DEVELOPMENT OF A WNT-SELECTIVE ONCOLOYTIC ADENOVIRUS FOR THE IMAGING AND THERAPY OF COLORECTAL CANCERS

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The work presented in this thesis is my own and was performed between October 2004 and March 2008 in the Molecular Oncology laboratory at The Institute of Cancer unless otherwise stated.

Inge D. L. Peerlinck

May 2008
I would like to thank my supervisor Georges Vassaux for giving me the opportunity to undertake this PhD and for his continuing support throughout the whole enterprise, including after he moved to France.

I would like to especially thank Professor Nick Lemoine for providing me with the necessary funds to carry out the remaining essential experiments to bring this work to a successful conclusion and for the effort and time he has sacrificed in his busy schedule to discuss issues with me.

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Abstract

Introduction: The concept of oncolytic adenoviruses has been validated in pre-clinical studies but clinical trials have demonstrated that the virus spread remains limited and the virus fails to infect all cancer cells in a tumour. Arming an oncolytic virus with a therapeutic transgene would enhance the antitumour effect of these viruses by killing adjacent non-infected cells. The aim of this thesis is to test armed oncolytic adenoviruses targeting a constitutive activation of the Wnt signalling pathway in pre-clinical models of colorectal cancer.

Methods: The Na/I symporter was inserted into the genome of Wnt-selective oncolytic adenoviruses to visualise adenoviral spread in the tumour and assess image-guided radiotherapy.

Results: In vitro testing of the virus has demonstrated that the Wnt-selectivity of the virus remains intact. The virus we generated has an equal or greater cytopathic effect than wild type adenovirus in Wnt-expressing cancer cell lines. The ability of the infected cells to take up iodine has been confirmed by iodine uptake assays. The virus has been injected into subcutaneous human tumour implants in nude mice. Images obtained with a SPECT/CT camera have demonstrated that viral propagation can be visualised in vivo. Finally, we have used the imaging data to determine the correct timing for the administration of therapeutic doses of $^{131}$I.

Conclusion: We have validated a non-invasive method to image viral propagation and transgene expression in a preclinical model of colonic cancer. Sequential imaging can provide information on the ideal time point for therapeutic intervention. In pilot experiments, the aim was to exploit the potential of the Na/I symporter for the concentration of radioactive iodine, but it did not lead to increased therapeutic efficacy in vivo in preclinical models. There is strong evidence that if these experiments were repeated, therapeutic efficacy could be demonstrated.
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<th>Description</th>
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<tr>
<td>5-FC</td>
<td>5-Fluorocytosine</td>
</tr>
<tr>
<td>5-FU</td>
<td>5-Fluorouracil</td>
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<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
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<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
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<tr>
<td>Ad</td>
<td>Adenovirus</td>
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<tr>
<td>ADP</td>
<td>Adenovirus Death Protein</td>
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<tr>
<td>AdWt</td>
<td>Wild-type adenovirus</td>
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<tr>
<td>AFP</td>
<td>Alpha-Foeto-protein</td>
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<tr>
<td>Ag</td>
<td>Antigen</td>
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<tr>
<td>AIT</td>
<td>Apical Iodine Transporter</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
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<tr>
<td>APC</td>
<td>Adenomatous Polyposis Coli</td>
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<tr>
<td>Asp</td>
<td>Asparagine</td>
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<tr>
<td>At</td>
<td>Astatine</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Tri Phosphate</td>
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<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackie Adenovirus Receptor</td>
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<tr>
<td>CBP</td>
<td>CREB-binding protein</td>
</tr>
<tr>
<td>CD</td>
<td>Cytosine Deaminase</td>
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<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>CEA</td>
<td>carcinoembryonic antigen</td>
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<tr>
<td>CK1α</td>
<td>casein-kinase 1 alpha</td>
</tr>
<tr>
<td>Chl</td>
<td>Chloramphenicol</td>
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<tr>
<td>Cl</td>
<td>Chlorine</td>
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<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
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<tr>
<td>CPE</td>
<td>Cytopathic effect</td>
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<tr>
<td>Cpm</td>
<td>counts per minute</td>
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<td>CR</td>
<td>Conserved Region</td>
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<tr>
<td>CsCl</td>
<td>Caesium Chloride</td>
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<tr>
<td>Ct</td>
<td>Cycle threshold</td>
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<tr>
<td>CT</td>
<td>Computed Tomography</td>
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<tr>
<td>CTLs</td>
<td>Cytotoxic T Lymphocytes</td>
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<tr>
<td>D2R</td>
<td>Dopamine-2 Receptor</td>
</tr>
<tr>
<td>DAPI</td>
<td>4',6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagle medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
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<tr>
<td>Dvl</td>
<td>Dishevelled</td>
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<tr>
<td>EC50</td>
<td>median effective concentration</td>
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<tr>
<td>EDTA</td>
<td>ethylene diamine tetraacetic acid</td>
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<tr>
<td>EGFR</td>
<td>Epidermal Growth Factor</td>
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<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<tr>
<td>EtBr</td>
<td>Ethidium Bromide</td>
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<tr>
<td>EtOH</td>
<td>Ethanol</td>
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<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>FAP</td>
<td>Familial Adenomatous Polyposis</td>
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FCS  Foetal Calf Serum
FESP  fluoro-ethyl-spiperone
FHBG  9-(4-18F-fluoro-3-[hydroxymethyl] butyl) guanine
FIAU  2'-fluoro-2'-deoxy-1-β-D-arabinofuranosyl-5-iodouracil
FITC  Fluorescein isothiocyanate
Fzd  Frizzled
GDEPT  Gene Directed Enzyme Prodrug Therapy
GFP  Green Fluorescent Protein
GSK3β  Glycogen Synthetase Kinase 3β
H&E  Hematoxylin and Eosin
His  Histidine
hNIS  Human Sodium Iodine Symporter
HSV  Herpes Simplex Virus
HSV-Tk  Herpes Simplex Virus Thymidine Kinase
HSG  Heparan Sulphate Glycosaminoglycan
hTERT  Human Telomerase Reverse Transcriptase
hTR  Human Telomerase
I  Iodine
IP  Intraperitoneal
IT  Intratumoural
IV  Intravenous
ITR  Inverted Terminal Repeat
K  Potassium
kDa  kilo-Dalton
kb  kilo-base
LacZ  β-galactosidase
LB  Luria Bertani
Lef  Lymphoid enhancer factor
MBq  Mega-Becquerels
mCi  milli-Curie
MHC-I  Major Histocompatibility Complex Class I
MLP  Major Late Promoter
MMP-7  Metaloproteinase matrylsin
MOI  Multiplicity of Infection
MTT  (3-(4,5-Dimethylthiazo1-2-yl)-2,5-diphenyltetrazolium bromide
MUC1  Mucin-1
Na  Sodium
NSCLC  Non Small Cell Lung Cancer
NIS  Sodium Iodine Symporter
NK  Natural Killer
PBS  Phosphate Buffered Saline
PCR  Polymerase Chain Reaction
PEI  Polyethylene eimine
PET  Positron Emission Tomography
PFU  Plaque Forming Units
PSA  Prostate Specific Antigen
Rb  Retinoblastoma
Re  Rhenium
RGD  Arginine-glycine-aspartic acid
RID  Receptor Internalisation and Degradation
<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>rNIS</td>
<td>Rat Sodium Iodine Symporter</td>
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<tr>
<td>ROI</td>
<td>Region of Interest</td>
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<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
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<tr>
<td>RT-PCR</td>
<td>Reverse Transcriptase Polymerase Chain Reaction</td>
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<tr>
<td>SAP</td>
<td>Shrimp Alkaline Phophatase</td>
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<tr>
<td>SCID-XI</td>
<td>X-linked Severe Combined Immunodeficiency</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>SPECT</td>
<td>Single Photon Emission Computed Tomography</td>
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<tr>
<td>T3</td>
<td>Tri-iodo-thyronine</td>
</tr>
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<td>T4</td>
<td>Tetra-iodo-thyronine</td>
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<tr>
<td>Tcf</td>
<td>T-cell factor</td>
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<tr>
<td>TCID-50</td>
<td>Tissue Culture Inhibitory Dose, 50%</td>
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<td>Tg</td>
<td>Thyroglobulin</td>
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<tr>
<td>TILs</td>
<td>Tumour Infiltrating Lymphocytes</td>
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<td>TNF</td>
<td>Tumour Necrosis Factor</td>
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<td>TP</td>
<td>Terminal Protein</td>
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<td>TPO</td>
<td>Thyroid peroxidise</td>
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<tr>
<td>TRAIL</td>
<td>TNF Related Apoptosis Inducing Ligand</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid Stimulating Hormone (Thyrotropin)</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>YPD</td>
<td>Yeast Peptone Dextrose</td>
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CHAPTER 1: Introduction

The aim of the work described in this thesis was to investigate a novel gene therapy for colorectal cancer.

I will start with the description of the statistics and molecular biology of colorectal cancer. Since I have received samples of desmoid tumours from patients, I will dedicate a subsection to desmoid tumours.

I will then carry on describing the different modalities available for gene therapy. Adenoviruses will be described in greater detail for they are the agents I will be working with.

A chapter will be dedicated to molecular imaging and more specifically the sodium iodine symporter which is the transgene I will use.

Lastly, I will discuss the potential benefits of combining imaging and molecular therapy.

1. Colorectal cancer and the Wnt-signalling pathway

1.1. The statistics of colorectal Cancer

Statistics were obtained from the National Statistics website from the U.K. government (2007/02/08).

Between 1971 and 2005, the incidence of cancer increased by around 21% in males and 44% in females. In women, colorectal cancer is the second most common cancer after breast cancer. In men, colorectal cancer takes third position after prostate cancer, which remains the number one and lung cancer, the number two cancer (figure 1.1).

England alone counted 238,977 new registered cases of cancer in 2005, out of which 29,565 were colorectal cancers (12.37%). Out of all new colorectal cancer cases 55% were males (16,282) and 45% were females (13,283). The incidence of colorectal cancer as rate per 100,000 population is 65.8 for males and 51.7 for females.
Figure 1.1: Overview of all newly diagnosed cancers in 2005 represented as percentage of total number of cancers for each sex (data source: www.statistics.gov.uk).

In 2005 malignant neoplasms accounted for 26% of all registered deaths in England and Wales (135,252 out of 512,692 total deaths). Colorectal cancer deaths represent approximately 10.5% (14,146). Over the last 50 years the cancer mortality rate has changed very little in England and Wales. However the mortality rate from other major causes like cardiovascular incidents and infectious diseases has decreased. Cancer has subsequently been the most common cause of death in women since 1969 and in men since 1995.

The five-year survival rate for colonic cancer in England and Wales is around 50%. A higher proportion of women than men survive for at least five years after diagnosis. For colorectal cancer patients diagnosed between 1999 and 2003 the 5-year survival was 49.6% in men and 50.8% in women. This is an increase of 2.7% and 2.9% respectively compared to the period between 1996 and 1999.

The considerable improvement of the survival rate for colorectal cancer is due to the recent development of new adjuvant therapies. The treatment of choice for stage I (superficial tumour) and stage II (invasion into subserosa, visceral peritoneum or neighbouring structures) colon cancer, where there is no lymph node involvement or distant metastasis, is surgery. Adjuvant therapy for stage II disease has not shown to significantly improve survival rate in random controlled clinical trials and is therefore controversial. Patients with stage III (lymph node metastasis) and IV (distant and/or lymph node metastasis) disease clearly benefit from adjuvant therapy (Wolpin et al., 2007). The basis of systemic treatment for colorectal cancer
is 5-fluoro-uracil (5-FU), an antimetabolite that interferes with pyrimidine nucleotide synthesis, in combination with Leucovorin, which is thought to stabilise 5-FUs interaction with thymidylate synthetase. Two newer cytotoxic drugs have recently been added as adjuvant agents. Irinotecan, a topoisomerase I inhibitor, which interferes with DNA synthesis by blocking the ligation step of the cell cycle and Oxaliplatin, an alkylating agent have shown to work synergistic when combined with 5-FU and improve disease free survival rate (Wolpin et al., 2007, Capdevila et al., 2007). The recent development of monoclonal antibodies that block signalling pathways has provided novel opportunities in the treatment of colorectal cancer. The most advanced drugs that have been tested in combination with chemotherapy in phase III clinical trials are Cetuximab, an anti-epidermal growth factor receptor monoclonal chimeric human/mouse immunoglobulin G1 and Bevacizumab, a humanised anti-Vascular endothelial growth factor antibody. These drugs have shown promising results and are currently being evaluated in larger scale randomised control clinical trials (Capdevila et al., 2007, Wolpin et al., 2007). Rectal cancer, due to its location in the pelvis is best treated by surgery, consisting of total mesorectal excision, in combination with chemoradiotherapy.

1.2. APC, FAP and Cancer

Familial adenomatous polyposis (FAP) is an autosomal dominant inherited disease characterised by the development of multiple adenomatous polyps in the colon and rectum and development of colorectal cancer by the age of 35-40 if left untreated. Adenomatous polyposis was first clinically described in the 18th century but it was not until recently that the molecular mechanisms were discovered (Kinzler and Vogelstein, 1996). The observation of a deletion of chromosome 5q in a patient with polyposis led to further studies that linked the disease to markers on chromosome 5q21. Mutation analysis in unrelated FAP families and sporadic colorectal tumours led to the identification, characterisation and cloning of the adenomatous polyposis coli (APC) gene (Kinzler and Vogelstein, 1996). Somatic mutations of APC occur in approximately 80% of sporadic colorectal cancers (Fearnhead et al., 2001).

APC is a tumour-suppressor gene located on chromosome 5q21 that encodes a large multidomain 312kDa protein, 2843 amino acids long (Fearnhead et al., 2001).
The main function of APC is that of a scaffolding protein affecting cell adhesion and migration. FAP patients have inherited one faulty APC allele. Spontaneous somatic mutation of the second (wild-type) APC allele occurs at a low frequency in colorectal epithelial cells and is a requirement for the development of neoplasia (two hit hypothesis of Knudson (Knudson, 1971) (Kinzler and Vogelstein, 1996). Additional genetic mutations in later life are required for adenomas (polyps) to develop into invasive cancer. Nearly all mutations in APC (>95%) result in a truncated protein with abnormal function (Fearnhead et al., 2001). In FAP patients there is a complex relationship between the position of the inherited APC mutation (genotype) and the clinical manifestation of disease (phenotype) (Fearnhead et al., 2001, Kinzler and Vogelstein, 1996, Galiatsatos and Foulkes, 2006).

In addition to the multiple colonic polyps other clinical manifestations of FAP are:

- **Congenital hypertrophy of the retinal pigment epithelium**: present in approximately 70-80% of patients with FAP (Galiatsatos and Foulkes, 2006). This condition is present at birth and does not impair sight. These ophthalmic lesions are specific to FAP. Screening of FAP families by ophthalmoscope can identify affected family members before the appearance of polyps (Fearnhead et al., 2001, Galiatsatos and Foulkes, 2006).

- **Upper gastrointestinal polyps** are present in nearly 90% of FAP patients by the age of 70 years with a median age of diagnosis of 38 years (Bulow et al., 2004). If left untreated these adenomas may progress and become cancerous. Periampullary carcinoma is a common cause of death in patients who have undergone prophylactic colectomy (Fearnhead et al., 2001) (Offerhaus et al., 1992). Routine endoscopic surveillance is therefore important.

- **Desmoid tumours** are large fibromatous lesions that usually arise from the abdominal wall, bowel mesentery or retroperitoneal space. They are a major cause of morbidity and the second leading cause of death in FAP patients after metastatic colorectal carcinoma (Galiatsatos and Foulkes, 2006). The overall prevalence of desmoid disease in FAP is 15% (Sturt et al., 2004). It is a general belief that desmoids are related to trauma, especially abdominal
surgery such as a prophylactic colectomy. Twice as many females develop desmoids compared to males (Sturt et al., 2004). Treatment today remains a challenge. Surgical incision involves the risk of bleeding and short bowel syndrome and includes high recurrence rates. Non-steroidal drugs have been used with varying success and the results of chemotherapy or radiotherapy have been disappointing.

- FAP patients have an increased risk of developing thyroid cancer. The incidence is estimated at 1-2% (Truta et al., 2003). The age of diagnosis is 25-33 years, affecting predominantly females (17:1). The majority of carcinomas are papillary, with a cribriform pattern. They appear multicentric, unilateral and with a predominance for the left lobe. Lesions are well-circumscribed, non-aggressive and have a low metastatic potential and 10-year mortality (Truta et al., 2003).
- Children of FAP families have an increased risk of developing hepatoblastomas (Fearnhead et al., 2001, Galiatsatos and Foulkes, 2006).
- Other extra-intestinal cancers associated with FAP are adrenal, pancreatic and biliary tract malignancies.

Two specified variants of FAP have been described. Gardner Syndrome is characterised as the association of gastrointestinal polyps with epidermal cysts, multiple soft-tissue adenomas and osteomas of the mandible and long bones (Fearnhead et al., 2001, Galiatsatos and Foulkes, 2006). Turcot Syndrome refers to the combination of primary central nervous system tumours with multiple polyps in the colon and rectum (Fearnhead et al., 2001, Galiatsatos and Foulkes, 2006).

1.3. The Wnt-signalling pathway

Inactivation of the APC gene product constitutes the initial step in the development of colorectal cancer in FAP patients and the majority of sporadic colorectal cancers (80%). APC is part of a protein complex that is regulated by the canonical Wnt-signalling pathway, which regulates the phosphorylation and consequent degradation of β-catenin (Fearnhead et al., 2001). The Wnt genes are defined by a sequence that is homologous to the Wingless gene in Drosophila, hence the name Wnt. The Wnt proteins are a large family of secreted ligands that are important for
tissue development in embryos and tissue homeostasis in the adult. The Wnt pathway is in control of the temporal and spatial regulation of cell growth, cell movement and cell survival. Continuous activation of the Wnt-signalling pathway leads to uncontrolled cell growth and survival and can lead to tumour genesis and cancer (Logan and Nusse, 2004) (Barker and Clevers, 2006). A simplified model of the Wnt-signalling pathway is presented in figure 1.2. A complete and detailed outline of the pathway can be found in the Kyoto encyclopaedia of Genes and Genomes (http://www.genome.jp/dbget-bin/www_bget?pathway+hsa04310).

In adult tissues, under normal circumstances, a Wnt signal is absent and β-catenin levels in the cell are kept low through continuous degradation by the actions of a protein complex. Within this complex APC and Axin form a scaffold that binds β-catenin and facilitates the phosphorylation of β-catenin by casein-kinase 1α (CK1α) and glycogen synthetase kinase 3β (GSK3β). Phosphorylated β-catenin is subsequently ubiquitinylated and degraded at the proteasome. Within the nucleus the DNA-binding T-cell factor/lymphoid-enhancer factor (Tcf/Lef) proteins interact with transcriptional co-repressors and effectively block transcription of target genes. In the presence of a Wnt signal, Wnt-signalling proteins bind to Frizzled (Fzd) receptors on the cell surface of target cells, which results in the formation of a Fzd-Dishevelled (Dvl) complex and inhibition of the phosphorylation of β-catenin. This results in the accumulation of β-catenin in the cytoplasm and its transfer to the nucleus where it binds to and interacts with Tcf/Lef proteins to activate the transcription of Wnt-target genes which include the proto-oncogene c-myc, cyclin-D, which regulate cell proliferation through cell cycle regulation (Logan and Nusse, 2004, Barker and Clevers, 2006) and Metalloproteinase matrylsin (MMP-7) (Oving and Clevers, 2002). Target genes affect the proliferation, differentiation, migration and apoptosis of cells.
**Figure 1.2**: Schematic overview of the Wnt signalling pathway. The left hand panel shows the situation in the absence of a Wnt signal. B-catenin is phosphorylated by the kinases CK1ε and GSK-3β and is subsequently ubiquitinylated and degraded in the proteasome which ensures that the nuclear DNA-binding proteins of the Tcf/Lef transcription family actively repress target genes. The right hand panel shows the situation in the presence of a Wnt signal. Interaction of a Wnt ligand with the Frizzled (Fzd) receptor results in the formation of Dishevelled (Dvl)-Fzd complexes and inactivation of the complex which results in the accumulation of β-catenin in the cytoplasm and its translocation to the nucleus where it binds to Tcf proteins converting them to potent transcriptional activators.

Approximately 90% of sporadic colonic cancers have the Wnt-signalling pathway continuously activated (Barker and Clevers, 2006). This is usually the result of mutations in APC (80%) but can also be caused by mutations in β-catenin or axin (Oving and Clevers, 2002, Barker and Clevers, 2006). The resulting accumulation of β-catenin in the nucleus drives the chronic activation and transcription of Tcf target genes which are considered to be the initiating event in colon cancer formation. Wnt activation forces the transformation of intestinal epithelial cells into adenomas that continue to grow and eventually acquire additional mutations that result in their progression to malignant, invasive and/or metastatic growths. Even in late stages, colonic cancer cells remain dependent on chronic Wnt-signalling activity to maintain their growth advantage, which makes the Wnt-signalling...
pathway a good candidate for therapeutic targeting (Barker and Clevers, 2006). *In vitro* experiments performed in colon cancer cell lines show that suppression of Wnt-signalling blocks cell growth and forces them to differentiate into epithelial cells (Barker and Clevers, 2006). Non-steroidal anti-inflammatory drugs are routinely used in FAP patients and reduce the number of intestinal polyps (Phillips et al., 2002). It is thought that these drugs act through suppression of Wnt-signalling activity (Barker and Clevers, 2006).

1.4. Desmoid tumours

Desmoid tumours are rare, benign, sometimes aggressive tumours resulting from unregulated proliferation of fibroblast-like cells (Sturt and Clark, 2006, Sakorafas et al., 2007). Desmoid tumours have a natural history of slow growth and progressive infiltration of surrounding soft tissues leading in some cases to strangulation of nerves and blood vessels. Abdominal desmoids are the most common and can be classified into sporadic and familial forms (Sakorafas et al., 2007). Inherited desmoid tumours are most frequently associated with FAP. The development of desmoid tumours has been associated with a constitutive activation of the Wnt-signalling pathway at the molecular level (Tejpar et al., 2005, Sturt et al., 2004, Wunder et al., 2007). FAP-associated desmoids are caused by germ-line APC mutations followed by somatic inactivation of the wild-type APC allele (as described above) (Tejpar et al., 2005, Sturt et al., 2004). Sporadic desmoid tumours are usually characterised by oncogenic mutations in the β-catenin gene.

The management of patients is difficult and these tumours are prone to local recurrence. Surgery is an obvious option but the development of 10-30% of sporadic abdominal wall desmoid tumours and 68-86% of FAP associated intra-abdominal desmoid tumours has been associated with surgical trauma (Tolan et al., 2007). As a result there is currently no satisfactory treatment for these tumours, especially in the context of FAP-related desmoid tumours which represent a major cause of morbidity and mortality (Sturt and Clark, 2006, Latchford et al., 2006). There is a need for complementary or alternative therapies that could delay surgery if not eradicate the tumour.
2. Cancer gene therapy

2.1. Definition

The Department of Health’s Gene Therapy Advisory Committee has defined Gene Therapy as: “The deliberate introduction of genetic material into human somatic cells for therapeutic, prophylactic or diagnostic purposes.” (GTAC, 2008) This includes techniques for delivering synthetic or recombinant nucleic acids (DNA and RNA) into humans. Such techniques include, but are not limited to, the use of:

- Genetically modified biological vectors (such as viruses or plasmids);
- Genetically modified stem cells;
- Oncolytic viruses;
- Nucleic acids associated with delivery vehicles;
- Naked nucleic acids;
- Antisense techniques (for example, gene silencing, gene correction or gene modification);
- Genetic vaccines;
- DNA or RNA technologies such as RNA interference;
- Xenotransplantation of animal cells (but not solid organs) (GTAC, 2008).

One of the major goals in cancer gene therapy is to target a toxic agent specifically and selectively to cancer cells and to spare the normal tissue from damage. This could be achieved by combining highly specific gene delivery with highly specific gene expression (Greco and Dachs, 2001). For gene therapy to be successful three important issues need to be addressed: (1) delivery of a gene to the tumour, (2) regulation of gene expression and (3) therapeutic efficacy (Greco and Dachs, 2001).

2.2. Different approaches to cancer gene therapy

2.2.1. Suicide gene therapy

Suicide gene therapy, has two approaches: toxin gene therapy, in which genes for toxins are transfected directly into the cancerous cells, and enzyme-activating prodrug therapy, where the cDNA for an enzyme is introduced into tumour cells
where it can convert an administered drug, which is not toxic per se (prodrug) into a potent cytotoxin that causes tumour cell death (Springer and Niculescu-Duvaz, 2000). In gene-directed enzyme/prodrug therapy (GDEPT), a DNA construct containing an enzyme encoding gene is delivered into the tumour. In an ideal situation, the selected prodrug should be inert, even at high concentrations, should diffuse freely through the tumour cells, should be chemically stable under physiological conditions and should have suitable pharmacological and pharmacokinetic properties (Greco and Dachs, 2001). In order to have a sufficient therapeutic effect, the released drug should be more toxic than the prodrug and have a sufficiently long half-life in order to allow diffusion into or active take up by the surrounding non-transfected cells (bystander effect) (Springer and Niculescu-Duvaz, 2000). The toxic effect should be independent of cell cycle phase or proliferation in order to kill a wide range of tumour cells (Greco and Dachs, 2001).

The enzyme should have a low molecular weight and not require glycosylation. In physiological conditions, the enzyme should have a high catalytic activity and activate the prodrug rapidly and efficiently, even at low concentrations of the substrate, without depending on further catalysis by other enzymes. The expression of the enzyme per se should not have any toxic effect on cells. The reaction catalysed by the transgene should be different from endogenous enzymes in order to avoid activation of the prodrug in normal tissues (Greco and Dachs, 2001). Figure 1.3 shows the mechanism of action of GDEPT.
Figure 1.3: The strategy of gene-directed enzyme/prodrug therapy.

The enzymes for GDEPT can be of xenobiotic origin, with or without a human homologue, or of human origin (Vassaux and Martin-Duque, 2004) (Springer and Niculescu-Duvaz, 2000). Enzymes of human origin should be absent or expressed at very low levels in normal cells.

A large variety of combinations of enzyme/prodrug have been developed over the years and some have shown therapeutic effects in vitro, in vivo and even in clinical trials (Vassaux and Martin-Duque, 2004). The majority of combinations do not meet all the “ideal” criteria stated above. The two most studied genes are the herpes simplex virus thymidine kinase (HSV-TK) gene and the bacterial cytosine deaminase (CD) gene. Cells expressing the HSV-TK gene are able to phosphorylate nucleoside analogues such as acyclovir and gancyclovir, leading to the accumulation of cytotoxic metabolites which intercalate into replicating DNA (Elion, 1983). Active DNA replication (S-phase) is an absolute requirement for cytotoxicity. The active substance is not membrane-permeable and depends on cell to cell contact to diffuse into neighbouring cells. Tumour cells expressing the CD gene as first described by Austin and Huber (Austin and Huber, 1993), can
selectively convert 5-fluorocytosine (5-FC) into the anticancer drug 5-fluorouracil (5-FU). 5-FU requires further metabolism by endogenous enzymes.

Several factors contribute to the success of suicide gene therapy. Enzyme/prodrug combinations have to be delivered to the tumour cells. The currently available gene delivery vectors (described later) fail to transduce all cells in a tumour. The bystander effect, killing cells adjacent to non-transfected cells is therefore very important for the success of this therapy. The nature and the extent of the bystander effect depend largely on the enzyme/prodrug combination used. The immune system could play an important role in the bystander effect by the induction of tumour-specific immunity. Apoptosis (e.g. activated gancyclovir induces apoptosis) and the phagocytosis of apoptotic bodies by neighbouring non-transfected cells could also play a role in the bystander effect (Vassaux and Martin-Duque, 2004, Springer and Niculescu-Duvaz, 2000).

2.2.2. Immunotherapy

Immunotherapy aims to generate an anti-tumour response through activation of the host immune defence mechanisms. It relies on the ability of the immune system to recognise and destroy tumour cells and to retain a long-lasting memory of this event (Wei et al., 2005). Different types of cancer immunotherapy can be employed (Wei et al., 2005).

1. **Passive specific immunotherapy:** specific antibodies or immune cells, such as cytotoxic T-lymphocytes, tumour infiltrating lymphocytes (TIL’s) or genetically modified TIL’s, are injected into the patient. An example of this type of therapy are monoclonal antibodies against the proto-oncogene HER2/neu (Trastuzumab, Herceptin®) which have become an accepted standard in the treatment of the subgroup of HER2/neu over expressing breast cancer patients (Untch et al., 2008).

2. **Passive non-specific immunotherapy:** transfer of lymphokine-activated killer cells.

3. **Active non-specific immunotherapy:** aims to induce an immune response by using microorganisms, microbial components (e.g. endotoxins) or immunomodulators (e.g. cytokines) or genetically modified
microorganisms. The first observed case of anticancer immunotherapy was in 1890 when injection of the bacterium Streptococcus Pyogenes into a tumour lead to tumour regression (Wei et al., 2005), original article for reference: (Coley, 1891).

4. Active specific immunotherapy: immunisation of the patient with anti-idiotypic or tumour-based vaccines or gene-transfer/gene-modified immune cells or vaccines. Cancer vaccines are able to induce an anti-tumour response mediated by immunological effector cells such as CD8+ T-lymphocytes, CD4+ lymphocytes and natural killer cells. The ideal antigen should be highly immunogenic, should be expressed at high levels in tumour tissue but either not or at low level in normal tissue. Ideally all the cells in a tumour should express the antigen (Jager and Knuth, 2005). Several anticancer vaccines have progressed to the stage of clinical trials and encouraging results have been reported especially for patients with limited disease (Stebbing et al., 2008).

