The role of stromal fibroblasts in lung carcinogenesis: a target for chemoprevention?

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This article summarises the interactions between lung cancer cells and cancer associated fibroblasts determined by use of 3-dimensional co-culture systems, alluding to a role for targeting of the desmoplastic microenvironment in lung cancer chemoprevention strategies.

Abbreviations:

α-SMA, alpha-smooth muscle actin; ACC, adenoid cystic carcinoma; bFGF, basic fibroblast growth factor; BMF, buccal mucosal fibroblasts; CA, carbonic anhydrase; CAF, cancer associated fibroblasts; Cav, caveolin; CCL, Chemokine ligand; CCN, connective tissue growth factor; CDKN1A, cyclin-dependent kinase inhibitor 1A; CRC, colorectal cancer; CTLA, cytotoxic T-lymphocyte-associated protein; DDR, discoidin domain receptor; DSB, double stranded break; EGCG, epigallocatechin gallate; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinases; FAP, fibrinogen activating protein; FGF, fibroblast growth factor; FOX, forkhead box; GFP, green fluorescent protein; HAS2, human hyaluronan synthase 2; HFL1, human foetal lung 1; HGF, hepatocyte growth factor; ICAM, intercellular adhesion molecule; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; IPF, idiopathic pulmonary fibrosis; JAK, janus kinase; JNK, c-jun N-terminal kinase; LFA, lymphocyte function-associated antigen; MAPK, mitogen activated protein kinase; KF, keloid fibroblasts; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; mTOR, mammalian target of rapamycin; NE, neutrophil elastase; NF, normal fibroblasts; NHLF, normal human lung fibroblasts; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor receptor; PGE, prostaglandin E; PIK3, phosphoinositide 3-kinase; PSC, pancreatic stellate cells; RAGE, receptor for advanced glycation endproducts; ROR, RAR-related orphan receptor; RAR, retinoic acid receptor; SMAD-2, Mothers against decapentaplegic homolog 2**;** STAT, signal transducer and activator of transcription; STR-3, stromelysin-3; TAF, tumour associated fibroblasts; TAT, tumor associated T cells; TCR, T cell receptor; TFPI, tissue factor pathway inhibitor; TGF, transforming growth factor; TKI, tyrosine kinase receptor; TSP, thrombospondin; u-PAR, urokinase-plasminogen activator receptor;

Abstract

The tumour microenvironment plays an essential role in the development and spread of cancers. Tumour cells interact with the surrounding extracellular matrix, embedded within which, are a variety of non-cancer cells including cells of the vasculature, immune system and fibroblasts. The essential role of fibroblasts in the cultivation and maintenance of an environment in which tumour cells are able to maintain their aggressive phenotypic traits is becoming increasingly well documented. Cancer associated fibroblasts are able to secrete a vast array of extracellular matrix-modulating factors, meaning that they have potential for a functional role in every step of the carcinogenic process. In particular, they are likely to have a role in early tumour-initiating inflammatory events, and so may provide a potential target for chemopreventive intervention.

This review summarises the known interactions between lung tumour cells and surrounding reactive fibroblasts, highlighting the need to further investigate cancer associated fibroblasts as therapeutic targets in lung cancer chemoprevention strategies.

Introduction

The lung is a highly perfused, hyper-oxygenated organ in which the microenvironment plays a key role in response to the many environmental insults that it is constantly exposed to. This includes the rapid recruitment of inflammatory cells following injury, with chronic inflammatory response altering microenvironmental stimuli in favour of a pro-carcinogenic environment. In established cancers, tumour cells interact with the complex milieu that is the tumour microenvironment, consisting of extracellular matrix (ECM), cytokines, vasculature-related cells (eg, smooth muscle cells), immune cells (eg, macrophages, lymphocytes) and fibroblasts [1]. Permissive within this environment, is the activation of the fibroblastic cellular component to gain a myofibroblast-like sub-type resulting from acquisition of pre-malignant changes within neighbouring epithelial cells. The pathways by which fibroblast activation occurs are not well described, but may be dependent upon αvβ6 integrin/ Transforming Growth Factor β (TGFβ) signalling, maintained via tumour cells expressing E-cadherin and Epithelial Cellular Adhesion Molecule (EpCAM) [2]. These tumour-activated or Cancer Associated Fibroblasts (CAFs) secrete many matrix re-modelling proteins including collagen, fibronectin, laminin and tenascin [3]. Further mechanisms by which CAFs are able to influence proliferation and survival of the adjacent epithelial network, include production of a number of powerful paracrine and autocrine mediators, promoting tumour growth and generation of extensive microvasculature. Several of these factors are directly implicated in carcinogenic progression, and include Hepatocyte Growth Factor (HGF), Fibroblast Growth Factor (FGF), Insulin-like Growth Factor (IGF), Epidermal Growth Factor (EGF), Nerve Growth Factor (NGF), Transforming Growth Factor β (TGFβ), Vascular Endothelial Growth Factor (VEGF) Matrix Metalloproteinases (MMP) interleukins such as IL-6, IL-22 and wnt ligands. The ability of CAFs to secrete such an array of ECM modulating, and paracrine mediating, factors means that they have a functional role in every step of the carcinogenic process, encompassing early initiating inflammatory events, tumour growth, local invasion and ultimately, metastasis.

