are previously gated on these variables, the use of histograms is justified to represent the single variable under investigation. In certain established cell lines, CXCR4 surface expression is clearly detectable in excess of levels of cell autofluorescence. However,
while some authors would advocate the use of subtraction histograms in these situations (Overton 1988; Lampariello 1994), these have not achieved widespread acceptance in the literature.

Flow cytometry yields data pertaining to the frequency of positive cells (percent positive) and the density of surface staining (mean fluorescence). For comparison between cell lines I have used the latter variable. This is because both the fluorescence distribution determined by flow cytometry and the qualitative findings of immunocytochemistry suggested that the staining within cell lines was homogenous. However, not all of the published literature is in agreement with other authors quoting the overall percentage of positive cells. My criticism of this is that these data condense the large number of cells analysed into one figure. The calculation of this figure is dependent on the (arbitrary) threshold of surface protein labelling at which a cell is termed “positive”. This does not take into account of variation in surface receptor density and the biological significance of this. Condensing these values into a single variable (mean fluorescence) is also highly reductionist, however, the histogram plots clearly better represent the biological variation between individual cells within the population examined. No summary data are perfect, but I felt that mean fluorescence was a better tool than percentage positive for descriptive purposes. However, comparison with published data using other variables is more difficult to interpret.

Regarding the choice of monoclonal antibody, the 12G5 clone was one of the first described and has been the most widely used. Recently its use in routine clinical practice for the staging of haematological malignancies has been advocated (Burger and Burkle 2007). However, binding of the 12G5 clone to the first and second extracellular loops of CXCR4 is conformation dependent (Brelot, Heveker et al. 1997). Consequently, as CXCR4 exists in at least two conformational subtypes, it has been argued that the 12G5 clone underestimates monoclonal antibodies and that clone 44717 (MAB#173, R&D Systems), which binds to many conformational shapes exclusive to CXCR4, is better suited to flow cytometry (Baribaud, Edwards et al. 2001). From these findings it was decided to use clone 44717 in these experiments.

Not all published studies routinely gate out cell without an intact cell membrane (Samara, Lawrence et al. 2004; Hosokawa, Hosokawa et al. 2005). However, omission of this step potentially skews results by the incorporation of non-viable cells. As
chemokine receptors are actively cycled to and from the surface membrane, when this membrane integrity is lost measurement of the intracellular “reservoir” of receptor protein may confound results. Several compounds have been used to probe membrane integrity: propidium iodide, ethidium homodimer-1, the cyanine Yo-Pro and the bizbenzimidazole Hoechst 33258. Of these, propidium iodide is the most common technique described in the literature with widespread use in flow cytometry associated with apoptosis assays (Smolewski, Grabarek et al. 2002) and fertility assessment (Gillan, Evans et al. 2005).

FACS techniques were employed to select transfected cells producing high levels of CXCR4 surface protein. This had the double benefit of ensuring that a “clean” population of high expressing cells was being used in the experiments (particularly important when performing functional assays) and speeding up the time required to create a pool of such cells (rather than wait for clonal expansion). One methodological modification might have been to include a GFP reporter in the transfection cassette. This would have allowed direct sorting by FACS (without the need for antibody labeling) and direct viewing of cells containing the cassette in culture. In this way cells that had expelled the transfection vector could be identified and excluded by a mechanism complimentary to antibiotic selection. However, in a mixed population of transfected cells with and without the CXCR4 construct, this methodology would be unable to distinguish between the populations.

A3.4.5 Immunocytochemistry and Immunohistochemistry

While tissue culture techniques are excellent tools by which individual experimental parameters may be manipulated, they do not provide data on the complex spatial arrangement between living cells in-vivo. Cell-cell relationships in-vitro can be investigated at the protein level using immunocytochemistry or at the mRNA level using in-situ hybridization. However, while potentially providing good quality qualitative data, both techniques have their limitations.

One drawback of immunocytochemistry is that the resultant image is inevitably a two-dimensional representation of a three-dimensional structure. To address this issue two strategies were employed: Firstly counterstains were used to delineate the nuclear
compartment of the cells. This allows a comparison of the distribution of protein staining throughout the cell with the outline of the nuclear structures. However, their relative relationship cannot be absolutely determined in this way using conventional microscopy. Consequently a second strategy using confocal microscopy was performed to divide the image into separate slices optically. The pattern of staining in images generated using this technique were less likely to suffer from the confounding effect of overlay and were therefore likely to be more representative of the actual intracellular protein distribution.

While this technique yields extraordinary optical clarity it still depends on visual resolution to determine the spatial relationship between CXCR4 localisation and cell ultrastructure. Further methodologies could have been used to define this relationship more clearly. For example, double-labelling of sections with antibodies against components of the cell membrane, nuclear membrane and other cell organelles would permit accurate visual co-localisation of CXCR4 staining within the cell. While the methodologies of these multi-labelling studies are well described, the precise protocols require meticulous optimisation of preparation and antibody incubation steps. However, once achieved, it is reasonable to suggest that the protocols would be similar for most cultured cells and the multi-channel image data generated would yield powerful results. This limitation of the current work is one that would lend itself well to further study.
Appendix 4:
Supplement to Chapter 4
- CXCR4 protein production in OSCC clinical specimens
A4.1 Preliminary investigation of CXCR4 staining in OSCC clinical specimens using immunohistochemistry

Preliminary experiments were carried out with tissue blocks selected from the clinical resection specimens of ten patients (Table A2.3, Appendix 2). Each specimen comprised of matched primary tumour and lymph node metastases excised from the same patient in continuity. Matched, adjacent, histologically normal mucosa was also examined where available. Lymph node parenchyma served as an internal positive control.

Three representative cases are reproduced here. Results from a pT2N1Mx tumour originating at the lateral border of the tongue are shown in Figure A4.1.64 CXCR4 staining of occasional basal keratinocytes together with some intra-epithelial and stromal cells (which had a lymphocytic morphology) was demonstrated in adjacent normal tissue (panels a & c). Endothelial cells within the adjacent normal mucosa were also stained positively (compared with isotype controls - panel e). Heterogeneous, moderate CXCR4 staining within the primary tumour (panels g, i & k) was found with a predominantly nuclear distribution. Some tumour islands demonstrated more intense cytoplasmic staining (black arrows, panel i). Within the associated lymph node metastases tumour cells of varying differentiation were seen. While the pathology report comments on the extracapsular spread of tumour from this lymph node, this is not represented on these sections. Once again a similar heterogeneous pattern of moderately intense nuclear CXCR4 staining of tumour cells (panels h, j, & l) was observed, although some cytoplasmic staining was also identified (black arrow, panel j). The intensity of CXCR4 staining did not obviously vary with the degree of tumour cell differentiation within the lymph node. Intense CXCR4 staining within the lymph node lymphocyte population was demonstrated (panel n).

64 pT2N1Mx denotes that the TNM staging was informed by histopathological examination of the resection specimen (primary lesion and associated modified radical neck dissection).
Figure A4.1: CXCR4 immunohistochemistry of a T2N1 OSCC clinical specimen (continued overleaf). Tissue sections taken from a single surgical specimen were stained with monoclonal antibodies to CXCR4 (a, c, g - l & n) or isotype controls (b, d - f, m) as outlined in Section 2.7. Sections from adjacent normal mucosa (a, c & e), primary tumour (b, g, i & m) and corresponding lymph node metastasis (d, f, h, j, l & n) are shown. Black arrows (panels i & j) demonstrated cytoplasmic staining of tumour cells in the primary lesion and lymph node secondary. For further details see text. Scale bar represents 100μm (a, b, d - h & m) or 50μm (c, i - l & n).
Figure A4.1 (continued)

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Sections from a pT2N2bMx lateral tongue OSCC with extracapsular lymphatic spread were also stained for CXCR4. Representative images are shown from the primary lesion and a lymph node metastasis in Figures A4.2a and A4.2b respectively. Within the primary lesion a heterogeneous CXCR4 staining pattern was observed with cells variably demonstrating predominantly cytoplasmic (black arrows), nuclear (white arrows) or no CXCR4 labelling. Within the associated lymph node metastasis (demonstrating extracapsular spread) CXCR4 staining was again observed in both the cytoplasmic and nuclear compartments. Staining with isotype control antibodies demonstrated no non-specific binding for either the primary lesion or associated lymph node metastasis (panels e, f, k & l).