2.2.3. Anti-angiogenesis

In the adult, neovascularisation is limited to the female reproductive system and healing wounds. In any other circumstance neovascularisation is the result of a pathological process such as cancer. Small tumours (up to 3mm) can depend on existing blood vessels for their blood supply. A tumour larger than 1-3mm needs additional vasculature in order to survive (Jain, 2005). Angiogenesis occurs when the effect of pro-angiogenic factors outweighs the effect of anti-angiogenic factors. This has been described as the “angiogenic switch”, which promotes the formation of new vessels and allows the tumour to survive and grow. Once this event has occurred, the tumour becomes invasive locally and/or systemically (Jain, 2005).

Numerous pro- and anti-angiogenic factors are involved in the different steps of vessel formation. These molecules can be grouped into three categories (Jain, 2005): (1) ligands and receptors, (2) molecules that manage cell to cell interactions and (3) molecules that manage cell to matrix interactions.

Depending on the hypothesis that tumour growth depends on angiogenesis, anti-angiogenic therapies aim to stop or inhibit the blood supply to a tumour, depriving cancer cells of nutrients and thus indirectly kill the cells (Jain, 2005).
A large number of anti-angiogenic agents are currently on the market and new ones are being developed continuously. Some agents have been especially developed for their anti-angiogenic property, others are drugs licensed for other treatment but with anti-angiogenic properties (Lenz, 2005). An anti-angiogenic drug currently undergoing further clinical trials to determine its efficacy in the adjuvant treatment of colorectal cancer in combination with conventional chemotherapy is the anti-vascular endothelial growth factor monoclonal antibody Bevaclusimab (Avastin®) (Wolpin et al., 2007).

Anti-angiogenic targeted gene therapy is directed at tumour endothelial cells and their micro-environment. This can be achieved by arming delivery vectors for anti-angiogenic genes with endothelial cell-specific promoters like endothelin I or with ligands to endothelial cell-surface specific receptors like alpha v beta 3 integrin (αvβ3) and alpha v beta 5 (αvβ5) integrin (Tandle et al., 2004).

### 2.2.4. Gene replacement therapy

The discovery that specific gene families play a role in the development of cancer has led to the idea that targeting these genes and their products may prevent or cure cancer (Roth et al., 1994). Gene replacement therapy therefore aims to repair the genetic defects caused by loss of tumour suppressor genes or activation of oncogenes.

The most famous example of gene replacement therapy is the replacement of the gene encoding the γc cytokine receptor subunit of interleukin-2, -4, -7, -9 and -15 receptors which are essential for the growth and differentiation of lymphoid progenitor cells. Patients with X-linked severe combined immunodeficiency (SCID-X1) syndrome are born with a mutation in the γc gene. Successful replacement was achieved by *ex vivo* delivery to CD34+ bone marrow cells with a retroviral vector (Cavazzana-Calvo et al., 2000). Despite the enormous therapeutic success, two out of ten treated patients developed a leukaemia-like disease within a year of treatment due to insertion of the retroviral delivery vector into the host DNA and the subsequent activation of an oncogene (Hacein-Bey-Abina et al., 2003).
In cancer, tumour suppressor genes play an important role in the control of cell proliferation by regulating the transcription of certain genes (Roth et al., 1994). The p53 gene is the most commonly mutated gene in human cancers. Under normal circumstances the gene plays a role in activating transcription of genes essential for the regulation of the cell cycle, for DNA repair, apoptosis and other regulatory molecules important for genetic stability (Roth et al., 1994). Abnormalities in the gene lead to the abrogation of its tumour suppressor function. Restoration of wild type p53 gene is able to suppress the growth of transduced cells in vitro and expression of wild type p53 in vivo may inhibit local tumour growth in nude mice (Roth et al., 1994).

To date p53 replacement has been extensively researched and several clinical trials have been conducted in a variety of cancers to study the feasibility of p53 replacement therapy. Phase 1 clinical trials conducted in non small cell lung cancer, head and neck cancer, bladder cancer, recurrent glioma and ovarian cancer all showed a reduction in tumour size or stabilisation of disease in a significant number of patients after intra-tumoural or loco-regional injection (Fujiwara et al., 2006b). Advexin®, an adenoviral-CMV-p53 is being evaluated in phase 2-3 clinical trials in the USA and Europe for intra-tumoural injection in head and neck cancer (March 2008). China has become the first country to licence replacement gene therapy as a regular treatment for head and neck cancer combining adenoviral delivery of the p53 gene by intra-tumoural injection with conventional radiotherapy (Peng, 2005, Garber, 2006).

2.2.5. Virotherapy

Oncolytic therapy or virotherapy will be described in more detail below.

2.3. Delivery vectors for gene therapy

Suicide gene therapy, replacement gene therapy, anti-angiogenesis and, to a lesser extent, immunotherapy require vectors or vehicles capable of efficient and selective delivery of the therapeutic genes to the cancerous cells. These vectors can be of viral origin (adenoviruses, adeno-associated viruses (AAV), herpes simplex virus (HSV), parvovirus, lentivirus, vaccinia virus and retroviruses), naked plasmid DNA
(with or without electroporation), antisense RNA, small interfering RNA, bacteria, bacteriophages, cationic lipids, liposomes, nanoparticles, polyethyleneimine (PEI), polyaminoacids, peptides and dendrimers.

Three issues are of major importance in selecting a delivery vector:
(1) The specific targeting to cancer cells, (2) the efficiency of transduction and (3) the safety of administration to human subjects.

Non-viral methods for gene delivery have the advantage that they are chemically defined, have the capacity to incorporate large genes and are easy to manufacture. The limitation in these methods is that transduction is transient and the level of expression is low. New and better synthetic vectors are being investigated and hold promise for the future (Young et al., 2006).

Viruses currently are the most efficient gene delivery system available for clinical purposes. They have evolved over millions of years to transfer genetic material in human cells and express their genes while evading the immune system. They can easily be manipulated to express the desired genes and/or to replicate only in the selected cells (Young et al., 2006). The choice of a specific virus depends on the target and the desired effect. Some viruses have a tropism for certain tissues, e.g. HSV for neuronal cells, while other viruses infect a wide range of different cell types, e.g. adenovirus. Retroviruses and lentiviruses integrate DNA stably into the host-cell genome, which is then transmitted to all subsequent cell generations (Young et al., 2006, Waehler et al., 2007).

Non-replicating viruses have been used safely in clinical trials, but there have been difficulties with inadequate distribution and delivery, insufficient levels of gene transfer and expression and short-term efficacy due to immune responses. Exploiting bystander effects can overcome these hurdles to a certain extent.

Another approach to circumvent the obstacle of successfully infecting all cells in a tumour is to use replication-competent viruses and exploit the ability of these viruses to replicate selectively in cancer cells, to kill the cells and to spread in the tumour. Such viruses are called oncolytic viruses.
2.4. Oncolytic viruses

One of the earliest reports on the ability of viruses to replicate in and kill tumour cells, was the case described by De Pace in 1912 (Sinkovics and Horvath, 1993) (and for reference (De Pace, 1912)). De Pace describes the regression of a fungating cervical tumour after the patient was bitten by a dog and inoculated with attenuated rabies vaccine.

In the 1940s and early 1950s clinicians started to infect cancer patients intentionally with live viruses, obtaining in some cases partial remissions (Huebner et al., 1956, Sinkovics and Horvath, 1993).

Over the last 20 years the advances in molecular biology have increased our understanding of the molecular mechanisms of carcinogenesis, viral infection, viral replication and viral toxicity. These advances have allowed the development of genetically engineered viruses with improved safety, cancer selectivity and efficacy (Sinkovics and Horvath, 1993).

The concept of oncolytic virotherapy is described in figure 4. When an oncolytic virus infects a normal, non-target cell, replication is inhibited and the infected cell is either recognised and destroyed by the immune system or undergoes apoptosis. In the target cancer cells, the virus is able to use the host cell machinery to replicate its genome. When the host cell is loaded with newly produced viral particles, the virus initiates cell death and viral progeny can spread to and infect neighbouring cells.
Figure 1.4: Oncolytic viruses are able to replicate in cancer cells and viral progeny spreads to infect, replicate in and kill neighbouring cells. Infection of normal, non-cancerous cells leads to apoptosis or immune destruction of the cell.

The ideal features of a candidate oncolytic virus are as follows:

1. Broad tissue specificity is required to allow the virus to deal with cancers originating from or metastasising to a variety of different tissues.
2. The virus needs to replicate to high titres so that an immune response can not be mounted until after infection is well established.
3. The virus must be lytic, killing infected cells.
4. The viral genome must be easy to manipulate and allow the insertion of large transgenes.

Several methods have been developed to target viruses selectively to tumour cells (Post et al., 2003, Hawkins et al., 2002).

1. Some viruses are innately tumour-specific and do not require additional genetic modifications. Examples of such viruses that have been used in clinical trials are: Reovirus that only replicates in cells with an activated Ras-signalling pathway, Newcastle disease virus and Vesicular stomatitis virus that only replicate in interferon-defective cells, Measles virus and Vaccinia virus (Norman and Lee, 2000, Liu and Kirn, 2007, Aghi and Martuza, 2005, Parato et al., 2005).
Viruses can be targeted to cancer cells through the use of promoters that are only functional in target cells. Examples of tissue-specific promoters that have been developed so far are alpha-fetoprotein (AFP), which is expressed in several tissues through development but is only expressed in tumours of hepatic and/or intestinal origin in adult life (Hallenbeck et al., 1999), prostate specific antigen (PSA) and rat probasin promoter for prostate cancer, mucin-1 (MUC1), which is over-expressed in breast carcinoma, human telomerase reverse transcriptase (hTERT) for a wide variety of cancers (Li et al., 2008), vascular endothelial growth factor VEGF, which is hypoxia-specific, carcinoembryonic antigen (CEA) for gastrointestinal cancer, the tyrosinase promoter, which is specific for melanoma and many more (McCormick, 2001).

Targeting can be achieved through discrete mutations in the viral genome. An example is the retinoblastoma (Rb) pathway. Viruses need to inhibit this protein in order to replicate in normal cells. Certain tumour cells are defective in the Rb pathway and as a result adenoviral E1A-deleted mutants that fail to neutralise Rb should therefore only replicate in targeted cells (Heise et al., 2000, McCormick, 2001).

The tropism of a virus can be manipulated in such way that the virus can only be taken up by cancer cells. In order to redirect a virus, all its native cell interaction mechanisms must be ablated. In case of adenoviruses, this includes ablating CAR, integrin and heparin sulphate glycosaminoglycan (HSG) binding and replacing them with a tumour-specific binding mechanism. This can be achieved by complexing viruses with bispecific molecules where one component blocks CAR-binding of the knob and another component assures receptor binding. Ligands that have been used include epidermal growth factor (EGFR) and angiotensin-converting enzyme (ACE) (Green and Seymour, 2002).

Another strategy involves genetically modifying viruses to ablate interaction with native receptors and incorporate targeting sequences. By altering the fibre protein for instance, the virus no longer recognises its receptor on normal cells but instead attaches to target specific cells receptors (Waehler et al., 2007). Insertion of the arginine-glycine-aspartic acid (RGD) motif or polylysine (pK7) into the fibre
protein enhances CAR independent binding to integrins and HSG respectively (Wu et al., 2002).

3. Adenoviruses

3.1. General considerations

Adenoviruses were first discovered in 1953 by Rowe et al. who extracted them from human adenoids (Young et al., 2006, Rowe et al., 1953). One year later, Hilleman and Werner found that extracts from respiratory secretions of people suffering from an acute respiratory illness were able to lyse human cells in culture (Hilleman and Werner, 1954). Clinically, adenoviruses are responsible for mild upper respiratory tract infections, mild gastrointestinal infections and eye infections.

3.1.1. Classification

The family of adenoviridae has been subdivided into four genera: Atadenovirus, Aviadenovirus, Mastadenovirus and Siadenovirus. The human adenovirus belongs to the genus Mastadenovirus and 51 serotypes have currently been identified based historically on immunological criteria. Human adenoviruses are grouped from A to F according to genome size, hemagglutination properties, oncogenicity in rodents, DNA homology and genomic organisation (Young et al., 2006, Zhang and Bergelson, 2005). The tissue tropism of viruses varies between the subgroups. Groups B1, C and E cause respiratory infections, group B2 infects the kidneys and urinary tract, group F causes gastroenteritis and group D viruses infect the eyes (Zhang and Bergelson, 2005).

The most commonly used vector for gene therapy is adenovirus 5 subgroup C.

3.1.2. Structure

Figure 5 shows a schematic drawing of an adenoviral capsid.
Adenoviral particles measure 80-110 nm, are not enveloped and consist of a capsid, fibers, a core and associated proteins.

The capsid has an icosahedral symmetry and is composed of 252 units called capsomers: 240 hexons and 12 pentons. Hexons are arranged in 20 triangular faces. Each hexon consists of a trimer of polypeptide II with a central cavity. VI, VIII and IX are minor polypeptides that are also associated with the hexon. They are thought to be involved in the stabilisation and/or assembly of the particle. The base of a penton is made up by a pentamer of peptide III and five molecules of peptide IIIa. Pentons are responsible for penetration of the cell membrane. Purified pentons can cause cell death in the absence of any other viral components. From each of the 12 vertices extends a fibre protein which is attached to the penton base proteins and consists of a trimer of peptide IV. The knob at the end of each fibre is responsible for recognising receptors on the cell membrane. The core contains the genome and at least four proteins. Terminal protein (TP) is attached to the 5’ends of the genome. Protein V and VII are proteins that are wrapped around the genome to form a “chromatin-like” structure. Mu is a small protein whose function is unknown.
The adenoviral genome is linear, double-stranded with a length of 30-40k base pairs (bp). At the end of each strand are inverted terminal repeats (ITR) of approximately 50-200 bp, which act as origins of replication (Buchen-Osmond, 2003) (http://microbiologybytes.com/virology/adenoviruses.html, 2007).

3.1.3. The adenoviral entry in the cell

The first step in adenoviral infection is the binding of the fibre knob with the cellular receptor. The main receptor for human adenoviruses of subgroup C is identical to the one for coxsackie virus and is therefore called the coxsackie/adenovirus receptor (CAR) but these viruses also bind to the major histocompatibility class I molecule (MHC-I) (Russell, 2000). CAR is a plasma membrane protein with extracellular, transmembrane and cytoplasmic domains. The extracellular domain is sufficient for attachment of the virus to the cell surface. CAR is expressed at variable levels in a broad variety of tissues in epithelial cells (Fechner et al., 1999).

The next step is the internalisation of the virus through receptor-mediated endocytosis, which is mediated by the interaction of the penton base proteins and αv proteins on the cell surface. The αvβ3 and αvβ5 integrins are known to facilitate
adenoviral internalisation. In the absence of integrins on the cell surface CAR-mediated uptake is possible but much slower (Fechner et al., 1999) (Russell, 2000).

In the endosome toxic activity of the penton base proteins is responsible for the disruption of the endosome membrane and the release of virus into the cytoplasm (Russell, 2000, http://microbiologybytes.com/virology/adenoviruses.html, 2007). In the cytoplasm the virus is partially disassembled and transferred to the nuclear membrane. The genome is passed through the nuclear pore and into the nucleus where its TP forms a tight complex with nuclear matrix components and viral transcription is initiated (Russell, 2000).

The adenoviral genes are grouped into transcriptional units which are expressed at different stages through the adenoviral life cycle: immediate early (E1A), early (E1B, E2A, E2B, E3, E4) and late genes (L1 to L5). Immediate early and early genes are encoded at various locations on both strands of the DNA (see figure 6). Each gene leads to multiple protein products through alternative splicing of mRNA transcripts (http://microbiologybytes.com/virology/adenoviruses.html, 2007).

Figure 1.7: Transcription of the adenovirus genome. The early transcripts are outlined in blue, the late in green. Arrows indicate the direction of transcription. MLP = Major late promoter.
3.1.4. Transcription
3.1.4.1. The function of EIA

The first gene to be expressed after the viral DNA has been transported to the nucleus is the EIA gene. The EIA gene contains three regions that are conserved across all adenoviral serotypes called CR1, CR2 and CR3. The gene encodes two proteins called 243R (or 12s mRNA transcript) and 289R (or 13s mRNA transcript) called after the number of amino acids. CR1 and CR2 are present in both proteins but CR3 is unique for the 289R protein. It is predominantly through these domains that EIA binds to key cellular proteins that play an important role in the transcriptional regulation of viral and host genes (Russell, 2000, Frisch and Mymryk, 2002).

The retinoblastoma family of genes include the retinoblastoma gene Rb, p107 and p130 which have common functional characteristics of which the most important function is the ability to control the cell cycle. Cell cycle control is mostly achieved through inactivation of transcription factors such as the E2F family which promote the cell entrance into S phase (Felsani et al., 2006). Binding of E1A proteins to the Rb-E2F complex disrupts the interaction between Rb and E2F proteins (figure 1.8). The released E2F proteins can activate transcription of target genes that are required for the induction of S phase of the cell cycle (Frisch and Mymryk, 2002). Target genes include Cyclin A, Cyclin E and cyclin-dependent kinase 2 (Felsani et al., 2006).

Inactivating retinoblastoma gene products also leads to induction of p53. This is one of the mechanisms by which E1A causes apoptosis in cells. E1A binds to p300/CBP (CREB-binding protein), which are co-activators of transcription. Binding of E1A to p300/CRB facilitates the acetylation of Rb by p300 which promotes its interaction with MDM2, which is known for its ability to bind p53. Rb forms a stable complex with p53 and MDM2 which prevents the degradation of p53, thus repressing the transcription of target genes and promoting apoptosis (Frisch and Mymryk, 2002).

The function of E1A proteins is complex and E1A interacts in many other ways both directly and indirectly with cellular proteins.
E1A products also play a role in immune modulation by inhibiting the activation of genes induced by interferon and interleukin-6 (Russell, 2000) (Weitzman and Ornelles, 2005).

Figure 1.8: E1A disrupts the interaction between retinoblastoma protein and E2F, allowing E2F to activate transcription of target genes that are required for S phase induction of the cell cycle. In addition to freeing E2F from Rb, E1A promotes the acetylation of Rb by p300/CBP which is known to promote its association with the MDM2 protein. RB and MDM2 form a complex with p53 which blocks p53 degradation, transcriptional activation and p53-mediated growth arrest and allows transcriptional repression and induction of apoptosis by p53 (Frisch and Mymryk, 2002).

3.1.4.2. The function of E1B

The expression of E1B genes is important to avoid cell death, which would inhibit viral production. Expression of E1A results in the stabilisation of p53 as described above. p53 interacts with p21 which results in cell cycle arrest in G1. p53 also activates cellular pro-apoptotic genes such as the bcl-2 family members Bax and Bak. Bak is also activated by the destabilisation of its binding partner MCL-1, a result of induction of a DNA damage response by adenovirus infection. Bak and Bax cooperate to form pores in the mitochondrial membrane, thereby facilitating the release of pro-apoptotic proteins (Berk, 2005). The E1B-19k protein is functionally similar to the cellular bcl-2 protein and binds to both Bak and Bax, thus preventing the release of pro-apoptotic proteins.
The E1B-55k protein binds to p53, thereby increasing the intracellular concentration and binding affinity of p53. The E1B-55k/p53 complex is a potent repressor of p53 target genes, which prevents transcriptional activation of cell cycle arrest and pro-apoptotic programs (Berk, 2005). The E4orf6 protein is involved in the interaction between E1B-55k and p53 and influences the stability of p53. The E1B-55k/E4orf6/ubiquitin ligase complex targets p53 for degradation at the proteasome (Berk, 2005). The E1B/E4orf6 protein complex also interacts with the cellular MRN complex which is involved in DNA repair. Degrading the MRN subunits is essential for viral DNA replication. When MRN is not inactivated, it treats the ends of linear viral DNA strands like a double-stranded DNA break, resulting in random stretches of viral DNA (Berk, 2005). E1B-55k and E4orf6 both play a role in the expression of late viral genes. They assist in the preferential transport of late viral mRNA from the nucleus to the cytoplasm in the late stages of infection (Russell, 2000, Weitzman and Ornelles, 2005).

3.1.4.3. The function of E2

Adenoviral E2 gene products provide the necessary machinery for the replication of viral DNA and the transcription of the late viral genes. The E2A gene encodes a DNA-binding protein that is required for viral DNA replication and that regulates the transcription and translation of late viral genes. The E2B gene encodes a DNA polymerase that synthesises viral DNA (Russell, 2000) (Weitzman and Ornelles, 2005).

3.1.4.4. The function of E3

The E3 gene products (figure 2.1) have been called the “stealth” genes (http://microbiologybytes.com/virology/adenoviruses.html, 2007) because they play an important role in the evasion of the host immune responses. E3 genes are not required for viral replication in cultured cells. The function of the E3-12.5k gene product has not yet been elucidated. The E3-6.7k protein plays a role as a general repressor of apoptosis and associates with the receptor internalisation and degradation (RID) protein complex which consists of RIDα (E3-10.4k) and RIDβ (E3-14.5k) proteins to internalise and
mediate degradation of cell surface receptors for tumour necrosis factor (TNF), Fas and TNF-related apoptosis inducing ligand (TRAIL). TNF is a cytokine that is released by activated lymphocytes and macrophages. T-lymphocytes and natural killer (NK) cells use TRAIL to induce apoptosis in virus infected and tumour cells. Thus by reducing cell surface receptors, the virus reduces immune-mediated destruction of infected cells. E3-6.7k is a membrane protein that is localised in the endoplasmic reticulum (ER) and the plasma membrane. In the ER it functions as a general repressor of apoptosis by maintaining calcium homeostasis in the cytosol (Lichtenstein et al., 2004). E3-6.7k is required in the plasma membrane in conjunction with the RID complex to internalise and degrade TRAIL receptor 2.

RIDA and RIDβ are co-expressed together in the plasma membrane but individually they are expressed in the Golgi apparatus (RIDA) or the Golgi apparatus and the ER (RIDβ). The RID complex mediates the internalisation and destruction of cell surface receptors such as Fas, TRAIL receptor 1, TRAIL receptor 2 and epidermal growth factor receptor (Lichtenstein et al., 2004).

The RID complex also plays a role in preventing TNF-mediated activation of the large cytoplasmic phospholipase A2 which cleaves arachidonic acid from membrane phospholipids. Arachidonic acid is a potent mediator of inflammation (Lichtenstein et al., 2004).

The E3-gp19k protein is localised in the ER membrane and binds to MHC class I proteins, preventing their transport to the cell surface where they would be recognised by cytotoxic T-lymphocytes (CTLs) (Russell, 2000). Viral antigens form complexes with MHC class I molecules in the membrane of the ER. CTLs recognise these complexes (Ag-MHC I) on the cell surface. This interaction leads to the release of perforin, resulting in cell lysis, thus eliminating the infected cell even before the viral life cycle has been completed. E3-gp19k thus prevents premature cell death induced by CTLs.

The 11.6k adenoviral death protein (ADP) is responsible for cell lysis after viral replication to allow virions to leave the cell and infect neighbouring cells.

The 14.7k protein protects the infected cell from apoptosis induced by tumour necrosis factor (TNF). The mechanism through which E3-14.7k achieves inhibition of TNF-mediated apoptosis is not clear (McNees and Gooding, 2002).
3.1.4.5. The function of E4

The adenoviral E4 is not needed for growth in tissue culture. While the E3 region is aimed at avoiding cell lysis by extracellular action of the host immune response, E4 gene products are important to counteract the cell's intracellular antiviral defences. They also promote viral protein synthesis and shut down host protein synthesis (Russell, 2000). Early in the infection a single E4 promoter is activated by E1A to produce the E4 transcript, which through alternative splicing results in the creation of several E4 proteins, called E4-orf1-6/7 (Weitzman and Ornelles, 2005).

E4orf6/7 binds the E2F that has been displaced from Rb by E1A and thereby stabilizes the binding of E2F to the E2 promoter and promotes DNA replication (Leppard, 1997). E4orf negatively regulates E1A expression and has a role in regulating late viral mRNA splicing and protein phosphorylation (Leppard, 1997). E4-orf4 can induce p53 independent apoptosis and this may contribute to lysis of infected cells at the end of the infectious cycle (Weitzman and Ornelles, 2005). E4-orf6 promotes the degradation of cellular proteins and acts in combination with E1B-55K as described above to prevent the initiation of the cellular DNA damage response and to promote nuclear export of late viral mRNAs for translation (section 3.1.4.2). E4-orf3 interacts with nuclear structures that are implicated in multiple cellular functions including DNA repair, transcriptional control, apoptosis and the interferon response (Weitzman and Ornelles, 2005). E4-orf3 is able to relieve the E1B inactivation of p53 (Russell, 2000).

3.1.4.6. The function of late viral genes

The late adenoviral genes encode structural viral proteins including hexon, penton and fibre, which are necessary for the assembly of progeny virions. The L4-100k protein blocks host translation and promotes late viral mRNA translation. Mature virus particle production precedes cell lysis.

3.2. Adenoviruses for cancer therapy

Adenoviruses are useful tools to deliver genetic material to host cells. They have a broad tissue tropism and can infect most mammalian cell types. They infect both
dividing and quiescent cells and they do not integrate in the host genome. The expression of the transgene is transient. Adenoviruses support simultaneous expression of multiple genes. The total amount of DNA that can be effectively packaged into virions is 105% of the wild type genome, which allows for the insertion of approximately 2kb of foreign DNA. The capacity of the adenovirus to accommodate foreign DNA can be increased by deleting non-essential viral genes. Adenoviruses possess a lytic life cycle, which can be exploited for oncolytic viral therapy. Because adenoviruses are stable and resistant to physical manipulations, they can be produced to high titres (up to $10^{13}$ viral particles/ml) and frozen for use at later times. Another advantage is that wild type virus causes only mild pathology. Intravenous injection of adenovirus generally is well tolerated and causes mild flu-like symptoms (Mathis et al., 2005).

A major disadvantage of adenoviral therapy is that after systemic injection the majority of viral particles are trapped in the liver by the Kupffer cells and to a lesser extent in the adrenal glands, lungs, pancreas and spleen (Fechner et al., 1999, Wang et al., 2003a, Groot-Wassink et al., 2002). Apart from being predominantly targeted to the liver, adenoviruses also interact with blood cells. Incubation of human and murine blood cells ex vivo demonstrated that more than 90% of the viral particles administered were associated with human (not murine) erythrocytes and in blood obtained from a patient infected with adenovirus during a clinical trial, more than 98% of the adenoviral genomes were associated with blood cells. This interaction may seriously reduce adenoviral delivery to extra-vascular target cells and tissues in humans (Baker et al., 2007). Furthermore adenoviruses were shown to bind to a number of plasma proteins, including coagulation factor IX, X and binding protein-4, which allow CAR-independent infection of liver cells (Waddington et al., 2008). Adenoviruses have therefore only been used intra-arterially or intra-venously for targeting the liver but in other organs intratumoural injection is preferred.

Systemic immune responses to viral capsid proteins may play a significant role in the outcome of adenoviral gene therapy and may prevent repeated administration (Chirmule et al., 1999). The extent to which an immune response is elicited is related to the amount of virus administered but also by individual variations of the immune response in humans. This became apparent when a patient died after administration of an adenoviral vector, while another patient receiving a similar
dose did not exhibit any side effects (Lehrman, 1999). Jesse Gelsinger developed a fever and disseminated coagulation within hours of intra-hepatic artery treatment with an adenovirus to correct partial ornithine transcarbamylase deficiency. The severe response by this patient may have been related to the amount of virus (3.5 x $10^{13}$ viral particles) and the pre-existing enzyme deficiency in his liver, which emphasises the importance of appropriate patient and dose selection (Chirmule et al., 1999). As a result of this event, the U.K. gene therapy advisory committee has issued several recommendations. These include the assessment of the patient’s immune status prior to treatment and the use of standardised methods to assess viral titre to ensure consistency through clinical trials (Relph et al., 2004).

It is therefore important to engineer viruses that in addition to containing the transgene, specifically target cancer cells (or target tissues) and evade the immune response.

### 3.3. Adenoviruses targeted to cancer cells

#### 3.3.1. Deletions

The prototype for oncolytic adenoviral therapy is the virus Onyx-015/dl1520. This mutant virus has a deletion for the adenoviral E1B 55kDa protein that plays a role in inactivating the cellular tumour suppressor protein p53 (Bischoff et al., 1996). Under normal circumstances the p53 tumour suppressor gene acts as a protective mechanism against DNA damage. Induction of p53 by such damage results in apoptosis or cell cycle arrest. More than 50% of human cancers have therefore inactivated the p53 pathway (Levine, 1997). The Onyx-015 virus was thought to replicate in and kill p53-deficient tumour cells only but unable to replicate in cells with a functional p53. It became clear that the virus is not specific for p53 null cells but the virus still kills cancer cells preferentially and exhibits significant antitumour activity (Mathis et al., 2005). The E1B-55k protein binds to E4-orf6 to mediate late viral RNA transport from the nucleus to the cytoplasm. Recent studies have confirmed that it is the loss of this function that restricts Onyx-015 replication in normal cells that are not able to take over the export function of E1B-55k (O'Shea et al., 2004). This virus has been tested extensively in Phase I, II and III clinical
trials as a single agent or in combination with conventional chemo- or radiotherapy with variable clinical responses (Mathis et al., 2005).

An almost identical virus H101, which contains an E3 deletion in addition to the E1B-55k deletion is the first oncolytic virus that recently has been licensed in China for head and neck cancer treatment in combination with conventional therapy (Garber, 2006).

The selectivity and safety of this virus have been encouraging for oncolytic viral gene therapy but durable objective responses with this virus as a single agent have been uncommon. Onyx-015 virus was severely attenuated due to the mRNA transport deficiency and possibly other functions attributed to E1B-55k. This has led to the development of tumour-selective adenoviruses in which other viral genes have been deleted. Deleting viral genes to increase tumour specificity can reduce the potency of the virus. It is important therefore to select those mutants that increase selectivity but leave viral replication and/or cytotoxicity unaffected. Deleting the adenoviral E3-gp19k gene for example results in enhanced intratumoural gene expression and/or replication in immunocompetent tumour models as compared to the common E3B (E3-RID genes + E3-14.7k) gene deletion (Wang et al., 2003b).