Within this review, we summarise the known interactions between lung epithelia and surrounding reactive fibroblasts, highlighting the need to further investigate CAFs as therapeutic targets in lung cancer chemoprevention strategies.

Interaction of fibroblasts with lung stem cells

In the healthy lung, lung stem cells are able to engage and recruit fibroblasts via paracrine signalling through stromal derived factor-1 (SDF-1). The fibroblasts are required by the stem cells to elicit their proliferative response, and to maintain a functional stem cell microenvironment. SDF-1 is itself regulated by fibroblast-secreted TNFα, which is in turn tightly regulated by p38α [4]. Similarly, lung cancer stem cells also rely on fibroblasts to maintain a functional stem cell niche in which they can maintain their self-renewing stem-like phenotype. It was recently observed that removal of CAFs from lung cancer stem cell co-cultures resulted in down-regulation of the characteristic stemness-associated genes *Oct3/4* and *Nanog*, resulting in much reduced tumour initiating frequency in the lung cancer stem cells. CAF-secreted cytokines that impacted on regulation of stemness genes included IGF-II, CD14 and HGF [5]. It is also likely that paracrine signalling via fibroblasts further contributes to the stem-like signature of lung cancer cells by induction of TGF-β-mediated epithelial to mesenchymal transition (EMT) [6].

CAFs in lung cancer

The origins of activated fibroblasts within the tumour stroma are ambiguous. It has been proposed that they may arise from a variety of originator cell types, including resident fibroblasts, bone marrow-derived progenitor cells, or epithelial cells that have undergone epithelial to mesenchymal transition to gain a myofibroblast-like phenotype [7, 8]. However, there is growing evidence that the origins of CAFs in lung cancer arise directly from reprogramming of resident fibroblasts, rather than from non-stromal sources or from a permissive stromal environment allowing for clonal expansion of rare fibroblast subsets exhibiting a CAF phenotype [9]. Characterisation of lung CAF ultrastructure reveals higher expression of intracellular α-smooth muscle actin (α-SMA) and extracellular bundles of fibronectin, associated with greater collagen gel contractility (a measure of matrix remodelling capacity) and invasive capacity compared to normal lung fibroblasts [10, 11]. CAFs also have higher basal levels of autophagy compared to their normal fibroblast counterparts, which may promote survival and protect against oxidative damage [12] or therapeutic intervention, allowing continued pro-carcinogenic signalling to adjacent tumour epithelium.

More extensive CAF characterisation has recently been undertaken using desmoplastic mouse mammary carcinoma models. Here, the mechanical stress-activated transcriptional regulator Yes-Associated Protein (YAP) exhibited nuclear localisation (maintained by Src) and was observed to be activated in CAFs, with the ability of CAFs to promote tumour cell invasion

dependent upon YAP activation [13]. Furthermore, nuclear translocation of YAP in CAFs was observed in pre-malignant models of breast cancer, suggesting a potential target for preventive strategies. Whilst this has yet to be observed in lung cancer models, growing evidence suggests that YAP-mediated ECM remodelling by CAFs is able to cause progressively stiffer matrices that drive tumour progression, and that this is likely to translate across a variety of tumour types [14].

