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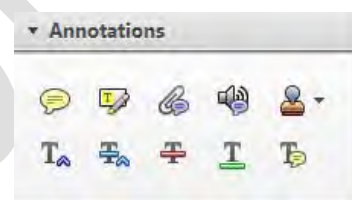
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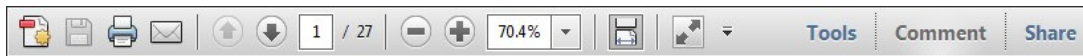
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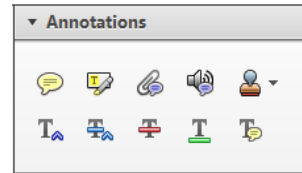
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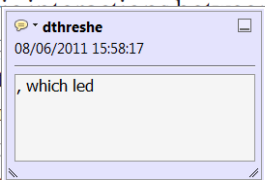## 1. Replace (Ins) Tool – for replacing text.

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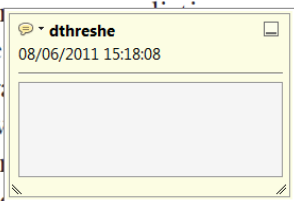
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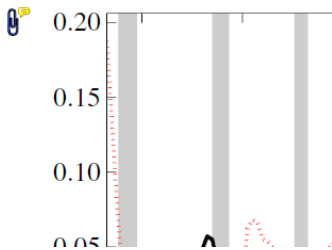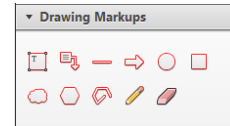
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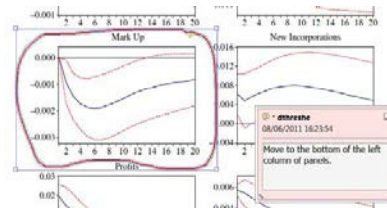
REVIEW ARTICLE

Fibroblast activation and senescence in oral cancer

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There is now compelling evidence that the tumour stroma plays an important role in the pathogenesis of cancers of epithelial origin. The pre-eminent cell type of the stroma is carcinoma-associated fibroblasts. These cells demonstrate remarkable heterogeneity with activation and senescence being common stress responses. In this review, we summarise the part that these cells play in cancer, particularly oral cancer, and present evidence to show that activation and senescence reflect a unified programme of fibroblast differentiation. We report advances concerning the senescent fibroblast metabolome, mechanisms of gene regulation in these cells and ways in which epithelial cell adhesion is dysregulated by the fibroblast secretome. We suggest that the identification of fibroblast stress responses may be a valuable diagnostic tool in the determination of tumour behaviour and patient outcome. Further, the fact that stromal fibroblasts are a genetically stable diploid cell population suggests that they may be ideal therapeutic targets and early work in this context is encouraging.

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Keywords: activation; cancer; fibroblasts; oral; senescence

Introduction

Head and neck cancer, including oral squamous cell carcinoma (OSCC), accounts for some 600 000 new cancer cases annually worldwide with a rising incidence in many countries. Only 50% of patients survive 5 years, a figure that largely reflects the tumour stage at presentation, locoregional invasion and recurrence, distant metastases and the development of 2nd primary tumours. Survival improves when tumours are detected early but the majority of patients present with advanced metastatic disease and have a poor prognosis (1). There is an urgent need, therefore, to develop new techniques for early diagnosis and to develop new therapeutic modalities.

Recently, Parkinson and colleagues developed a new paradigm of OSCC and identified genetically stable (GS-OSCC) and genetically unstable (GU-OSCC) epithelial subtypes in cell culture, with the latter being associated with marked LOH and gene copy number alterations, together with loss of *TP53* and inactivation of *CDKN2A* (p16^{INK4A}) (2–5). Therefore, with respect to changes in *TP53* and *CDKN2A*, the molecular characteristics of the GU-OSCC cell lines resemble the recently published data on smoking-related head and neck cancers (6). Diploid cancer-associated fibroblasts (CAFs) from isotypic biopsies of GS-OSCC, GU-OSCC and fibroblasts from normal oral mucosa have been isolated from these tissues and have served as useful tools to investigate the interaction between stromal fibroblasts and cancer epithelium.