An example of newer second-generation adenoviral mutants is the adenoviral mutant d1922-947, which has a deletion in the E1A gene. Under normal circumstances the E1A-CR2 protein interacts with the host cell retinoblastoma protein and is important for inducing S-phase entry in the infected cell. The retinoblastoma pathway is abnormal in the majority of human cancers and therefore this d1922-947 virus should replicate in cancer cells but not in quiescent normal cells (Heise et al., 2000) (Lockley et al., 2006).

3.3.2. Promoters and markers

Placing the adenoviral E1A and or E1B gene under the control of cancer-specific promoters is another way of controlling adenoviral gene expression and replication. In the case of prostate cancer, the rat probasin promoter and the prostate-specific antigen (PSA) promoter or a combination of both have been used to drive the expression of E1A and/or E1B in oncolytic adenoviruses. Synergistic effects between prostate-specific replicating adenoviral vectors and chemo- or
radiotherapy have been demonstrated in animal models and phase I-II clinical trials (Young et al., 2006) (DeWeese et al., 2001).

Other cancer specific promoters include the telomerase promoter that is deregulated in a wide range of cancers, the α-fetoprotein promoter in liver cancer, the MUC-1 promoter in breast cancer (see also section 2.4) (Young et al., 2006). Placing cancer-specific markers under the control of adenoviral promoters is another way to target viruses to cancer cells. In the context of colonic cancer, Richard Iggo and co-workers have constructed a series of Wnt-specific replicating adenoviruses by inserting Tcf-binding sites in multiple early adenoviral promoters. Viruses containing Tcf-responsive elements in the E1A, E1B and E4 gene promoters were selective for cells with an activated Wnt-signalling pathway and were active in most of the colon cancer cell lines studied in cytopathic effect assays (Fuerer and Iggo, 2002, Homicsko et al., 2005).

3.3.3. Receptors

Another retargeting strategy consists of diverting the adenovirus away from its binding on the CAR receptor by introducing changes in the fibre or knob domain. CAR, integrin and heparin sulphate glycosaminoglycan binding must all be abolished and replaced with a tumour targeting mechanism (Green and Seymour, 2002) in order to guarantee tumour specificity. Several mechanisms can be used to achieve altered viral tropism. These have been discussed in section 2.4.

3.3.4. Conclusion

Clinical studies conducted so far with tumour-specific replicating adenoviruses have demonstrated that these agents are safe but convincing clinical responses have been obtained only in studies where they were administered in combination with conventional therapies (Russell, 2000, Green and Seymour, 2002, O'Shea, 2005, McCormick, 2005). The observation that oncolytic adenoviruses lack efficiency when used in monotherapy has led to the conclusion that additional features or combination therapies were necessary. Arming oncolytic viruses with transgenes is therefore one way to increase the efficacy of viral oncolysis.
4. Molecular imaging of gene expression

In clinical trials the endpoints for measuring the effect of oncolytic viral therapy are limited to tumour size measurement and biopsies. Alternative endpoints are required to direct future development of new oncolytic viruses. In this context, non-invasive imaging of gene expression could provide information about the location, magnitude and kinetics of gene expression and provide the explanation as to why these oncolytic adenoviruses have only limited efficacy.

Molecular imaging has been defined broadly as: “the in vivo characterisation and measurement of biological processes at the cellular and molecular level using remote imaging detectors” (Sharma et al., 2002).

Molecular imaging methods employed to detect and visualise gene expression in vivo in animal models and humans require expression of a reporter gene that alters the distribution of a radioactive tracer compound, activates a substrate or concentrates a contrast agent (Vassaux and Groot-Wassink, 2003).

The devices used for molecular imaging need to be able to detect very low amount of reporter probes that are designed to accumulate in cells that express a reporter ectopically (Penuelas et al., 2005b). The ideal molecular imaging technique needs to be highly specific, highly sensitive, have a high temporal-spatial resolution and be non-invasive (Rudin et al., 2005). A number of technologies have been developed for non-invasive molecular imaging.

4.1. Optical imaging

Optical imaging can be performed when the reporter genes encode bioluminescent (firefly luciferase is an enzyme that transforms a substrate into a light-emitting product) or fluorescent proteins (e.g. green fluorescent protein) or when fluorescent dyes are used as ligands to label a biologically interesting molecule. The emitted light energy can be detected externally by the use of sensitive photon detection systems (Contag et al., 2000).

The emission of light photons in cells expressing the transgene transmits through the tissues after activation of substrate by the enzyme (in the case of luciferase) or upon gene expression (in the case of fluorescent transgenes) in the absence of background noise since mammalian cells do not normally express light-emitting
proteins. The disadvantage of this technique is the poor spatial resolution. The anatomical definition is high at the surface but deteriorates quickly with increasing depth due to the scattering of light in tissues (Rudin et al., 2005).

While in small animals optical imaging techniques can provide informative data, it is highly unlikely that these techniques could be used for *in vivo* gene imaging in human subjects (Penuelas et al., 2005a).

In addition to the poor spatial resolution, the reporter genes described are of xenogenic origin. They will be recognised by the immune system as “non-self” and the cells expressing these genes may be destroyed by cytotoxic immune cells, which in turn may prevent viral replication and oncolytic activity.

### 4.2. Radionuclide based imaging

Radionuclide-based imaging technologies are Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT). Radionuclides or radiolabelled molecules are injected intravenously in trace quantities and are retained in tissues as a result of binding to a receptor, cell entrapment due to enzyme-catalysed conversion or intracellular uptake through a transporter (Penuelas et al., 2005a).

These modalities can be performed repeatedly *in vivo* in humans, are highly sensitive, have a high spatial resolution and can give precise quantitative data (Gambhir et al., 2000b). A non-invasive, clinically applicable method for quantitative imaging of transgene expression in human subjects would enable clinicians to define the location(s), magnitude and persistence of gene expression over time (Gambhir et al., 2000b).

Positron-emitting isotopes are required for PET scanning. A positron is a particle with the same mass as an electron but with a positive charge. Positron-emitting radionuclides have a short half life ($^{11}$C: 20 minutes, $^{18}$F: 2 hours) and are thus generally produced in cyclotrons in or near the laboratory or hospital where the scanning takes place. Emitted positrons interact with atoms they encounter in their path. They travel for some distance depending on their energy and the density of the surrounding matter. When the velocity is low, they may combine with an
electron in their vicinity and annihilate. During the process of annihilation two photons with energy of 511 MeV are released simultaneously in opposite directions at an angle of 180° (provided positron and electron are at rest when they combine). Detectors are arranged in a ring around the subject and register only photon pairs that arrive within a certain time span of each other at an angle of 180°. The acquired data are analysed by computer and a three-dimensional image is reconstructed (Levin, 2005).

Single photon-emitting isotopes (used for SPECT) are isotopes that result in the emission of gamma ray photons or high energy X-ray photons. The photon is emitted directly from the radioactive atom. Single photons are emitted in all directions and are attenuated as they travel through the body. The photons that leave the body can be detected by rotating detector panels around the subject. The exact provenance of the photon can be determined by the use of collimators consisting of a well defined configuration of holes for the photons to enter. Photons that hit the collimator at the wrong angle will not make it through the hole and are filtered out. The acquired data are analysed by a computer and reconstructed to create three-dimensional tomographic images. The spatial resolution depends on how well the photons can be collimated. High spatial resolutions (1-2 mm) have been obtained by using magnifying collimators e.g. pinhole collimator (Levin, 2005).

C. S. Levin (Levin, 2005) has written an extensive review of all the imaging modalities including technical specifications, drawbacks and advantages. PET and SPECT both provide accurate quantitative information on gene expression in vivo in small animal studies. Both imaging modalities are available for use in humans. However, since human subjects have a greater mass, spatial resolution is reduced and the information obtained is thus less accurate.

In order to increase the amount of information obtained from images, molecular imaging modalities can be combined with conventional radiography. PET/Computed tomography (CT) and SPECT/CT have become available for clinical use combining the exact, high-resolution anatomical information from the CT with the molecular information obtained from PET or SPECT.
In addition to the radioactive tracer, molecular imaging of gene expression requires a reporter gene that will concentrate the tracer in the transduced tissue (Vassaux and Groot-Wassink, 2003). Currently three types of reporter genes are available: enzymes, receptors and transport proteins.

### 4.2.1. Enzymes

Upon intravenous injection, a radiotracer can freely diffuse into the subject and cross the plasma membrane of all cells. In cells which express the transgene, the radiotracer is metabolised through enzymatic reaction (usually phosphorylation). As a result the tracer can no longer cross the cell membrane and remains trapped within the cell (figure 1.9A). The degree of accumulation of radiolabelled substrate reflects the level of enzyme activity and thus the level of gene expression (Vassaux and Groot-Wassink, 2003).

**Figure 1.9:** General principles of radionuclide-based imaging: A. enzymes: the radiotracer can freely cross the cell membrane. In cells expressing the enzyme the radiotracer is metabolised (usually phosphorylated) and as a result becomes trapped in the cell. B. Receptors: expression of the transgene leads to expression of an intra- or extracellular receptor to which the radiolabelled ligand binds. The receptor ligand can be internalised. This leads to the accumulation of detectable ligand in transduced tissues. C. Transporter: upon expression of the transporter in transduced cells the radiotracer is selectively transported into the cell where it concentrates. The tracer is then either trapped in the cell or released when the extracellular concentration decreases, leading to a transient signal.

![Figure 1.9](image)

The herpes simplex virus 1 thymidine kinase (HSV1-tk) gene is an example of a reporter gene encoding an enzyme. Several thymidine analogues have been designed that are preferential substrates for the HSV1-tk rather than the cellular thymidine kinase. Radiolabelled derivatives can be used for imaging and therapy.
5-iodo-2'-fluoro-2'-deoxy-1-β-D-arabino-furanosyl-uracil (FIAU) can be labelled with several different radionucleotides including \(^{99m}\)Tc, \(^{123}\)I, \(^{124}\)I and \(^{131}\)I, which makes it appropriate for imaging and therapy with PET and SPECT (Vassaux and Groot-Wassink, 2003). Several investigators have created or are in the process of creating and evaluating other reporter probes with improved sensitivity and specificity (Penuelas et al., 2005a). The group of Ghambir, S. S. (Gambhir et al., 2000a) has investigated mutant HSV1-tk reporter genes, which present a higher specificity to the reporter probe 9-(4-[\(^{18}\)F] fluoro-3-hydroxymethylbuthyl) guanine (FHBG), providing a further increase in the sensitivity.

Studies in cell cultures and small animals have demonstrated that the \(^{18}\)F FHBG PET \textit{in vivo} signal correlates with transgene expression as determined by mRNA and protein levels (Gambhir et al., 1999). For a review of animal studies see (Min and Gambhir, 2004).

PET images monitoring the \textit{in vivo} expression of mutant HSV1-tk gene in xenografts of stably transfected cell lines in nude mice were able to predict the response to therapy (Yaghoubi et al., 2005).

PET imaging with \(^{18}\)F FHBG has been used to monitor HSV1-tk expression after intratumoural injection of Ad-CMV-tk in patients with hepatocellular carcinoma (Penuelas et al., 2005b). Radiotracer accumulation was detected in all lesions treated with an adenoviral dose above \(10^{12}\) viral particles. Fused PET/CT images provided precise anatomic-metabolic correlation between the tumour location and the site of HSV1-tk gene expression. These data provide evidence that PET imaging could monitor transgene expression in cancer patients. It was also suggested that PET imaging of gene expression could be used as a non-invasive tool to predict the efficacy of gene therapy in patients.

### 4.2.2. Receptor

Intra- or extracellular receptors can be expressed as transgenes and specific radiolabelled ligands can be developed that bind reversibly or irreversibly to the receptor (figure 1.9 B).

An example of such a receptor is the Dopamine D2 receptor (D2R), which is expressed in the plasma membrane of cells and is endogenously present in the striatum of the brain and the pituitary gland. Several ligands have been developed
that allow imaging by PET or SPECT (Sharma et al., 2002, Gambhir et al., 2000b) (Vassaux and Groot-Wassink, 2003).

3-(2’-[18F] fluoroethyl) spiperone ([18F] FESP) can bind to D2 receptors, leading to the accumulation of the ligand in the cells expressing the transgene, and can be imaged by PET. The levels of [18F] FESP accumulation thus reflect the level of transgene expression. Because ectopic expression of the D2 receptor may sensitise cells to circulating adrenergic signals a mutant receptor (D80RA) has been developed that uncouples receptor occupancy from intracellular signalling (Sharma et al., 2002, Gambhir et al., 2000b, Vassaux and Groot-Wassink, 2003).

In vivo imaging studies in mice after intravenous injection of a replication-deficient adenovirus carrying a D2R reporter gene driven by the CMV promoter with [18F] FESP-PET have shown very high expression of the reporter gene in the liver. The amount of radiotracer retained in the liver as determined by region of interest measurements from the PET images was proportional to the amount of hepatic FESP present and to D2R mRNA levels (Gambhir et al., 2000b, Vassaux and Groot-Wassink, 2003).

4.2.3. Transport protein

Transport proteins can be expressed in the plasma membrane of cells and act as reporter genes. They have a high specificity for certain compounds and use active transport mechanisms to concentrate a specific radiotracer into a defined compartment such as the cell cytosol (Figure 1.9 C) (Vassaux and Groot-Wassink, 2003).

The sodium/iodine symporter is such a transport protein and will be discussed in greater detail.
5. The human sodium iodine symporter (hNIS)

5.1. What is the hNIS?

E. Bauman reported in 1896 for the first time the ability of the thyroid cells to concentrate iodine (Dohan et al., 2003) (for reference (Baumann, 1896)). Iodine is scarce in the environment and consequently dietary intake is low. The capacity of the thyroid follicular cells to concentrate iodine up to 40 times the plasma concentration is essential for the production of thyroid hormones.

Over the past 60-70 years radioactive iodine isotopes and technetium-\(^{99m}\) have been used for the diagnosis of thyroid diseases, the treatment of hyperthyroidism and benign thyroid diseases and the diagnosis and treatment of thyroid cancer. Thyroid cancer was treated for the first time with radioiodine in 1941 (Chung, 2002), but it was not until 1996 that Dai et al. isolated the rat cDNA that encodes the protein involved in the uptake of iodine and technetium in the thyroid, the sodium iodine symporter (NIS) (Dai et al., 1996). In the same year the human homologue was isolated by Smanik et al. (Smanik et al., 1996).

The human NIS gene (hNIS) is localised on chromosome 19p12-13.2 and consists of an open reading frame of 1929 nucleotides. The coding region comprises 15 exons interrupted by 14 introns and it codes for a 3.9-kb mRNA, which in its turn generates a glycoprotein of 643 amino acids with a molecular mass ranging from 70 to 90 kDa as a result of variable levels of glycosylation (Smanik et al., 1997).

The secondary structure of the hNIS is thought to consist of 13 transmembrane segments of which the amino-terminus is located extracellularly and the carboxyl terminus intracellularly. Three of its Asp residues, position 225, 485 and 497 are glycosylated but glycosylation is not essential for the function, stability or targeting to the cell membrane of the NIS (Levy et al., 1998).

5.2. The function of hNIS in the thyroid

The hNIS mediates iodine uptake in the thyroid and several other extra-thyroidal tissues like stomach, salivary glands and lactating mammary glands. The transport of iodine across the basolateral membrane of the thyroid follicular cells is the first step in the production of thyroid hormones T3 and T4 (Dohan et al., 2003).
Figure 10 is a graphic depiction of the function of hNIS in the thyroid.

Two sodium ions are translocated inward together with inward translocation of one iodine ion across the basolateral membrane, which makes the sodium iodine transporter a symporter as both substrates are transported at the same time in the same direction. The driving force is the sodium gradient generated by the Na/K adenosine triphosphatase (De La Vieja et al., 2000) (Dohan et al., 2003). NIS activity in the body is blocked by the specific competitive inhibitors perchlorate and thiocyanate. It was in 1936 that Barker et al. (Dohan et al., 2003) discovered for the first time that patients treated with thiocyanate for hypertension develop hypothyroidism and/or goitre.

Figure 1.10: The function of the NIS in thyroid cells. AIT: Apical Iodine Transporter, ATP: Adenosine Tri Phosphate, Cl: Chlorine, K: potassium, NIS: Sodium (Na) Iodine (I) Symporter, Tg: Thyroglobulin, TPO: Thyroid Peroxidase, TSH: Thyroid Stimulating Hormone.

In the thyroid, in contrast to other tissues, iodine is translocated from the cytoplasm of the cell across the apical membrane to the colloid by pendrin, a chloride/iodine transporter and by the apical iodine transporter (AIT) (Dohan et al., 2003). Iodine is then linked to thyrosyl residues within thyroglobulin (Tg). This step is catalysed by the thyroid specific enzyme thyroid peroxidise (TPO) and is called iodine organification. Thyroid hormones T3 and T4 are synthesised by pairing of two
iodothyrosine residues and stored in the colloid until they are needed. Iodine accumulation in the thyroid in contrast to other tissues is stimulated by thyroid stimulating hormone (TSH) (Dohan et al., 2003). This organification is the key to the success of radioiodine therapy in thyroid cancer.

5.3. hNIS in extra-thyroidal tissues

Scintigraphic studies have demonstrated that iodine is also accumulated in tissues other than the thyroid. Iodine concentrating extra-thyroidal tissues include: the salivary and lacrimal glands, the gastric mucosa, the lactating mammary gland, the choroid plexus, the thymus, the adrenal gland, the lung, the heart and the ciliary body of the eye (Dohan et al., 2003). Iodine uptake in these tissues is also inhibited by thyocianate and perchlorate and iodine concentration gradients of the same magnitude are generated (Dohan et al., 2003). In contrast to the thyroid, non-thyroidal tissues lack the ability to organify iodine and TSH does not influence iodine uptake in the cells. Gastric mucosa and salivary glands concentrate thiocyanate in contrast to the thyroid where it is metabolised and eliminated after uptake (Dohan et al., 2003).

The isolation of NIS and the production of monoclonal antibodies have permitted investigators to study the distribution of NIS in tissues. Surprisingly, iodine uptake appears to be mediated by the same NIS, which is regulated and processed differently in each tissue (Dohan et al., 2003).

Radioiodine and iodine analogues used for thyroid scintigraphy continue to play an important diagnostic and therapeutic role in thyroid disease and thyroid cancer. The recent discovery of hNIS has permitted the investigation of new strategies that extend the use of iodine therapy through the ectopic expression of hNIS in the cell membranes of cancer cells with the use of gene delivery vectors. Ectopic expression of hNIS in cancer cells allows the entry of iodine in these cells and renders them susceptible to radioiodine therapy. Specific targeting of the hNIS gene to malignant cells can be achieved by the use of tissue-specific promoters thereby maximising cancer specific therapeutic effect and minimising toxic side effects in non-malignant cells (Dohan et al., 2003).
The hNIS is a unique imaging tool for cancer gene therapy. The hNIS is not a foreign protein since it is expressed endogenously in the thyroid and several other tissues and is thus not immunogenic. The endogenous expression is limited to a small number of tissues, which results in limited background interference for exogenous hNIS function. Iodine and other chemical elements (e.g. Tc$^{99m}$, Rhenium$^{188}$, Astatin$^{211}$) transported by the hNIS are in themselves the tracers and radiochemistry is therefore not required, which offers significant logistic and cost-effective advantages. Decaying isotope does not produce cold tracers (which could compete with the radiotracer) but disappears rapidly from the circulation. The radionuclides are specific to hNIS-expressing cells, which further reduces unwanted background noise (Vassaux and Groot-Wassink, 2003) (Marsee et al., 2004). Radioisotopes transported by hNIS are available for in vivo imaging by both PET and SPECT.

Shimura et al. (Shimura et al., 1997) performed the first imaging experiment with NIS. Undifferentiated rat thyroid cancer cells were stably transfected with the NIS gene and transplanted into rats. $^{125}$I was injected intraperitoneally and radioiodine accumulation was visualised by autoradiography. Iodine uptake in the tumours peaked 90 minutes after injection and diminished rapidly attaining 50% after 6 hours. Following this first experiment a large number of in vivo preclinical animal experiments have been performed using the same principles.

In our laboratory, Thomas Groot-Wassink has performed adenoviral biodistribution studies in nude mice using hNIS as a reporter gene (Groot-Wassink et al., 2002). Intravenous injection of a non-replicating Ad-CMV-hNIS construct and subsequent (after 48h) injection of $^{125}$I lead to the accumulation of radioactivity, measured by post mortem $\gamma$-counting of organs, mainly in the liver and the adrenal glands and to a lesser extent in the lungs, spleen and pancreas. These data were confirmed by Northern blot analysis of total hNIS RNA. In vivo imaging of gene expression was obtained with PET after injection of $^{124}$I.

In a second experiment, it was demonstrated that the combination of $^{124}$I and PET can provide quantitative information on gene expression in vivo (Groot-Wassink et al., 2004b). They found a correlation between the intensity of the PET signal and

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iodine uptake measured by post mortem γ-counting and between the PET signal and hNIS mRNA amount determined by quantitative real time PCR and the number of hNIS positive cells seen by immunohistochemistry using an hNIS specific antibody. These data validate the iodine uptake induced by ectopic expression of hNIS as a quantitative reporter system to evaluate gene expression. They also assessed the feasibility of using the hNIS reporter gene to visualise cancer selectivity of different promoters incorporated in non-replicating adenoviral vectors with $^{124}$I/PET imaging (Groot-Wassink et al., 2004a). Intravenous injection of a non-replicating adenoviral vector encoding the human telomerase promoters (Ad-hTR-NIS and AdhTERT-NIS) driving hNIS expression in tumour free animals does not yield a signal with $^{124}$I/PET imaging suggesting that the promoters are not functional in normal tissues infectable by adenovirus. This conclusion was confirmed by post mortem γ-counting of tissues, immunohistochemistry and quantification of hNIS mRNA levels. When injected in subcutaneous experimental human tumours in nude mice, the Ad-hTR-hNIS virus injected tumours showed increased iodine uptake as measured by quantitative analysis of PET images and post mortem radioactive counting compared to tumours injected with a control virus and similar to Ad-CMV-hNIS injected tumours, suggesting that the hTR promoter is a potent cancer-specific promoter. Altogether these data suggest that PET imaging of the hNIS reporter gene could be applied to measure the activity of cancer-selective promoters in humans, providing unique information on the pattern of gene transfer and transgene expression in patients (Groot-Wassink et al., 2004a).

6. Combining imaging with therapy

The hNIS is not only an imaging reporter gene. The extensive experience in the treatment of thyroid cancer provides sufficient evidence that the hNIS can also serve as a therapeutic transgene. The vectors that are currently available for cancer gene therapy have, so far, failed to transduce every single cell in a tumour. In mouse experiments intratumoural injection of a NIS-expressing adenovirus showed heterogeneity of expression, with areas of high level expression adjacent to regions of low expression (Groot-Wassink et al., 2004a).
The combination of hNIS as a transgene with the use of radioisotopes has the advantage that non-transduced cells can still be eradicated by the bystander effect caused by the radioactivity. $^{131}$I and other isotopes transported by NIS like $^{188}$Re-perrhenate and $^{211}$At (astatin) kill non-transduced cells by radiation emission. These radioisotopes are used in clinical settings in the treatment of patients and the technology and experience are readily available.

Dwyer et al. (Dwyer et al., 2005a, Dwyer et al., 2005b, Dwyer et al., 2006b, Dwyer et al., 2006a) have extensively investigated the possibility of adenoviral hNIS gene transfer, imaging and therapy in ovarian, breast and pancreatic cancer in preclinical mice models. Intravenous injection of a non-replicating adenovirus where a tumour specific promoter (MUC-1) drives the expression of hNIS did not yield an imaging signal or liver toxicity in comparison to the control virus where hNIS was driven by the CMV promoter demonstrating cancer-selective expression of the virus. Repetitive imaging with a gammacamera after injection of the virus in subcutaneous xenografts in nude mice allowed the investigators to determine the time of maximal iodine uptake. Treating the mice with $^{131}$I showed a marked reduction in tumour size.

The same authors have also carried out a study in large animals in preparation of a phase 1 clinical trial of prostate cancer using a non-replicating Ad-CMV-hNIS (Dwyer et al., 2005c). The viral vector was injected directly into the prostate of male dogs. Serial imaging with SPECT/CT allowed the investigators to visualise transgene expression and to predict the dose of $^{131}$I required to obtain a therapeutic effect. The dogs did not show side effects to other organs from the administered therapy. All these experiments were carried out with a non-replicating adenoviral vector.

One of the most promising results for hNIS gene therapy was obtained by Faivre et al. (Faivre et al., 2004). They have created an animal model of liver cancer. Hepatic carcinomas were induced in immunocompetent Wistar rats by diethylnitrosamine administration in the drinking water. The adenoviral non-replicating CMV-rNIS vector was administered directly into the portal vein. Pretreating the rats with thyroxine inhibited thyroid uptake of iodine. They found a long-term retention of iodine in normal and cancerous liver ($572 \pm 110\%$ hours and $294 \pm 50\%$ hours) after transfer of the rNIS gene, which was mainly attributable to recirculation and reuptake in the transduced liver cells. Iodine was cleared rapidly from the
hepatocytes when NIS activity was blocked by perchlorate. They also showed that tumour growth was strongly inhibited and survival improved by $^{131}$I therapy (40Gy) after rNIS gene transfer in hepatocarcinoma bearing rats. In some cases complete regression could be obtained. The authors did not observe any $^{131}$I related liver damage in 2 months follow up in Wistar rats despite the high radiation dose suggesting that liver carcinoma cells are more sensitive to radiation than normal hepatocytes. These results suggest that intra-arterial NIS based gene therapy for hepatocellular carcinoma is an approach worth investigating in humans.

7. Research strategy

Richard Iggo has created a series of Wnt-specific replicating adenoviruses containing multiple Tcf binding sites in several adenoviral promoters (Fuerer and Iggo, 2002, Fuerer and Iggo, 2004, Homicsko et al., 2005). These viruses were evaluated in a series of in vitro experiments by Richard Iggo’s team. I received an aliquot of the plasmid vpKH1, encoding the Wnt-specific adenovirus KH1 containing Tcf binding sites in E1A, E1B and E4. I also received an aliquot of pMB20, encoding the wild-type adenovirus 5 genome. I incorporated the hNIS gene into these viruses. In order to accommodate such a large gene, space had to be created by deleting the E3-6.7k gene and E3-gp19k gene, which has been shown to result in enhanced intratumoural gene expression and/or replication in immunocompetent tumour models when compared to the commonly deleted E3B genes (Wang et al., 2003b). Replacing the E3-gp19k with the hNIS resulted in viruses with 105% of the wild type genome size, which still allowed adequate packaging into virions. It was decided to use the yeast vector system for manipulation of the adenoviral genome following the experience of my supervisor with this technique (Vassaux et al., 1997) (Vassaux and Huxley, 1997).

Firstly, the constructed viruses were validated in vitro in different colonic cancer cell lines and in primary cultures of desmoid tumours.

Secondly, the imaging of the propagation of virus inside a tumour in vivo was validated in subcutaneous xenografts in nude mice.
Finally, I explored the therapeutic potential of the hNIS by combining imaging with therapeutic radioiodine therapy in vivo in nude mice.

Figure 1.11 shows a diagram of the constructed viruses.

**Figure 1.11**: Overview of created viruses. AdWt = wild-type adenovirus, AdIP1 = Wild-type adenovirus with hNIS replacing E3-6.7k and E3-gp19k, AdIP2 = Wnt-specific virus with Tcf-responsive elements driving the expression of E1A, E1B and E4 and hNIS replacing E3-6.7k and E3-gp19k, AdIP3 = wild-type adenovirus with and multiple cloning site (MCS) replacing E3-6.7k and E3-gp19k, AdIP4 = Wnt specific adenovirus with Tcf-responsive elements driving expression of E1A, E1B and E4 and MCS replacing E3-6.7k and E3-gp19k.
CHAPTER 2: Materials and Methods

1. Molecular Biology techniques

1.1. Standard PCR

Polymerase chain reaction was used to amplify the desired sequences of the adenoviral genome which were needed for the construction of shuttle vectors. The strategy for the construction of shuttle vectors can be found in the figure below. Our aim is to replace the adenoviral E3-6.7k and E3-gp19k with hNIS.

Figure 2.1: A. Representation of the early transcripts of the adenoviral genome. ITR=Inverted Terminal Repeat. ψ=packaging signal. B. Detailed representation of the transcripts of the adenovirus E3 region. 6.7k and gp19k overlap with 3bp. C. The cloning strategy aims at replacing the adenoviral overlapping regions E3-6.7k and E3-gp19k by hNIS.

Each PCR was optimized with the Opti-Prime™ PCR Optimization Kit (Stratagene, UK). PCR reactions were set up in 50μl volumes containing 100ng template DNA, 1μl Master Mix Buffer (obtained from the Opti-Prime™ Kit), 1mM dNTP’s, Forward and Reverse primers (provided by Sigma-Genosis) at a final concentration of 0.5 μM, 3U Taq DNA polymerase (Roche Diagnostics) and one of each of the 12 Opti-Prime™ buffers provided by the Opti-Prime™ Kit. A control
lacking DNA was run in parallel. PCR was performed in a PTC-200 Peltier Thermal Cycler (MJ Research).

The PCR products were confirmed by electrophoresis analysis (see below) on a 0.8% agarose gel (Invitrogen, Paisley, UK) and the Opti-Prime™ buffer leading to the best product was selected for future PCR reactions.

The hNIS cDNA was provided by S. Jiang (Smanik et al., 1996) and hNIS was cloned into a pShuttle based vector by Thomas Groot-Wassink (Groot-Wassink et al., 2002) to obtain the plasmid pTGW10. This vector was used as the source for hNIS (nt 220-2151) amplification by PCR. A map of this vector can be found in the appendix. Primers used were:

Forward: Xba1-hNIS: CTCTAGAGATGGAGCCGTGGAGACCGGGGAACGGGGAACCGG
Reverse: Xho1-hNIS: CCTCGAGGTCAGAGGTTTGTCTCCTGTCGGTCTCG.