In vivo evidence of a role for CAFs in driving tumour development arises from orthotopic and xenograft mouse models. Here, CAFs in co-culture with epithelial tumour cells exhibit significantly larger tumour volumes and faster growth rates compared with xenografts of tumour cells alone [9]. Additionally, xenograft co-culture models exhibit strong pro-inflammatory and angiogenic paracrine signalling [15], with the CAF component promoting metastatic deposition of circulating tumour fragments [16]. CAF-induced functional alterations are thought to occur in the leading edge of lung cancer cells, promoting not only their invasive capacity, but also their proliferative potential. The exact mechanism by which this occurs is unkown, but it has been suggested that CAFs are able to upregulate genes associated with regulation of cellular adhesion such as integrin-β3 and laminin-γ3, and anti-apoptotic proteins including Bcl-2, mediated via TGF-β [17]. Adhesion molecules such as the integrins, play a key role in cellular migration, which in mesenchymal-like migration, occurs via the leading edge of cells which undergo cyclical events of protusion and adhesion formation [18]. Integrins facilitate cellular migration by binding to proteins within the extracellular matrix such as fibronectin, collagen and laminin, increased deposition of which, can also be regulated by CAFs. Further evidence for the role of paracrine TGF-β signalling in tumour invasion and transition to an EMT-phenotype in epithelial lung carcinoma cells is observed from TGF-β-induced up-regulation of N-cadherin, vimentin and concurrent migratory properties in A549 lung adenocarcinoma cells. Furthermore, TGF-β cross-talk between lung cancer cells and fibroblasts, appeared to be regulated via IL-6 [19], with both *IL-6* and *CLCF1* (cardiotrophin-like cytokine factor) genes up-regulated in CAFs vs normal fibroblasts (NFs) [9]. Epithelial-mesenchymal interactions within the lung can also be regulated via a number of transcription factors including Forkhead box F1 (FoxF1), which has an essential role in normal lung development [20]. HGF and FGF-2 are fibroblast- secreted regulators of tumour cell proliferation and invasion, which are both up-regulated by FoxF1 as are α–smooth muscle actin and PDGFRα. Transcriptionally active FoxF1 therefore increases the paracrine signalling ability of fibroblasts to promote proliferation and invasion of neighbouring lung

epithelium, as well increasing the motility and contractility of the fibroblasts themselves [21]. ECM degradation by CAF-associated production of matrix remodelling proteins is key in allowing invasion of tumour cells into surrounding tissue areas. Furthermore, it is thought that motile fibroblasts provide invasive tracks down which tumour cells are able to migrate [22] [23]. This 'tracking' of the tumour cells may also be driven by mechanical stresses caused conversely by increased CAF-induced matrix deposition, raising interstitial pressures which force the tumour cells into less dense surrounding areas [24].

CAFs, and their ability to facilitate pro-carcinogenic signalling cascades, can also be affected by gross tumour morphology, which is observed in the case of hypoxia. Within the hypoxic stromal microenvironment, Hypoxia-Inducible Factors (HIFs) are stabilised and promote expression of CAF Membrane Metallo-endopeptidase (MME), which can be released into the microenvironment via exosomes [25]. Hypoxia-induced upregulation of MME results in elastin degradation and thus may enhance invasive capacity of hypoxic tumours [26]. Investigating interaction of CAFs with tumour cells and other cellular components of stromal matrices has increasingly been undertaken using co-culture models to demonstrate the extensive cross-talk between the cell types, and are summarised in Table 1 [5, 11, 21, 27-60], with an overview of these interactions in figure 1.

Relevance of CAFs in prognosis

Clinically, there is accumulating evidence which implies prognostic relevance for CAFs in several malignancies including Non-Small Cell Lung Cancer (NSCLC) [61-63], colorectal cancer [64] and breast cancer [61]. In lung cancer specifically, podoplanin [65], TGF-β1 and α-smooth muscle actin have been associated with poor prognosis in NSCLC [66] [67], with high stromal CD99 associated with improved long term survival in NSCLC [68]. Fibroblasts also interact with other cellular components of the ECM such as regulatory T cells (T_{reg}), high levels of which give rise to a poor prognostic signature. CAFs induce T_{reg} cells via TGF-β signalling, and thus have the facility to act in an immune-regulatory capacity [69]. Gene expression signatures of NSCLC CAFs vs Normal Fibroblasts (NFs) were used to develop an 11 gene prognostic signature (*ICAM-1*(intracellular adhesion molecule-1), *THBS2* (thrombospondin 2), *MME (*membrane metallo-endopeptidase), *OXTR* (oxytocin receptor), *PDE3B* (phosphodiestaerase 3B), *CLU* (clusterin), *B3GALT2* (UDP galactosyltransferase

polypeptide 2), *EVI2B* (ecotropic viral integration site 2B), *COL14A1* (collagen type XIV α1), *GAL* (galanin prepropeptide) , *MCTP2* (multiple C2 domains, transmembrane 2)) which was significantly associated with patient survival [45].