It is now recognised that solid tumours are not simply clonally evolved epithelial cells that have accumulated a critical number of cancer-driving or cancer-facilitating mutations but rather operate as dysfunctional tissues where the mesenchymal component plays an important role in tumour pathogenesis (7). The stromal compartment consists of a variety of different cell types (fibroblasts, pericytes, smooth muscle and endothelial cells, pre-adipocytes and cells of the immune system) but fibroblasts are pre-eminent. The present report focuses on two common stress responses in CAFs, namely activation and senescence, because despite having distinct morphologies, both cell types have tumour-promoting capabilities. We believe that it is now timely to review the recent advances that demonstrate that fibroblast activation and senescence are fundamental to the pathogenesis of oral cancer and have clinical importance. A glossary of terms where abbreviations have been used is included for clarification (Table 1).

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Table 1 Glossary of terms

Abbreviation	Name	Abbreviation	Name
α-SMA	α Smooth muscle actin	IGF2	Insulin-like growth factor 2
NG2	Neural/glial antigen 2	BMP4	Bone morphogenic protein 4
PDGF	Platelet-derived growth factor	MMP	Matrix metalloproteinase
FSP	Fibroblast-specific protein	PGE2	Prostaglandin E2
FAP	Fibroblast-activating protein	KGF	Keratinocyte growth factor
TGF-β	Transforming growth factor-β	CSL	CBF1; Suppressor of hairless; Lag-1
TNF-α	Tumour necrosis factor-α	AP-1	Activator protein 1 transcription factor composed of c-Fos, c-Jun, ATF and JDP family members
IL-1; Il-33	Interleukin 1/33	NF-κB	Nuclear factor κ-light chain-enhancer of activated B cells
CCL7	Chemokine (C-C motif) ligand 7	NRF2	NFE2; Nuclear factor (erythroid-derived 2)-like 2
SDF-1	Stromal cell-derived factor 1	TERT	Telomere reverse transcriptase
BDNF	Brain-derived growth factor	VEGF	Vascular endothelial growth factor
HGF	Hepatocyte growth factor	ROS	Reactive oxygen species

Fibroblast activation

Identification and origin

In 1971, the presence of modified fibroblasts was described in healing wounds and due to their similarity to smooth muscle cells, they were termed myofibroblasts (8). In cancer, these modified fibroblasts have been termed tumour-associated fibroblasts, activated fibroblasts and CAFs, the names being used interchangeably.

CAFs have been identified by several different markers including α-SMA, tenascin-C, periostin, NG-2, PDGF receptor-α/β, FSP (S100A4) and FAP. Markers such as vimentin, type I collagen, prolyl 4-hydroxylase and fibroblast surface protein are also used to detect various types of mesenchymal cells including CAFs. The likelihood is that these fibroblast markers, expressed alone or in combination, identify distinct subpopulations within CAFs (9). In oral cancer, we have shown that fibroblasts from GS-OSCC, GU-OSCC and normal oral mucosa express distinct transcriptional profiles (10), findings that overlap with observations by Partridge and colleagues who identified two distinct CAF populations in OSCC that induced epithelial invasion *in vitro* by different mechanisms (11).

Several cell types have been described as possible progenitors of CAFs (Fig. 1). Recently, a transgenic mouse model has been used to explore the cellular origins of CAFs; FSP-1+ and FAP+ fibroblasts were largely derived from bone marrow cells including mesenchymal stem cells, whereas α-SMA+ and NG2+ myofibroblasts originated from resident tissues primarily of adipocyte origin (12).