The use of these primers introduced engineered restriction sites Xba1/Xho1 into the PCR product to facilitate further sub-cloning into a shuttle vector.

The settings were 5' at 94°C, 26 cycles: 1' at 94°C, 1' at 55°C, and 2' at 72 °C and 10' at 72 °C to finish.

The adenoviral regions E3-12.5k and E3-ADP were obtained and amplified by PCR from the plasmid MB20, a generous gift from Richard Iggo (St. Andrews University, Scotland). This plasmid contains the entire wild-type adenovirus-5 genome, a Chloramphenicol resistance gene, and a Histamine gene.

The reaction was optimized with the Opti-Prime PCR Optimization Kit (Stratagene, UK) according to the manufacturer’s guidelines (see above).

Primers for the E3-12.5k gene (nt27801-28533) were: Forward: SacII-12.5k: GCCGCGGCGATCAATTTATTCCTTTGACGC and

Reverse: Xba1-12.5k: CTCTAGAGCTGGCCTTTGGCCTAATAC introducing engineered restriction sites SacII and Xba1 respectively.

The conditions used were: 5' at 94°C, 26 cycles of: 45” at 94°C, 45” at 58°C, 1’ at 72°C and 10’ at 72°C to finish.
The nucleotide sequence spanning E3-ADP (nt29355-29755) was amplified using Xho1/Kpn1-engineered primers.

Xho1-ADP: Forward: CCTCGAGGCAATTGACTCTATGTGGGATATGCTCC

and

Kpn1-ADP: Reverse: GGGTACCCGAACTGTGTTTCAGTCCGTCCAATC.

The conditions used were: 5’ at 94°C, 26 cycles of: 45” at 94°C, 45” at 58°C, 45” at 72°C and 10’ at 72°C to finish.

(Nucleotide numbers refer to the sequence location within the Ad5 genome and hNIS genome which can be consulted on the NCBI website: http://www.ncbi.nlm.nih.gov/entrez accession number for Ad5 is AC_000008, gi: 56160529 and for hNIS is D87920, version D87920.1, gi: 2887404)

1.2. Analysis by Agarose Gel Electrophoresis

Gel Electrophoresis was used to confirm successful PCR, cloning and ligation. 10 μl of PCR product or restriction-digest was run on a 0.8%-1% (w/v) agarose gel [0.8g-1g agarose in 100ml 1x Tris-Borate-EDTA (TBE) buffer with 10μl ethidium bromide (EtBr) at 0.5μg/ml] @ 160V in a tank containing 1x TBE buffer. Samples were run alongside a 1kb DNA stepladder (Invitrogen™). Visualisation under UV light ensured that the PCR products were the expected size and of sufficient quality (buffer selection).

1.3. Cloning

1.3.1. Cloning of PCR products

The selected PCR product, obtained from the hNIS amplification, was cloned into a commercial plasmid pcDNA3.1/V5-His®-TOPO® (Invitrogen™, USA) (see appendix) with the TOPO-TA Cloning® kit (Invitrogen, USA) according to the manufacturers’ protocol and plated on Luria-Bertani (LB) agar plates [LB broth supplemented with 1.5% (w/v) agar] (Cancer Research UK Research Services) containing Ampicillin at a concentration of 60μg/ml LB agar.
The selected PCR products of E3-12.5k and E3-ADP were cloned into a commercial plasmid pCR®T7/CT-TOPO (Invitrogen™, USA) (see appendix) with a TOPO-TA® Cloning Kit and plated on LB agar plates containing Ampicillin at a concentration of 60µg/ml LB agar.

Plasmids thus obtained were called pIP1 (E3-12.5k-TOPO), pIP2 (E3-ADP-TOPO) and pIP3 (hNIS-TOPO).

1.3.2 Sub-cloning by restriction digestion

Consequently, the hNIS, E3-12.5k and E3-ADP were sub-cloned into the multi-cloning site (MCS) of plasmid pGV1, a URA3 [Orotidine-5'-phosphate (OMP) decarboxylase] integrating shuttle vector, donated by Dr. Georges Vassaux, my supervisor. This vector sequence is based on pRS406 (NCBI Accession U03446, gi: 416317), in which the HindIII site within the MCS has been inactivated. The map of pGV1 can be found in the appendix.

The E3-12.5k was cloned in the MCS of pGV1 by parallel Sac II and XbaI restriction-digestion of pIP1 and pGV1. The plasmid obtained was called pIP4. The E3-ADP was subcloned into pIP4 by parallel restriction digestion of pIP2 and pIP4 with XhoI and KpnI. The plasmid obtained was called pIP5. hNIS was cloned into pIP5 by parallel restriction-digestion of pIP3 with EcoRV and XbaI and of pIP5 with SmaI and XbaI. Digestion with SmaI and EcoRV enzymes leads to blunt ends, which can be joined with T4 Ligase. The restriction sites SmaI and EcoRV in the resulting construct however are lost for further use in later screening experiments. The resulting plasmid was called pIP7 (map below).
Restriction enzymes and T4 Ligase were purchased from New England Biolabs®, Hitchin, UK and reactions carried out in buffers supplied by the manufacturer. Restriction enzyme digestions were set up in 20μl volumes. Samples were digested with 2.5U of required restriction enzyme(s) in 10x buffer. 1μg of backbone DNA was digested at the same time as 5μg of the plasmid containing the relevant insert. Digestion was carried out for 2 hours in a warm water bath at 37°C.

Nucleotides were removed using a nucleotide removal kit (Qiagen, Crawley, U.K.) in accordance with the manufacturer’s instructions and the DNA was digested with the second enzyme if appropriate.

The digestions were run on a 0.8% agarose (Invitrogen, Paisley, UK) gel and the bands required for further ligation steps were excised with a clean scalpel under the guidance of ultraviolet light illumination in the dark room. The DNA was purified from the gel with a QIAquick® Gel Extraction kit (Qiagen®) according to the manufacturers’ instructions.

1.3.3. Alkaline Phosphatase treatment

The digested backbone plasmid DNA was modified by incubation with 2U Shrimp Alkaline Phosphatase (SAP) (USB, Ohio, USA) in 1x buffer.
SAP removes 5’ phosphates from plasmid and bacteriophage vectors that have been cut with a restriction enzyme. In subsequent ligation reactions, this treatment prevents self-ligation of the vector and thereby greatly facilitates ligation of other DNA fragments into the vector.

Reactions were set up in 20μl reaction volumes and incubated at 37°C for 15 minutes before further incubation at 65°C for 15 minutes in order to denature the modifying enzyme. Reactions were allowed to cool to room temperature before setting up ligation reactions.

1.3.4. DNA ligation

Ligation reactions were set up in 25μl volumes using 2U T4 DNA Ligase, 2.5 μl 10x T4 Ligase buffer with differential ratio’s of backbone to insert. 3μl of the dephosphorylated backbone and 17μl or 5μl of insert respectively were ligated together. Controls included unmodified backbone – no insert, dephosphorylated backbone – no insert and no backbone – insert only. Ligation reactions were incubated overnight at 16°C in a PTC-200 Peltier Thermal Cycler (MJ Research).

1.4. Generation of chemically competent bacteria

DH10B *E.Coli* bacteria were made competent by CaCl₂ treatment by using a variation of the method of Cohen et al. (Cohen et al., 1972).

Glycerol stocks of DH10B available in the laboratory were streaked onto Luria-Bertani (LB) agar [LB broth supplemented with 1.5% (w/v) agar] (Cancer Research UK Research Services) plates. The following day a single colony was picked and inoculated into 5ml of LB [1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% sodium chloride] broth and grown over night in a 50ml falcon tube at 37°C. The following day the culture was added to 100ml of LB broth in a 11 flask and the bacteria were incubated at 37°C with vigorous shaking (300 cycles/minute in a rotary shaker) until a density of 10⁸ bacteria/ml was reached. The cultured cells were then transferred into sterile 50ml ice-cold Falcon tubes and cooled to 0°C by placing the tubes on ice for 10 min. The bacteria were then recovered by centrifugation at 4000rpm for 10 minutes at 4°C. Supernatant was discarded and each bacterial pellet was then gently resuspended in 10ml of ice cold 0.1M CaCl₂.
and stored on ice. Cells were recovered by centrifugation at 4000 rpm for 10 min at 4°C. After discarding the supernatant each pellet corresponding to 50ml original culture was resuspended in 2ml of 0.1M CaCl₂. 200μl of the suspension of competent cells was added to a 1.5ml sterile Eppendorf tube ready for transformation. Long-term stocks were made by storing aliquots of 200μl of bacteria in a 20% (w/v) glycerol solution at -80°C.

1.5. Chemical transformation into competent bacteria

5 μl of the overnight ligation reactions was used to transform one aliquot of competent DH10B. DNA-extracts (method described below) obtained from the colonies selected after TOPO-cloning (no more than 50ng DNA in a volume of 10μl or less) were also added to 200μl of competent cells. The contents of the tube were mixed and stored on ice for 30 minutes. The tubes were then transferred to a rack and placed in a water bath preheated to 42°C for exactly 90 seconds. Thereafter the tubes were rapidly transferred on ice and allowed to chill for 1-2 minutes. 800μl of LB broth was then added and the bacteria were allowed to recover in a water bath at 37°C for 45 minutes. 200μl of transformed cells were then transferred to an LB agar plate containing the appropriate antibiotic and incubated over night at 37°C.

1.6. Extraction of DNA from bacteria

Colonies were chosen from the agar plates and added to 5ml LB containing the appropriate antibiotic selectable marker. After overnight incubation at 37°C in a shaking incubator the bacterial cultures were harvested by centrifugation at 13,000 rpm and plasmid DNA purified from the pellet by standard protocol, using a QIAPrep® Spin Miniprep Kit (Qiagen®). Constructs that were selected were inoculated in larger volumes of antibiotic selective LB and grown over night. A QIAPrep® Maxiprep Kit was used to purify these larger amounts of plasmid DNA according to the manufacturer’s instructions. DNA concentrations of successful constructs were determined using a Nanodrop® ND-1000 photospectrophotometer (NanoDrop Technologies Inc.). Samples were
assessed at 260nm versus a blank control of the elution buffer and DNA concentration was calculated from the formula: DNA concentration = OD260nm x 50μg/ml.

1.7. Glycerol stocks of bacterial plasmids

Long-term stocks of bacterial plasmids were made and stored at -80°C. A fresh colony was picked and incubated over night in 5ml of LB broth containing the appropriate antibiotic. The following morning 800μl of LB broth containing the bacterial plasmids at the appropriate density (approximately 10^8 bacteria /ml) was mixed with 400μl of 50% glycerol in water to obtain a final concentration of 20% glycerol and stored in cryovial tubes at -80°C until required.

1.8. Sequencing and analysis of colonies

The Cancer Research UK sequencing service was used to screen the DNA extracts from selected colonies after cloning and transformation of E3-12.5k and E3-ADP into TOPO cloning vectors. The primers used were T7 Seq/Invitrogen: Forward: TAATACGACTCATAAGGG and V5 (Rev) Seq/Invitrogen: Reverse: ACCAGAGAGAGGTAGGGAT. The desired DNA sequence was selected by comparing each clone with the original DNA sequence by BLAST analysis using the DNA Strider™ 1.3f11 computer program.

Analysis of hNIS clones was not performed by sequencing. The DNA extracts from the colonies obtained from the hNIS-TOPO cloning reaction were transfected into 293 cells. After 24h an Iodine Uptake assay was performed and the most functional clone was selected (See in vitro manipulations below for further details).

1.9. Homologous recombination in yeast

Adenoviral genomes were modified by two-step homologous recombination in yeast (Gagnebin et al., 1999).

Co-transformation of the relevant URA3 integrating shuttle vector and the adenoviral genome into the yeast resulted in the first part of the gene replacement
event, called "pop-in". These intermediate constructs contain a duplication of the target sequence (figure 3.1).

Removal of the selective requirement for URA3 resulted in a second spontaneous recombination event called "pop-out", which excised the integrated vector. Recombinants which had lost the URA3 integrating vector were selected on -HIS plates (Q-Bio-Gene, U.K.) supplemented with 5-Fluoro-orotic acid (5-FOA) at 0.1% (w/v). 5-FOA is converted into a toxic metabolite 5'-Fluoro-Uracil (5-FU) by the URA3 gene product (orotidine-5'-phosphate decarboxylase).

1.9.1. Transformation in yeast

The shuttle vector was co-transformed into yeast with plasmids pMB20 or vpKH1 (both generous gifts from Richard Iggo, St. Andrews University). The pMB20 has been described earlier. The vpKH1 plasmid is similar to the pMB20 but contains a Wnt-specific adenoviral genome instead of the wild-type Ad5 genome. Tcf-4 binding sites are present in E1A, E1B and E4 of the viral genome (for complete description see (Homicsko et al., 2005)).

In a first step Saccharomyces cerevisiae YPH587 (ATCC 76628): genotype MAT alpha ura-3-52 lys2-801 ade2-101 trpl-Δ200 leu2Δ1, provided by Richard Iggo (St. Andrews University, Scotland), was transformed with pMB20-Wild type Ad5 or vpKH1 respectively using the Alkali-Cation Yeast transformation Kit (Q-Bio-Gene, UK) following manufacturers’ instructions.

A fresh single colony of YPH587 was picked from YPD (YPD supplemented with 1.7% agar) agar plates (Q-Bio-Gene, UK) and inoculated into 100ml YPD [2% (w/v) peptone-Y, 1% (w/v) yeast extract and 2% (w/v) dextrose] broth (Q-Bio-Gene) and grown with aeration at 30°C for 12-24 hours. At the same time pMB20 or vpKH1 were linearised by PacI restriction digestion over night. 2μg of plasmid DNA was digested in a final volume of 20μl at 37°C. Digestion was followed by an enzyme denaturing step at 65°C for 15min. Yeast cells were made competent by treatment with Lithium/Cesium Acetate Solution, according to the manufacturer’s protocol. Transformation reactions were set up using four different concentrations of DNA from 0.1μg to 1μg in a maximum volume of 10μl. DNA was combined with 5μl carrier DNA and 5μl Histamine Solution (provided by the manufacturer)
and mixed gently with 100μl of competent yeast cells. Transformation was performed by heat shock at 42°C for 10 minutes. Cells were plated onto synthetic defined dropout media, –HIS [0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulphate and 2% (w/v) dextrose supplemented with 1.7% agar] agar plates and incubated at 30°C for 48-72 hours, until colonies appeared.

In a second step, a fresh colony of y-MB20 or yKH1 was picked and inoculated into 100ml –HIS broth (Q-Bio-Gene) and incubated for 12-24 hours at 30°C with aeration. At the same time the relevant shuttle vector pIP5/pIP7 was linearised by NotI restriction digestion over night. 2μg of plasmid DNA was digested in a final volume of 20μl at 37°C. Digestion was followed by an enzyme denaturing step at 65°C for 15min. Yeast cells were transformed the following day using the Alkali-Cation Yeast Transformation kit (Q-Bio-Gene) as described above. Transformation reactions were set up as described above. Transformation was performed by heat shock at 42°C for 10min. Cells were plated onto synthetic dropout media –HIS –URA [0.17% (w/v) yeast nitrogen base, 0.5% (w/v) ammonium sulphate and 2% dextrose supplemented with 1.7% (w/v) agar] agar plates and incubated at 30°C for 48-72 hours, until colonies appeared.

The yeast resulting from co-transfection of pMB20 and pIP7 was called yIP1.

The same reaction was repeated to transform vpKH1 and pIP7 into YH587 and the resulting yeast was called yIP2. Co-transfection of pMB20 and pIP5 was called yIP5 and co-transfection of vpKH1 and pIP5 was called yIP6.

### 1.9.2. Yeast DNA extraction

Yeast DNA was extracted from overnight cultures following enzymatic digestion of the cell wall resulting in spheroplast formation.

After transformation colonies were selected from the plates and grown in 10ml of selective media (provided by Q-Bio-Gene, UK) at 30°C over night. Each colony selected was recorded and numbered on a reference plate of the same media.

The next day the yeast cells were spun down at 7500rpm for 5 to 10 minutes. The supernatant was discarded and the pellets washed with 20ml of YI solution [250ml of 2M sorbitol (final concentration 1M), 100ml 0.5M ethylenediaminetetraacetic acid (EDTA) (final concentration 0.1M) supplemented with dH2O to 500ml]. After 10 minutes centrifugation at 7500rpm, the supernatant was discarded and the cells
spheroplasted by enzymatic digestion in 500μl yeast lytic enzyme solution (YI solution containing 2mg/ml of yeast lytic enzyme and 0.1% β-mercapto-ethanol for 60 minutes at 30°C. After 1 hour of incubation the spheroplasting process was observed under a microscope as cell lysis on addition of 1 drop of 10% Sodium Dodecyl Sulphate (SDS). Spheroplasts were pelleted by centrifugation for 5 minutes at 7500rpm and resuspended in 300μl of TE pH8 containing RNase (50μl/ml) and transferred to an eppendorf tube. Spheroplasts were then lysed by adding 30μl of 10% SDS to each tube and incubated for 1 hour at 65°C. Proteins, genomic DNA and debris were precipitated by adding 100μl of 5M potassium acetate (CH₃COOK) followed by centrifugation at maximum speed for 30 minutes at 4°C. The supernatant was transferred to a new tube and an equal volume of room temperature isopropanol (CH₃CHOH) added. The DNA was recovered by spinning the tubes at maximum speed for 15 minutes at 4°C. The DNA pellet was gently washed with 1ml of room temperature 70% ethanol (EtOH) and re-spun for 10 minutes at 4°C maximum speed. The DNA was air-dried and resuspended in a suitable volume of distilled water. DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc).

1.9.3. Generation of electrically competent bacteria

One colony of DH10B EColi bacteria was picked from a freshly grown LB plate and inoculated into 10ml of LB broth and grown over night in a 50ml Falcon tube. The following morning 2ml of the starter culture was added to 400ml of LB broth and transferred to a 2l flask. The cells were grown with gentle shaking at 37°C for 2.5-5 hours until the optical density at 550nm reached 0.8. The cell suspension was divided into 8 x 50ml disposable plastic tubes and left on ice for 1 hour, after which the cell pellets were recovered by centrifugation at 3000rpm for 10 min at 4°C. The supernatant was removed and four pellets were collected and transferred to a single 50ml Falcon tube, resuspended in 50ml of ice-cold WB [10% (w/v) ultrapure glycerol and 90% distilled water] solution. Subsequently both tubes were centrifuged at 3000rpm for 30min at 4°C, the supernatant removed and the cells resuspended in 50ml WB. This centrifugation was repeated and then each pellet was resuspended in 10ml WB and both pellets were pooled into one tube. Cells were kept on ice as much as possible. In a final centrifugation step at 3000rpm for
10 min at 4°C the pellet of competent cells was recovered, the supernatant removed and the cells resuspended in 1.5 ml of WB solution. 50 µl of bacteria was used for each transformation reaction. The remaining bacteria were divided in 50 µl aliquots in eppendorf tubes and stored at -80°C until required.

1.9.4. Transformation of electrically competent E. Coli with yeast DNA

One tube of 50 µl electrically competent bacteria was thawed on ice and up to 5 µl of yeast DNA extract was added. The mixture was transferred to a pre-chilled 2 mm electroporation cuvette (Thermo Scientific) and electroporated using a BioRad GenePulser® II Electroporator, under the following conditions; 2.5 kV, 200 Ω, 25 µF. The electroporated bacteria were resuspended with 450 µl of LB broth and incubated at 37°C for 30 minutes. 50 µl - 100 µl and 350 µl of bacteria were plated onto LB agar plates containing the relevant antibiotic and incubated over night at 37°C with aeration. The efficiency of home made electrically competent bacteria was inconsistent and therefore the yeast DNA extracts were predominantly transformed into commercially available electro-competent DH10B E. Coli bacteria (ElectroMAX™, Invitrogen) according to the manufacturer’s instructions to facilitate further PCR screening and confirmation by restriction digestion fingerprinting. No more than 100 ng of DNA in a final volume of 1 µl was used to transform competent cells by electroporation using a BioRad GenePulser® II Electroporator, under the following conditions; 2.0 kV, 200 Ω, 25 µF. Cells were resuscitated in 1 ml SOC (Invitrogen™) medium at 37°C for 1 hour. 100 µl of this suspension was plated onto LB agar plates containing Chloramphenicol (Chl) (25 µg/ml) and incubated overnight at 37°C with aeration.

1.9.5. DNA extraction for BAC low copy plasmids

Colonies selected from plates were grown over night in 5-10 ml of LB containing the appropriate antibiotic at 37°C. DNA was extracted according to a modified miniprep protocol (Qiagen®), which included a second alkaline lysis step to eliminate bacterial chromosomal DNA.

The next morning, the bacteria were spun down at 5000 rpm for 10 minutes at room temperature. The supernatant was discarded and the pellet re-suspended in 300 µl of P1 supplemented with 50 µg/ml RNase A and transferred to a 1.5 ml eppendorf tube...
(P1, P2 and P3 were home made according to the recipe found in the Qiagen® MaxiPrep Kit handbook and autoclaved). 300μl of normal alkaline lysis reagent P2 was then added to the solution, the tube inverted 4-6 times and incubated at room temperature for 5 minutes. 300μl of pre-chilled alkaline lysis reagent P3 was then added and the solution incubated on ice for 30 minutes. The tubes were centrifuged at maximum speed in a refrigerated Eppendorf tabletop centrifuge for 10 minutes at 4°C. The supernatant was collected, transferred to a 2ml eppendorf tube and an equal volume of room temperature isopropanol was added. The tubes were then centrifuged for 10 minutes at maximum speed at room temperature. The supernatant was removed; the pellet resuspended gently in 50μl of distilled water and transferred to a sterile 1.5 ml eppendorf tube. 200μl of room temperature 70°C ethanol was added and the tube was centrifuged at room temperature at maximum speed for 10 minutes. The supernatant was then discarded and the pellet re-suspended in 30 μl of Elution Buffer (EB) (obtained from Qiagen® miniprep kit).

1.9.6. Screening of constructs

The DNA extracts from the pop-in constructs were screened by analysing their restriction digestion fingerprint profile when compared against pMB20 or vpKH1 and pIP5 or pIP7, following digestion with XbaI/XhoI. Successful constructs were prepared for large-scale DNA extraction using the Large Con MaxiPrep Kit (Qiagen®).

Successful integrative recombinant yeasts or “pop-in” were inoculated from their reference plate into 10ml of –HIS broth and incubated for 12-24 hours at 30°C with aeration. Following this incubation period, 100μl of culture was plated onto –HIS 5-FOA plates (described above) and incubated for 48-72 hours at 30°C to select for “pop-out” recombinants. Individual colonies were selected, referenced on –HIS plates and electroporated into commercially available electro-competent Electromax™DH10BTM E. Coli (Invitrogen™). BAC low copy DNA was extracted and successful constructs selected by analysing their restriction-digestion fingerprint profile (see above) and by PCR. Successful constructs were prepared for large-scale DNA extraction using the Large Con MaxiPrep Kit (Qiagen®).
Table 2.1: Names of yeast plasmids produced: The yeast plasmids, the provenance and how they were produced.

<table>
<thead>
<tr>
<th>Names of yeast plasmids</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>yLP1</td>
<td>yMB20+IP7</td>
</tr>
<tr>
<td>yLP2</td>
<td>yKH1+IP7</td>
</tr>
<tr>
<td>yLP3</td>
<td>yMB20hNIS</td>
</tr>
<tr>
<td>yLP4</td>
<td>yKH1hNIS</td>
</tr>
<tr>
<td>yLP5</td>
<td>yMB20+IP5</td>
</tr>
<tr>
<td>yLP6</td>
<td>yKH1+IP5</td>
</tr>
<tr>
<td>yLP7</td>
<td>yMB20-gp19k+MCS</td>
</tr>
<tr>
<td>yLP8</td>
<td>yKH1-gp19k+MCS</td>
</tr>
</tbody>
</table>

Table 2.2: Names of bacterial plasmids: The bacterial plasmids and their structure.

<table>
<thead>
<tr>
<th>Names of bacterial plasmids and their DNA-extracts</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>plP1 12.5k-TOPO</td>
<td></td>
</tr>
<tr>
<td>plP2 ADP-TOPO</td>
<td></td>
</tr>
<tr>
<td>plP3 hNIS-TOPO</td>
<td></td>
</tr>
<tr>
<td>plP4 12.5k-GV1</td>
<td></td>
</tr>
<tr>
<td>plP5 12.5k-ADP-GV1</td>
<td></td>
</tr>
<tr>
<td>plP6 faulty plasmid</td>
<td></td>
</tr>
<tr>
<td>plP7 12.5k-hNIS-MCS-ADP-GV1</td>
<td></td>
</tr>
<tr>
<td>plP8 MB20+hNIS</td>
<td></td>
</tr>
<tr>
<td>plP9 KH1+hNIS</td>
<td></td>
</tr>
<tr>
<td>plP10 MB20-Δgp19k+MCS</td>
<td></td>
</tr>
<tr>
<td>plP11 KH1-Δgp19k+MCS</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.3: Primers used for cloning and screening of clones: The primers, description and sequence.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Description</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>P1</td>
<td>ADP-Xho1 F</td>
<td>CCTCGAGGCAATTGACTCTATGTGGGATATGCTCC</td>
</tr>
<tr>
<td>P2</td>
<td>ADP-Kpn1 R</td>
<td>GGGTACCCGAACATGTGTTCAGTCGGTCCAATC</td>
</tr>
<tr>
<td>P3</td>
<td>12.5-SacI F</td>
<td>GCCCGCGGATCAATTATCTATCTATGCTCTGAGG</td>
</tr>
<tr>
<td>P4</td>
<td>12.5-Xba1</td>
<td>CTCTAGAGCTGCGCTTTGCGCTTAC</td>
</tr>
<tr>
<td>P5</td>
<td>hNIS-Forward-Xba1</td>
<td>CTCTAGAGATCGAGCCGGGTGGAGACGGGGAACCG</td>
</tr>
<tr>
<td>P6</td>
<td>hNIS-Reverse-Xho1</td>
<td>CCTCGAGGTCAGAGGTGTGTCTCATGTCCTCTG</td>
</tr>
<tr>
<td>P7</td>
<td>NIS-screen-R</td>
<td>AGCGCTTCATGCGGCGGCGAAGAGGAGGGAACGG</td>
</tr>
<tr>
<td>P8</td>
<td>T7Seq/Invitrogen</td>
<td>TAATACGACTCACTATAGGG</td>
</tr>
<tr>
<td>P9</td>
<td>V5(Rev)Seq/Invitrogen</td>
<td>ACCGAGGAGAGGTAGGGAT</td>
</tr>
</tbody>
</table>
2. In vitro work

2.1. Maintenance of Cells

Immortalised cell lines were obtained from Cancer Research UK Research Cell Services Department. Primary desmoid tumour cells were obtained from St. Mark's Hospital, London, UK (Desmoid 5.2) and UZ-leuven, Belgium (Desmoid 1-5), with the appropriate ethical approvals.

Cells were maintained as adherent monolayers in sterile tissue culture flasks T75 or T175 containing the appropriate medium (both obtained from CR-UK Research cells services) supplemented with 10% foetal calf serum (FCS) (Sigma-Aldrich Chemie, GmbH, Germany) (immortal cell lines) or 20% FCS (primary cell lines). All FCS was heat inactivated (HI) by placing it in a water bath for 30 min at 55°C, unless otherwise stated. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5%/10% carbon dioxide (CO₂) to allow cell attachment and growth.

When approximately 70% confluent, cells were sub-cultured. The medium was aspirated and the cells were washed with 12ml (for T175) of Phosphate buffered saline (PBS). After aspiration of PBS, cells were incubated with 3ml of 0.083% v/v trypsin (CR-UK Media Services) in PBS at 37°C. When the cells were detached from the flask, trypsin was neutralised with 15ml of the appropriate medium containing 10% FCS and the cells resuspended by gentle pipeting. 2.5-4ml (depending on cell line) of the cell suspension was then added to a sterile T175 flask containing 25ml of growth medium supplemented with 10% FCS and the cells were allowed to adhere at 37°C.

To generate long-term stocks, cell suspensions were centrifuged after trypsin neutralisation at 1000g for 5 minutes. The supernatant was removed and the pellet was resuspended in 2ml of freezing medium consisting of 90% FCS supplemented with 10% dimethyl sulphoxide (DMSO; BDH, distributed by VWR, UK) and split into 2 x 1ml in cryovials (Falcon), which were frozen at -80°C over night and the next day transferred into liquid nitrogen for long-term storage. Vials retrieved from long-term storage were defrosted on ice, transferred to a 37°C water bath and then re-suspended in the appropriate warm growth media and plated in culture flasks.
All immortal cell lines were tested at regular intervals for mycoplasma contamination.

2.2. Transient transfection of DNA into CYMR293 cells

Screening of pIP3 (CMV-hNIS-TOPO) recombinants was performed by transfection with recombinant DNA of CYMR 293 cells and by subsequent Iodine Uptake Assay. Viral production of recombinant plasmids pIP8-11 was also performed by transfection of CYMR293 cells and was carried out in a category 2 tissue culture lab.

The day prior to transformation, CYMR 293 cells were plated into 24-well plates for plasmids containing pIP3 clones and 6-well plates for plasmids containing the adenoviral genome (pIP8-11).