Role of CAFs in therapeutic resistance

Lung cancers that exhibit EGFR activating mutations are treated with small molecule EGFR tyrosine kinase inhibitors (TKI) such as gefitinib or erlotinib. TKIs are also often used in maintenance therapy to improve progression free survival and may have potential in the adjuvant setting. However, some patients exhibit intrinsic resistance to these TKIs, and most individuals will eventually acquire TKI resistance, ultimately resulting in treatment failure. Co-culture of lung cancer cell lines with fibroblasts has been shown to induce gefitinib resistance in gefitinib-sensitive PC9 lung cancer cells, which was ablated following treatment with an anti-HGF neutralising antibody [49]. Resistance to the anti-EGFR IgG1 monoclonal antibody, cetuximab, was also induced by HGF, likely via HGF-mediated constitutive phosphorylation of Met (HGF Receptor), Grb2-associated binder-1 (Gab1) and Akt [70]. Combination of tyrosine kinase inhibition with ionising radiation was unable to overcome TKI resistance in CAFs [71]. In addition, CAFs isolated from EGFR-TKI resistant tumours may further contribute resistance to EGFR-TKI-mediated blockade of the EGFR pathway and have been shown to exhibit tumourigenic properties in their own right in xenografts models [72]. Examination of extracellular vesicles (EV) shed from gefitinib-resistant NSCLC (PC9 R cells) adds weight to the two-way interaction between the tumour and surrounding microenvironment. Here, secreted EVs contained Akt, mTOR and EGFR-activating components which may act upon the microenvironment to further enhance resistance-inducing properties of stromal components [73]. EML4-ALK fusions in lung cancers are now specifically targeted using Met/ ALK kinase inhibitors such as crizotinib [74]. However, acquired resistance via mutation gain, render these new targeted treatments less effective. Acquisition of resistance to crizotinib is enhanced by CAF-secretion of HGF, mediated via increased Akt signalling, which is abrogated in the presence of a Met tyrosine kinase inhibitor [75]. Whilst potential for chemopreventive strategies are less clear within this tertiary setting, CAF signalling plays a clear role in the contribution to therapeutic resistance, tumour recurrence and metastasis. Thus, targeting of CAF signalling in lung cancer may contribute to prolonged sensitivity of tumour cells to current interventional modalities.

Targeting of CAF-related signalling pathways

Variable responses of CAFs to a variety cytotoxic drugs have reported, dictated by many factors including cancer type and microenvironment composition [76]. Dense desmoplastic stromal environments such as that observed in lung cancer exhibit poor drug penetrance properties, thus the tumour microenvironment plays a critical role in dictating efficacy of interventional drugs. If the extracellular matrix of the tumour could be modified, then drug penetration into the tumour might be facilitated. Table 2 [77-86] alludes to effects of various treatment modalities on CAFs.