Induction and function

The induction of the activated CAF phenotype and the potentiation of tumour epithelial cell behaviour involves crosstalk between the tumour epithelium and stroma (13–15). Epithelial cancer cells activate CAFs, an interaction that is mediated by ligands such as TGF-β, TNF-α and IL-1α/β. CAFs, in turn, release molecules such as TNF-α, IL-1α/β, IL-33, CCL7, SDF-1, BDNF, Collagen 1, HGF, IGF2, BMP4, MMPS, PGE2, KGF, Activin A and PDGF, amongst others. Taken together, the pre-neoplastic cell growth (15), tumour cell migration and invasion, metastases, angiogenesis and immune escape (13, 14) and cancer therapy resistance (16) are promoted. For head and neck cancer, the molecular basis of the interaction between cancer cells and CAFs has been extensively reviewed recently by Leef and Thomas (13).

The observation that the activated fibroblast phenotype is maintained after the removal of the fibroblasts from their tissue of origin and after prolonged cell culture suggests that there are irreversible genetic changes associated with this cell type. Whilst CAFs from GS-OSCC and GU-OSCC show no copy number alterations (10), CAFs are likely to sustain their protumorigenic phenotype by epigenetic mechanisms and/or miRNA-mediated gene regulation.

Epigenetic alterations, somatically heritable changes that are not due to DNA sequence conversions, have been reported in CAFs and include altered DNA methylation patterns and global hypomethylation (17). By contrast, micro-RNAs (miRNAs) are small (19–25 nucleotides) non-coding RNAs that regulate gene expression at the post-transcriptional level by hybridising to the complimentary, or partial complimentary, sites predominantly in the 3′UTR of their target genes; this results in translational inhibition or miRNA degradation. In so doing, miRNAs regulate diverse biological functions and have both tumour suppressive and oncogenic functions depending on their gene target (18). Whilst miRNAs are differentially expressed in ageing and cancer, there have been few studies that have examined the role of miRNAs in the development and maintenance of fibroblast stress responses. What is known suggests that miR-210, miR-21, miR-146a, miR-27, miR-155 and miR-200b are involved with fibroblast activation (19–21) whereas miR-210 and miR-146a are thought to play a role in fibroblast senescence (21, 22). In OSCC, the expression of miR-21 in the tumour stroma appears to be an independent biomarker of disease-free survival (23). Interestingly, one of the major risk factors of OSCC (cigarette smoke) also regulates specific miRNAs (24). The study of miRNA in malignancy and particularly in stromal cells, however, is in its infancy. The nature of the miRNA profile in isotypic cancer cells and CAFs has not been explored, the temporal nature of miRNA expression during the different stages of tumour progression is unknown, and data relating specifically to OSCC are only now starting to emerge. Our unpublished data reveal widespread changes in miRNA expression in both CAFs and senescent fibroblasts (D Lambert, personal communication).
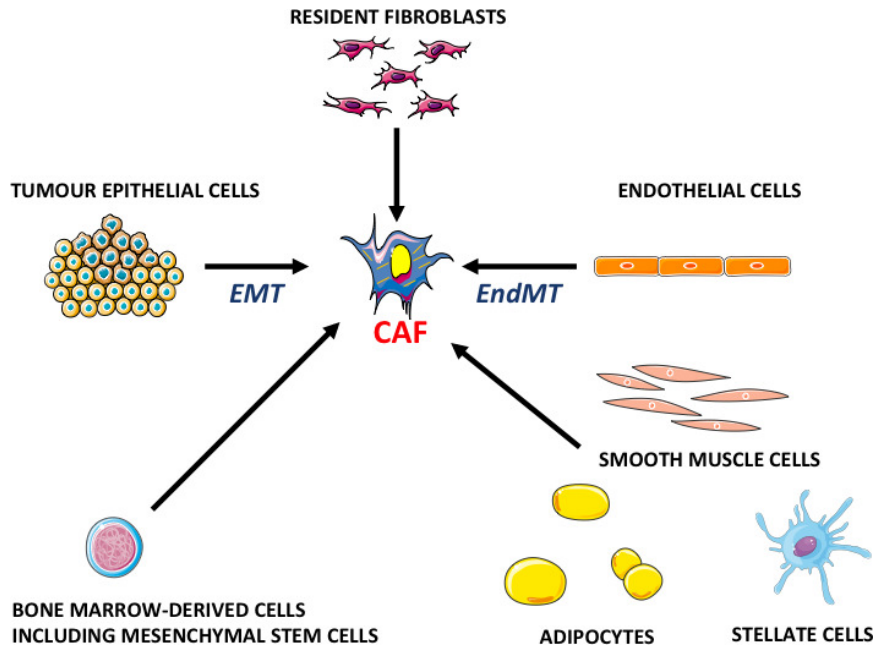
RESIDENT FIBROBLASTS