2.2.1 Adenoviral genomes

Adenoviral recombinant plasmids were digested overnight with PacI. 10μg of plasmid DNA was digested at 37°C in a final volume of 50μg, with 2U of PacI to release a replication-competent Ad5 genome. The following day DNA the 36kbp fragment was purified using phenol-chloroform-isoamyl alcohol. 10μl (2μg DNA) was removed and saved in an eppendorf for later use as a non-purified transfection. The remaining 8μg was purified by adding an equal volume (40μl) of phenol/chloroform/isoamyl alcohol followed by centrifugation at 13,000rpm for 5 minutes at room temperature. The upper layer was collected as the purified fraction and 2μg of purified DNA was used to transfect one of four wells in a 6-well plate containing 2x10^5 CYMR 293 cells/well. 2μg of DNA was mixed with 100μl OptiMEM™ (Invitrogen Ltd, Paisley, UK) and in parallel 100μl of OptiMEM was mixed with 6μl Lipofectamine (Invitrogen). A control was set up without DNA. The mixtures were incubated separately at room temperature for 45min after which the Optimem/Lipofectamine and Optimem/DNA mixtures for each well were combined and incubated for another 30 minutes at room temperature. Following this 800μl Optimem was added to each mixture and each mixture added to one well of CYMR293 cells that were washed with 2ml Optimem. Cells were incubated for 4h at 37°C under standard conditions after which the Optimem/DNA mixture was
aspirated replaced with 2-3ml of growth medium supplemented with 10% FCS. Cells were incubated for 10-11 days until detachment. The culture medium containing virus and cells was collected and stored in a 15ml falcon tube at -80°C until required for further expansion of the virus.

2.2.2. topo-vectors

The standard protocol was adjusted as follows for the CMV-hNIS plasmids. 2μg DNA of each clone was dissolved in Optimem™ (Invitrogen, Paisley, UK) to a total volume of 125μl. In a separate tube 6μl of lipofectamine (Invitrogen, Paisley, UK) was added to 119μl of Optimem for each clone. Both tubes were incubated for 45 minutes at room temperature. Each tube containing DNA was then mixed together with a tube containing lipofectamine and incubated for another 30 minutes at room temperature. During incubation the medium was aspirated from the 24-well plate containing CYMR 293 cells at 5x10^4 cells per well and 1ml of Optimem was added to each well. At the end of the 30 minutes incubation 600μl of Optimem was added to each tube. The Optimem was aspirated from the cells and 200μl of the mixture DNA/lipofectamine/Optimem was then added to each well (four wells for each clone) and incubated for 4 hours at 37°C. After incubation the mixture was aspirated and 500μl of E4 (DMEM, Dulbecco’s modification of Eagle’s medium) medium supplemented with 10% FCS was added to each well. The following day, the medium was changed and fresh medium added to the cells. On the second day after transfection (after 48h), an Iodine uptake assay was performed.

2.3. Iodine Uptake Assay

Iodine uptake assays were performed to demonstrate the presence of functional hNIS in vectors and adenoviruses. Control plasmid was pTGW10 and control virus was Ad10, a non replicating (E1A deleted) CMV-Ad5 virus, previously produced in our laboratory by Thomas Groot-Wassink (Groot-Wassink et al., 2002). Cells were seeded in 24-well plates at 5-7.5 x 10^4 cells per well depending on cell type. The following day, cells were infected with recombinant adenovirus at different multiplicity of infection (see below) or transfected with recombinant plasmids, pIP3 clones and pTGW10 (see above). After 24-48 hours the growth
medium was removed and 500μl of growth medium containing $^{125}$I (10μl of 1mM Na$^{125}$I in 100ml medium) (purchased from Amersham Biosciences, UK). Controls wells were set up by adding perchlorate, a hNIS inhibitor to the $^{125}$I-containing medium (10μl of 1M Perchlorate added to 100ml $^{125}$I-medium). The source has a specific activity of 740MBq/mmol. The plate containing cells and radioactive media was incubated at 37°C for one hour, after which the radioactive media was removed and the cells washed with ice-cold PBS. Cells were subsequently lysed in 500μl of 0.1% sodium dodecyl sulphate (SDS). 400μl of each well was added to 3.6ml of Biodegradable Counting Scintillant (BCS®, Amersham Biosciences, UK Ltd) and counted in a LS6500 multipurpose scintillation counter (Beckman-Coulter). The readings were normalised against background noise as measured by counting 4ml of scintillation (BCS®) fluid and against a sample of the radioactive incubation medium.

For each well the protein content was determined by adding 25μl of each well to a well of a 96-well plate (in duplicate) and performing a standard bicinchoninic acid (BCA™) Protein Assay (Pierce Biotechnology Inc., part of Perbio Science, Tettenhall, UK) according to the manual provided by the manufacturer. The radioactivity was described as cpm (counts per minute)/μg protein.

### 2.4. Cell lysate protein concentration determination

Colorimetric detection and quantitation of total protein content was performed with the BCA™ Protein Assay Kit (Pierce Biotechnology Inc).

Preparation of diluted Albumin (BSA) Standards was performed according to the manufacturer’s protocol. Bovine Serum Albumin 2mg/ml (v/w) was diluted in 0.1% SDS in order for standards to have the same diluent as the samples.
Preparation of Diluted Albumin (BSA) Standards for BCA™ Assay and BCA™ Reducing Agent-Compatible Assay

Preparation of Diluted Albumin (BSA) Standards for BCA™ Assay

Dilution Scheme for Standard Test Tube Protocol and Microplate Procedure
(Working Range = 20-2,000 µg/ml)

<table>
<thead>
<tr>
<th>Vial</th>
<th>Volume of Diluent</th>
<th>Volume and Source of BSA</th>
<th>Final BSA Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0 µl</td>
<td>300 µl of stock</td>
<td>2,000 µg/ml</td>
</tr>
<tr>
<td>B</td>
<td>125 µl</td>
<td>375 µl of stock</td>
<td>1,500 µg/ml</td>
</tr>
<tr>
<td>C</td>
<td>325 µl</td>
<td>325 µl of stock</td>
<td>1,000 µg/ml</td>
</tr>
<tr>
<td>D</td>
<td>175 µl</td>
<td>175 µl of vial B dilution</td>
<td>750 µg/ml</td>
</tr>
<tr>
<td>E</td>
<td>325 µl</td>
<td>325 µl of vial C dilution</td>
<td>500 µg/ml</td>
</tr>
<tr>
<td>F</td>
<td>325 µl</td>
<td>325 µl of vial E dilution</td>
<td>250 µg/ml</td>
</tr>
<tr>
<td>G</td>
<td>325 µl</td>
<td>325 µl of vial F dilution</td>
<td>125 µg/ml</td>
</tr>
<tr>
<td>H</td>
<td>400 µl</td>
<td>100 µl of vial G dilution</td>
<td>25 µg/ml</td>
</tr>
<tr>
<td>I</td>
<td>400 µl</td>
<td>0 µl</td>
<td>0 µg/ml = Blank</td>
</tr>
</tbody>
</table>


25µl of each standard A-I or unknown sample replicate was added to a 96-well plate. Working Reagent (WR) was prepared by mixing 50 parts of BCA™ reagent A with 1 part of BCA™ reagent B. 200µl of the WR was added to each well and the plate mixed thoroughly on a plate shaker for 30 seconds. The plate was then covered with tin foil and incubated for 30 minutes at 37°C. After allowing the plate to cool to room temperature the absorbance was measured at 560nm on a plate reader. The average 560nm absorbance measurement of the blank standard replicates was subtracted from all other individual standard and unknown sample replicates. A standard curve was prepared by plotting the average blank-corrected measurements for each BSA standard versus its concentration in µg/ml. The standard curve was used to determine the concentration of each unknown sample.

2.5. Viral amplification

CYMR 293 cells transfected with adenoviral plasmids were left in 6-well plates for approximately 10 to 11 days at which time cells were dying due to viral infection and were floating in the medium (= cytopathic effect (CPE)). The viral suspension (medium, virus and cells) from each well was collected separately and freeze-thawed 3 times by transferring the tubes from the -80°C freezer to a 37°C water bath. After conclusion of the last cycle, the entire content of one tube was used to infect a 10cm diameter culture dish containing 5 x 10⁵ CYMR 293 cells seeded the
previous day under standard conditions in E4 medium supplemented with 10% FCS. Prior to infection the media was removed from the cells and replaced with 10-15ml E4 supplemented with 2% FCS. After approximately 3-4 days CPE became evident and the cells and supernatant were collected and stored at -80°C until required. In preparation for further viral amplification the suspension was subjected to 3 cycles of freeze-thaw action as described before. 3-5ml of the cell suspension was used to infect a T175 flask containing CYMR 293 cells at 70%-80% confluency. Full CPE became evident after 48-72h and the cell suspension was again collected from the flasks and frozen at -80°C. The cell suspension was again subjected to three freeze-thaw cycles and 8-15ml of the freeze-thawed suspension was then used to infect a CF10™ cell factory (Fisher).

Viruses based on Ad5 Wild-type were produced in CYMR293 cells and viruses based on the Wnt-specific Ad5 genome were produced in SW480 or SW620 colonic cancer cells.

Four T175 flasks at 70-80% confluency are required to seed a single CF10™. The supernatant was removed from the flasks and the cells were washed with 12ml of warm PBS. The PBS was aspirated from the cells and 5ml of trypsin was added to each flask. Once the cells had detached 5ml of growth medium supplemented with 10% FCS was added to each flask. The four flasks were pooled together into one T175 flask and 400ml of growth medium supplemented with 10% FCS was added to the flask. The CF10™ was then unpacked and sterile tubing and a glass funnel were used to transfer the cells from the flask into the CF10™. 600ml of growth medium supplemented with 10% FCS was then used to flush the remaining cells from the tubing. The cell factory was then incubated under standard conditions for 48-72 hours at 37°C. On the day of infection, the medium was removed from the cell factory and replaced with 400ml of growth medium supplemented with 2% FCS to which 8-15ml of the freeze-thawed viral suspension was added. The tubing was flushed with 600ml of growth medium supplemented with 2% FCS.

After approximately 3-4 days CPE became evident and the cells suspension was collected from the CF10 tower in two sterile 500ml centrifuge tubes and centrifuged at 2000 rpm for 10 minutes at 4°C. After the supernatant had been discarded the cell pellet was resuspended in 15ml of ice cold PBS, transferred to a 50ml tube and centrifuged at 1000 rpm for another 10 minutes. After aspiration of the supernatant the remaining cells were resuspended in 24ml of cold 10mM Tris-
HC1, pH 8, transferred to a single labelled sterile 50ml tube and stored at -80°C until ready for Caesium Chloride (CsCl) banding.

### 2.6. CsCl banding

Each virus was purified by CsCl (Sigma) banding as described by He et al. (He et al., 1998) and (Graham and Prevec, 1995). The viral concentrate collected from the cell factory was defrosted at 37°C and then freeze-thawed three times by transferring the sample between liquid nitrogen and a 37°C water bath. The virus suspension was spun for 10min at 6000rpm at room temperature and the supernatant removed and layered onto the CsCl for banding. The virus was collected by ultracentrifugation following two successive rounds of purification on a CsCl gradient. Subsequently the virus was dialysed to remove CsCl using the slide-a-lyser system (Pierce Biotechnology Inc part of Perbio Science, Tettenhall, UK). Dialysis solution consisted of 10mM Tris-HCl pH7.4, 1mM MgCl2, 150mM NaCl and 10% glycerol (w/v). After dialysis the virus solutions were divided into 100μl aliquots and stored at -80°C in eppendorf tubes.

<table>
<thead>
<tr>
<th>viruses produced</th>
<th>Structure</th>
<th>Provenance</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdWt</td>
<td>Ad5-Wild type</td>
<td>Claudia Montiel-Equihua</td>
</tr>
<tr>
<td>AdKH1</td>
<td>Ad5-Wnt Specific</td>
<td>vpKH1</td>
</tr>
<tr>
<td>AdIP1</td>
<td>Ad5-Wild type + hNIS</td>
<td>pIP8</td>
</tr>
<tr>
<td>AdIP2</td>
<td>Ad5-Wnt Specific + hNIS</td>
<td>pIP9</td>
</tr>
<tr>
<td>AdIP3</td>
<td>Ad5-Wild type - Δ6.7k-gp19k</td>
<td>pIP10</td>
</tr>
<tr>
<td>AdIP4</td>
<td>Ad5-Wnt Specific - Δ6.7k-gp19k</td>
<td>pIP11</td>
</tr>
</tbody>
</table>

### 2.7. Viral yield determination

The viral titre was determined by titration in CYMR 293 cells in 96-well plates in duplicate. Cells were seeded at 10,000 cells in 100μl of E4 medium supplemented
with 2% FCS per well and incubated under standard conditions at 37°C with aeration. The following day, ten-fold serial dilutions of virus were made (ranging from \(10^{-5}\) to \(10^{-12}\)) in E4-2% FCS and 100µl of diluted virus was added per well. For each dilution ten wells per 96-well plate were infected. Plates were incubated under standard conditions at 37°C for 10 days and then the titre was determined according to the Tissue Culture Infectious Dose 50 (TCID50) method of Reed-Muench (Http://gbiogene.com/technical/protocols/gene-expression/pdf/madeasy.pdf). The titre was calculated using the Kärber statistical method \(T=10^{d+0.5}S\) were \(d\) is log10 of the dilution and \(S\) is the sum of ratios. TCID50 values are adjusted as they are 0.7 Log higher than standard plaque assay enabling viral titres to be expressed as PFU/ml. (i.e. \(1\times10^5\) TCID50/ml is adjusted to \(1\times10^{4.7}\) PFU/ml). The average of the duplicate titration was used as the definitive titre.

### 2.8. Infection of cells with adenovirus

Cell lines were seeded in 24-well plates in the appropriate medium at 50-75,000 cells per well or 6-well plates at 200,000 cells per well and incubated under standard condition at 37°C. The following day viruses were diluted in FCS-free medium at different multiplicities of infection (MOI) (i.e. \(50,000\) PFU/\(50,000\) cells = MOI 1) to assess cell killing (see MTT assay below) or at the required MOI for other experiments (i.e. Western Blot). 500µl (1ml for 6-well plates) of diluted virus or FCS-free medium for controls was added per well and the cells incubated ad 37°C under standard conditions for 30 minutes. 1.5ml (2ml for 6-well plates) of medium supplemented with 15% FCS was then added to each well so the final concentration of FCS would be 10% and the plates were incubated under standard conditions at 37°C until ready for further processing.

### 2.9. Cell viability (MTT) assay

MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (sigma. UK) assays first described by Mosmann (Mosmann, 1983) were used to determine the cytotoxic effect of viruses on a panel of cell lines.
This assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which can not permeate the cell membranes, thus resulting in its accumulation within healthy cells. Lysis of the cells by the addition of a detergent results in the liberation of the crystals which are solubilised. The number of surviving cells is directly proportional to the level of the formazan product created. The color can then be quantified using a simple colorimetric assay.

4-6-8 days after infection, depending on the virus and cell line, MTT solution at 5mg/ml (v/w) was added to the medium of cells to obtain a total concentration of 10% (v/v) (e.g.150μl in 150ml medium in each well of a 24-well plate). The plates were incubated at 37°C under standard conditions for two hours after which the supernatant was aspirated and 200μl of DMSO was added to each well of a 24-well plate. The plates were left on a shaker for 15 minutes. 50μl of each well was transferred to a well of a 96-well plate containing 100μl of DMSO (each well was performed in duplicate) and the plate was read at 560nm in an OPSYS MR (Dynex Technologies) micro-plate reader. The average 560nm absorbance measurement of non infected wells (medium without virus) was used as the reference value = 100% cells. CPE of each virus in each cell line was expressed as % of cells surviving as compared to the reference.

2.10. Western Blot analysis of protein extracts

The selective activity of Wnt-specific viruses in Wnt-positive colonic cancer cell lines as compared to Wnt-negative cancer cell lines was verified by detection of the adenoviral E1A protein in extracts from infected cells using the Western Blot technique developed by W. Burnette (Burnette, 1981).

Cells were seeded under standard conditions and infected the next day with adenoviruses at different MOI. 24-48 hours later cells were detached with trypsin, the trypsin neutralised with medium containing 10% FCS and the cells pelleted by centrifugation (5 minutes at 1000rpm). Supernatant was aspirated; the pellets resuspended in 300μl lysis buffer (1% SDS) containing 1x complete protease inhibitor (Roche Diagnostic GmbH, Lewes, UK) (1 tablet dissolved in 2ml dH2O is
25x solution) and put on ice in eppendorf tubes. 25μl was used to determine protein concentration using BCA protein assay (described above).

20μg of protein was loaded onto a 12% SDS-PAGE (polyacrylamide gel electrophoresis) gel and was run at 100V. The gel was then transferred onto a nitrocellulose membrane using semi-wet transfer at 15 V for 45 minutes. Transfer buffer consists of 10% 10x Tris-Glycine-electro-blotting buffer (CR-UK Research Services), 20% Methanol and 70% distilled water.

The membrane was then blocked over night with 5% milk in PBS with gentle agitation on a rocking plate in the cold room at 4°C. The next day the membrane was washed with PBS 3 x 5 minutes on a shaker with gentle agitation at room temperature and mouse monoclonal anti E1A (Invitrogen or BD Pharmingen) primary antibody added at a concentration of 1/1000 in 5% milk in PBS. After having rocked for 1.5 hours at room temperature the antibody was poured away and the membrane washed with 10ml of 0.1% Tween20 (Sigma) in PBS (3 x 5 minutes). The secondary antibody, peroxidase conjugated goat anti-mouse IgG (Dako Cytomation Inc, Ely, UK), was added at a concentration of 1/5000 in 5% milk in PBS for 1 hour, after which the membrane was washed with 0.1% tween20 in PBS (3 x 10 minutes). The membrane was then developed by chemical luminescence (ECL™ reagent from Amersham Biosciences Ltd, Little Chalfont, UK).

A loading control was performed to ascertain that the same amount of protein was loaded in each well.

The membrane was stripped by adding 10ml of stripping buffer (0.2M Glycin at pH 2.5, 0.05% Tween20 in PBS) and was put in the oven at 80°C for 30 minutes. The buffer was then removed, the membrane washed and blocked with 5% milk in PBS over night with gentle agitation in the cold room at 4°C. The next day, the milk was removed and primary antibody goat monoclonal anti KU70 (Santa Cruz Biotechnology) was added at a concentration of 1/1000 in 5% milk in PBS. After having rocked for 1.5 hour at room temperature, the milk was poured away and the membrane washed 3 x 5 minutes with 10ml of 0.1% Tween20 in PBS. The secondary antibody, donkey anti goat IgG (Santa Cruz Biotechnology) was then added at a concentration of 1/2000 in 5% milk in PBS and the membrane incubated for 1 hour after which the membrane was washed with 0.1% tween20 in PBS (3 x
10 minutes). The membrane was then developed by chemical luminescence (ECL™ reagent from Amersham Biosciences Ltd, Little Chalfont, UK).

2.11. RT-PCR

Real-time PCR (RT-PCR) was carried out to amplify and simultaneously quantify the target molecule (E1A or hNIS). It enables both detection and quantification (absolute number of copies or relative amount compared to housekeeping genes) of a specific sequence in a sample. To quantify relative gene expression at a particular time RNA was extracted from cell homogenates. An additional step of reverse transcription was required to convert mRNA into cDNA. To determine the copy number of the target gene DNA was extracted from cell homogenates.

Total RNA was purified from cells using Trizol® Reagent (Invitrogen™) (see below). Total DNA was purified from cells using QIAamp® DNA Micro Kit (Qiagen) and from tissue using the DNeasy Tissue Kit (Qiagen) following the manufacturers guidelines. All RT-PCR was performed using SYBR green PCR Master Mix (Applied Biosystems). SYBR green is a fluorescent dye that intercalates with double stranded DNA amplicons generated during the PCR. When bound to dsDNA the intensity of fluorescent emissions increases.

RT-PCR detects the accumulation of amplicon during the reaction. The fluorescence is measured during the exponential phase of the reaction. Fluorescence is plotted against cycle number on a logarithmic scale so an exponentially increasing quantity will give a straight line. The threshold is determined as the point at which the fluorescence level of a reaction exceeds background fluorescence. The cycle at which a sample reached this threshold is called the cycle threshold, C_t. The quantity of DNA doubles every cycle during the exponential phase so relative amounts of DNA can be calculated, e.g. a sample whose C_t is 4 cycles earlier than another's has 2^4 = 16 times more template (http://www3.appliedbiosystems.com/cms/groups/mcb_marketing/documents/generaldocuments/cms_042484.pdf).

The total amount of DNA in a sample was determined by absolute quantification by comparing the results to a standard curve produced by RT-PCR of serial
dilutions of a known amount of DNA of the amplified gene (plasmid or PCR-product). Standards were prepared and stored at -20°C.

To quantify gene expression from RNA samples, the measured amount of RNA from the gene of interest was divided by the amount of RNA from a housekeeping gene (18s) measured in the same sample to normalise for possible variation in the amount and quality of RNA between samples.

2.11.1. Reverse transcription

Reverse transcription of total RNA extracts was carried out with TaqMan® Reverse Transcription Agent Kit (N808-0234) (Applied Biosystems). 2µg of total RNA, diluted in a total volume of 10.4µl with diethylpyrocarbonate (DEPC)-treated water, was added to a reaction mix containing 3µl TaqMan® RT buffer (10x), 6.6µl MgCl₂ (25mM), 6µl dNTP (2.5mM each), 1.5µl random hexamers (50µM), 0.6µl RNase inhibitor (20U/l) and 1.88µl Multiscibe Reverse transcriptase. All reagents were provided by the manufacturer. Conditions used were 10min @ 25°C, 60min @ 37°C, 5min @ 95°C and 4°C to finish.

2.11.2. Quantitative PCR

SYBR® Green PCR Master Mix (Applied Biosystems) was used to perform all RT-PCR. Samples obtained through reverse transcription of mRNA were normalised by amplifying 18S ribosomal RNA (rRNA) from diluted samples.

In early experiments standard curves were borrowed from other researchers in our group. The standard curve for hNIS was made by serial dilution of a known concentration of plasmid pShuttle-TK-hNIS (previously made in our laboratory by Ms Korin Knight); the standards for E1A were obtained by serial dilution of pShuttle-hTERT-E1A (previously made in our laboratory by Andrew Merron, also called pAM10) and the standards for 18s were produced from reverse transcription of standard Universal U-RNAs (Applied Biosystems).

In subsequent experiments standard curves were produced by serial dilutions of PCR-products. PCR reactions were optimised with Opti-Prime™ PCR Optimization Kit (Stratagene, UK) as described above.
hNIS PCR-product was obtained from pIP3. Primers used were P5 and P6. Conditions were 5min @ 94°C, 27 cycles of 1min @ 94°C, 1min @ 55°C, 2min @ 72°C and 10min 72°C).

E1A-PCR product was obtained from pAM10. Primers used were E1A-F and E1A-R. Conditions were 10min @ 95°C, 40 cycles of 10sec @ 95°C, 15sec @ 58°C, 8sec @ 72°C and 5min @ 4°C to finish.

18S-PCR product from reverse transcribed U-RNA. Primers used were 18S-F and 18-R. Conditions were 2min @ 95°C, 10min @ 95°C, 40 cycles of 30sec @ 95°C, 1min @ 60°C and 5min @ 4°C to finish.

PCR products were run on a 0.8% agarose gel to verify the quality and the correct size of the product.

RT-PCR reactions were set up for standard curve dilutions, reverse transcribed products and purified DNA by adding the SYBR® green mix (2x final concentration) and primer mix (final concentrations of primers for hNIS and 18s were 300nM forward and 50nM reverse and 100nM forward and 100nM reverse for E1A). In each reaction 19.4 μl of reverse transcribed product was used or 50ng of total genomic DNA for extracts from cell monolayer or 500ng for extracts from tissue diluted in DEPC water to a total volume of 19.4μl. 32.3μl of SYBR® Green mix and 12.9μl of primer mix was added to obtain a total volume of 64.6μl. Optimisation of primer concentrations for hNIS and 18s had previously been performed by Thomas Groot- Wassink. Optimisation for E1A primer concentrations was carried out using RT-PCR analysis on a primer matrix using E1A-PCR product with different concentration ratios of forward and reverse E1A primers.

20μl of each sample was loaded in triplicate to a 96-well Optical Reaction plate (Applied Biosystems). Non-template (NTC) wells were added to determine potential template contamination. The plate was sealed with Optical Caps (Applied Biosystems) and quantitative RT-PCR performed on either ABI PRISM 7700 or ABI PRISM 7500 sequence detection systems (Applied Biosystems). Reaction conditions used for hNIS and 18s amplification were 2min @ 95°C, 10min @ 95°C, 40 cycles of 30sec @ 95°C, 1min @ 60°C. Cycling conditions for E1A on ABI PRISM 7700 were 10min @ 95°C, 40 cycles of 10sec @ 95°C, 15sec @ 58°C, 8sec @ 72 °C and on ABI PRISM 7500: 10min @ 95°C, 40 cycles of 10sec @ 95°C.
15 sec @ 58°C, 33 sec @ 72°C. A primer-DNA dissociation stage was performed on each plate after template amplification under the following conditions: 15 sec @ 95°C, 1 min @ 60°C and 15 sec @ 95°C. All RT-PCR analysis was carried out using sequence detection software (SDS) 7500 version V.1.3 (Applied Biosystems). The primers used for RT-PCR analysis can be found below (table).

**Table 2.5:** Quantitative real-time PCR primers: primer names, description and sequence are shown for hNIS, E1A and 18S. F, forward; R, reverse.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Description</th>
<th>Sequence 5'-3'</th>
</tr>
</thead>
<tbody>
<tr>
<td>P10</td>
<td>hNIS RT-PCR-F</td>
<td>TCATGTTTGTGTTTCTACACTGAC</td>
</tr>
<tr>
<td>P11</td>
<td>hNIS RT-PCR-R</td>
<td>TGCTGAGGTTGGGCACTGAAG</td>
</tr>
<tr>
<td>P12</td>
<td>E1A-F</td>
<td>CCTGCTGCTAGGACATGCAA</td>
</tr>
<tr>
<td>P13</td>
<td>E1A-R</td>
<td>ACAGCTCAAGTCCAAAGGTT</td>
</tr>
<tr>
<td>P14</td>
<td>18S-F</td>
<td>CGCCGCTAGAGGTGAATT</td>
</tr>
<tr>
<td>P15</td>
<td>18S-R</td>
<td>CATTCTGGAATGCTTTCG</td>
</tr>
</tbody>
</table>

### 2.12. Nucleic acid extraction from adenovirus infected cells

#### 2.12.1. Total RNA extraction

Extraction of total RNA from cells was carried out using Trizol® Reagent (Invitrogen™) according to the manufacturer’s guidelines. Total RNA was then used to perform reverse transcription and RT-PCR for target genes to quantify relative gene expression at a specific time.

Cells were seeded with a density of 2x10^5 cells per well in 6-well plates (immortal cell lines) or 12-well plates (primary cells) with the appropriate medium [E4 supplemented with 10% FCS for cell lines, RPMI-1640 (developed by Moore et. al. at Roswell Park Memorial Institute) 2% supplemented with 20% FCS for primary cells] and incubated under standard conditions at 37°C. The following day, cells were infected with the required adenovirus(es) as described before. Depending on the experiment total RNA extraction was performed 1-2-3-4-5 days post infection.

The medium was aspirated from the cells and the cells washed with warm PBS. PBS was aspirated and under a fume hood 1 ml Trizol® was added to each well to lyse the cells. The total RNA was then recovered by following the manufacturer’s protocol. Samples were stored at -20°C until required.
Extraction of total RNA from animal tissue was performed with the RNeasy Kit (Qiagen) according to the manufacturer's guidelines. Fresh samples were stored in RNAlater RNA Stabilization Reagent (Qiagen) at -20°C until extraction was performed. RNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc). RNA quality was verified by gel electrophoresis (see below).

2.12.2. Total DNA extraction

Total DNA was extracted from cells using the QIAamp DNA Micro Kit (Qiagen). The protocol used was a modified version of the DNeasy tissue Kit (Qiagen) protocol. Cells were seeded and infected as described above for RNA-extraction. On the day of collection, the medium was aspirated and the cells washed with warm PBS. Cells were detached with 300µl trypsin and the trypsin was neutralised by gently resuspending the cells in 1ml of warm medium. Cells were transferred to a sterile eppendorf tube and centrifuged at room temperature for 5min at 300 x g. The medium was aspirated and the cells resuspended in 200µl PBS. 20µl of proteinase K and 200µl of Buffer AL (both provided in the kit) were added to the sample, mixed thoroughly by vortexing and incubated at 70°C for 10 minutes. 200µl of ethanol (96-100%) was added to the sample and mixed thoroughly by vortexing. The tubes were briefly centrifuged to remove drops from the lids and the mixture of each tube was transferred to a QIAamp MinElute Column. Columns were spun for 1min at room temperature at 8000rpm. The column was then transferred to a clean 2ml collection tube (provided by the manufacturer) and the collection tube containing the flow-through discarded. 500µl of buffer AW1 was added to the column and the column spun at room temperature at 8000rpm for 1min. The column was transferred to a clean collection tube and the collection tube containing the flow-through discarded. 500µl of buffer AW2 was added and the column spun at room temperature at 8000rpm for 1min. The column was transferred to a clean collection tube and the collection tube containing the flow-through discarded. The columns were then spun at room temperature for 3min at full speed to dry the membrane.
completely. The QIAamp MinElute Column was transferred to a clean 1.5ml eppendorf tube and 20μl of buffer AE was added to the centre of the membrane. incubated at room temperature for 5min and then spun at full speed for 1min. This step was repeated so a final volume of 40μl DNA was obtained for each sample. Total DNA was extracted from tissue using the DNeasy Tissue Kit (Qiagen) following the manufacturer’s guidelines. DNA concentrations were determined using a NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies Inc).

2.13. Viral replication kinetics

2.13.1. By titration

In order to assess replication competence of the viruses produced, compliant cells were infected with virus at a low MOI so the cells could survive long enough in order for the virus to be able to replicate within. SW480/SW620 (Wnt-positive colonic cancer cells) cells were seeded in 6-well plates and incubated for 24 hours under standard conditions. The following morning the 6-well plates (3 wells per virus per day) were infected with Adenovirus (AdIP1 and AdIP2) at MOI 0.01 as described above and incubated under standard conditions at 37°C. Cell homogenates from three individual wells for each virus were collected every day for 7 consecutive days by scraping the plates and transferred to a 15ml sterile Falcon tube. The collected homogenates were stored at -80°C until required for titration. The viral titre of each cell homogenate was determined by TCID50 method in CYMR293 cells as described above. Cell homogenates were freeze-thawed three times before serial dilutions were made to infect 96-well plates for titration.