Examination of a larger and locally invasive primary tumour was made. Results from the pT4N2bMx tumour originating from tongue and floor of mouth are shown in Figure A4.3. In a similar fashion to before, isotype controls demonstrated no evidence of non-specific binding (panel d). Nuclear and cytoplasmic CXCR4 staining was evident in tumour cells (panels a and b). The vascularity of the tumour was demonstrated on H&E staining (panel c) and CXCR4 labelling was noted in the endothelial cell cytoplasm. A number of cells in the epithelium overlying the tumour stained positive for CXCR4. Similar results were observed in the other seven cases examined.
Figure A4.2a: CXCR4 immunohistochemistry of a T2N2b OSCC specimen showing the primary lesion (A4.2a) and associated lymphatic metastases (A4.2b) with extracapsular spread. Tissue sections taken from a single surgical specimen were stained with monoclonal antibodies to CXCR4 (a - d) or isotype controls (e & f) as outlined in Section 2.7. Sections from the primary tumour (a - f) and corresponding lymph node (g - l) were prepared. For details see text. White and black arrows highlight areas of nuclear and cytoplasmic staining respectively. Scale bar represents 100µm (a, c, e, f, g, k & l) or 50µm (b, d, & h -j).
Figure A4.2b: CXCR4 immunohistochemistry of a T2N2b OSCC specimen showing the primary lesion (A4.2a) and associated lymphatic metastases (A4.2b) with extracapsular spread. Tissue sections taken from a single surgical specimen were stained with monoclonal antibodies to CXCR4 (a - d) or isotype controls (e & f) as outlined in Section 2.7. Sections from the primary tumour (a - f) and corresponding lymph node (g - l) were prepared. For details see text. White and black arrows highlight areas of nuclear and cytoplasmic staining respectively. Scale bar represents 100µm (a, c, e, f, g, k & l) or 50µm (b, d, & h -j).
Figure A4.3: CXCR4 immunohistochemistry of a locally invasive T4N2bMx OSCC specimen. Tissue sections taken from a primary tumour resection specimen were stained with monoclonal antibodies to CXCR4 (a & b), isotype control (d) or H&E counterstain (c) as outlined in Section 2.7. For details see text. Scale bar represents 250µm (c), 100µm (a & d) or 50µm (b).
A4.2 Design, validation and discussion of a semi-quantitative method for analysing immunohistochemistry images.

A4.2.1 Methodology

The aim of these experiments was to compare immunohistochemistry staining using a robust, reproducible and unbiased method of assessment. Tissue arrays were taken and stained for CXCR4 expression as per the optimised protocol outlined in Section 2.7. All sections were examined using a PC-driven conventional light microscope with dry and oil-immersion lenses (Zeiss). Computer-controlled algorithms for focus, illumination and shutter speed were selected. Photomicrographs of tissue array specimens were taken sequentially under standardised conditions and a 5µm graticule was photographed at all magnifications to define scale. Images were assigned a randomised study number, carefully labelled, transferred to computer database and analysed using the Photoshop CS suite of software (Adobe). All computer-based analysis was performed blind to the pathological diagnosis to obviate observer bias.

A methodology was developed to standardise the measurement of staining intensity to permit comparison between specimens located on the same slide. Firstly all photomicrographs were taken under standard conditions of magnification, colour balance and illumination (Figure A4.4a). Secondly all isotype control slides were reviewed to ensure that no background or non-specific binding had occurred (Figure A4.4c). Thirdly each immunostained photomicrograph was enlarged to a full screen size and nine separate locations were defined using a grid overlay (Figure A4.4b). The single tumour cell on (or nearest) to each intersection point was selected and the nuclear and cytoplasmic components defined. The yellow and magenta colour saturations for each selected cell (nucleus and cytoplasm) were assessed using the software colour analysis tool and tabulated in Microsoft Excel. Nine areas of background (no tissue present) were selected using the grid system and analysed in a similar way. In this way yellow and magenta colour saturations were obtained in a standardised fashion for nucleus, cytoplasm and background from 9 positions on each photomicrograph (sample data is represented in Figure A4.4d). The variability of the raw data obtained was compared with the normal distribution using P-P plots to determine its suitability for summarisation and analysis with parametric statistical
methods. Finally the mean background colour saturations for each slide were subtracted from each measured variable to give a background corrected score.

Preliminary analysis revealed that the correlation between magenta and yellow colour saturation across the entire array was high: for background, cytoplasmic and nuclear staining $R^2$ linear regression values were 0.521, 0.731 and 0.640 respectively (Figure A4.5). For cytoplasmic and nuclear staining the mean ratio of yellow/magenta saturation was calculated to be 1.532 and 1.367 respectively suggesting that yellow saturation was the most sensitive marker of peroxidase activity. The robustness and reproducibility of this method was tested in two ways: Firstly observer consistency was measured by the selection of twenty slides at random. Data were collected in the same manner as described previously and the results paired with their original counterparts. For each variable recorded a scatterplot was generated linear regression performed and a $R^2$ value calculated. Secondly each clinical tumour sample was represented by two sections on the array. A plot was made of the mean and standard deviation of the data derived from each replicate to give an indication of how robust the technique was. To interpret the results, the blinding of samples was removed and background-corrected yellow saturation data was combined for each replicate pair. The mean and standard deviation was calculated for both nuclear and cytoplasmic components for each sample. A scatterplot of nuclear staining against cytoplasmic staining was produced. Subgroups were identified according to their tissue / tumour stage of origin.

Data were generated from all of the images and P-P plots confirmed that these conformed to the normal distribution. The repeat analysis of 20 images selected at random showed high levels of correlation ($R^2 > 0.95$ for all variables). When the blinding of samples was removed, data from those represented twice on the array were used to generate a scatterplot and regression line (Figure A4.6). There was an excellent correlation between replicate data sets for both the cytoplasmic and nuclear compartments with $R^2$ values of 0.85 and 0.68 respectively.
Figure A4.4: Image analysis and data tabulation for a semi-quantitative method of immunohistochemistry results. Standardised individual images from CXCR4-stained tissue arrays were obtained as outlined in Sections 2.7 and A4.1 (panel a) and compared with isotype controls (panel b). A grid with 9 intersections was overlaid and the magenta and yellow saturations were recorded for the background, cytoplasm and nuclear components nearest each intersection (panels b & d). The mean background saturation was calculated and subtracted from each measured component. Legend: M = % saturation of magenta; Y = % saturation of yellow; scale bar represents 250µm.
Figure A4.5: Scatterplots of magenta and yellow colour saturations. Areas were selected on each slide on the tissue array (as outlined in Section 2.7). Measurements of magenta and yellow colour saturation were made using a software colour analysis tool, tabulated and a scatterplot prepared for background (a), cytoplasm (b) and nuclear (c) components. The conformity to a regression line was analysed and the gradient, intercept and R² values calculated for each component (d).
Figure A4.6: Scatterplot of colour saturations from replicate samples represented on tissue arrays. Colour saturation data were collected blind to tissue of origin as described in Section 2.7. Mean values (points) and standard deviations (error bars) from replicate samples were plotted for the cytoplasmic (a) and nuclear (b) compartments. $R^2$ values were 0.85 and 0.68 respectively. The respective line gradients were 0.912 and 0.993.
A4.2 Discussion of Experimental Procedures

A4.2.1 Selection of techniques

Although complex *in-vivo* monitoring techniques (such as intravital microscopy) are described and available, immunohistochemistry remains the mainstay of investigative techniques that focus on tissue morphology and the cellular interactions within it. To build up a robust “picture” it is necessary to examine a number of repeat samples with this technique. However, there are a number of methodological difficulties associated with immunohistochemistry that may significantly bias the results it generates. These can be broadly grouped into two main categories: 1) experimental methodology; and 2) interpretation of results.

A4.2.2 Immunohistochemical experimental methodology

Methodological problems associated with immunohistochemistry are predominantly due to the wide number of experimental factors that may influence the pattern and intensity of colour generation. These include variations in specimen selection, sample processing, slide preparation, antigen unmasking, antibody concentration, wash efficiency and duration of colour development. For repeat experiments these variables can be difficult to standardise precisely.