TUMOUR EPITHELIAL CELLS

EMT

CAF

EndMT

ENDOTHELIAL CELLS

SMOOTH MUSCLE CELLS

**BONE MARROW-DERIVED CELLS
INCLUDING MESENCHYMAL STEM CELLS**

ADIPOCYTES

STELLATE CELLS

Figure 1   Source of carcinoma-associated fibroblasts.

What other factors regulate the tumour stroma? Interference with the Notch/CSL pathway (CSL is a DNA-binding protein with intrinsic repressive function that is converted into a transcriptional activator by activated Notch) in cancer cells, a common event in OSCC leads to the development of a CAF-like phenotype and fibroblast senescence suggesting that CSL may be acting as a repressor of multiple CAF and senescence effector genes. Concurrently, there is upregulation of pro-inflammatory cytokines and MMPs and ultimately widespread dermal atrophy, expanding areas of inflammation and carcinoma *in situ* formation (25). Other factors that are known to regulate the tumour stroma include AP-1 family members, NF-κβ and NRF2, but their significance in the pathogenesis of OSCC has yet to be determined.

Senescence in fibroblasts
Characteristics
In 1961, Hayflick (26) made the seminal observation that cultured human fibroblasts could not proliferate indefinitely and lost their ability to divide over many cell doublings despite being viable and metabolically active. The permanent growth arrest state is referred to as senescence and is distinguished from quiescence by elevation of $p16^{INK4A}$. Senescence is induced by a wide variety of stimuli other than exhaustive cell replication. Persistent DNA damage is a common theme, and factors that induce these changes include oxidative stress arising from mitochondrial dysfunction, overexpression of oncogenes leading to over-replication of the genome and uncontrolled cell division (27) and anticancer treatments such as chemotherapy and irradiation. The precise molecular mechanisms of senescence are well documented and beyond the scope of the present review (28).

Senescence is associated with shortening of telomeres, the six base repeats in DNA that protect the ends of chromosomes with each round of replication. When telomeres reach a critical length, DNA double-strand breaks occur and DNA damage foci assemble. Telomere dysfunction can drive the activation of senescence. Mutations in telomere-specific structural proteins, for example, lead to premature ageing (29). Conversely, telomere length is maintained by the enzyme telomerase and the gene encoding the catalytic domain of human telomerase, TERT, can reconstitute telomerase activity, block telomere erosion and immortalise human fibroblasts without changing their karyotype or cell cycle checkpoints (30). The majority of tumours, irrespective of origin, overexpress telomerase, and the recent observation showing somatic mutations in the TERT promoter of OSCCs of the lateral border of the tongue (31) suggests that in this subset of cancers, telomerase is deregulated rather than being selected from the stem cell compartment, a phenomenon that occurs early in tumour development (32). The remaining human tumours, largely of mesenchymal origin, use an alternative mechanism (ALT) (33), but there is no evidence for a role of ALT in the pathogenesis of OSCC.

Function
In cancers of epithelial origin, senescence occurs when the damage to a cell is irreparable or overwhelms the DNA repair machinery and in this capacity, senescence acts as a valuable tumour suppressor. Tumour cells overcome this barrier in a process that involves inactivation of the p53 and $p16^{INK4A}$/pRB signalling pathways (34). By contrast, senescence in fibroblasts of the tumour stroma is tumour promoting. Coculture of either human senescent prostate or lung fibroblasts with their premalignant epithelial counterparts, for example, stimulates cell growth, migration and invasion (35).