2.13.2. By qPCR for E1A

Viral replication was also evaluated by extracting total RNA (gene expression) and total DNA (copy number of genes) from infected cells at different time points and performing real time PCR for E1A. The methods employed are described above. It was assumed that an increase in gene expression or copy number signified
replication of virus. A non-replicating E1A-deleted virus (Ad10) was used as negative control.

2.14. RNA gel

The quality of RNA extracts was verified by running them on a 1% agarose (w/v)/formaldehyde gel. The gel was made by mixing 1% agarose with 1x running buffer (RB) at pH7 [3-(N-Morpholino)propanesulfonic acid (MOPS) 25mM, anhydrous Na-Acetate (CH$_3$COONa) 6.25mM, ethylene-diamine-tetraacetic acid (EDTA) 0.125mM pH8 in DEPC water] and 7.5% formaldehyde 37-40%. Samples were mixed with three volumes of sample buffer [1x RB, 50% ultrapure formamide, 5.5% formaldehyde 37-40%, 20% Bromophenol Blue (10mg/ml), 1% Ethidium Bromide (10mg/ml) in DEPC water]. The gel was run at 80V in 1x RB and the bands checked under UV light when opportune.

2.15. Immunofluorescence of adenoviral proteins

2.15.1. Immunocytochemistry

Immunocytochemistry was used to determine whether the cells infected with Adenovirus-hNIS (AdIP1 and AdIP2) were expressing the hNIS protein correctly within the cell membrane. Negative controls were non-infected cells and Wild-type Ad5 adenovirus (AdWt). The same method was employed to demonstrate the selectivity of the Wnt-hNIS adenovirus (AdIP2) for Wnt-positive cells. Negative controls were non-infected cells, a non Wnt-expressing cancer cell line and AdIP4. Cells were seeded onto microscope cover slips in 6-well plates and incubated over night under standard conditions at 37°C. Cover slips were prepared for use as follows: 30min wash in 60:40 ethanol:concentrated HCl, 3x 10min rinse with dH$_2$O and stored in 70% ethanol. The following day, cells were infected with virus at MOI1 as described above and incubated for 24h at 37°C with aeration. The medium was removed, cells were washed with warm PBS and infected cover slips were then fixed with 2ml of 3-4% para-formaldehyde in PBS for 15min at room temperature. The paraformaldehyde was then removed and the cells washed 2x10
minutes with 2ml warm PBS. Cells were permeabilised with 0.1% Triton® X-100 (a non-ionic surfactant) in PBS for 5 minutes at 4°C. Cells were then washed 2 x 10 minutes with PBS after which the primary hNIS mouse antibody Ab1 FP5A (Neomarkers, Lab Vision Corporation) 1:150 in 500μl PBS was added to each well and incubated at room temperature with gentle shaking for 30 minutes. The primary antibody was then removed and the slides washed with PBS for 3 x 10 minutes at room temperature. Secondary antibody, goat anti-mouse IgG (2mg/ml) 488nm Alexa Fluor® Conjugate (Molecular Probes, Invitrogen), a dye which fluoresces around 488nm, was diluted 1:1000 in 5% heat inactivated goat serum in PBS and incubated for 30min in the dark before addition to the wells. Cells were incubated with secondary antibody for 30 minutes at room temperature with gentle shaking. Cells were washed for 3 x 10 minutes with PBS at room temperature after which cells were treated with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen), a blue fluorescent dye that binds to double stranded DNA 1:500 in PBS for 5 minutes at room temperature with gentle shaking. Cells were then washed for 2x 10 minutes with PBS after which the cover slips were removed from the wells, excess liquid removed and mounted on microscope slides with a drop of PermaFluor™ (Thermo Scientific) mounting media. The slides were then stored in the dark at 4°C until the Laser Scanning Microscope (ZeissLSM510 Meta) became available. DAPI was scanned with laser diode 405 (blue) and revealed the nucleus of cells and Alexa Fluor® with Argon/2 laser (green), which revealed hNIS.

2.15.2. AdGFP infection

To determine whether desmoid cells could be infected with adenovirus, infection with a non-replicating Ad5 containing the gene for green fluorescent protein (GFP) was performed in conjunction with flow cytometry analysis for quantification. Infected cells express the GFP gene and auto-fluoresce.

Desmoid cells were seeded in 6-well plates at 2x10⁵ cells per well in RPMI2% medium supplemented with 20% FCS and incubated at 37°C over night under standard conditions. The following day cells were infected with AdGFP (provided by Sandra Martinico) at different MOI and incubated for 48h at 37°C with aeration. Cells were visualised and auto-fluorescence of infected cells detected with an Olympus® CKX41 microscope fitted with a fluorescence illuminator for GFP. Cells
were subsequently treated with trypsin and after neutralisation with growth medium spun for 4min at room temperature at 1200rpm. Cells were resuspended in 400μl FACS Buffer [FCS 20% (v/v), 0.5mM EDTA pH8 in PBS] and stored on ice until required for flow cytometry analysis.

2.16. Flow Cytometry analysis

Flow cytometry analysis was performed on a BD FacsCalibur System (BD Biosciences) for desmoids cells infected with AdGFP and desmoid cells stained with antibodies for CAR, Integrins and CEA and the results analysed with CELLQUESTPro software (BD Biosciences) provided with the machine. Green fluorescing cells were detected with FL1 a blue argon laser (488nm) detecting fluorescein isothiocyanate (FITC) and GFP.

Desmoid cells were seeded in 6-well plates at 2x10^5 cells per well in RPMI2% medium supplemented with 20% FCS, incubated over-night and either infected with AdGFP as described above or stained with antibodies as described by Fuxe et al. (Fuxe et al., 2003).

Cells for staining were collected by trypsinisation, spun down for 4 minutes at room temperature at 1200rpm, the medium removed and the cells washed with pre-cold FACS Buffer. Cells were spun down, FACS buffer removed and the pellet incubated with primary monoclonal mouse antibody 1:500 in FACS buffer containing 5% normal goat serum (Dako Cytonation, Denmark) for 90min on ice. Every 15 minutes the each eppendorf tube was shaken gently. Cells were spun down for 3 minutes at room temperature at 2000rpm and the primary antibody washed away by three wash-spin cycles with pre-cold FACS buffer. After the last wash the pellet was incubated for 30 minutes at room temperature in the dark with secondary antibody goat anti-mouse FITC 1:30 in FACS buffer. The cells were spun down and the pellets washed by three wash-spin cycles. The final pellet was resuspended in 400μl FACS buffer and stored on ice until required. Primary antibodies used were CAR, CEA-CAM6 (both received from Dr. Y.Wang in our laboratory), Integrin Alpha-V (1mg/ml) (abcam), Integrin AlphaV/Beta3
(Chemicon® International) and Integrin AlphaV/Beta5 clone P1F6 (obtained from the Tumour Biology laboratory).

### 2.17. Establishment of primary cultures of desmoid cells

Desmoid tumour cells were obtained following surgical resection. The protocol was used in conjunction with the ethical approval from the Harrow or Leuven (Belgium) Research Ethical Committee. The tumours were macerated in small pieces with a scalpel and digested over night at 37°C in RPMI containing 20% FBS (AutogenBioclear, Ltd, Calne, UK), 100 units/ml penicillin, 100μg/ml streptomycin, 0.2μg/ml Butyl-p-hydroxybenzoate supplemented with 1mg/ml of Collagenase D (Roche Diagnostic GmbH, Lewes, UK). The digest was then filtered through a 100μl nylon cell strainer and centrifuged twice at 1000rpm. Cell pellets were washed twice with PBS, re-suspended in RPMI 20% FBS containing antibiotics (see above) and seeded. Primary cultures were maintained in a 5% CO₂ atmosphere at 37°C. Erythrocytes and cell debris were washed away with PBS 24h later. Long term stocks of cells were made as described above and frozen at -80°C over night and in liquid nitrogen there after. Germ-line and somatic mutations were identified.

Desmoid 1: β-catenin mutation: substitution TTT for TCT resulting in a Ser to Phe change at codon 45.

Desmoid 2: β-catenin mutation: substitution of TTT for TCT resulting in a Ser to Phe change at codon 45.

Desmoid 3: Mutations in APC or β-catenin not detected.

Desmoid 4: APC mutation: exon 15 c.3927_3931del AAAGA (pGlu1309AspfsX4).


Desmoid 5.2: APC mutation: germline exon 6 c.646 C>T (p.R216X), somatic exon 15H: c.4385delA.

The above described collection of cells and identification of mutations was performed in Leuven for desmoids 1-5 and by Sandra Martinico in our lab for desmoid 5.2. Cryo-preserved samples were shipped to London from Leuven.
3. In vivo investigations

All animal experiments were conducted with appropriate ethical approval and in accordance with the Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 (House of Commons 1990). Heterozygous female Balb-c Nu- mice were used for the tolerability experiment, homozygous female Balb-c Nu/Nu mice (Harlan) or homozygous female CD1 Nu/Nu mice (Charles River) were ordered for all other experiments. Mice were left to recover for one week after transport from the supplier before experiments were initiated. All mice were kept in individually ventilated cages and were only handled under sterile conditions in a safety cabinet. Mice were removed from the IVC cages and transported into the SPECT/CT room where they were kept behind a lead wall until the experiment was finished and mice were terminated. Mice were disposed off after the radioactive decay by passing them through a macerator.

3.1. Tolerability experiment

Heterozygous female Balb-c Nu/- mice were injected intravenously via the tail vein with $10^9$ PFU of virus in 100µl in PBS or with PBS. Six mice were injected with AdIP1, four mice were injected with AdIP and four mice with PBS. After 3 and 6 days respectively three mice were killed for AdIP1 and two for AdIP2 and PBS and the livers were collected and split in half. One half was snap-frozen by dipping it for 5 minutes in isopentane and then for 5 minutes in liquid nitrogen and stored at $-80^\circ$C until immunohistochemistry for hNIS and E1A was carried out on tissue sections (performed by the Institute of Cancer Pathology Services). The other half was stored in RNA later (Qiagen) at $-20^\circ$C until RNA extraction (Qiagen RNeasy kit) and RT-qPCR.

3.2. Generation of subcutaneous tumours

Subcutaneous tumours were created by injection of $2\times10^6$ HCT116 cells suspended in 100µl PBS. Cells were seeded in T175 flasks in E4 medium supplemented with 10% FCS in the tissue culture facility and incubated under standard conditions until
the flasks were 80-90% confluent. On the day of injection, cells were trypsinised and the trypsin neutralised with growth medium. Cells were counted and the pellet was spun for 5 min at 1000 rpm at room temperature. The medium was removed, the cells resuspended in an appropriate volume of PBS and transported on ice to the animal facility. The mice were injected subcutaneously with 100 \mu l containing 2x10^6 cells under sterile conditions in a biology safety cabinet. A 1 ml syringe (BD, Plastipak™) and a 23G needle (BD, Microlance™3) were used. Tumour growth was determined by calliper measurement. After approximately 14 days tumours reached the required size of 5x5 mm.

### 3.3. Adenoviral intra-tumoural injections

When the subcutaneous tumours reached the appropriate size, viruses were injected using Ultra-Fine™ Insulin Syringes (BD). Viruses were defrosted on ice and remained on ice for transfer and until injection. Viruses were diluted to a total volume of 100 \mu l in PBS and injected evenly in the centre of the tumour through one single entry point by moving the needle in all directions. If tumours were small, two doses of 50 \mu l were injected with a 1 hour interval to assure the full dose was delivered inside the tumour. Doses used were 5 x 10^8 PFU and 1 x 10^9 PFU, depending on the experiment. All injections were carried out in a biological safety cabinet.

### 3.4. Non-invasive imaging *in vivo*

Imaging of mice was carried out with a four-headed multiplexing multi-pinhole single-photon emission computed tomography (SPECT), NanoSPECT/CT™ *in vivo* animal imager (Bioscan Inc, Washington D.C.). Acquisition time depended on the specific radioactivity level measured in each mouse. 100,000 counts were required in order to obtain a good quality image.

Mice were transferred to the scanning room and anaesthetised via intra-peritoneal injection (IP) injection for experiments until June 2007 and via gas anaesthesia for experiments thereafter.
**IP injections:** Anaesthesia was induced by administration of 20-40μl of a Ketamine (100mg/ml):Xylazine (20mg/ml) 2:1 mixture. When the anaesthesia was deep enough the mouse was positioned on its side and 18.5MBq (=500μCi) of Technetium 99m (Tc$^{99m}$) (obtained from Barts Hospital, London) in a volume of 100μl was injected intra-venously (IV) via the tail vein. The mouse was then wrapped in cling film to keep her warm and positioned in a prone position on the bed of the NanoSPECT/CT™ *in vivo* animal imager and a scan performed. When the scan was finished, the animal was transferred to an individual cage positioned under a heating lamp and left to recover before it was allowed to join its litter-mates.

**Gas anaesthesia:** Each mouse was first placed in a restrainer and 18.5MBq of Tc$^{99m}$ in a volume of 100μl was injected IV in the tail vein. The mouse was then moved to the ventilated chamber of an anaesthetic machine with a non-rebreathing circuit for induction. The gas-oxygen mixture for induction was: Halothane: dial setting 4, N$_2$O 600ml/min, O$_2$ 1l/min. The animal was then transferred to the bed of the imager and a nose cone applied. The maintenance anaesthesia was: Halothane: dial setting 2, N$_2$O 600ml/min, O$_2$ 1l/min. After the scan was performed, the mouse was transferred to an individual cage heated with a warm lamp and allowed to recover. Once recovered the animal was allowed to join its litter-mates.

Animals that were scanned on five successive days were kept in a lead-shielded cage until the next scan was performed. After the last scan the mouse was culled while still under influence of the anaesthetic.

Animals that were scanned only once were culled while still under the influence of the anaesthetic and the tumour collected. Half of the tumour was kept at -20°C until DNA-extraction with a DNEasy Kit (Qiagen) and the other half was stored in 10% formaldehyde to be processed for immunohistochemistry (Pathology Services).

After acquisition, the data obtained from the NanoSPECT/CT™ *in vivo* animal imager were reconstructed using the HiSPECT software (Bioscan Inc., Washington D.C., USA) and Interview XP® (Mediso Ltd., Budapest, Hungary) provided with the machine. Post-processing software used to fuse SPECT and CT images was
3.5. Radiotherapy

The therapeutic experiment including radiotherapy with $^{131}$I was carried out in the Departamento de Bioquimica, Facultad de Ciencias Biosanitaria, Universidad de Francisco Vitoria, Madrid, Spain and repeated in INSERM, CIC-04 Biothérapies Hepatiques, Université de Nantes, France.

Mice were injected with colon cancer cells HCT116 to create subcutaneous tumours (method described above). When the tumours reached the appropriate size, the mice were given intra-tumoural injections of adenovirus as described above. Mice were fed levo-thyroxine (5mg/ml) in their drinking water in order to suppress thyroidal iodine uptake. After 48h, mice in the treatment arm were injected IP with 3mCi (111MBq) $^{131}$I according to the local institute radiation safety guidelines. Irradiated mice were kept separate from the control mice and were shielded with lead plates. They were kept in isolation until the radioiodine had sufficiently decayed. Tumour size was assessed by calliper measurements in two dimensions every other day. Mice were culled when tumour size reached the limit of 144mm$^2$, which is the maximum allowed by the Home Office regulations.

4. Statistical analysis

All graphs were created and analysed with using GraphPad Prism 4 software (GraphPad, San Diego, C.A., USA).

4.1. t-test

The $t$-test was used was chosen for biological data when they were expected to follow an approximately Gaussian, bell-shaped distribution. A two-tailed $p$-value was chosen because it was assumed that for every of the two groups being compared either could have the largest mean.
4.2. Kaplan-Meier percentage remaining curves

Percentage remaining curves presented were compared by generating a p-value testing the null hypothesis that the intervention did not alter survival and therefore that the survival curves are identical in the populations. Each analysis asks whether intervention $a$ alters survival compared to intervention $b$ as well as asking whether intervention $b$ changes survival compared to intervention $a$. Median survival is the time at which half the mice have died.

4.3. ANOVA one way analysis

Anova one way analysis of variance was used to test for differences between two or more treatment groups.

Similarly, for the comparison of means of three or more treatment groups where the data was normally distributed, one way analysis of variance (ANOVA) was performed to test for significance in variation between these treatment groups. ANOVA utilizes positively skewed f distribution and works better in reducing the type 1 error (false positive) in multiple comparisons of means.
CHAPTER 3: Results

1. Molecular biology

1.1. Introduction

The aim of this PhD project was to incorporate the cDNA of the human NIS into the genome of a Wnt-specific oncolytic adenovirus. This virus, AdKH1, was donated by Dr. Richard Iggo and had previously been characterised and its specificity and selectivity determined (Fuerer and Iggo, 2002).

We chose to incorporate the hNIS cDNA into the E3 region of the adenoviral genome. Deleting E3-6.7k and E3-gp19k creates the necessary space to introduce the hNIS cDNA and has the additional benefit of increasing the antitumour activity in immuno-competent tumour models (Wang et al., 2003b).

We opted for homologous recombination in yeast by "pop-in"/"pop-out" replacement as the method of gene transfer for its high recombination efficiency (Rothstein, 1991) and because of the previous experience with this technique in our laboratory (Vassaux et al., 1997), (Vassaux and Huxley, 1997).

Homologous recombination is very efficient in yeast when a DNA fragment containing free ends is introduced into these cells and if a homologous sequence is already present. To create these free ends in the shuttle vector, a unique restriction site is required and the homologous recombination will be directed at this exact site, leading to the integration of the whole shuttle vector in the targeted site. This event is selected by the presence of an URA-3 gene in the shuttle vector that will provide auxotrophy for uracil in the previously uracil-dependent yeast. In our case, a unique restriction site is present in the ADP sequence, allowing integration into the adenoviral genome already present in the yeast (see figure 3.1A).

The strategy of this system is to introduce a transgene (hNIS) into a region of interest (adenoviral E3 region). The transgene is cloned into a shuttle vector containing the URA-3 gene. When creating the shuttle vector, it was important to leave as much homology as possible on both sides of the insertion. It was decided to flank hNIS by E3-12.5 and E3-ADP. The restriction site for targeting should be
between the insertion site (hNIS) and the shortest stretch of homology (ADP) to increase the probability that the pop-out event will occur in the longer stretch of homology (E3-12.5), resulting in successful replacement (Rothstein, 1991). E3-ADP contains the unique restriction site NotI. The adenoviral plasmid contains a HIS gene and can be linearised by PacI.

---

**Figure 3.1A**: The yeast containing the adenoviral genome (either wild-type or AdKH1) is selected for growth on plate without histidine. This yeast is transformed with the shuttle plasmid linearised in the ADP region. The resulting recombinants are selected onto plates without uracil and Histidine. The most frequent event is a duplication containing the wild type and the mutant copy of the gene flanked by the plasmid sequences. This is called "pop-in".
**1.2. Construction of shuttle vectors**

E3-12.5 and E3-ADP DNA was amplified by PCR from the plasmid pMB20 using the Opti-Prime PCR Optimisation Kit (Stratagene). The PCR products were cloned into the commercial plasmid pCR®-T7/CT-TOPO (Invitrogen, USA) (see appendix for map). The resulting plasmids were called pIP1 for E3-12.5-TOPO and pIP2 for E3-ADP-TOPO. Glycerol stocks were made of each bacteria clone containing the plasmids.
Figure 3.2: On the left hand side an agarose gel showing the PCR product of E3-12.5 together with a clone of E3-ADP-TOPO digested with XbaI and HindIII, which excises the cloned product from the backbone vector. On the right hand side an agarose gel showing the selected E3-12.5k-TOPO clones. Correct clones (2, 8, 11, and 12) were sequenced and clone 12 contained the correct sequence and was used for large-scale DNA purification.

To facilitate molecular biology manipulations, the hNIS cDNA was amplified by PCR from the plasmid pTGW10 (Groot-Wassink et al., 2002) by Opti-Prime PCR Optimisation Kit (Stratagene) and cloned into plasmid pcDNA3.1/V5-His-TOPO (Invitrogen, USA). Functional clones were selected using an iodide uptake assay after transfection into 293 mammalian cells. As a positive control pTGW10 was used and pIPl as a negative control. An iodine uptake assay was performed after 24 hours and the clones showing the highest iodine uptake (1 and 21) were transformed into chemo-competent DH10B bacteria. Large scale DNA purification (maxi Kit, Qiagen) was performed and glycerol stocks were made. The plasmid was called pIP3.

Figure 3.3: Example of agarose gel on which hNIS-TOPO clones were run. The clones containing the insert were used for transfection into CYMR293 cells and iodine uptake assay.
Figure 3.4: Iodine Uptake Assay performed on CYMR293 cells transfected with hNIS-TOPO clones. Clone 1 and Clone 21 have the highest iodine uptake and have been selected for large-scale DNA purification.

Subsequently pIP1, pIP2 and pIP3 were sub-cloned into the pGV1 (for map see appendix) to create the shuttle vector.

(1) In the first step, a SacII/XbaI-digested fragment of pIP1 containing the E3-12.5k gene was ligated into SacII/XbaI-digested pGV1 backbone to create pIP4. Confirmation digests were performed and a correct clone selected for large-scale DNA purification to be used for the next cloning step. Glycerol stocks were made.

Figure 3.5: Digestion of selected miniprep clones with XbaI, linearises the shuttle vector and should only represent one band on the agarose gel of approximately 5kb (pGV1+E3-12.5k).
Figure 3.6: A. Representation of pIP4 showing the restriction sites. B. Further verification of correct clones was performed by multiple restriction digestions.

![Figure 3.6](image)

(2) In a second sub-cloning step, a KpnI/XhoI fragment of pIP2 containing the E3-ADP gene was ligated into KpnI/XhoI-digested pIP4 to create pIP5. Restriction digestion was performed and the correct clone selected for large-scale DNA purification (Maxi Kit, Qiagen). Glycerol stocks were made.

Figure 3.7: A. Representation of pIP5 showing restriction sites. B. Digestion of the selected clone with NotI, which is a unique restriction site and linearises the vector, with SacII, which cuts out the MCS and part of the inserted DNA fragments and uncut pIP5 DNA to compare.

![Figure 3.7](image)
A previous cloning attempt digesting the backbone with XhoI/XbaI failed so we opted to blunt both ends by digestion with blunting restriction enzymes. An EcoRV/XbaI fragment of pIP3 containing the hNIS gene was ligated into SmaI/XbaI digested pIP5 to create pIP7. Confirmation digests were performed and a clone was selected and large-scale DNA purification performed. Glycerol stocks were made.

Figure 3.8: A. Map of pIP7 showing restriction sites and inserts, B. Restriction digestion with multiple enzymes to confirm correct clones were selected.

1.3. Transformation in yeast

The plasmids containing the adenoviral genome (pMB20 and vpKH1) were transformed into yeast yPH587 using the Alkali-Cation Yeast Transformation Kit (Q.Biogene). The yeasts were plated onto -HIS selective plates (adenoviral plasmids express the HIS-gene). Individual colonies were selected and glycerol stocks were made. Yeasts were called yMB20 (Wild-Type Adenovirus-5 genome) and yKH1 (Wnt-specific Adenovirus-5 genome).

In a second step, pIP7 linearised to direct integration was co-transfected into the yeast using the same method and plated onto -HIS -URA plates (pIP7 contains the URA-gene). These intermediate constructs contain a duplication of the target sequence and this step is called “pop-in”. To verify and select correct clones, DNA was extracted from the individual yeast colonies (see M&M) and reference plates
were made. DNA was then transformed into commercial DH10B electro-competent bacteria (ElectroMAX™) and plated onto LB agar plates containing Chloramphenicol (Chl) (12.5μg/ml). Individual colonies were selected from these plates and DNA extracted (see M&M). DNA was digested with restriction enzymes XbaI/XhoI and NotI and run on a 0.8% agarose gel.

Clones containing the duplication pMB20/vpKH1 and pIP7 were selected.

**Figure 3.9:** A. Digestion with XbaI/XhoI: comparing restriction digestion profiles of pIP7 and vpKH1 with the individual clones. Backbone and insert of pIP7 are both present in the selected clones. B. Digestion with NotI, a unique restriction site, which linearises the shuttle vector. Restriction digestion profiles are compared and should not differ.

The selected clones were taken from their yeast reference plates and grown over night in -HIS broth. (This removes the selective pressure for URA-3). The following day cultures were plated onto -HIS plates containing 5-FOA (1mg/ml) and the plates incubated at 30°C until colonies appeared. Individual colonies were selected and reference plates were made. DNA was extracted from individual clones and transformed into commercial electro-competent DH10B bacteria, which were grown on Chl LB agar plates. DNA was extracted from individual bacterial colonies and digested with XbaI + XhoI and NotI and restriction digestion profiles of the clones compared those of pMB20 and vpKH1.

The selected clones were called pIP8 for wild type adenoviral-hNIS plasmid (pMB20-hNIS) and pIP9 for Wnt-specific adenoviral-hNIS plasmid (vpKH1-hNIS).
Figure 3.10: A. Restriction digestion with XbaI-XhoI. Selected clones contain the mutation (hNIS), but not the backbone of the shuttle vector. Restriction digestion profiles match the profiles of parent adenoviral plasmids. B. Restriction digestion with NotI: Restriction digestion profiles of selected clones match the profiles of parent plasmids and do not contain the shuttle vector.

The same yeast transformation process was repeated with pIP5 (hNIS-negative) as a shuttle vector. Adenoviral plasmids created with this vector contain a deletion of the E3-6.7k and E3-gp19k, which lie between E3-12.5 and E3-ADP. “Pop-in” bacterial clones were screened by restriction-digestion with XbaI/XhoI and NotI as described before and “pop-out” bacterial clones were screened by PCR. Primers used were P3 and P2 (see M&M). The reaction was optimised with the Opti-Prime PCR-optimisation kit. The reaction conditions were the same as for ADP-TOPO cloning (see M&M). The amplified samples were run on a gel and the clones with the deletion selected. Plasmids were called pIP10 for the wild type adenoviral plasmid with E3-Δ6.7k, Δgp19k (parent pMB20) and pIP11 for the Wnt-specific viral plasmid with E3-Δ6.7k, Δgp19k (parent vpKH1).
1.4. Generation of adenoviruses

The plasmids vpKHI, pIP8, pIP9, pIP10 and pIP11 were transfected into CYMR293 cells. The viral amplification and purification protocol and the titration method are described in the M&M section. All viruses obtained were stored at -80°C in 100μl aliquots. Viruses were called AdIP1 (Wild type-hNIS), AdIP2 (Wnt-specific adenovirus-hNIS), AdIP3 (Wild type-Δ6.7k, Δ gp19k), AdIP4 (Wnt-specific adenovirus- Δ6.7k, Δ gp19k) and AdKH1.

An aliquot of wild type Adenovirus 5 was obtained from Claudia Montiel (Molecular Oncology Unit) and an aliquot of Ad10 (non-replicating adenovirus with CMV promoter driving hNIS) from Thomas Groot-Wassink (Groot-Wassink et al., 2002). These aliquots were also amplified by direct infection of T175 flasks containing CYMR293 cells. Cell homogenates from these flasks were used to infect CF10 cell factories. The viruses were used as controls in cell assays.

A few aliquots of AdLacZ, a non-replicating adenovirus where the CMV promoter drives the expression of the β-galactosidase gene were obtained from Sandra Martinico in our laboratory.
2. *In vitro analysis of constructed viruses*

2.1. **Comparison of the cytotoxic effects of the different viruses**

2.1.1. **AdWt compared to AdIP1 (AdWt-hNIS) and AdIP3 (AdWt-Δ6.7kΔgp19k)**

Cell survival assays were performed to assess the cytotoxic properties of the mutant viruses in comparison to the wild type virus. Assays were performed on a variety of cell lines in quadruplicate. The data presented are representative of three independent experiments. Statistical analysis was performed with the two-tailed unpaired t-test. Differences are significant when $p<0.05$.

The viruses AdWt, AdIP1 and AdIP3 were compared and it was evaluated whether the mutant viruses were able to kill cancer cells to the same extent as wild-type virus.

From these assays it was observed that insertion of the hNIS gene appears to compromise the cytotoxic effect of the adenovirus. In the cell lines represented, cell death is significantly less ($p<0.001$ at MOI 1 in all cell lines except MCF-7) in the hNIS-containing virus pIP1 as compared to the wild-type virus and the virus with the deletion in the E3 region (pIP3). Deletion of E3-Δ6.7k, Δgp19k does not seem to affect the cell-killing potential of the virus in colon cancer cell lines with Wnt activation. However, in other non-Wnt-activated cancer cell lines deletion of E3-6.7k and E3-gp19k affects the toxicity of the virus and makes it less powerful.

We conclude that the insertion of hNIS or deletion of 6.7k and gp19k seems to have an effect on the cytotoxic potential of the virus.

Cell lines SW480 and SW620 are related human colorectal cancer cell lines, which were derived from the primary tumour and a lymph node metastasis respectively from the same patient.
Figure 3.12: MTT assay for SW480 (a), SW620 (b), HCT116 (c) colonic cancer cells infected with AdWt, AdIP1 and AdIP3. 100% corresponds to the value obtained in the non-infected control wells. EC50 values are represented in a table with each graph.

a.

**SW480**

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<td>AdIP3</td>
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b.

**SW620**

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c.

**HCT116**

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<tr>
<td>AdIP3</td>
<td>1.11</td>
</tr>
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</table>
Figure 3.13: MTT assay for HPAF (a) (pancreatic), SKBR3 (b) (breast), HeLa (c) (cervical) cancer cells infected with AdWt, AdIP1 and AdIP3. 100% corresponds to the value obtained in the non-infected control wells. EC50 values are represented in a table with each graph.

a. 

![Graph of HPAF cells](image)

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b. 

![Graph of SKBR3 cells](image)

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c. 