One of the limitations of using archived tissue is that the investigator must make several assumptions that cannot be tested empirically. These include methodological steps such as tissue processing and handling, as well as clinical and pathological diagnoses. For example, is tissue labelled as “normal oral mucosa” really normal oral mucosa? It is possible that this tissue was derived from specimens taken for diagnostic purposes (probably with a visible lesion) but in which no histological abnormality could be detected by conventional light microscopy. However, short of harvesting control tissue from healthy, age- and sex-matched consenting adults, this limitation is difficult to overcome. Assumptions regarding the processing of tissues must also be made. Including the provision of research samples in routine patient management would help standardise this process and facilitate access by more sensitive techniques such as laser-capture microdissection with sqRT-PCR or in-situ hybridisation.
Although immunohistochemistry techniques are not so sensitive to variations in tissue handling, the standardisation of surgical sample processing will undoubtedly reduce variability in the results (Werner, Chott et al. 2000).

Immunohistochemistry slides derived from formalin-fixed paraffin-embedded (FFPE) tissue blocks are often dependent on techniques of antigen retrieval. These require careful optimisation against known positive and negative controls. From the early literature pertaining to CXCR4 many techniques of antigen retrieval were described including microwave heating (Schrader, Lechner et al. 2002), pressure cooking (Sun, Wang et al. 2003; Katayama, Ogino et al. 2005) or other unspecified methods (Muller, Homey et al. 2001). Using tonsillar sections it was determined empirically that the pressure cooking method using commercially available antigen-unmasking solutions produced the most reproducible results for antigen retrieval. One method employed to reduce variability in this stage was to prepare samples in batches. This has the benefit that separate slides were exposed to identical conditions but would not overcome variations in the later stages of washing, antibody incubation or staining. To achieve this multiple samples must be mounted on single slides. The presentation of samples in this format is provided by tissue arrays.

The majority of my immunohistochemical results were derived from tissue arrays. The processing of multiple tissue samples on one slide removes the effect of the majority of potentially confounding experimental factors listed above. The problem of sample processing (the transfer from patient to stabilization in fixative) remains difficult to address in samples not specifically taken for research purposes.

A4.2.3 Interpretation of immunohistochemistry

The interpretation of immunohistochemistry results has been the subject of much debate within the literature. Numerous methodologies have been described regarding the assessment and grading of CXCR4 immunohistochemistry results although these are predominantly for tumour tissues other than OSCC. Unfortunately, not all authors are assiduous with their description of their experimental methodologies: particularly

65 Derived from the report of the first meeting of the International Consensus Group on Standardisation and Quality Control (ICGSQC), Nice, France, 2000
the immunohistochemical protocols, rationale for interpretation, justification of scaling system and minimisation of observational bias. Most of the published work on CXCR4 in OSCC falls into this category which makes its credibility difficult to establish (Uchida, Begum et al. 2003; Delilbasi, Okura et al. 2004; Uchida, Begum et al. 2004). Other work defines a dichotomy (either positive or negative) dependent on whether greater than a defined percentage of cells demonstrate visible staining (Ishikawa, Nakashiro et al. 2006). My criticisms of this approach are that it is over-simplistic and the threshold is completely arbitrary.

My *in-vitro* results have shown that different OSCC cell lines demonstrate variable degrees of CXCR4 protein production in their nuclear and cytoplasmic compartments as well as on the surface membrane. Whether this reflects normal protein assembly and transport, receptor cycling or pathological processes is unclear from these data. However, at a simplistic level, it can be hypothesised that the cell responsiveness to exogenous SDF is, at least partly, defined by the density of functional CXCR4 protein on the cell surface membrane. While immunohistochemistry does not give insight into protein functionality, it does yield qualitative data on protein localisation.

Many authors have described systems by which immunohistochemical image data can yield quantitative results. These scoring systems range from a simple four-point scale (Katayama, Ogino et al. 2005) to complex systems that attempt to combine parameters for both staining location (nuclear versus cytoplasmic) and intensity (Cabioglu, Yazici et al. 2005; Retz, Sidhu et al. 2005; Su, Zhang et al. 2005; Su, Wu et al. 2005). All of these scoring systems can be criticised due to their subjective interpretation. Using multiple observers either concurrently or consecutively is a strategy by which the subjectivity is shared but not eliminated (Maxwell and McCluggage 2000). The system I have described was developed to try and address this lack of objectivity and allow these biological systems to be analysed with parametric (rather than categorical) variables. Objective measurements of colour saturation were determined using points defined by a standardised grid. The mean and standard deviation were calculated for each data set. One drawback of this technique might be if the staining pattern was highly heterogeneous. This would result in data sets that did not conform to the normal distribution and had wide variability relative to the mean. Probability plots suggested that all data sets conformed to the normal distribution although it is important to stress
that the numerical results generated are complimentary to (but do not replace) the qualitative interpretation of the slides.

Concerns regarding the reproducibility of the technique were tested using a second random sample set of 20 images. R-squared\textsuperscript{66} values exceeding 0.95 for all variables tested suggested the method was both robust and highly reproducible. Some authors aim to address the issue of subjectivity by multiple assessments by “independent” observers (Katayama, Ogino et al. 2005)\textsuperscript{67} and this technique could have been used to test for inter-observer variation in these experiments. Another potential source of bias (especially in more subjective methods) is awareness of the clinicopathological details when assessing the staining pattern. Most authors describe blinding techniques to overcome this and this was also performed in these experiments.

**A4.2.4 Alternative techniques**

Techniques to identify mRNA species within histological sections of clinical tissues are well described. These include *in-situ* hybridization and laser-capture microdissection (LCMD) in association with sqRT-PCR. The former yields qualitative data on the spatial distribution of mRNA transcripts and is a useful compliment to immunohistochemistry. The latter can be used to identify multiple transcripts (with the use of multiplexed PCR) and provides (semi) quantitative data on cellular subsets identified visually.

It was my intention to perform LCMD with multiplexed sqRT-PCR on the same archived FFPE sections of OSCC investigated with immunohistochemistry. However, preliminary experiments revealed that the yield of housekeeping gene mRNA was too small and too variable to allow meaningful comparison or further investigation in combination with laser-capture. Although these techniques are well described in the literature (Stanta, Bonin et al. 1998; Lehmann and Kreipe 2001; Macabeo-Ong, Ginzinger et al. 2002), they are highly dependent on the speedy handling of the clinical

\textsuperscript{66} Linear regression was selected as the technique to investigate measurement reproducibility because of the parametric nature of the data. Kappa statistics are inappropriate in this case for two reasons: Firstly the data are not categorical; and secondly it examines observer independence (not valid in this case because the same person did the evaluation both times).

\textsuperscript{67} No kappa-correlation coefficients were published in this paper.
samples *ex-vivo* to prevent degradation of mRNA species by RNases. The most likely interpretation of my preliminary results was that the timing of tissue fixation was highly variable between specimens. It was therefore likely that *in-situ* hybridisation techniques would be difficult to optimise in these FFPE specimens.

Without doubt clinical material is fundamental to the interpretation of results derived from *in-vitro* experiments. However, ethical and tissue storage issues aside, the interface between clinician and laboratory investigator is key in securing material of use to researchers. The potential conflict between diagnostic interpretation and the early stabilisation of research samples must be addressed at a local level if highly technique sensitive methodologies (such as LCMD and microarrays) are to be able to produce meaningful results.
Appendix 5: Supplement to Chapter 5

- Establishing a stable CXCR4-transfected OSCC cell line
A5.1 Transfection optimisation

A5.1.1 Characterisation of the donated plasmid

Experiments were performed using the methodology outlined in Section 2.4.11. Optimisation of the transfection reaction was performed using the pcDNA3.1-lacZ control plasmid. Initially, JM109 competent cells were transformed with the plasmid with a calculated efficiency of $5.8 \times 10^6$ cfu/µg of DNA added. Single colonies were selected on the basis of blue staining and the DNA was extracted following overnight incubation. Restriction enzyme digestion was performed to confirm the presence of the pcDNA3.1-lacZ plasmid (Figure A5.1). A panel of restriction enzymes were used: BCl1 was predicted to cut once at 8540 base pairs although two bands were seen on the gel at 9333 and 25600 bp. It is likely that the former represents plasmid that has been cut once and made linear, while the latter, smaller band would be consistent with plasmid that has either retained or re-annealed to form its original circular structure. EcoR1 was expected to cut the plasmid twice, yielding fragments of 3023 and 5517 base pairs. Sal1 was also predicted to cut twice (fragments 2188 and 6352 base pairs) and NcoI was expected to cut three times (fragments 735, 3347 and 4458 base pairs). Results obtained correlated very closely with predicted values confirming the presence of the correct plasmid in the clone tested.