The mechanism by which senescent cells in the microenvironment stimulate epithelial tumour progression has been

the focus of intense scrutiny in recent years. Significantly, senescent cells secrete a wide variety of protumorigenic proteins collectively referred to as the senescence-associated secretory phenotype (SASP) (36). The SASP is made up of soluble signalling factors consisting of receptors and ligands, chemokines, interleukins, inflammatory factors, ROS, growth factors and proteases, together with extracellular matrix proteins such as fibronectin, collagen and laminin. Among the myriad of factors that constitute the SASP, IL6 and IL8, MMPs, VEGF and osteopontin (37, 38) have all been associated with epithelial tumour progression in different systems (Fig. 2). In this context, fibroblast senescence has been shown to contribute to epithelial proliferation, differentiation, cellular metabolism and genetic instability, amongst others, and ultimately leads to the promotion of tumour cell invasion, metastases and therapeutic resistance. Interestingly, whilst fibroblast senescence induces an epithelial–mesenchymal transition (EMT) (37), recent data have shown that EMT is dispensable for invasion, metastases and the generation of cancer stem cells suggesting that it has a redundant role in these latter functions (39).

Our own work (40, 41), and that of others (42), have demonstrated that TGF-β is also a key mediator in senescent fibroblast–tumour cell interactions. We have shown that tumour-derived epithelial TGF-β acts in concert with ROS to induce fibroblast activation and senescence; fibroblasts from GU-OSCC appear to be particularly susceptible to oxidative DNA damage because of the high levels of fibroblast-derived ROS together with downregulation of antioxidant genes and upregulation of pro-oxidant genes (40). Once CAF senescence has been induced, CAF-derived TGF-β, in conjunction with MMP2, induces EMT in the tumour cells and downregulates a broad spectrum of epithelial cell adhesion molecules (e-cadherin, desmoglein 1 and 3, desmoplakin, desmocollin, β-catenin and

plakophilin) to cause keratinocyte discohesion and the promotion of epithelial invasion *in vitro* (N. Cirillo, manuscript submitted). Taken together with the recent findings that EMT is redundant for invasion and metastases (39), our findings suggest that whilst the loss of adhesion molecules is a characteristic of epithelial cancer cells and likely facilitates the general spread of a tumour, it is not the primary driving force in the capacity of these cells to gain access into lymphatics and blood vessels.

As stated, downregulation of the CSL/Notch pathway leads to fibroblast activation and senescence in both pre-malignant lesions (actinic keratosis) and overt carcinomas (25). It is only in carcinomas, however, where it is also associated with downregulation of *CDKN1A* and p53, the latter requiring additional paracrine influences (43). Extensive work by Campisi and colleagues has shown that the loss of p53 actually augments at least a subsection of the SASP including the interleukins (37), but the complete ablation of the senescence programme by the dual knockout of p53 and the p16[INK4A] locus in mice suppresses the inflammatory response (44). Further, recent findings show that p38MAPK also controls the stability of SASP miRNAs (45). With regard to our own work, we have shown overexpression of p16[INK4A] in senescent fibroblasts from GU-OSCC but not GS-OSCC (40) but the status of CSL/Notch and p38MAPK in these cells has not been investigated to date.

The fibroblast senescent metabolome

Recently, we completed a detailed, unbiased screen of the extracellular senescence metabolome (ESM) of oral CAFs (46) and identified alterations in several metabolites including increased levels of alanine, citrate, molecules involved in oxidative stress, a sterol, monohydroxylipids, tryptophan metabolism, phospholipid and nucleotide
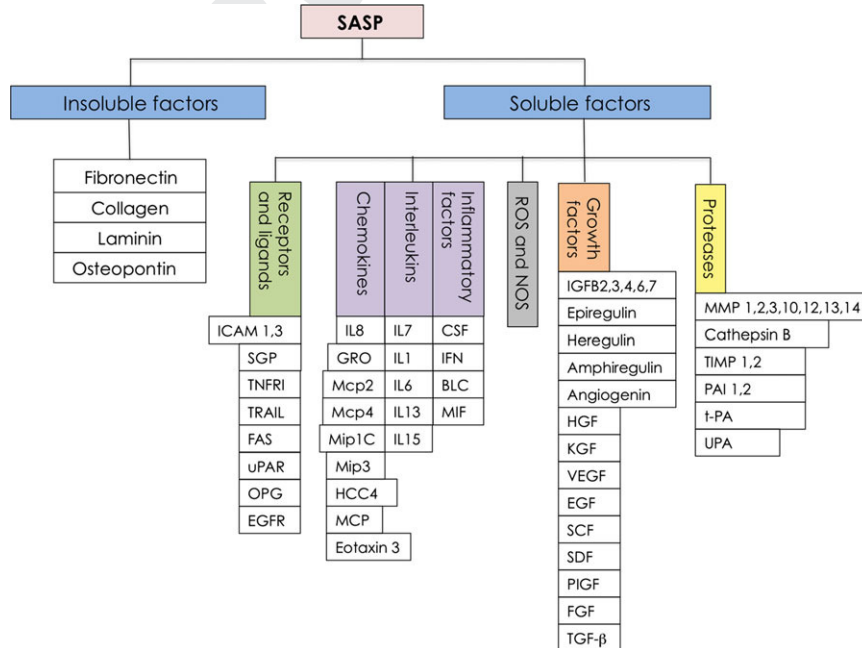