![Graph of HeLa cells](image)

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<td>AdIP3</td>
<td>4.09</td>
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HT29 is a colonic cancer cell line and MCF-7 a breast cancer cell line. Adenoviral infection increases the intensity of the MTT signal and appears to stimulate cell growth at non-lethal multiplicities of infection. Other investigators in our laboratory have described this before (Claudia Montiel-Equihua, PhD, 2006) but to our knowledge the mechanism behind it has not yet been elucidated. It is beyond the scope of this thesis to investigate this further, but it could be that viral infection increases the metabolic activity in the infected cells and as a result leads to a higher MTT signal. The increased intensity measured may thus not be due to cell duplication or cell growth but merely reflect increased mitochondrial activity. If this were the case, viral infection may not lead to tumour growth in vivo but rather tumour swelling before cell death and tumour destruction occurs.

Infection of these cell lines with AdWt, AdIP1 and AdIP3 at different MOI shows that insertion of the hNIS gene into the E3 region reduces the potency of the virus compared to wild type. In the breast cancer cell line the deletion in the E3 region also results in reduced efficacy.

Figure 3.14: MTT assay for HT29 (a) (colonic) and MCF-7 (b) (breast) cancer cells infected with AdWt, AdIP1 and AdIP3. 100% corresponds to the value obtained in the non-infected control wells. EC50 values have not been calculated due to the shape of each graph.
2.1.2. AdWt compared to AdIP1 and AdIP2

In a further series of MTT assays in non-permissive cell lines we verified whether the insertion of hNIS into the Wnt-specific adenovirus affected the selectivity of the virus to replicate only in Wnt-activated cells. Assays were performed in quadruplicate and the data presented are representative of at least two independent experiments. HPAF is a pancreatic cancer cell line, MCF-7 and SKBR-3 are breast cancer cell lines and HeLa is a cervical cancer cell line.

AdIP2 kills cells significantly less than the wild-type viruses. In HPAF cells the Wnt-specific virus is 25 times less toxic while in SKBR-3 cells and MCF7 cells AdIP2 is more than 100-fold less toxic than wild-type virus. AdIP2 is 100-fold less toxic in HeLa cells.

We observe that the insertion of hNIS preserves the selectivity of the virus for Wnt-activated cell lines.

Figure 3.15: MTT assay for HPAF (a) (pancreatic), HeLa (b) (cervical), SKBR3 (c) and MCF7 (d) (breast) cancer cells infected with AdWt, AdIP1 and AdIP2. 100% corresponds to the value obtained in the non-infected control wells. EC50 values are represented in a table with each graph except for MCF-7 cells due to the shape of the graph.

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<td>AdIP2</td>
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2.1.3. AdWt compared to AdIP1 and AdIP2 in Wnt activated cell lines

In the next experiment we verified the potency of the Wnt-selective virus for Wnt-permissive cell lines. We selected a panel of colonic cancer cell lines and one Wnt-activated, non small cell lung cancer cell line (NSCLC), A549.

Table 3.1 shows the genomic modifications in the different cell lines that result in the activation of the Wnt signalling pathway.

Table 3.1: Genetic profile of Wnt-activated cell lines. LOH = loss of heterozygosity, NL = normal.

References: (Rowan et al., 2000) (Ikenoue et al., 2002).

<table>
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<th>β-catenin</th>
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<td></td>
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<td>APC upregulated</td>
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In colonic cancer cell lines there is a varying effect of the virus on the cells. In SW480, AdIP2 is more potent than AdWt but in other colonic cancer cell lines
AdIP2 is equally or less effective than AdWt. The cell lines SW480 and SW620 have high Tcf activity whereas the cell lines HCT116 and HT29 have weak Tcf activity, which could explain why the viruses have a reduced cytotoxic effect. The same differences have been described by Fuerer et al. (Fuerer and Iggo, 2002). In the NSCLC cell line AdIP2 kills the cells but less effectively than AdWt.

Figure 3.16: MTT assay for SW480 (a), SW620 (b), DLD1 (c), HCT116 (d), CACO2 (e) and HT29 (f) colonic cancer cell lines and A549 (g) NSCLC cell line infected with AdWt, AdIP1 and AdIP2. 100% corresponds to the value obtained in the non-infected control wells. EC50 values are represented in a table with each graph except for HT29 cells due to the shape of the graph.

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<tbody>
<tr>
<td>AdWt</td>
<td>1.33</td>
</tr>
<tr>
<td>AdIP1</td>
<td>Approx. 10</td>
</tr>
<tr>
<td>AdIP2</td>
<td>4.77</td>
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2.1.4. AdIP2 compared to AdIP4

We also verified the effect of hNIS mutation on the potency of the Wnt-selective adenovirus. We therefore compared AdIP2 (hNIS) and AdIP4 (deletion E3-6, 7-gp19k) in permissive and non-permissive cell lines. MTT assays were performed in quadruplicate and repeated at least twice.

In all colonic cancer cell lines presented, deletion of E3-6.7k and E3-gp19k without insertion of hNIS increases the cytopathic effect of the virus.

In HeLa and SKBR3 cells the AdIP4 virus is less potent than the virus containing hNIS.

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<thead>
<tr>
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<th>EC50 (pfu/cell)</th>
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</thead>
<tbody>
<tr>
<td>AdWt</td>
<td>0.44</td>
</tr>
<tr>
<td>AdIP1</td>
<td>6.67</td>
</tr>
<tr>
<td>AdIP2</td>
<td>0.79</td>
</tr>
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</table>
Figure 3.17: MTT assay for SW480 (a), SW620 (b), HCT116 (c) and HT29 (d) colonic cancer cell lines and HPAF (e) (pancreatic), HeLa (f) (cervical), SKBR3 (g) and MCF7 (h) breast cancer cell lines infected with AdIP2 and AdIP4. 100% corresponds to the value obtained in the non-infected control wells. EC50 values are represented in a table with each graph except for HT29 HeLa and MCF cell lines due to the shape of the curve.

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<thead>
<tr>
<th>Virus</th>
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<tr>
<td>AdIP2</td>
<td>0.12</td>
</tr>
<tr>
<td>AdIP4</td>
<td>0.002</td>
</tr>
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</table>

<table>
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<tr>
<th>Virus</th>
<th>EC50 (pfu/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdIP2</td>
<td>0.25</td>
</tr>
<tr>
<td>AdIP4</td>
<td>0.008</td>
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</table>

<table>
<thead>
<tr>
<th>Virus</th>
<th>EC50 (pfu/cell)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AdIP2</td>
<td>4.67</td>
</tr>
<tr>
<td>AdIP4</td>
<td>0.008</td>
</tr>
</tbody>
</table>
2.1.5. Comparing AdIP2 and AdIP4 to the original Wnt-specific virus AdKH1

Deleting the E3-6.7k and E3-gp19k makes the virus significantly more potent than the original Wnt-specific virus without changes in the E3 region. Inserting hNIS into the E3 region reduces the cytotoxic effect of the Wnt virus *in vitro* in permissive cell lines.
Figure 3.18: MTT assay for SW480 (a) and SW620 (b) colonic cancer cell lines infected with AdKH1, AdIP2 and AdIP4. 100% corresponds to the value obtained in the non-infected control wells. EC50 values are represented in a table with each graph.

\[
\begin{array}{|c|c|}
\hline
\text{virus} & \text{EC50 (pfu/cell)} \\
\hline
\text{AdIP2} & 0.12 \\
\text{AdIP4} & 0.002 \\
\text{AdKH1} & 0.04 \\
\hline
\end{array}
\]

2.3. The expression and functionality of the hNIS protein

To evaluate hNIS expression in infected cells, SW620 and HeLa cells were infected with AdIP1, AdIP2, AdIP4 or Ad10 at MOI 10. At 24h after infection, cells were stained for the presence of the hNIS protein. An hNIS-specific signal was observed at the periphery of the infected cells, suggesting that the hNIS is correctly transported to the cell membrane (figure 3.19). The Wnt-selective virus AdIP2 does not show hNIS expression in HeLa cells, which do not have an active Wnt-signalling pathway. A signal is absent in cells infected with AdIP4 which does not have the hNIS (negative control). Ad10 is a non-replicating adenovirus where the CMV promoter drives hNIS (positive control).
Figure 3.19: Immunocytochemistry DAPI (staining the nucleus blue) and hNIS (green) in HeLa cells (top) and SW620 cells (bottom) infected with Ad10, AdIP1, AdIP2 or AdIP4.

To test the functionality of hNIS, HeLa and SW480 cells were infected at different MOI with AdWt, AdIP1, AdIP2 and Ad10. $^{125}$I uptake was monitored 48h after infection in the presence and absence of the hNIS inhibitor sodium perchlorate. A dose-dependent perchlorate sensitive $^{125}$I uptake was seen for all hNIS-expressing viruses.

Figure 3.20: Iodine uptake for HeLa cells (left) after infection with Ad10 (a), AdIP1 (b) and AdIP2 (c) at different MOI and Iodine Uptake for SW480 cells (right) 48h after infection with Ad10 (a), AdIP1 (b) and AdIP2 (c). I = Iodine, P = Perchlorate, a competitive inhibitor of hNIS.
The amplitude of the uptake was similar for Ad10 (CMV promoter driving hNIS expression) and AdIP2. This demonstrates that hNIS expression driven by the endogenous adenovirus E3 promoter can provide high levels of transgene expression.

Reduction of iodine uptake at MOI 100 with viruses Ad10 and AdIP2 is due to cell toxicity. After 48h cells have started dying and no longer take up iodine.

### 2.4. Viral replication

Viral replication was first assessed by titration in CYMR293 cells of cell homogenates collected at different time points after infection. By 48 hours the amount of virus produced in the cells had exceeded the initial input dose of 0.1 pfu /cell. Both the wild-type viruses (AdWt and AdIP1) increase 100-fold between 24 and 48 hours. The Wnt-specific viruses increase less than 50-fold, which may signify that the Tcf promoters are weaker than the wild-type promoters in this colon cancer cell line with weak Tcf activity. Viruses that have insertion of the hNIS gene in the E3 region (AdIP1 and AdIP2) replicate to lower titres than wild-type adenovirus, which might be due to the size of the transgene.
Figure 3.21: Graph showing the titres of AdWt, AdIP1, AdIP2 and AdIP4 cell homogenates on successive days. Cells were infected on day 0 with 0.1 PFU per cell (dotted line).

Even in cells with a high Tcf activity, the AdIP2 virus does not replicate more than approximately 50-fold between 24 and 48 hours (figure 3.22).

Figure 3.22: Virus production in SW620 cells after infection with AdWt and AdIP2 at input dose of 0.01 pfu / cell (dotted line).

The E1A adenoviral gene is transcribed early and is a key mediator of viral replication. The kinetics of the E1A gene were determined by quantitative real-time PCR.
In a first experiment, total RNA (tRNA) was extracted from cells infected with different viruses. Total RNA was reverse-transcribed and quantitative real-time PCR performed. Control virus was Ad10 which does not replicate. The same experiment was repeated but instead of total RNA, total DNA (tDNA) was extracted.

Figure 3.23: Left: real-time quantitative PCR for E1A on total RNA extracts from cells. Right: real-time quantitative PCR for E1A on total DNA extracts from cells. The E1A values are plotted at day 1 and day 3. In all cases except the non-replicating control virus there is an increase in the amount of E1A at day 3 compared to day 1.

In both t-RNA and t-DNA extracts there is an increase in E1A for all oncolytic viruses tested on day 3 compared to day 1, indicating that viral replication ensues. There is no increase in the control virus which is a non-replicating virus.

In the next experiment we evaluated the viral replication from day to day. SW480 cells were infected at MOI 0.01 and total DNA extracted every day. SW480 cells are very sensitive to viral infection and even at MOI 0.01 cells are dying, especially cells infected with AdIP2.
The experiment was repeated and HCT116 cells were infected with AdIP2 at MOI 0.1. DNA was extracted from cell homogenates daily and quantitative real-time PCR performed for E1A and hNIS. The amount of viral gene DNA relative to total DNA continues to increase up to day 5 since there is no cell lysis. It was important to perform this experiment since HCT116 cells will be used in in vivo experiments and the viral dose administered to tumours may not be lethal to all cells within a tumour.

2.5. Selectivity of Wnt-specific viruses for Wnt activated cell lines

To verify whether insertion of hNIS in the E3 region of the Wnt-selective virus does not have a detrimental effect on the selectivity of the virus a Western Blot was performed to detect the expression of the adenoviral E1A protein after of Wnt-
activated and Wnt-negative cell lines. High expression of E1A viral protein was detected in SW480 cells infected with AdIP2 while no E1A expression was detected in Wnt-negative HeLa cells up to at least MOI 10 (figure A). E1A expression in SW480 cells is stronger than in control 293 cells.

The experiment was repeated in HeLa cells alone and AdIP2 virus was compared to AdWt virus (figure B). A small amount of E1A is expressed at MOI 100 in cells infected with AdIP2, which corresponds to the cell killing effect seen in the MTT assays (figure 3.15). No expression of the late adenoviral gene Hexon is detected in HeLa cells infected with AdIP2.

The experiment was repeated in the cell line wi38, which is derived from normal human embryonic lung tissue and the cells are fibroblast-like. There is some expression of E1A at MOI 100 in cells infected with AdIP2 but much weaker than expression in cells infected with wild-type virus.

Figure 3.26: A. Western Blot for E1A and KU70 on HeLa and SW480 cells infected with AdIP2 at different MOI. B. Western Blot for Hexon, KU70 and E1A on HeLa cells infected with AdWt and AdIP2. C. Western blot for E1A and KU70 in wi38 normal human fibroblasts.
3. In vitro analysis of the effect of constructed viruses on primary cells

3.1. Evaluation of cell killing potential

To determine the effect of adenoviral infection on primary desmoid tumour cells, cells from sporadic (Desmoid 1-3) or FAP-related (Desmoid 4, 5, 5.2) desmoid tumours were infected at different MOI with AdIP2 or AdKH1. Primary desmoid cells were obtained from St. Mark’s Hospital (Desmoid 5.2) and the UZ Gasthuisberg, Leuven, Belgium (Desmoid 1-5). The effect of adenoviruses on these cells was assessed by performing MTT assays. Each assay was performed in triplicate.

Figure 3.27: Cell survival assays performed after infection of primary desmoid cells with AdIP2 and AdKH1. Des1 (a), Des 2 (b), Des 3(c), Des 4 (d), Des 5 (e) and Des 5.2 (f). 100% represents the value obtained in non-infected control wells. The dotted line represents 50% cell death.

a.  

![Desmoid 1](image1)  

b.  

![Desmoid 2](image2)  

c.  

![Desmoid 3](image3)  

d.  

![Desmoid 4](image4)
Different levels of sensitivity to oncolytic or wild-type adenoviral treatment (not shown) were observed in the desmoids. The strongest effect was noticed in desmoid 2 (sporadic) > desmoid 5, desmoid 5.2 (familial) > desmoid 3 (sporadic) > desmoid 1 (sporadic), desmoid 4 (familial). Statistical analysis showed a highly significant difference (p<0.0001) between uninfected cells and cells infected with AdKH1 and AdIP2 at MOI 100 for desmoids 2, 3, 5 and 5.2. These results demonstrate that the sensitivity to oncolytic adenoviral treatment is not related to the type of mutation in the Wnt signalling pathway, or the sporadic or familial nature of the desmoid tumours.

3.2. Evaluation of infectivity of primary cells

The infectability of primary cells extracted from the different desmoid tumours was determined by infection with a replication-deficient adenovirus encoding the green fluorescent protein (GFP) and measurement of GFP-positive cells by flow cytometry analysis (figure 3.28).

Figure 3.28: Flow cytometry analysis of GFP positive cells. The infectivity corresponds to the cytopathic effect seen in MTT assays. Ec50 values are presented in the graph.
Figure 3.29: Desmoid cells infected with AdGFP at MOI 100, % of positive cells as measured by flow cytometry (left y-axis and left bar) compared to the amount of dead cells as measured by cell death assay (right y-axis and right bar).

The range of infectability is similar to that obtained for the sensitivity to adenoviral infection.

3.3. Evaluation of viral replication in desmoids cells

To determine whether the reduced cell viability observed upon adenoviral infection of cells from desmoids 2, 3, 5 and 5.2 was associated with viral replication, DNA from cells infected with AdIP2 was collected one day or four days after infection. This DNA was subjected to quantitative PCR to titrate the amount of adenoviral E1A DNA compared to total cell DNA. In all cases amplification of the adenoviral genome was observed between day 1 and day 4, suggesting replication of the oncolytic virus in these cells (figure 3.30).

Figure 3.30: qPCR for E1A in DNA extracts from primary desmoid cells. Des1 was infected at MOI 10, all other desmoids at MOI 5 because at MOI 10 cell death was apparent after 4 days.
3.4. Evaluation of a panel of known adenoviral receptors on cell membranes

I wanted to verify whether there was a correlation between the infectability of the cells and the expression of CAR receptor and integrins on the surface of the cells. The coxsackie and adenovirus receptor (CAR) has been shown to act as a primary receptor to adenovirus and to play a critical role in viral entry into the cell. Surprisingly, CAR staining was negative in all desmoid cell samples. This is in sharp contrast to the highly positive A549 cells (97.16%) used as a positive control and known to express high levels of CAR.

Integrins play a role in the internalisation of adenovirus and gene delivery to the cell. The main integrins that play a role in adenoviral infection are αvβ3 and αvβ5.

Figure 3.31: Desmoid cells were stained with antibodies against adenoviral CAR-receptor, integrins and CEA-Cam6, which are all known to influence adenoviral uptake into cells. The percentage of cells positive for each receptor was measured with flow cytometry.

<table>
<thead>
<tr>
<th></th>
<th>Desmoid 1</th>
<th>Desmoid 2</th>
<th>Desmoid 3</th>
<th>Desmoid 4</th>
<th>Desmoid 5</th>
<th>Desmoid 5.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAR</td>
<td>1.16</td>
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<td>1.89</td>
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</tr>
<tr>
<td>CEA</td>
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<td>AlphaV</td>
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<td>AlphaVBeta3</td>
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<td>56.09</td>
<td>0.97</td>
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<td>2.14</td>
<td>1.85</td>
<td>3.22</td>
<td>9.66</td>
</tr>
</tbody>
</table>

There does not appear to be a correlation between the infectability of cells and the expression of CAR and/or integrins on the surface of the cell membrane suggesting that a CAR- and integrin-independent mechanism is responsible for entry of the virus into the cells.

3.5. Additional toxic effect of the hNIS transgene

In cells from desmoids 2, 5 and 5.2 infected at MOI 100 the AdIP2 hNIS-positive virus is statistically more efficient (p<0.0001) than the AdKH1 (hNIS negative) virus (figure 3.27). To determine whether the presence of hNIS in the viral genome and its expression are toxic for desmoid cells, independently from the oncolytic
action of the virus, primary cells were infected at MOI 100 with a replication-deficient recombinant adenovirus encoding either the β-galactosidase gene (AdLacZ) or hNIS under the control of the immediate early CMV promoter (Ad10). The toxicity of Ad10 is not observed with AdLacZ suggesting a toxic effect of the hNIS gene product in desmoid tumour cells (Figure 3.33).

Figure 3.32: Effect of replication incompetent adenoviruses Ad10 and AdLacZ on desmoid tumour cell survival. Desmoid tumour cells were infected at MOI 100 with replication incompetent adenoviruses encoding lacZ or hNIS. The number of surviving cells was assessed after 6 days using MTT assay. 100% corresponds to the number of cells in control non infected wells. Statistical analysis was performed using a two-tailed student t-test. P values are < 0.0001 in all the conditions compared.
4. In vivo analysis of constructed viruses

4.1. Toxicity of the virus

Heterozygous balb-c nude (Nu/-) mice were injected intravenous with PBS, AdIP1 or AdIP2. Livers were collected on day 1 and day 5 and half of the tissue was stored in “RNA later” and the other half was snap-frozen and sent to the pathology unit where H&E staining and immunohistochemistry for E1A were performed. Although the sample size of this experiment (two-three mice per group) is not large enough to perform statistical analysis, the results are intriguing. Repeating this experiment would also pose ethical problems as one of the viruses is evidently toxic for the mice.

All animals injected with AdIP2 remained healthy throughout the study. Mice injected with AdIP1 had either died or were seriously ill by day five. H&E staining showed a normal liver architecture for all animals injected with AdIP2, although there is evidence of a mild inflammatory infiltrate. The liver architecture of mice injected with AdIP1 was seriously damaged and even destroyed (diffuse necrosis) and there is massive infiltration of inflammatory cells. Staining for E1A showed a few positive cells in the liver of mice injected with AdIP2. The livers of mice injected with AdIP1 showed massive, diffuse staining for E1A. Real time PCR for E1A was performed on the DNA extracts from liver tissue. The amount of E1A present in mouse liver for each condition correlates with the amount seen in immunohistochemistry. These results are comparable to the ones seen by Homicsko et al. after IV injection of Wnt-specific viruses compared to Wild-type virus (Homicsko et al., 2005).
Figure 3.33a: Immuno-histochemistry of liver tissue for E1A (top) compared to H&E staining (bottom) for mice injected with PBS, AdIP1 and AdIP2 at 48h. The arrows point out cells staining for E1A. Arrows = cells staining positive for E1A.
Figure 3.33b: Immuno-histochemistry of liver tissue for E1A (top) compared to H&E staining (bottom) for mice injected with PBS, AdIP1 and AdIP2 at 120h. The arrows point out cells staining for E1A. Arrows = cells staining positive for E1A.
Figure 3.33c: Real-time PCR for E1A on the DNA extracted from the livers of mice injected with PBS, AdIP1 and AdIP2. Next to the graph are the exact quantities with standard deviation.

![Graph showing real-time PCR results](image)

<table>
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<th>DNA (fg/AU/100ng total DNA)</th>
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<tbody>
<tr>
<td>PBS</td>
<td>4.51e-06 +/- 0.0000008</td>
</tr>
<tr>
<td>AdIP1-48h</td>
<td>0.11 +/- 0.003</td>
</tr>
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<td>AdIP2-48h</td>
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<tr>
<td>AdIP1-120h</td>
<td>0.027 +/- 0.0006</td>
</tr>
<tr>
<td>AdIP2-5</td>
<td>0.00021 +/- 0.000016</td>
</tr>
</tbody>
</table>

### 4.2. Molecular imaging

Subcutaneous xenografts of the colonic cancer cell line HCT116 in nude mice were injected with adenovirus at a dose of $5 \times 10^8$ PFU on two consecutive days. Mice were anaesthetised and scanned on day 1 and day 5.

Figure 3.34 shows representative images of hNIS expression 24h after the last injection of virus. A signal is detected in the thyroid and the stomach as a result of endogenous expression of hNIS and in the tumour. No signal was observed in the liver after intratumoural injection of virus. Five days after virus administration (figure 3.34b) the tumours did not accumulate $\text{99mTc}$, suggesting an absence of hNIS expression. To confirm these data, tumours were collected and immunohistochemically stained with anti-hNIS antibody. Figure 3.34c shows intense brown staining in the tumour at day 1, which had completely disappeared at day 5 (figure 3.34d).
Figure 3.34: hNIS expression in vivo. (a) and (b) nano-SPECT/CT fused images of mice with subcutaneous HCT116 tumours (arrows) were intratumorally injected with $5 \times 10^7$ PFU AdIPI on two consecutive days. A total of 18.5 MBq of 99-mTc was injected i.v. for scanning on day 1 (a) and day 5 (b). (c) and (d) corresponding hNIS immunohistochemistry of tumours ($\times 20$) from (a) and (b) respectively.

Tumours were excised after scanning and stained with hNIS-specific antibody. Strong hNIS staining at day 1 (c) has decreased to non-specific background staining at day 5 (d). The data presented are representative of three animals at each time point. hNIS, human sodium iodide symporter; SPECT, single photon emission computed tomography.
In addition real-time quantitative PCR on total RNA extracts from tumours (experiment performed by Andrew Merron) showed a signal at day 1 that dramatically decreased by day 5 (figure 3.36).

**Figure 3.35**: Quantitative real-time PCR on total RNA extracts from tumours infected with AdIP1 and AdIP2 at day 1 and day 5. The amount of hNIS total RNA is decreased at day 5.

These data confirm that it is possible to image gene expression after adenoviral gene transfer of hNIS with SPECT/CT imaging.

In a next series of experiments we wanted to image adenoviral spread within subcutaneous xenografts in nude mice. A single dose of $5 \times 10^8$ PFU was injected into each of the subcutaneous HCT116 xenografts. The same animals were imaged daily, starting 24h after infection. To obtain a greater resolution and as transgene expression was exclusively localised in the tumour (see previous experiment), the scans were focused on the tumour region. Radiotracer uptake reflecting expression of hNIS transgene can be seen spreading through the tumour for both viruses AdIP1 and AdIP2. The peak of transgene expression as seen by $^{99}$m-Tc uptake was detected after 48h for both viruses (figure 3.36 and 3.37). No signal was detected on scans performed after 8 days (not shown).
Figure 3.36: Successive scans of subcutaneous xenografts injected with a single dose of $5 \times 10^9$ PFU AdIP1. The maximal signal is detected 48h after infection of the tumour.
Figure 3.37: Successive scans of subcutaneous xenografts injected with a single dose of $5 \times 10^8$ PFU AdIP2 virus. The maximal signal is detected 48h after infection of the tumour.

These data confirm that viral propagation can be imaged *in vivo* by SPECT/CT.

Next we wanted to find out whether quantitative information could be obtained from these scans. A region of interest (ROI) was determined and the amount of radioactivity in MBq calculated. The activity measured in the tumour was standardised by dividing it by the amount of activity measured in the background to account for the differences in the administered dose.
Figure 3.38: Determination of the region of interest in the tumour (green) and the background (blue). The software calculates the amount of radioactivity in MBq present in the ROI and also the volume in mm$^3$ (see screen snapshot quantifications). CT = computed tomography, NM = NMR = Nuclear Magnetic Resonance.

These data confirm that the maximum amount of activity can be seen in the tumour 48 hours after injection (figure 3.39). We can therefore conclude that SPECT/CT is able to image viral replication and that quantitative information of viral gene expression can be obtained from the images.
Figure 3.39: Relative intensity of radioactivity as expressed by dividing intensity in the tumour per mm$^3$ by the intensity in the background per mm$^3$.

4.3. Viral replication *in vivo*

Subcutaneous HCT116 tumours were created in nude mice and tumours injected with AdIP2. Scans were obtained at different time points and mice sacrificed after scanning. The tumours were collected and total DNA extracted from half of the tumour. The other half was fixed in formaldehyde. Quantitative real-time PCR was performed on DNA extracts for E1A and hNIS to determine viral DNA and transgene content. Immunohistochemistry for E1A and hNIS was performed on formalin-fixed tissue.

Figure 3.40: SPECT/CT fused image of mouse with HCT116 tumour in the left flank, scanned 24 hours (a), 48h (b), 72h (c), 96h (d) and 120h (e) after intratumoural injection of $5 \times 10^8$ PFU AdIP2. A total of 18.5 MBq of $^{99m}$Tc was injected intravenously approximately 20min before scanning. Below each scan are the corresponding pathological slides (10x) with H&E staining, E1A and hNIS immunohistochemical staining. The data presented are representative of seven animals at each time point.
The intense brown staining is mostly nuclear for E1A and at the plasma membrane for hNIS. The maximum intensity in the scans is after 48 hours of intra-tumoural injection as seen before. The intense staining in the pathological slides remains present until the fifth day. The amount of positive cells is reduced by the fifth day.

Figure 3.41: A. qPCR for E1A of DNA extracts from tumours infected with AdlP2. B. q-PCR for hNIS of DNA extracts from tumours infected with AdlP2.

The amount of E1A and hNIS in the tumour continues to rise until the fourth day and to decline from the fifth day onward.

4.4. Therapeutic experiment virus alone

In a first experiment we wanted to evaluate whether there is a difference in vivo between each of the wnt-specific viruses. Subcutaneous tumours were created in nude mice with HCT116 tumour cells. When the tumours reached a size of approximately 5 x 5 mm², they were injected with either 100µl PBS, 5 x 10⁸ PFU of either virus in 100 µl in PBS on three consecutive days. Tumours were measured every other day for 100 days. Mice were killed when the tumour reached a size of approximately 144mm². The data were plotted as a “% remaining” curve and Kaplan-Meier analysis performed.
Figure 3.42: "percentage remaining" curve. 100% corresponds to all mice alive, 0% = all mice dead. Total number is 6 mice per group. Arrow = intratumoural injection of virus on day 15, 16 and 17. Day 0 = day of injection of $2 \times 10^6$ HCT116 cells.

Kaplan-Meier statistical analysis show that there is a significant difference between non-treated and AdIP2 ($p = 0.0014$) and treatment with AdIP4 and AdIP2 ($p = 0.0055$). The difference between non-treated and treated with AdIP4 is not significant ($p = 0.058$). The Wnt-specific adenovirus containing hNIS appears to be better than the gp19k-deleted mutant in vivo in xenografts in nude mice.

Figure 3.43: Tumour size plotted over time for therapeutic groups ($0 =$ initial size at the time of injection). Injection of PBS or viruses was effectuated at day 14. All non-treated mice (PBS) were dead at day 44. All AdIP4 treated mice were dead at day 50. The last AdIP2-treated mouse was killed at day 113.

Figure 3.43 shows the tumour size plotted against time in days for all three treatment groups. Statistical analysis with ANOVA one-way non-linear regression confirms a statistically significant difference ($p<0.0001$) between treatment groups. Comparing the individual treatments with an unpaired t-test analysis does not show a significant difference between non-treated and AdIP4 but shows a significant
difference (p<0.0001) between non-treated and treatment with AdIP2, and between treatment with AdIP4 and AdIP2 (p = 0.0002). This confirms the data obtained from the survival curve.

4.5. Therapeutic experiment virus and iodine

In the next experiment we want to correlate the data obtained from the imaging with radiotherapy by administration of $^{131}$I.