A5.1.2 Charge Ratio

The effect of charge ratio on transfection efficiency was investigated. For the charge ratios 1:1, 1.5:1 and 2:1 (TransFast reagent volume to DNA volume) there was a trend towards the 1:1 ratio being the most efficient, although the difference was not statistically significant (Figure A5.2). This occurred over a range of DNA concentrations between 0.2 and 0.8 µg per well. Consequently the 1:1 TransFast to DNA ratio was used for further experiments. Interestingly, an approximately linear relationship between β-Galactosidase reporter activity and DNA added was noted for the concentration range used in this experiment. Further experiments were performed to investigate the effect of adding more than 0.8 µg of DNA per well.
5.1.3 Amount of DNA added

Further experiments were performed to determine the relationship between the amount of DNA added per well and the overall efficiency of the transfection reaction (Figure A5.3). From a range of starting DNA concentrations (0 to 2.0 µg per well) it was shown that β-galactosidase reporter activity was approximately linear over the range of 0.2 to 1.5 µg of DNA per well. Further addition of DNA did not result in significantly greater transfection efficiency (p>0.05). Qualitative evidence supporting this finding is provided by direct visualisation of β-galactosidase reporter activity in H357 cells transfected with pcDNA3.1-lacZ DNA in concentrations ranging from 1.0 to 2.0 µg per well (Figure A5.4). A very low level of background or endogenous β-galactosidase activity as seen in pcDNA3.1vB transfected negative controls confirms the results from the quantitative methods used.

5.1.4 Exposure Time

A final set of experiments were done to determine the relationship between transfection incubation time and transfection efficiency (Figure A5.5). A range of incubation times from 30 minutes to 2 hours were investigated. Greatest β-Galactosidase reporter activity was shown for one hour incubations. Shorter or longer incubation steps resulted in significant reductions in transfection efficiency (p<0.05).

A5.2 Antibiotic Selection

The antibiotic G418 was found to be toxic to wild-type H357 cells in a dose-dependent manner (Figure A5.6). At lower concentrations (<800 µg/ml) the effects on wild-type H357 cells were less at 72 hours than at 48 hours when compared with no-treatment controls. However, at concentrations of 1200 µg/ml and above further reductions in cell survival were seen at the 72 hour time point. A G418 concentration of 1600 µg/ml achieved the aim of 90% wild-type cell killing after 72 hours. Consequently, 1600 µg/ml was the concentration of G418 used to apply positive selection towards the transfected clones.
Restriction enzyme digestion products from the pcDNA3.1-lacZ plasmid run on 0.75% agarose gel against a 1kbp DNA ladder. Results show two bands for the restriction enzyme BCl at 25600 and 9333 base pairs (predicted one band at 8540 bp). The remaining digestions are as predicted: EcoR1 reveals two bands (5300 and 3200 base pairs), Nco1 three bands (4214, 3233 and 717 base pairs), and Sal1 two bands (5500 and 2181 base pairs). All fragments correspond closely in size with those predicted in-silico. Legend: LD = GeneRuler 1 kbp DNA ladder, BCl = BCl1, Eco = EcoR1, Nco = Nco1, Sal = Sal1.

<table>
<thead>
<tr>
<th>Restriction Enzyme</th>
<th>BCl</th>
<th>Eco</th>
<th>Nco</th>
<th>Sal</th>
</tr>
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<tbody>
<tr>
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<td>25600</td>
<td>9333</td>
<td>5300</td>
<td>3200</td>
</tr>
<tr>
<td></td>
<td>3200</td>
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<td>717</td>
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<td>3023</td>
<td>3347</td>
<td>735</td>
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Figure A5.1: Gel electrophoresis and tabulation of observed and expected band sizes following restriction enzyme digestion of the pcDNA3.1-lacZ plasmid.
Figure A5.2: The effect of Charge Ratio on Transfection Efficiency over a range of DNA concentrations: Plot of Beta-Galactosidase activity against the amount of DNA added per well. Beta-galactosidase activity was measured as a reporter of transfection efficiency. Wild-type H357 cells were incubated with 1:1, 1.5:1 and 2:1 charge ratios of TransFast reagent volume to pcDNA3.1-lacZ volume for one hour over a range of DNA amounts added per well. Bars represent the mean (standard deviation) of β-Galactosidase activity from six wells each measured in duplicate. The 1:1 ratio appears to give the greatest transfection efficiency over a range of DNA concentrations, although statistical significance was not achieved (p>0.05, ANOVA with Bonferroni correction). The graph shows one representative experiment of three performed.
Figure A5.3: The effect of DNA concentration on Transfection Efficiency: Plot of Beta-Galactosidase activity against the amount of DNA added per well. Beta-galactosidase activity was measured as a reporter of transfection efficiency following incubation of wild-type H357 cells for one hour with a range of pcDNA3.1-lacZ DNA amounts added per well. A 1:1 charge ratio of TransFast reagent to DNA volume was used. Bars represent mean (standard deviation) of β-Galactosidase activity from six wells each measured in duplicate. Transfection efficiency increases approximately linearly with the amount of DNA added up to 1.5 µg/well. Further increases in DNA concentration do not result in statistically significant increases in transfection efficiency (p>0.05, ANOVA with Bonferroni correction). The graph shows one representative experiment of three performed.
Figure A5.4: The effect of starting pcDNA3.1-lacZ concentration on transfection efficiency: Visualisation of Beta-galactosidase reporter activity in H357 cells. Beta-galactosidase activity was visualised in H357 cells transfected with the pcDNA3.1-lacZ plasmid compared with mock-transfected pcDNA3.1vB negative controls. The effect of starting DNA concentration per well on β-galactosidase reporter activity is demonstrated. Cells were transfected with pcDNA3.1-lacZ DNA at concentrations of: a) 1.0 µg per well; b) 1.5 µg per well; and c) 2.0 µg per well. A qualitative increase in reporter activity can be appreciated with an increase in starting DNA concentration up to, but not above, 1.5 µg per well. The pcDNA3.1vB negative control well (d) confirms little background or endogenous β-galactosidase reporter activity. Scale bar represents 100µm.
Figure A5.5: The effect of Incubation Time on Transfection Efficiency: Plot of Beta-Galactosidase Activity against Incubation Time. Beta-galactosidase activity was measured as a reporter of transfection efficiency following incubation of wild-type H357 cells with 1.5 µg/well of pcDNA3.1-lacZ DNA for periods varying from 30 minutes to 4 hours. A 1:1 charge ratio of TransFast reagent to DNA volume was used. Bars represent mean (standard deviation) of β-Galactosidase activity from six wells each measured in duplicate. Transfection efficiency is maximal after one hour incubation. Incubation for shorter (30 minutes) and longer (4 hour) periods results in a statistically significant reduction in transfection efficiency (p<0.05, ANOVA with Bonferroni correction). The graph shows one representative experiment of three performed.
Figure A5.6: The Effect of G418 Concentration and Time on the Killing of wild-type H357 Cells as determined by MTT Cell Viability Assay: Plot of Percentage Survival (compared with untreated control) against G418 Concentration by time point. Wild-type H357 cell survival after exposure to varying concentrations of G418 (100 to 1600 µg/ml) for periods of 24, 48 and 72 hours. Bars represent mean (standard deviation) cell survival as determined by MTT assay for each G418 concentration (n=12) and time point calculated as a percentage of untreated control (n=12). Exposure of untransfected H357 cells to 1600 µg/ml of G418 resulted in the required 90% cell killing in 72 hours. The graph shows one representative experiment of three replicates performed.
Appendix 6: Supplement to Chapter 6
- The effects of CXCR4 function on cell proliferation and apoptosis in established OSCC cell lines and CXCR4 transfectants
A6.1 Supplemental figures