Figure 2 List of molecules that constitute the senescence-associated secretory phenotype.

catabolism, as well as reduced levels of dipeptides containing branch chain amino acids. Few of these metabolic products, however, have been revealed in studies of cancer serum metabolomics perhaps because of the great variation in cancer patient metabolism and other confounding factors such as age, body mass index, blood pressure, drugs and smoking. Further, most clinical studies on the serum metabolome have to date been seriously underpowered. Whilst serum alanine is emerging as a common biomarker of human cancer (47), elevated saliva alanine has been detected in only one study of oral malignancy and pre-malignancy and was not detected in a much larger study of serum from similar patient groups using different technology (48). Elevated saliva glycerphophorylcholine was reported to be able to discriminate between oral cancer and periodontal disease as well as controls (49) but the number of samples was rather small. If this area is to be taken forward, it will be necessary to perform a metabolomics analysis of saliva in a large number of individuals in an analogous way to the Husermet project (Husermet characterised the human serum metabolome using mass spectrometry, chromatographic techniques, infrared spectroscopy and NMR to identify biomarkers of Alzheimer's disease and ovarian cancer.

What is also unclear at present is whether any of the ESM actually transfers phenotypes from senescent cells to other non-senescent cells. Lisanti and co-workers have provided persuasive evidence that lactate and ketones from glycolytic fibroblasts induced to undergo autophagy and senescence can provide an energy source for cancer cells in mixed cultures of the two cell types and in so doing, promote tumorigenicity and invasion (50). In our study, the most reliable senescence marker in the ESM was citrate although it was unable to induce alterations in growth or senescence on its own (46). Extracellular citrate is also induced by irreparable DNA damage and accumulates with advanced

age in humans; it is reduced in some mouse models of longevity (47). Another interesting role for citrate within the cell is to act as a substrate for ATP citrate lyase to produce acetyl CoA and this, in turn, is used as a source of lipids to generate membranes during senescence (51). Acetyl CoA is also used as a substrate for histidine acetylation, and so it is possible that intracellular citrate may contribute to the gradual epigenetic changes that occur in ageing tissue and senescent cells (52).

A unified programme

Mechanisms linking fibroblast activation and senescence are unknown. Nevertheless, there is circumstantial evidence that fibroblast activation and senescence may reflect different stages of the same pathway (40; M. Mellone, manuscript submitted). First, the gene expression profile of TGF-β-treated fibroblasts (activated) overlaps significantly with radiation-treated fibroblasts (senescent); the former are characterised by the expression of genes associated with the elaboration of the extracellular matrix, and the latter show enrichment of metabolic pathways. Second, treatment of oral fibroblasts with TGF-β leads to first activation and then senescence. Third, both activated and senescent CAFs upregulate cytoskeletal markers and develop a contractile SMA-positive phenotype. Fourth, CAF activation and senescence share common tumour-promoting activities. And fifth, the tumour-promoting capabilities of these cell phenotypes may be mediated through similar signalling pathways (25, 43, 45).