Drugs such as the anti-hypertensive Losartin have known anti-fibrotic effects, via ability to down-regulate TGF-β activators such as thrombospondin-1, thus decreasing CAF-associated collagen deposition into the ECM [77]. Receptor tyrosine kinases have also been targeted in an attempt to inhibit the pro-carcinogenic interactions between CAFs and tumour cells. PDGF Receptors (PDGFR) α and β are highly expressed in CAFs, the tyrosine kinase activity of which can be inhibited by current molecular targeting agents such as Imatinib mesylate (Gleevec). Imatinib blocks PDGF-BB-induced activity of PDGFRβ in fibroblasts, abrogating PDGFR-mediated activation of Akt and extracellular-related kinase 1/2 (ERK1/2), preventing PDGF-induced fibroblast proliferation [78]. Similar findings were also observed for Dasatinib, Nilotinib and Sorefenib [79]. Stromal production of IL-6 causes tyrosine phosphorylation and activation of Signal Transducer and Activator of Transcription-3 (STAT3) which is often constitutively activated in NSCLC, and has a role in oncogenesis and resistance in particular, to targeted therapies [87]. The IL-6 neutralising antibody Siltuximab was observed to suppress fibroblast IL-6-induced STAT3 phosphorylation and activation in NSCLC cell lines *in vitro* and *in vivo* [80, 88], but lacked observable clinical activity in a number of different solid tumours [89]. Similarly, Sibrotuzumab, a promising humanised monoclonal antibody targeting fibroblast activation protein [81], lacked observable clinical efficacy in a phase II trial for metastatic colorectal cancer [90]. Inhibitors directed against Met activation by HGF blockade, include the anti- HGF antibody Rilotumumab (AMG 102), which has recently been evaluated in oesopho-gastric cancers in combination with epirubicin, cisplatin and capecitebine (ECX). Here, greater efficacy was observed in the Rilotumumab + ECX group than placebo + ECX group [82].

The potential for chemopreventive strategies to influence fibroblast-mediated models of disease, remains relatively unexplored. Chemoprevention models for breast cancer have utilised the mTOR inhibitor, rapamycin, to decrease stromal content of mammary tumours,

rendering the microenvironment less suitable for tumour growth and progression [83]. Curcuminoids (<100 nM) were able to block ECM deposition and TGF-β/p-SMAD-2 signalling pathways in keloid, a fibrotic disease characterised by the abnormal accumulation of ECM in the dermis [84], with further evidence for anti-fibrotic effects observed following inhibition of the bleomycin-induced fibrotic progression in the mouse lung [85]. Another compound with potential anti-fibrotic activity is the tea polyphenol Epigallocatechin Gallate (EGCG), which inhibited TGFβ-mediated oral submucous fibrosis via suppression of p-38 mitogen activated protein kinase (p-38 MAPK) and c-jun NH2-terminal kinase (JNK) phosphorylation [86]. Recently, the Src kinase inhibitor Saracatinib (AZD0530) was shown to prevent TGF-β –induced Src activation in human lung fibroblasts, preventing transition to a myofibroblast phenotype [91].

Many agents being investigated for their putative cancer chemopreventive properties, have been shown to exert effects on the signalling pathways described above, across many tumour models. Such agents, which may have potential for utility in lung cancer chemoprevention strategies include statins, non-steroidal anti-inflammatories (NSAIDS), metformin, tea polyphenols, curcumin and carotenoids [92-97]. To date, the mechanistic focus for these agents has been on their ability to directly inhibit pro-carcinogenic signalling pathways within the tumour cells themselves, whereas there is great potential for chemopreventive strategies to target ECM remodelling capabilities of fibroblasts. This in turn would decrease pro-carcinogenic paracrine signalling to adjacent epithelia, in addition to preventing cultivation of ECM niches permissive for tumour growth and invasion.

Ultimately, primary prevention strategies for lung cancer must be targeted towards smoking cessation, yet chemoprevention via pharmacological means remains attractive for those cohorts at high risk for either primary lung cancer, or lung cancer recurrence and metastatic spread. Targeting desmosplasia and the complex paracrine signalling networks between the epithelia and fibroblastic constituents in inflammatory fibrotic or malignant disease offers an attractive target for evaluation in future pharmacologic prevention strategies.

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Table 1: Studies utilising lung fibroblasts in model co-culture systems.