Figure A6.1: The effect of serum-starvation on transfected and untransfected OSCC cell lines. Three established OSCC cell lines (CAL27, SCC4 and H357), three CXCR4-transfected H357 clones (H357-X4.1, H357-X4.2 and H357-X4.4) and mock-transfected empty-vector cells (H357-EV) were incubated for 72 hours under optimum conditions (10% FCS) and in serum-reduced conditions (0.1% FCS) with or without 100 ng/ml of SDF. This figure represents the effect of serum reduction on proliferation in the absence of SDF. The effect of SDF stimulation is represented in Figure 6.3 (Chapter 6). Bars represent the mean (standard deviation) of combined data from three identical experiments each containing twelve replicate wells per cell treatment. Statistical analysis was performed using the Student t-test. All comparisons were not statistically significant unless indicated. Serum reduction significantly reduced proliferation in SCC4 and untransfected H357 cells. No significant effect was demonstrated in transfected H357 cells with or without the CXCR4 insert. Legend: ** = p<0.01. The figure represents pooled data from three identical experiments.
Figure A6.2: Representative raw data for Figure 6.6. Each well was photographed in three positions for both BrdU labelling (panels a), c) and e)) and DAPI (panels b), d) and f)). No signal was detected when an isotype primary antibody (panel (g)) was compared with the DAPI control (panel (h)). Scale bar represents 250µm.
Figure A6.3: The effect of staurosporine on apoptosis. Results demonstrated that caspase 3/7 activity was proportional to staurosporine concentration over the range of concentrations tested and that the enzyme kinetics of the fluorescence assay were stable over a wide time window ensuring robust and reproducible findings.
Figure A6.4: The effect of serum starvation on apoptosis in H357 cells. Untransfected H357 cells were incubated with varying concentrations of FCS supplementation (0.1, 0.5 and 10%) for 24 hours. Caspase 3/7 activity and MTT absorbance were measured. Bars represent mean (standard deviation) of 12 replicate wells calculated as a percentage of the positive control (10% FCS). Cell viability was reduced in 0.5% and 0.1% FCS (p=0.016 and p<0.001 respectively) compared with positive controls. Apoptosis was significantly increased in conditions of 0.1% FCS (p=0.011), but not 0.5% FCS, compared with positive controls. Legend: * = p<0.05, *** = p<0.001 (Student t-test). Figure represents cumulative data from two identical experiments.
A6.2 Determination of the optimal experimental conditions for the investigation of the effect of SDF on cell proliferation

A6.2.1 Overview

The overall aim of this set of experiments was to investigate the effect of SDF stimulation on the proliferation rate of CXCR4-positive OSCC cells and transfected clones. A number of potentially confounding variables were identified.

5. Cell seeding density
6. Duration of incubation
7. Cell culture supplementation
8. SDF concentration
9. Variability across cell lines

These included the effect of basic culture conditions as well as growth media components (for example mitogenic factors such as EGF). In order to investigate these variables simple and repeatable assays were required. For this purpose the MTT and resazurin-based assays were performed (as outlined in Chapter 2, Sections 2.8.2 and 2.8.3 respectively). However, both these techniques require the enzymatic conversion of a substrate to a detectable end-product. Therefore these techniques are, more accurately, determinants of cell viability rather than proliferation *per se*. Consequently, once the experimental conditions had been optimised, results were obtained using measurements of BrdU incorporation as a direct measure of cellular proliferation. A statistical power calculation was also performed to determine the number of replicate wells required to detect likely differences in cell proliferation rates.
A6.2.2 Determination of Replicate Numbers

In order to determine suitable replicate numbers for further experiments using a statistical power calculation a sample data set was generated by a preliminary series of experiments that investigated the effect of cell media supplementation and SDF concentration on cell proliferation (Figure A6.5). The data generated conformed to the normal distribution and was therefore amenable to statistical tests for parametric variables. Results showed the trend that CAL27 cells were responsive to SDF gradients (in a roughly dose-dependent manner) when cultured with supplementation with FCS, but not BSA. Although overall proliferation was greater in cells cultured in 10% FCS, compared with 0.5% FCS, the response to SDF concentration was demonstrated in both arms of the experiment. However, multiple statistical testing revealed that only the comparison between cells treated with 100 ng/ml of SDF and untreated controls reached significance in the 0.5% FCS arm (p=0.007) but not the 10% FCS group (p=0.101).

From these preliminary experiments, the data were suggestive of a trend but were not statistically conclusive. At this point, for the purposes of a statistical power calculation, it was assumed that SDF stimulation altered cell proliferation but that the direction (positive or negative) was unknown. Consequently (based on the data derived from the FCS arm) it was calculated that twelve replicates per treatment would give the experiment sufficient statistical power. Interestingly if similar assumptions were made for the results for 1% BSA, then it was calculated that a sample size of over 100 would be required to power the experiment to the same degree.

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68 A P-P plot of expected against observed cumulative probability for data from the three experimental conditions investigated conformed well to a straight line confirming that the data were normally distributed (as outlined in Figure A2.5, Appendix 2).
69 ANOVA with Bonferroni corrections for multiple testing.
70 The working hypothesis was that SDF stimulation increased cell proliferation. However, if the power calculation was altered to a one-way test to reflect this assumption there was a risk that the experimental arms would have insufficient replicates to demonstrate an effect either way.
71 The statistical power calculation was performed as outlined in Chapter 2, Section 2.8.5. Briefly, variances were compared and found to be equal (Levene’s test) and the number of replicates calculated to detect differences of p<0.05 on 90% of occasions using a two-way test.
Figure A6.5: Preliminary experiment to determine proliferation assay replicate numbers. CAL27 cells were seeded at equal density and grown for 24 hours under optimal conditions with supplementation of the basal growth media with: 1) 10% FCS; 2) 0.5% FCS; and 3) 1% BSA. Cells were also treated with SDF in concentrations of 0, 10, 50 and 100 ng/ml. Relative proliferation at 24 hours was determined by MTT assay. Bars represent the mean (standard deviation) of relative proliferation from six replicate wells per treatment. Statistical comparisons were performed using one-way ANOVA with Bonferroni correction for multiple testing. Cells grew best in media supplemented with 10% FCS and least well in 1% BSA. There was a trend that increasing concentrations of SDF were associated with increasing proliferation of cells grown in 10% and 0.5% FCS (compared with untreated controls). These results did achieve statistical significance in latter group (p=0.007) but not the former (p=0.101). No difference in proliferation with SDF stimulation was demonstrated in cells grown in 1% BSA.
A6.2.3 Determination of Incubation parameters

A preliminary study was performed to determine if the compounds used to supplement the growth media in future experiments would interfere with the fluorescence-based resazurin assay. It was found that growth media, FCS, and all compounds used in the cell stimulations (including SDF, AMD3100 and desferrioxamine) did not cause reduction of resazurin in the absence of cells. A second experiment was performed to determine the effect of resazurin on intracellular fluorescence: Cells incubated overnight in resazurin-supplemented growth media showed similar background fluorescence readings when the media was removed compared with cells grown in normal media. Finally, a very slow auto-reduction of resazurin with time was noted in cell-free culture and this was accounted for by the subtraction of media-only blanks from each reading for each time point.