It is important to emphasise that what is common to the different fibroblast stress responses is the protumorigenic nature of their secretome. Interestingly, the expression of the SASP does not require cellular senescence. Cells defective in the p53 and pRB, pathways, for example, retain the ability to express the SASP in response to persistent DNA
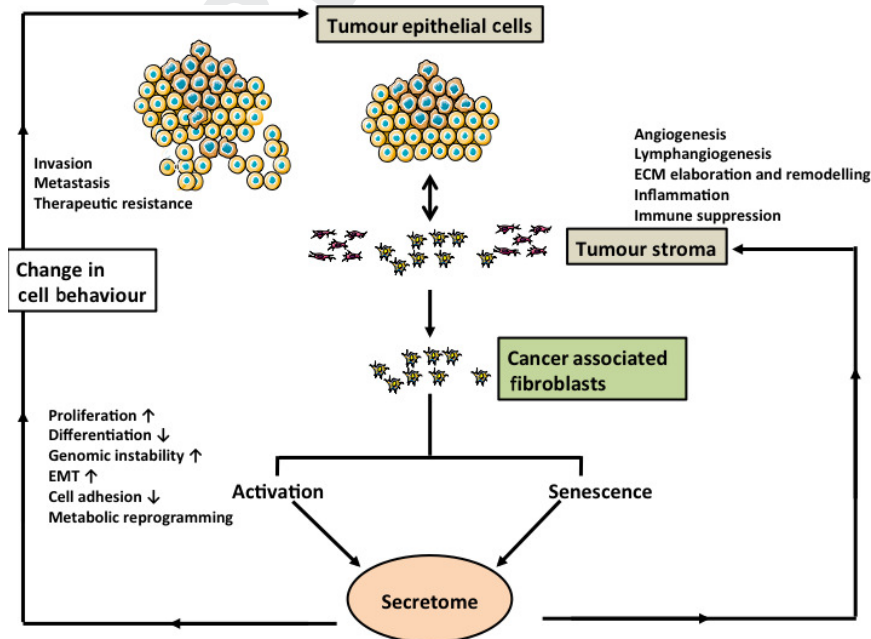
Figure 3 Function of fibroblast stress responses in the promotion of epithelial cancer and stromal development.

damage signalling (36) and senescence induced by ectopic expression of p16^{INK4A} or p21$^{Cip1/WAF1}$ fails to activate the SASP (53). Nevertheless, the consequences of a fibroblast stress response are epithelial tumour progression and the promotion of stromal angiogenesis/lymphangiogenesis, ECM remodelling, cancer-associated inflammation and metabolic reprogramming. The concept is summarised schematically in Fig. 3.

Clinical implications

In 2004, CAFs were demonstrated for the first time in the stroma of OSCCs (54) and since then, a number of studies have emphasised their importance. When the data are taken together, CAFs occurring in high frequency in OSCCs are associated with invasion, disease progression, tumour recurrence and poor patient prognosis (55, 56). We have shown that fibroblast activation is also strongly associated with patient mortality regardless of disease stage and appears to be a better predictor of prognosis than TNM staging (57). Diagnostically, therefore, the identification of activated CAFs in tissue specimens of OSCC is an important guide to tumour behaviour and patient outcome.

Unfortunately, very little is known about the practical clinical implications of fibroblast senescence in OSCC. What we do know, however, is that fibroblast senescence is induced by the primary risk factors associated with OSCC including tobacco, alcohol and betel nut alkaloids and that fibroblast senescence precedes dysplasia in the pre-malignant condition oral submucous fibrosis (58). We are unaware of work examining fibroblast senescent markers in relation to OSCC tumour behaviour or outcome.

Conclusion

There is now compelling evidence that fibroblast stress responses promote epithelial cancer.

The fact that stromal fibroblasts are a genetically stable diploid cell population suggests that they may be ideal therapeutic targets. Our own work (Mellone, submitted for publication) and that of others (59) is encouraging in this context and may lead to the development new therapeutic modalities for the management of this debilitating disease.

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Conflict of interest statement

As far as we are aware, there is no conflict of interest in this manuscript.

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