From the imaging experiments we learned that the time point of maximal viral replication and transgene expression was 48 hours. Subcutaneous tumours were created in nude mice with HCT116 colonic cancer cells. When the tumours reached a size of approximately $5 \times 5\text{mm}^2$ they were injected with one dose of either PBS (100 µl), AdIP2 or AdIP4 ($1 \times 10^9$ PFU in 100 µl in PBS). After 48 hours a single dose of $3\text{mCi}^{131}$I was injected intraperitoneally. Tumour size was measured every other day. Due to logistical circumstances (the host institution was unable to house the mice for longer), mice had to be culled after 8 days. In this pilot experiment there appears to be a difference between treatment with virus alone and treatment with AdIP2 and radioiodine. Statistical analysis performed with one-way non-linear ANOVA analysis shows the difference is not significant between groups. Statistical analysis with unpaired t-test does not show a significant difference between any of the treatment groups.

Figure 3.44: Pilot experiment: tumour size plotted against time for the 8 days post-radiotherapy. There is no statistical difference between treatment groups.
A second therapeutic experiment was performed in France. Due to poor hygienic conditions in the host institution which compromised the health of the experimental animals as well as different housing facilities for radiotherapy-treated and control mice no definite conclusions can be drawn from these results but they are included here for completeness.

Figure 3.45: Mice treated with either PBS, AdIP2, AdIP2 + iodine, AdIP4 and AdIP4 + iodine. Tumour size in mm$^2$ is plotted against time for each treatment.

Statistical analysis with ANOVA one-way, linear analysis shows that there is a statistically significant difference between treatment groups. Due to the fact that irradiated mice were kept in a separate animal house, groups with virus alone can not be compared to groups with virus and radiotherapy for statistical analysis. Statistical analysis with unpaired t-test shows a significant difference between PBS-treated and treatment with AdIP2 ($p = 0.0002$) and between PBS-treated and AdIP4 ($p = 0.01$). The same difference exists between treatment with PBS and AdIP2+iodine ($p = 0.002$) and PBS and AdIP4+iodine ($p = 0.05$). All we can conclude is that treatment with AdIP2 appears to be better than treatment with AdIP4 ($p = 0.0003$) and treatment with AdIP2 and radioiodine is better than treatment with AdIP4 and iodine ($p = 0.0001$).
CHAPTER 4: Discussion

1. Characterisation of the viruses

The hNIS transgene was successfully cloned into the E3 region of replicating adenoviruses. The endogenous gene expression machinery (promoter, polyadenylation and splicing signals) was used to express the foreign gene. The maximum viral genome size that can be packaged is limited to 105% of the wild type adenoviral genome (Bett et al., 1993). The hNIS gene has a size of approximately 2kb and therefore it was necessary to delete adenoviral genes to create the necessary space. The endogenous genes E3-6.7k and E3-gp19k were deleted together as they are adjacent in the adenoviral genome, are encoded by the same mRNA and have overlapping open reading frames. The gp19k protein complexes with the MHC class I molecules and inhibits their transport to the cell surface, preventing the detection of the infected cell by the immune system and thus preventing its destruction and clearance by adenovirus-specific cytotoxic T lymphocytes (CTLs). Malignant transformation of cells is often associated with altered HLA class I expression and/or function (Hicklin et al., 1999). The function of the gp19k gene may thus be dispensable in tumour cells. The E3-6.7k-gp19k region is non essential for viral replication in vitro and in vivo (Ono et al., 2005) (Wang et al., 2003b). It has been demonstrated that when substituting E3-6.7k and E3-gp19k for another gene, transgene expression mimics the substituted endogenous gene (Hawkins et al., 2001). Immunocytochemistry has demonstrated that the hNIS transgene is expressed and correctly presented at the cell membrane in infected cells (figure 3.19) and iodine uptake assays have confirmed that the level of gene expression is comparable to the level seen after infection of cells with a non-replicating virus where the CMV promoter drives gene expression (figure 3.20).

Hawkins et al have also demonstrated that the expression of the surrounding E3 genes can be maintained when substituting E3-6.7k and E3-gp19k for another gene (Hawkins et al., 2001) however, we have not verified this point experimentally. In my constructs the E3-ADP remains intact, this is important since in the late stages...
of adenoviral infection it is responsible for cell lysis and the release of viral progeny.

The viruses with the insert hNIS exhibit a reduced potency in cell toxicity assays when compared to wild type adenovirus (figures 3.12, 3.13, 3.14, 3.15, 3.16). This could be due to the critical size of the viral genome after insertion of the transgene. It has been demonstrated that larger inserts reduce replication efficacy, which may in its turn result in lower cytotoxic effect (Bett et al., 1993). Replication assays performed by infection of 293 cells have confirmed that the titres obtained with these viruses are lower than the ones obtained with wild type virus (figure 3.21, 3.22).

Another reason could be that insertion of the transgene reduces the expression of the surrounding E3 genes and the virus's ability to induce CPE (Hawkins et al., 2001). However in all the viruses constructed the E3 y-leader sequence has been maintained. Hawkins et al. demonstrated that while the y-leader sequence is not coding it is spliced onto the E3-ADP mRNA. Deletion of this sequence resulted in weaker expression of ADP and reduced ability of the virus to induce CPE.

Deleting the E3-6.7k and E3-gp19k has no effect on the efficacy of the wild-type virus in colonic cancer cell lines (figure 3.12, 3.14a) in vitro but a reduced efficacy can be seen in other cancer cell lines (figure 3.13, 3.14b). This observation has not been further investigated.

The majority of colonic cancers have activation of the Wnt signalling pathway. The group of Richard Iggo has created and tested a series of Wnt-specific replicating adenoviruses. The vKH1 virus with E1A, E1B and E4 under the control of Tcf is specific for Wnt-activated cell lines leaving normal cells intact (Homicsko et al., 2005).

Substituting E3-6.7k and E3-gp19k for hNIS in this virus has resulted in a virus that is as powerful as wild-type virus in cell death assays in colonic cancer cell lines with high Tcf activity (figure 3.16a-c) but slightly less active in colonic cancer cell lines with low Tcf activity (figure 3.16d and 3.16f). I have clearly demonstrated that hNIS insertion does not affect this selectivity. The virus is at least 100x less toxic than wild-type virus in non-Wnt activated cancer cell lines.
(figure 3.15) and normal embryonic lung fibroblasts (figure not shown) in cell death assays. Western Blot analysis for E1A and Hexon has confirmed these results (figure 3.26). While expression of E1A and Hexon in HeLa cells 48h after infection is high for cells infected with wild-type virus, expression of E1A protein is very weak for the Wnt-hNIS virus and expression of Hexon protein is absent (figure 3.26A-B). The results obtained in normal lung fibroblast were similar (figure 3.26C).

Deleting E3-6.7k and E3-gp19k in this virus enhances the cytotoxic effect of the virus in colonic cancer cell lines when compared to the hNIS-substituted virus (figure 3.17a-d) but also compared to the parent virus (figure 3.18). Viral replication assays have shown that the titres obtained with this virus are comparable to titres obtained with wild-type virus but titres of the hNIS substituted virus remain lower (figure 3.21). As stated above, the size of the transgene could be responsible for the reduced replication and reduced CPE.

The enhanced efficacy of the deleted virus compared to the parent virus has been described before by Wang et al. (Wang et al., 2003b) although the mechanism behind it remains unclear.

In non-Wnt-activated cell lines the deleted virus is less active than the hNIS-substituted virus (figure 3.17f,g). Possibly high levels of hNIS transgene expression are toxic to cells. In primary cells infection with a non-replicating virus where CMV drives the expression of hNIS is lethal to cells at MOI 100 (figure 3.32). Infection of the same cells with a non-replicating virus where CMV drives the expression of the β-galactosidase gene is not lethal.

2. Effect of the viruses on primary desmoids tumour cells

Ideally we would have preferred to work on fresh colorectal cancer tissue rather than only desmoid tumours. Unfortunately it was not possible to access such a tissue and therefore desmoids tumours were a practical alternative.

Constitutive activation of the Wnt-signalling pathway is a hallmark of sporadic and familial desmoid tumours (Tejpar et al., 2005). Normal tissues where the Wnt
pathway is active are stem cells in the skin, haematopoietic system and intestine and neurons in several regions in the brain, including subventricular zone, cortex and hippocampus (Homicsko et al., 2005). Therapeutic strategies targeting the Wnt-signalling pathway are expected to provide therapeutic benefits with minimal side effects in patients with this pathology. Selectively replicating oncolytic adenoviruses represent a novel class of therapeutic agents. Preclinical and clinical studies have demonstrated that these agents are safe and that they can be used in conjunction with classical cancer treatments such as chemotherapy and radiotherapy (Khuri et al., 2000, Alonso et al., 2007, Cheong et al., 2008). A limitation of adenoviral vectors is that after systemic administration the majority of the virus is sequestered in the liver and only a minimal proportion of the injected dose reaches the tumour. Considering the size and location of tumours and the lack of metastasis in patients with desmoids local intratumoural injection of oncolytic adenovirus represents a practical approach that could be implemented in conjunction with chemo- and/or radiotherapy.

Our data clearly demonstrate that primary cells from some desmoid tumours allow replication (figure 3.30) and are sensitive to the action of oncolytic adenoviruses (figure 3.27). However, in two cases the cells were completely refractory (figure 3.27a and 3.27d). The difference between these cells and the responding cells is the infectability as demonstrated by infection of cell samples with a non-replicating virus expressing GFP (figure 3.28). Analysis of the expression of the key molecules involved in adenovirus infection (CAR and the integrins αvβ3 and αvβ5) proved puzzling (figure 3.31). Low or no CAR expression has already been reported in desmoids (Gu et al., 2004) and we confirm that CAR was absent in all the cellular samples tested whether responding to adenoviral treatment or not. These data suggest that an alternative CAR-independent mechanism is responsible for viral entry in the cell. Integrins are present on the surface of all cell samples when tested with αv antibody but the highest level of αvβ3 and αvβ5 was observed in non-responding cells. Other mechanisms of infection may be involved (Nicklin et al., 2005) in these cells and further studies will be required to elucidate these mechanisms. Mechanisms that have been suggested involve heparan sulphate proteoglycans (Dechecchi et al., 2001) or coagulation factors (Waddington et al., 2007, Waddington et al., 2008).
In these primary desmoid cells there appears to be a direct effect of the hNIS transgene on the viability of cells (figure 3.32). This is the first time that such an effect has been reported.

This thesis demonstrated that gene therapy for desmoid tumours is feasible and that the success of treatment is dependent on the infectivity of the cells. In a clinical setting, direct injection of the oncolytic virus into the tumour under radiological guidance could be envisaged. Even a relatively modest effect could have an important clinical implication by making a previously unresectable intra-abdominal tumour amenable to surgery. Imaging of hNIS gene transfer could be performed to visualise viral spread and additional radiotherapy could extend the effect of the viral therapy (described below). Although it has been suggested that desmoid tumours are relatively radioresistant, there is evidence in the literature, as reviewed by Tolan et al. (Tolan et al., 2007) and Sakorafas et al. (Sakorafas et al., 2007) to suggest that these tumours can respond to external beam radiotherapy, which supports the use of localised radiotherapy by hNIS gene transfer.

3. Effect of the oncolytic viruses in vivo

*In vivo* intravenous administration of wild-type replicating adenovirus 5 is lethal in mice after IV injection of $10^{10}$ particles (Homicsko et al., 2005) and acute hepatic necrosis was also seen after injection of $10^9$ PFU of wild-type adenovirus expressing hNIS as confirmed by pathology (figure 3.33a-b). The acute hepatotoxicity that was seen is probably related to the expression of adenoviral E1A in the liver cells (Engler et al., 2004) since adenoviruses fail to replicate efficiently in mouse cells. Immunohistochemistry of livers infected with AdIP1 showed massive E1A expression and this was confirmed by real time PCR for E1A on DNA extracted from mouse livers (figure 3.33c). Intravenous injection of Tcf viruses is well tolerated in nude mice (Homicsko et al., 2005) and this was no different for intravenous injection of the hNIS expressing Tcf virus. Histology showed an intact liver architecture and mild inflammatory infiltration at 48h which increased after 120h. This inflammation subsides after 20 days (Homicsko et al., 2005) but in our small scale experiment, mice were not kept for such a long period of time. Very
few cells stain positive for E1A when immunohistochemistry is performed and this was confirmed with real time PCR on liver extracts. Normal liver cells do not have the Wnt signalling pathway active and reduction in toxicity is probably due to Tcf regulation of early viral promoters. Although this experiment together with the experiments performed by Homicsko et al. suggest that intravenous administration of Tcf-regulated adenoviruses would be safe, only a minimal proportion of the injected dose reaches the tumours (Homicsko et al., 2005). Intratumoural injections of virus are therefore advised.

*In vivo*, the hNIS-encoding viruses AdIP1 and AdIP2 demonstrate functional transgene expression (figure 3.34) and the kinetics of viral replication can be monitored by molecular imaging using SPECT/CT (figure 3.36 and 3.37). A signal can be detected 24h after intratumoural injection of virus, which increases to a maximum after 48 hours and then sharply reduces thereafter. Measurements of radioactivity normalised against background confirmed maximum activity 48 hours after infection (figure 3.38 and 3.39). The sequence of events after injection of virus into the tumour is firstly internalisation into the cell, followed by transcription of viral DNA and viral replication and possibly packaging of viral progeny in virions that will infect neighbouring cells upon cell lysis and release. An imaging signal can be detected when hNIS protein is being produced and translocated to the cell membrane where it can take up radiotracer. Real-time PCR for hNIS and E1A on DNA extracted from tumour samples (figure 3.41) shows an increase of transgene expression up to day four. Positive staining for E1A and hNIS antibody is present in immunohistochemistry up to day 4 and only reduces at day 5. A possible explanation for the discrepancy between the maximum imaging signal and the qPCR data is that although viral replication continues and hNIS is still being produced in the tumour after 48h, radiotracer is no longer taken up by the cells due to cell death. The marked necrosis that can be seen from day 3 onwards in the pathology slides of tumours supports this theory.

Surprisingly, viral spread stops as early as four days post-infection and no significant signal can be detected. At this point the remaining life cells in the tumour may be inaccessible to the virus due to surrounding necrosis. Possibly reducing the viral load and preventing early necrosis may allow the virus to spread
further in the tumour. Ono et al. inserted the EGFP gene in the E3 region of a replicating wild-type adenovirus 5 and were able to monitor viral replication through EGFP expression with fluorescence-based optical imaging. The signal increased during the first week and reduced thereafter. A signal was visible up to day 19 post-infection. The viral dose injected was comparable ($10^{10}$vp) but the tumour size on injection was at least twice as large. The slight discrepancy between my data and these observations may be due to the difference in reporter gene. EGFP may have a longer half life than hNIS. In addition, to be detectable by SPECT imaging, hNIS has to be expressed in a cell that maintains its ability to accumulate iodide. Therefore, it is likely that a tumour positive for hNIS immunohistochemistry may not be detectable by SPECT if hNIS is present on dead or apoptotic cells.

Animal models are important to evaluate and approve new anti cancer-therapies. The human adenovirus infects murine cells, viral proteins are synthesized and viral DNA replicates but the production of new infectious viral particles is limited. This leads to difficulties in interpreting the results of nude mice human xenograft tumour models. Another hurdle is that although the tumour cells are human, the supporting cells (e.g. stromal and vascular cells) are murine. This is likely to alter the dynamics of viral spread in the tumour. Last but not least, nude mice are immunocompromised. Most humans have been exposed to adenovirus and have antibodies to various human adenovirus serotypes. It has been reported, however, that neutralising antibodies predominantly play a role after intravenous but not after intratumoural injection of virus (Bramson et al., 1997). The immunity may have an effect on the elimination of virus and virus infected cells. However specific cytotoxic T-cell responses develop after a week or more, which should allow sufficient time for initial viral propagation. Immunity may also play a role in viral toxicity as was demonstrated by the Gelsinger case (Lehrman, 1999) as described in the introduction (p48).

In a clinical setting therefore, one would hope that the virus spread continues for longer than 48 hours to avoid repeated administration of virus. Although clinical trials have provided evidence that repeated administration is feasible (Fujiwara et
al., 2006a) one has to be aware of the possibility of inducing an immune response, which could reduce the efficacy of the virus.

One clinical trial conducted in patients with hepatocellular carcinoma has confirmed that it is possible to monitor gene expression in vivo with PET imaging in humans (Penuelas et al., 2005b). The viruses that have been constructed for this thesis could behave differently when injected in human subjects. It would be feasible to monitor gene expression by PET or SPECT with or without CT to determine the time of maximal gene expression. Our mouse experiments have highlighted that it is very important in oncolytic virotherapy to determine the optimal viral dose. The administered dose should be high enough to obtain maximal viral spread and transgene expression but should not be too high in order to prevent early elimination of the cells by oncolysis which would prevent further viral spread in necrotic tumour tissue and make additional radioiodine therapy less effective.

In vivo experiments conducted on mice have highlighted the possibility of an added effect of the hNIS gene in the Wnt-specific virus and injection of radioiodine could extend the effect of the viral therapy to the surrounding cells thus maximising the effect of oncolytic viral therapy.

Since the completion of this work, further in vivo experiments have been successfully conducted by my supervisor in France leading to the submission of a manuscript. The therapeutic experiment as described in figure 3.45 was repeated in duplicate as follows: Balb-c nude mice were injected with HCT116 cells subcutaneously (2x10^6 cells). When the tumours reached an average size of 1cm^2, tumours were injected with 10^9 PFU of AdIP2 or AdIP4. Forty-eight hours later, a single dose of ^131I (1.5mCi) was administered intra-peritoneally. The data below represent means +/- SEM of the tumour measurement, with 6 animals per group. Two-way ANOVA statistical analysis was performed.
These data show that a single injection of a therapeutic dose of radioiodine 48h after a single injection of AdIP2 resulted in a significant decrease in tumour size that is not obtained with the hNIS-negative AdIP4. These data demonstrate that hNIS-mediated viro-radiotherapy is relevant to oncolytic adenoviruses.

4. Future developments

Endogenous expression of hNIS in differentiated thyroid carcinoma has led to extensive utilisation of radioiodine for the diagnosis and treatment of this pathology. Based on this experience, ectopic expression of hNIS in extrathyroidal cancers and subsequent treatment with radioiodine could provide a powerful new gene therapy strategy. The thyroid could be protected by administering thyroid hormone prior to treatment with radioiodine. The thyroid is the only organ which stores iodine in the colloid where it is used for the production of thyroid hormones. In other organs which express the hNIS, like the gastric mucosa, salivary glands, adrenal glands and lactating breast, iodine is not stored and is dependent on the concentration of iodine in the extracellular fluid. The uptake of iodine in the thyroid is regulated by thyroid-stimulating hormone (TSH) also called thyrotrophin. When the amount of thyroxine in the blood increases, TSH in the circulation decreases and the iodine uptake in the thyroid is down-regulated by recycling the hNIS from the cell membrane into the cell (Dohan and Carrasco, 2003). Other factors that down-regulate TSH are sex hormones, iodine, steroids, somatostatin, IL-1α, TNF-α, IFN-γ and protein kinase C. It has been shown in animal studies that administering iodine (NaI) six days prior to radiiodine treatment greatly reduces radiiodine uptake in the thyroid (Ferreira et al., 2005).
Pre-treatment with thyroid hormone or even sodium iodide could therefore be used to down-regulate the expression of hNIS in the thyroid and consequently suppressing the uptake of $^{131}$I in the thyroid thus protecting the thyroid from radiation damage.

But, even if radioiodine uptake in the thyroid would cause damage to the gland, patients could be easily and inexpensively treated by routine thyroid hormone replacement therapy and the lost thyroid function might then be a small price to pay in the treatment of cancer.

The extensive experience (more than 50 years) with radioiodine treatment for thyroid disease and cancer provides evidence that damage to extra-thyroidal tissues that express the hNIS remains limited. Possible side effects to radioiodine treatment depend on the administered dose and include nausea and vomiting (although very rare), dry mouth, temporary change in taste (in up to 60% of patients, typically lasting several weeks), salivary gland swelling and pain, drop in blood counts and other effects related to the radiation (Alexander et al., 1998). The most common long-term side-effects are due to radiation damage to the salivary glands. Several drugs have been reported that could provide protection for the salivary glands. They include Amifostine, Pilocarpine and/or Lidocaine (Hakim et al., 2005).

The combination of oncolytic adenoviruses and hNIS could overcome the limited efficacy of oncolytic adenoviruses alone. In an ideal clinical situation (figure 4.1), the adenovirus would then be delivered to the tumour where it would penetrate the tumour cells and replicate. In a next step, cell lysis and the release of progeny would allow for the virus to propagate in the tumour. Due to the presence of hNIS, patients could be monitored by SPECT or PET imaging and viral propagation in the tumour closely followed. The time-point of maximal propagation could be determined and dosimetry performed to calculate the ideal dose that would be required to obtain a maximum biological effect. Once this information is established, phase 1 clinical trials could be initiated and patients could be treated with a combination of virus and radioiodine. The efficacy of this treatment could then be compared to other more conventional therapies.
Although adenoviral vectors are highly potent gene delivery vehicles, can be produced to high titres, are able to infect both dividing and quiescent cells, do not integrate in the host genome and are stable, a few important hurdles still need to be overcome.

Firstly, there is the problem of delivery. Intravenous administration of adenoviruses results in high liver transduction and relatively little virus reaches the target tissue. In the liver the macrophages or Kupffer cells interact with the virus and activate a strong immune response. Studies have suggested that the activation of the innate immune response can be largely attributed to the capsid proteins. Another important factor in gene delivery is the fact that most people have been previously exposed to adenoviruses and may have circulating anti-adenovirus antibodies. Oncolytic adenoviruses although they do not replicate in normal cells, can still infect a wide variety of cells since the two most important receptors, CAR and integrins are expressed on many different cell types. Circulating adenoviruses are also able to interact with coagulation factors, platelets and plasma proteins in the circulation, which further reduces the amount delivered to the target tissue (Kreppel and Kochanek, 2008). Chemical modification of adenovirus vectors with
synthetic polymers could reduce innate and adaptive immune responses and subsequent liver toxicity (Kreppel and Kochanek, 2008). PEGylation of viral particles can overcome neutralisation by anti-adenoviral antibodies and significantly reduces neutrophil infiltration of liver cells after gene transfer due to reduced sequestration by Kupffer cells, which are responsible for initiating an immune response and has been shown to prevent interaction of the viral capsid with platelets. It has also been suggested to administer glucocorticoids before treatment with adenovirus to ablate further the innate immune response. However, although PEGylation reduces liver toxicity, it also results in ablation of the CAR tropism of adenoviruses, which could prevent delivery to tumour cells. Experiments revealed that the RGD motif in the penton base protein was still partially accessible even after heavy PEGylation which would allow for binding to integrins. Another strategy would be to combine PEGylation with ligands coupled to the vector surface. Another possibility would be to genetically modify the capsid proteins to prevent binding to CAR, which could be used in combination with chemical modification.

An entirely different strategy to allow for intravenous administration and target delivery of adenoviral vectors would be to incorporate adenoviral DNA into synthetic vectors. A wide variety of synthetic vectors have been developed that are able to deliver genetic constructs to cancerous tissues (Wolff and Rozema, 2008). In our laboratory Dr. Ed Chisholm and Jérôme Burnet have successfully attached the adenoviral plasmid containing AdIP1 to polypropylenimine dendrimers. Polypropylenimine dendrimer generation 3 are small molecules that when mixed with plasmid DNA form nanoparticulate complexes which when intravenously administered are able to deliver the plasmid DNA to tumour xenografts in mice (Dufes et al., 2005). Furthermore, these dendrimers appear to possess an intrinsic antitumour activity which acts together with the delivered DNA to provide a stronger effect than can be obtained with either component alone (Dufes et al., 2005). Animal experiments performed in our laboratory have demonstrated that the intravenously administered adenoviral DNA (AdIP1) attached to the dendrimers was delivered exclusively to xenografts in nude mice as seen by SPECT/CT imaging. These data have not yet been published. Dufes et al. have also demonstrated that it is possible to use tissue specific promoters like the telomerase
promoter to further increase the specificity of these delivery vectors (Dufes et al., 2005). To increase the efficiency of gene delivery, physical methods such as ultrasound or hydrodynamic injection could be added to these synthetic vectors (Wolff and Rozema, 2008).

Although promising data have come out of the experiments previously described, to date no clinical experiments have been performed to confirm these data in human subjects. If we would plan a clinical trial with the virus produced in this thesis, intratumoural or intra-hepatic injection would be envisaged. Possible indications would then be for example multiple colonic cancer liver metastases. Currently the primary treatment for colorectal cancer and solitary metastases remains surgery. Multiple liver metastases pose a more complicated problem. Laparoscopic coagulation or laser treatment is available for the metastases that are accessible by this technique but metastases deep in the liver or posterior located may not be amenable to this technique. Adenoviral injection into the hepatic artery would be a good alternative and could be combined with radioiodine therapy. Animal studies performed by Faivre et al. (Faivre et al., 2004) and the clinical study performed by Penuelas et al. (Penuelas et al., 2005b) have provided evidence that this approach is possible.

Another indication could be in FAP patients. The treatment of desmoids poses a challenging dilemma. Surgery increases the risk of the development of desmoid tumours but if left untreated they can become so large that surgical intervention becomes impossible and death is unavoidable. Injection of oncolytic adenovirus could reduce the size of tumours and make a previously unresectable tumour amenable to surgery.

The majority of FAP patients also develop duodenal polyps. If these polyps progress and become cancerous, the surgical procedure (Whipple’s procedure or pancreaticoduodenectomy) to remove them is associated with a high morbidity and mortality rate and could trigger the development of desmoid tumours in these patient that have already undergone total colectomy. One could envisage intratumoural injection of adenovirus via endoscopy with or without treatment with radioiodine.
The work presented in this thesis has shown that it would be possible safely to inject Wnt-specific adenovirus into cancerous growths. *In vitro* treatment of primary cells has demonstrated that two out of six samples were refractory to treatment. Possibly the infectability of cancer cells and the Tcf status should be determined before therapy is envisaged. In the future, adenoviruses could be further modified as described above to enhance their potency in the treatment of cancer.

Who would have thought 20 years ago that a surgeon would be sipping coffee in front of a computer console while operating a patient in an operating theatre 1000 miles away? Today this is a reality (www.intuitivesurgical.com). Genetically modified adenoviruses with or without therapeutic transgenes may one day become a routine therapy for a wide range of cancers.


BRAMSON, J. L., HITT, M., GAULDIE, J. & GRAHAM, F. L. (1997) Pre-existing immunity to adenovirus does not prevent tumor regression following intratumoral administration of a vector expressing IL-12 but inhibits virus dissemination. Gene Ther. 4, 1069-76.


HTTP://WWW.CEPHB.FR/GACCC/TABLE2.PHP Mutations of oncogenes and tumour suppressor genes in colorectal cancer cell lines.


MONTEIRO, R. Q., BAROUCH, D. H., VAN ROOIJEN, N., NAPOLI, C.
serotype 5 hexon mediates liver gene transfer. *Cell*, 132, 397-409.

WADDINGTON, S. N., PARKER, A. L., HAVENGA, M., NICKLIN, S. A.,
fundamental involvement of coagulation factors and redundancy of CAR


APPENDIX

1. Plasmid pTGW10
2. Plasmid map of pcDNA3.1/V5-His-TOPO

pcDNA3.1/V5-His-TOPO®

The figure below summarizes the features of the pcDNA3.1/V5-His-TOPO® vector. The vector is supplied linearized between base pairs 953 and 954. This is the TOPO® Cloning site. The complete nucleotide sequence is available for downloading from our World Wide Web site (www.invitrogen.com) or from Technical Service (page 23).
3. Plasmid map of pCR\textsuperscript{T7/CT-TOPO}

![Plasmid Map of pCR\textsuperscript{T7/CT-TOPO}](image)

**pCR\textsuperscript{T7/CT-TOPO}**

2702 bp

Comments for pCR\textsuperscript{T7/CT-TOPO}:

- **2702 nucleotides**
- **T7 promoter**: bases 21-37
- **T7 promoter priming site**: bases 21-40
- **Ribosome binding site**: bases 85-91
- **TOPO\textsuperscript{I} Cloning site**: bases 100-101
- **V5 epitope**: bases 122-163
- **V5 (C-term) Reverse priming site**: bases 131-151
- **Polyhedrin (6xHis) region**: bases 173-190
- **T7 transcription terminator**: bases 240-287
- **Zeocin\textsuperscript{R} resistance gene**: bases 367-830
- **ORF**: bases 456-830
- **Ampicillin resistance gene**: bases 834-1711
- **ORF**: bases 851-1711
- **pUC origin**: bases 1696-2529
4. Plasmid map of pGV1 created with vector NTI
5. Publications arising from this thesis


Dr. INGE PEERLINCK, SAIED NIK, DR. ROBIN PHILLIPS, DR. RICHARD IGGO, DR. NICHOLAS LEMOINE, DR. SABINE TEJPAR, DR. GEORGES VASSAUX. Therapeutic potential of replication-selective oncolytic adenoviruses on cells from familial and sporadic desmoid tumors. Accepted for publication in Clinical Cancer Research.


MOHAN HINGORANI, CHRISTINE WHITE, ANDREW MERRON, INGE PEERLINCK, MARTIN GORE, ANDREW SLADE, SIMON SCOTT, CHRISTOPHER NUTTING, HARDEV PANDHA, ALAN MELCHER, RICHARD VILE, GEORGES VASSAUX, KEVIN HARRINGTON: Inhibition of repair of radiation-induced DNA damage enhances gene expression from replication-defective adenoviral vectors. Accepted for publication in Cancer Research.

INGE PEERLINCK, ANDREW MERRON, PATRICK BARIL, SOPHIE CONCHON, PILAR MARTIN-DUQUE, JEROME BURNET, MIGUEL QUINTANILLA, MOHAN HINGORANI, RICHARD IGGO, NICHOLAS LEMOINE, KEVIN HARRINGTON, GEORGES VASSAUX: Combining the imaging and therapeutic potential of the Na/I symporter using a Wnt-targeted replicating adenovirus. Revised version awaiting approval for publication in Cancer Research.