Although not contact inhibited, the proliferation of OSCC cells is influenced by the physical constraints of the culture environment. A series of experiments were performed to optimise seeding density by allowing the cells to proliferate unhindered over the duration of the experiment. The effect of seeding density on H357 cells is shown in Figure A6.6(A). Increases in starting cell densities up to $5 \times 10^3$ per well were associated with a proportional increase in fluorescence measured at the 48 hour time point. Similar proportionality was not demonstrated with further increases in seeding density. These data suggested that wells initially seeded with more than $5 \times 10^3$ cells demonstrated a confounding limitation on proliferation within a 48 hour time period. This was possibly due to the accumulation of toxic waste products and/or the generation of over-confluent cultures caused by the physical constraints of the 96-well-plate. The latter was confirmed by visual inspection using light microscopy.
Figure A6.6: The effect of cell seeding density and incubation time on cell proliferation. Untransfected H357 cells were seeded at densities ranging from 1.25 to 20 x 10³ per well and incubated for 48 hours (A). Mean (standard deviation) fluorescence values were plotted against seeding density. A starting density of 5 x 10³ cells per well or less resulted in a proportional increase in proliferation at 48 hours. This was not the case for seeding densities greater than 5 x 10³ cells per well. A proliferation time course was performed for the same cell line (B). Cells were seeded at 2.5 x 10³ (red line) and 5 x 10³ (blue line) cells per well and incubated for 72 hours. Mean (standard deviation) relative fluorescence values multiplied by the number of cells seeded per well (for twelve replicate wells) were plotted against time. The rates of fluorescence accumulation (black lines) remained linear up to 48 hours in both cases ($R^2 >0.995$). The accumulation rate for wells seeded with 5 x 10³ cells was 2.04 times (95% CI 2.03-2.05) that for wells seeded with 2.5 x 10³ cells. Legend: RFV = Relative Fluorescence Value; Cw = Cells seeded per well; AU = arbitrary units. Both figures present data from a single representative experiment of three performed.
The relationship between the accumulation of fluorescence and time was determined for the same cell line seeded at two different starting densities (Figure A6.6-B). Results showed that, for cells seeded initially at either $2.5 \times 10^3$ or $5 \times 10^3$ per well, proliferation proceeded at a linear rate for the first 48 hours with excellent correlation of the multiple readings taken ($R^2$ value >0.995). For wells initially seeded with $5 \times 10^3$ cells, the rate of fluorescence accumulation reduced between the 48 and 72 hour time points and the relationship with time became non-linear. This was not demonstrated in the replicate wells seeded with $2.5 \times 10^3$ cells. Overall, these findings were consistent with the results from the experiments investigating the effect of cell seeding density and proliferation (demonstrated in Figure A6.6-A). The rate of accumulation of fluorescence in wells starting with $5 \times 10^3$ cells was 2.04 (95% CI 2.03 - 2.05) times greater than in those seeded with $2.5 \times 10^3$ cells. This is very close to the twofold increase predicted by the numerical relationship between the starting densities.

Consequently, as shown in Figure A6.6(B), the difference in rates of fluorescence accumulation correlates closely with the amount of cellular material present. When care is taken to ensure that all replicates start with similar cell numbers, changes in the rates of fluorescence accumulation can be assumed to represent variation in the rates of cellular viability. In these experiments it was assumed that this was due to changes in proliferation rates although this was tested empirically using a complimentary BrdU-based technique (vide infra). Therefore, for fluorescence-based assays using time-series data, it was decided to measure proliferation rates over the first 48 hours of culture for wells with seeding density of $5 \times 10^3$ cells per well.

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72 As outlined in Chapter 2, Section 2.8.3 all experimental plates were analysed at the start of the experiment ($T_0$) for parity of cellular material. Plates that did not conform to this quality control measure were discarded.
A6.2.4 The effect of cell culture supplementation on cell proliferation.

Another series of preparatory experiments were performed to investigate the influence of culture media supplementation on cell proliferation. Initially the influence of serum was determined (Figure A6.7). Results demonstrated that the proliferation of CAL27 and SCC4 cells, and to a lesser extent H357 cells, was dependent on FCS concentration. For CAL27 and SCC4 cells a reduction of serum supplementation below 10% (final concentration) resulted in a significant decrease in cell proliferation after 72 hours, compared with baseline controls. Interestingly, a plot of absorbance value against log_{10}[FCS concentration] for CAL27 and SCC4 cells suggest that the relationship is linear (R^2>0.95) over the range of values tested. In contradistinction, the relationship between H357 cell proliferation and FCS concentration was less clear-cut: FCS concentrations of 1% and 0.5% resulted in no significant difference in proliferation compared with 10% FCS. Only incubation with 0.1% FCS significantly reduced H357 proliferation compared with 10% FCS. Furthermore, plots of absorbance value against log [FCS concentration] did not produce a linear relationship for H357 cells. Finally, over 72 hours, H357 cells demonstrated significantly more growth in serum-free conditions than both CAL27 and SCC4 cells. Collectively, these findings demonstrate that all three OSCC cell lines tested proliferated better with serum supplementation to a final concentration of 0.5% or greater.
Figure A6.7: The effect of FCS on OSCC cell proliferation. Cells from three OSCC cell lines (CAL27, SCC4 and H357) were seeded at $5 \times 10^5$ cells per well and incubated in normal growth media supplemented with varying concentrations of FCS (0, 0.1, 0.5, 1.0 and 10% by total volume). MTT assay was performed after 72 hours. Bars represent mean (standard deviation) of twelve replicate wells per treatment. Statistical comparisons were performed using one-way ANOVA with Bonferroni correction for multiple testing. The proliferation of CAL27 and SCC4 cells demonstrated a dramatic response to FCS concentration with a significant ($p<0.001$ respectively) reduction in proliferation with decreasing FCS. However, the proliferation of H357 cells was less responsive to FCS concentration, with changes from 10 to 0.5% showing no significant reduction in MTT absorbance. H357 cells grown in 0.1% FCS showed significantly less proliferation than those grown in 10% ($p<0.001$). Interestingly, H357 cells demonstrated significantly more growth than CAL27 and SCC4 cells in serum-free conditions ($p<0.001$, Student t-test). Legend: ns = not significant; * = $p<0.001$. Figure shows pooled data from three identical experiments performed.
The results from the previous experiment (Figure A6.7) are suggestive that, while CAL27 and SCC4 cells are highly dependent on FCS concentration for growth, H357 cells are able to proliferate more independently of serum supplementation. This important finding was investigated further with a time-course of cell proliferation against FCS concentration for H357 cells (Figure A6.8-A) and the SCC4 cell line (Figure A6.8-B). The results obtained were consistent with earlier findings: The reduction of serum concentration in SCC4 cells was associated with a significant reduction in absorbance values at the later time points of 48, 72 and 96 hours. However, after incubation for only 24 hours there was no statistically significant difference in SCC4 proliferation in conditions of reduced FCS compared with controls cultured with 10% FCS. Towards the end of the time course, SCC4 cells in 0.1% and serum-free environments demonstrated a loss of cellular material from the wells. One explanation was that, under these conditions, cells were dying and becoming detached from the culture surface: Aspirates of the media taken just prior to performing the MTT assay and inspected by light microscopy showed that this was indeed the case.

The behaviour of H357 cells (Figure A6.8-A) under similar conditions was quite different. Compared with controls cultured with 10% FCS, H357 cells demonstrated no statistically significant reduction in proliferation when the serum supplementation was reduced to 0.5% or 1% (final concentration). Only incubation in 0.1% and serum-free conditions resulted in a significant reduction in proliferation ($p<0.01$) when compared with 10% FCS control wells. Furthermore, H357 cells grown in media supplemented with 0.1% or no FCS also demonstrated a significant increase ($p<0.01$) in absorbance values over the entire 96 hour time course of the experiment. This is suggestive that a slow rate of proliferation was still occurring with a net accumulation of total cellular material (confirmed by light microscopy).
Figure A6.8: The effect of FCS on the proliferation of H357 and SCC4 cells. Five thousand (A) H357 and (B) SCC4 cells per well were incubated in normal growth media supplemented with varying concentrations of FCS. MTT assay was performed at 5 time points up to 96 hours. Bars represent mean (standard deviation) of twelve replicate wells per treatment. Statistical comparisons were performed against the 10% FCS group for each time point using ANOVA with the *post-hoc* Bonferroni correction. FCS concentration was a clear determinant of SCC4 cell proliferation with significant differences demonstrated at the later time points (p<0.01). However, the proliferation of H357 cells in FCS concentrations ≥ 0.5% was statistically indistinguishable in the later time points. While H357 cells incubated in serum-free conditions continued to grow over the 96 hour time period, SCC4 cells did not. All statistical results were not significant (p>0.05) unless indicated (* = p<0.01). Figure represents pooled data from three identical experiments.
The results from the previous experiment (Figure A6.8) demonstrate that untransfected H357 cells are more resistant to serum-starvation than SCC4 cells. It was hypothesised that differences in the basal growth media used for H357 and SCC4 cells were responsible for the effect shown. H357 growth media (outlined in Table A2.1, Appendix 2) contains additional factors (such as Epidermal Growth Factor, EGF) that are pro-proliferative. A time-course experiment was performed to determine the effect of EGF stimulation on transfected and untransfected H357 cell proliferation with and without serum-starvation. For clarity the results for the 72 hour time point are represented in Figure A6.9. The results showed that EGF supplementation had no significant effect on untransfected, empty-vector or CXCR4-transfected H357 cells grown under conditions of 0.1% FCS concentration. Interestingly, the only statistically significant comparison was found in the 10% FCS control group where CXCR4-transfected H357 cells showed reduced proliferation in response to EGF supplementation. However, this difference was not reproduced across the time points and it was felt that this finding had statistical but not biological significance. Given that the aim of the experiment had been to determine the effect of EGF at the low serum concentrations to be used in the chemokine stimulation experiments, it was concluded that EGF was not a significant influence on H357 proliferation at FCS concentrations of 0.1%.
Figure A6.9: The effect of EGF supplementation on H357 cell proliferation. Three H357 clones (wild-type, empty-vector and CXCR4-transfected) were seeded at equal densities and incubated with or without EGF supplementation in media either supplemented with 10% or 0.1% FCS. Serial fluorescence measurements were taken, of which only the 72 hour time point is represented for the sake of clarity. Bars represent mean (standard deviation) of twelve replicate wells per treatment. Statistical analysis was performed using the Student t-test. Essentially, EGF supplementation had no significant effect on the proliferation of any of the three H357 clones tested, except for CXCR4-transfected cells in conditions of 10% serum (p<0.05). This finding was replicated at both 10% and 0.1% FCS concentration. Legend: WT = wild-type; EV = empty-vector mock transfected; X4 = CXCR4 transfected; ns = not significant; * = p<0.05. Figure represents pooled data from three identical experiments performed.
A6.3 Discussion of Experimental Methodologies

A6.3.1 Proliferation assays

A6.3.1.1 Resazurin-based assays

The use of resazurin-based assays for the determination of cytotoxicity in real-time was originally described in 1993 (Fields and Lancaster 1993). However, their usefulness in monitoring cell growth and survival is greater than that for measuring the toxic effect of particular compounds (Carter, Ericsson et al. 1998). This is due to the fact that the fluorescence measurements obtained by the assay are due to the accumulation of the reduced substrate with time. Consequently, although the experiments reported here initially incubated cells with 5% resazurin and 10% FCS overnight, this was removed and replaced with fresh media at the time of the addition of cell treatments. However, considering the hypothesis that the pre-incubation of cells with resazurin may affect subsequent results, this experimental design might attract criticism. Importantly, the findings from preliminary experiments determined that this was not the case; and evidence from the literature supports this finding by describing that very little fluorescence is retained intracellularly (O'Brien, Wilson et al. 2000). Another criticism of these fluorescence assays, described by the same authors, is that possible redox reactions between resazurin and cell treatments may yield confounding effects if these are not initially determined empirically (O'Brien, Wilson et al. 2000). Meticulous preliminary experiments were performed to ensure the accuracy of these experimental procedures by the empirical determination of any interference between cell treatments and resazurin.

Another consideration is the impact of the experimental methodology on cell growth. Serial measurements requiring prolonged removal of cultures from optimal conditions for growth might have a confounding effect on results. Consequently it was decided to measure at spaced time-points over the duration of the experiment to reduce the effect of cooling and atmospheric change on growth profiles. Other authors describe techniques using similar principles although the effect of multiple measurements is impossible to quantitate (Katayama, Ogino et al. 2005).
A6.3.1.2 Selection of complimentary assays

Many techniques are described in the literature under the loose heading of “proliferation assays”. These include fluorescence-based real-time assays, measures of DNA synthesis (such as BrdU incorporation), single measures of cellular activity (such as MTT assays) and cell counts either performed manually or by flow cytometry (Murdoch, Monk et al. 1999). It is important to remember what these assays actually measure and, consequently, their relative strengths and weaknesses. BrdU, [3H]-thymidine and MTT assays provide a single measure of DNA synthesis or cellular activity over a short, defined period of time at the end of treatment. Cell counts provide serial measurements over time, but not of the same wells. Many authors use cell counting to substantiate results derived by other methods (Scotton, Wilson et al. 2002; Chen, Yu et al. 2006; De Falco, Guarino et al. 2007; Yang, Lee et al. 2007; Zhang, Lu et al. 2007). However, the inherent assumption (as with the other single measurement techniques) is that all replicate wells are set up with exactly the same amount of starting material. Results from my real-time, resazurin-based assays have demonstrated that this premise is not valid. The experimental data showed that, despite assiduous technique, individual wells varies by as much as 10%. Consequently, a comparison of data from two or more techniques provides the most robust and reliable results.

A6.3.1.3 Incubation times

For anchorage-dependent cells there is an inverse relationship between seeding density and incubation duration in vessels of fixed volume. To reduce the accumulation of waste products within the media the protocol of an optimum number of cells measured serially over 48 hours was adopted. These time-course data could be represented graphically; replicates checked against a normal distribution and compared statistically using parametric tests. Other authors opted for lower seeding densities and prolonged incubation (Katayama, Ogino et al. 2005; Ueda, Neel et al. 2006; Yasumoto, Koizumi et al. 2006; Wen, Zhu et al. 2007; Zhang, Lu et al. 2007; Kajiyama, Shibata et al. 2008). The concern with these methodologies would be the confounding effects of waste accumulation within the media or the effect of contact-inhibition inhibiting growth. Attempts to address the former by changing the media at regular intervals were described by some authors, although the effects of these changes
on cell growth were not investigated. My preliminary data suggested that changing the cell media has a significant impact on cell growth kinetics.

A6.3.1.4 Serum supplementation

The influence of culture media supplementation on cell proliferation is an important question to address. This is because FCS has a significant impact on cell growth and contains many soluble factors that may impact on cell behaviour and thereby act as confounding variables when investigating the impact of cytokines and chemokines on cell function. Consequently the ability to minimise FCS concentration while still maintaining cell viability is key, not only to proliferation assays, but also to all assays of cell function.

My results show the profound effect of serum supplementation on cell growth. Other groups have reported proliferation data derived from experiments in which serum supplementation varied greatly. Results in two pancreatic cell lines demonstrated a proliferative effect of SDF in the presence of serum in one cell line but not another (Marchesi, Monti et al. 2004). This suggests that the interaction between SDF and serum is idiosyncratic. However the majority of papers reporting a positive association between SDF concentration and cell proliferation incubated cells in base media supplemented with low concentrations of either BSA (Scotton, Wilson et al. 2002; Katayama, Ogino et al. 2005; Yasumoto, Koizumi et al. 2006) or FCS (Chen, Yu et al. 2006). Interestingly a number of studies reporting no effect used either full (10% FCS) or no supplementation at all (Uchida, Begum et al. 2003; Pan, Mestas et al. 2006). Supplemental serum has a permissive effect on cell growth and low levels are required by all cells. However, high levels may mask the (subtle) effects of SDF.

A6.3.2 Apoptosis Assays

The process of apoptosis involves highly regulated intracellular processes that may be triggered by intrinsic or extrinsic events. Consequently there are a number of methodologies by which apoptosis can be measured – each focussing on different elements of these pathways. The caspase 3/7 assays system used for these
experiments was selected for two principal reasons: Firstly, the mechanism by which SDF might affect apoptosis was unknown and so measurement of the final “executioner” steps was necessary. Secondly, in order to ensure that quantitative results were not skewed by cell number, an apoptosis assay that used the cell supernatant was preferred as it allowed contemporaneous measurements of cell viability. Therefore methodologies such as labelling of cell proteins (such as PARP or cytochrome C), plasma membrane components (Annexin V) or DNA fragmentation would not have permitted the concurrent measurement of cell viability.

The caspase 3/7 assay system used was validated empirically using staurosporine-induced apoptosis in untransfected H357 cells. It was evident from this preliminary work that, because of the enzyme kinetics, cell measurements should take place in batches. Consequently, this technique was dependent on having hardware that could acquire the data sufficiently quickly so as to negate the impact of delay in measurement between samples. Formatting the experiments in opaque 96-well plates facilitated this. According to the manufacturer’s instructions, the resazurin-based viability assay and the apoptosis assay could be multiplexed within the same well. However, this was not done due to the complex set of preliminary experiments that would be required to ensure that no interactions between the cell treatments and the assay components would confound the results.

A6.3.3 Data handling and statistical tests

A fluorescence-based assay of cell proliferation has several advantages and some disadvantages. Although it might be thought that the acquisition of readings at multiple time points affords greater accuracy through sheer volume of data, this is not necessarily the case. Statistical handling of “time series” data has attracted much debate. However, there is general consensus that, if the multiple readings for a single replicate can be represented by a single value, then the data from experimental replicates can be combined using simple statistical methods (Matthews, Altman et al. 1990). In these experiments, fluorescence values (reflecting the accumulation of cellular material) from each single replicate (represented by individual wells on the 96 well plate) could be collected at different time points and plotted against time. It was found that, using certain empirically-derived setup parameters (such as cell seeding
density and incubation duration), the rate of accumulation of fluorescence was linear with time over an initial 48 hour period. Consequently, for each individual well, it was appropriate to summarise the time series data generated as a rate (defined by the gradient of the line) and handle the calculated slopes from each replicate as parametric data as long as they fitted the normal distribution (as tested by P-P plots).

At the outset the effect of SDF stimulation on cell proliferation was unknown. Consequently, calculations of statistical power were based on the possibility of distinguishing two-tailed (bi-directional) responses each with equal variance. However, from the preliminary data it was possible to hypothesise that SDF stimulation would increase proliferation rather than decrease it. The experimental model was then changed to investigate a unidirectional response for which a one-tailed comparison was sufficient. Maintaining twelve replicates per treatment therefore increased the statistical power of the experiment.

Resazurin-based assays of cell viability have the advantage that multiple replicates can be performed with ease. In this way a large volume of parametric data was produced yielding significant statistical power. Conversely, statistical handling of the categorical data generated by BrdU incorporation assays (cells label positive or not) is more problematical. At the simplest level a chi-squared test for each individual comparison may be performed. This has the significant disadvantage that the statistical power of multiple testing (dose-response) is lost.73 One test covering the range of SDF concentrations would be preferred to serial comparisons of individual results. However, in agreement with data from the cell viability experiments, the relationship between SDF concentration and proliferation is not linear. Consequently, as the dose increases (and the biological response plateaus) the effect is to reduce the statistical significance of the result. An alternative would be to apply the Mantel Haenzsel trend test or to calculate the Kendall rank correlation coefficient. However, again, arbitrary values of significance for the effect of dose would have to be tested for fit.

73 The Chi-squared test employed for BrdU incorporation assays requires a difference of approximately 12% to reach statistical significance at the p<0.05 threshold. This was approximately the magnitude of the effect observed.
Appendix 7:
Supplement to Chapter 7
- The effects of CXCR4 function on cell motility in established OSCC cell lines and CXCR4 transfectants
Figure A7.1: The effect of SDF on cell chemokinesis: Raw data for Figure 7.1 (Chapter 7). Transfected cells with (H357-X4.1 & H357-X4.2) and without (H357-EV) the CXCR4 insert were incubated for 20 hours on sterile coverslips coated with gold colloid at an initial density of 4,000.
cells per well. Growth media was supplemented with SDF to yield final concentrations varying between 0 and 100ng/ml. Cells were washed, fixed, mounted and photographed under oil immersion. Panels represent H357-EV (a, c, e & g) and H357-X4.1 cells (b, d, f & h) exposed to SDF at concentrations of 0 (a & b), 1 (c & d), 10 (e & f) and 100 ng/ml (g & h). Areas were recorded for 10 cells per experimental condition and the mean and standard deviations represented in a histogram and the same mean values were plotted against SDF concentration on a logarithmic scale (Figure 7.2, Chapter 7). Legend: EV=H357 Empty Vector, X4.1= H357-X4.1, Scale bar represents 50µm.

A7.2 Discussion of Experimental Methodologies

Three complimentary methodologies were performed to determine the effect of SDF stimulation on cell movement. Chemokinesis (with and without directional stimuli) and chemotaxis were investigated using gold colloid, scratch and Transwell assays respectively. Gold colloid assays provided an indirect measurement of chemokinesis (without directional stimuli) by tracking the total surface area covered by single cells at the end of a defined time point. More recent, fluorescence-based adaptations of this phagokinetic assay have been described to investigate the motility of tumour cell lines (Gu, Pellegrino et al. 2007). An alternative technique would to be to collect real-time data using time-lapse photography. This would also obviate the need for pre-coating with gold colloid, the ingestion of which may impact on cell motility. Time-lapse photography would also be a useful technique by which to ascertain the movements of individual cells in the scratch assay system. In this way the direction and velocity of cell movements could be defined and compared. Data collected using this technique would provide a useful adjunct to the results already achieved and therefore could be usefully included in the scope of further work. Time-lapse photography would also allow chemotaxis chambers to be used for the investigation of chemokinetic and chemotactic responses. The mechanism by which SDF stimulation mediates increased cell motility was not addressed in this work. It has been demonstrated that immediate actin polymerisation and cytoskeletal rearrangement with the formation of stress fibres in SCLC (Burger, Glodek et al. 2003) and hepatoma (Sutton, Friand et al. 2007) cell lines. Similar methods could be applied to OSCC-derived cell lines using conventional and confocal immunofluorescence microscopes.
Cell proliferation was a potential confounding variable for all three methodologies employed. Previous results have shown that SDF does influence the rate of cell proliferation (Chapter 6). Various techniques were therefore employed to minimise the effect of this confounding variable: In gold colloid assays the movements of single cells were analysed. In scratch assays, growth arrest was induced by pre-incubation with mitomycin C. Results showed that scratch wounds healed under both mitomycin C treated and untreated conditions. The closure rate was greater without mitomycin C treatment, providing indirect evidence that proliferation contributes to wound healing under these conditions. In the Transwell assays, although incubation times were kept short to minimise the effect of proliferation, no direct steps were taken to induce growth arrest prior to SDF stimulation. This would be an interesting line of further work but these experiments would require careful controls to ensure that the methods used to induce growth arrest had no direct effect on cell motility or adhesion.

Other in-vitro methodologies investigating cell motility and invasion were considered. Preliminary experiments using organotypic cultures of H357 cells on dermal fibroblasts were performed. The aim of these assays was to determine the feasibility of the technique and to ascertain the usefulness of the H357 model under these conditions. The technique was found to be robust and reproducible although its time-consuming nature conferred a long lag time between replicates. Transfected and untransfected H357 cells were established on the dermal collagen and stimulation with SDF was performed. However, unlike the Transwell Matrigel assays, no striking differences between treatments were observed in the preliminary experiments and it was decided that subsequent use of this technique should be restricted to further works.
Appendix 8:

Associated presentations and published abstracts
<table>
<thead>
<tr>
<th>Year</th>
<th>Conference</th>
<th>Title and Abstract Reference</th>
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<tr>
<td>2009</td>
<td>BAOMS Bournemouth</td>
<td>A role for CXCR4 in OSCC cancer stem cells</td>
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<tr>
<td>2007</td>
<td>QMUL William Harvey Day</td>
<td>Overexpression of the receptor CXCR4 results in an aggressive OSCC tumour phenotype in-vitro</td>
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<tr>
<td>2007</td>
<td>BAOMS Aviemore</td>
<td>Overexpression of the chemokine receptor CXCR4 is associated with lymph node metastasis in patients with HNSCC. BJOMS 2007 45(7) e60 Characterisation of chemokine receptor expression by oral squamous cell carcinoma cells in-vitro. BJOMS 2007 45(7) e3 Overexpression of the receptor CXCR4 results in an aggressive OSCC tumour phenotype in-vitro, BJOMS 2007 45(7) e3 Winner of the BAOMS research poster prize</td>
</tr>
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<td>2004</td>
<td>BSDR Birmingham</td>
<td>Expression of the chemokine receptor CCR7 in oral squamous cell carcinoma cell lines <a href="http://iadr.confex.com/iadr/bsdr04/techprogram/abstract_49383.htm">http://iadr.confex.com/iadr/bsdr04/techprogram/abstract_49383.htm</a></td>
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Legend: BAOMS = British Association of Oral & Maxillofacial Surgeons Annual Scientific Meeting; BJOMS = British Journal of Oral & Maxillofacial Surgery; IADR = International Association for Dental Research Annual Scientific Meeting; BSDR = British Society for Dental Research Annual Scientific Meeting; ICOOC = International Congress on Oral & Oropharyngeal Cancer; QMUL = Queen Mary University London.
References


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Cheng, Z. J., J. Zhao, et al. (2000). "beta-arrestin differentially regulates the chemokine receptor CXCR4-mediated signaling and receptor internalization, and this