Year	Author	Fibroblasts Used/Source	Purpose	Findings/Comments	Ref
2014	Nazareth MR et al.	Primary human NSCLC tissue	Characterization of human lung tumour associated fibroblasts (TAF) and their effect on the activity of tumour associated T cells (TAT) cells	1) Fibroblasts expressed Thy1, α-SMA and fibroblast activating protein. 2) Co-cultures increased the levels of IFN-γ via T cell receptor (TCR) activation 2) TAF have the capacity to modulate the function of TAT cells derived from the same tumour microenvironment	[27]
2014	Prasad S et al.	Normal human lung fibroblasts (NHLF) and idiopathic pulmonary fibrosis (IPF) fibroblasts from surgical lung biopsies	Examine effect of fibroblast phenotype on epithelial repair	1) NHLFs and IPF fibroblasts stimulate a differential epithelial repair response 2) IPF fibroblasts exhibited reduced expression of PDGFRα compared to NHLFs 3) Co-culture of epithelial cells with IPF fibroblasts led to marked increase in the levels bFGF and PDGF 4) Increased migration and faster wound closure observed in co-cultures with IPF fibroblasts	[28]
2014	Amann A et al.	SV-80	Development of 3D cell culture system to study tumour - stroma interactions in non-small cell lung cancer cells	1) Promising tool for the generation of tumour spheroid co-cultures 2) Tumour-stroma interactions can be studied 3) Better reflection of *in vivo* cancer cell microenvironment	[29]
2014	Xiao Y et al.	HFL-1	To examine antitumor activity of gefitinib on lung fibroblasts co-cultured with non-small cell lung cancer (NSCLC) cells	1) Geftinib inhibited proliferation of co-cultured lung cancer cells 2) Presence of fibroblasts decreased the anti-invasive and anti-migratory effect of gefitinib on co-cultured NSCLC cells 3) Geftinib did not affect mRNA and protein levels of vimentin and MMP2 when tumour cells were in co-culture with fibroblasts.	[30]
2014	Kobayashi T et al.	HFL-1 Murine lung	To evaluate the role of endogenously produced MMP-	MMP-9 regulates fibroblast contraction of 3D collagen gels mediated through the generation of active TGF-β1	[31]

		matrices	9 in fibroblast contraction of 3D collagen gels		
2014	Chen WJ et al.	CAFs resected from NSCLC patients	To find how cancer stem cell plasticity is maintained *in vivo*	1) IGF1R signalling is activated in cancer cells in the presence of CAFs expressing IGF-II which induces Nanog expression and promotes stemness 2) IGF-II/IGF1R signalling blockade inhibits Nanog expression and attenuates cancer stem cell features	[5]
2013	Varzavand A et al.	MRC-5	To define Integrin α3β1 functions in tumour cells *in vivo*	α3 Integrin suppresses tumour cell growth in response to paracrine signalling from stromal cells	[32]
2013	Loubaki L et al.	Human bronchial fibroblasts isolated from patients	To investigate the role of bronchial fibroblasts obtained from asthmatic subjects and healthy controls in regulating Th17 response	1) Coculture of bronchial fibroblasts with $CD4^+$ T cells stimulated RAR-related orphan receptor (RORc) expression and induced a significant increase in Th17 cells 2) IL-6, IL-17, IL-22 IL-1β, TGF-β and IL-23 were significantly elevated in fibroblasts from asthmatic subjects upon co-culture with $CD4^+$ T cells	[33]
2013	Conte E et al.	NHLFs from patients undergoing surgery	To evaluate functional modifications induced by NHLFs in co-cultured $CD4^+$ T lymphocytes	1) Fibroblasts induced a significant increase in $CD25^+$ cells in co-cultured activated $CD4^+$ T lymphocytes 2) Fibroblasts treatment with a COX2 inhibitor abrogated the increment in $CD25^+$ cells whereas exogenous PGE_2 restored it 3) $CD25^+$ subpopulation was characterized by increased Fox-P3, Cytotoxic T lymphocyte associated protein-4 (CTLA-4), IL-10 and TGF-β positive cells	[34]
2013	Choe C et al.	Tissues from patients with resected NSCLC	To investigate the differential contribution of direct cell-cell contact and paracrine signalling factors to NSCLC metastases	1) CAFs potently induce EMT in NSCLC H358 cells through direct contact 2) H358 cells in direct contact with CAFs up-regulate the expression of the pan-mesenchymal markers α-SMA, FAP, TGFβ, SMAD3 and hedgehog signalling effector GLI family zinc finger-1 (GLI1) 3) Snail family zinc finger-1 (SNAI1) and SNAI2 are up-regulated, suggesting that the hedgehog signalling pathway is active in direct co-culture	[35]

2013	Kim SH et al.	Tissues from patients with resected NSCLC	To examine the role of CAFs in NSCLC tumour progression	1) CAFs exhibited greater expression of α-SMA than normal fibroblasts (NFs) 2) CAFs were more potent in inducing the EMT phenotype than NFs which led to increased motility and decreased proliferation of NSCLC cells via SMAD3 dependent up-regulation of p21(CIP1), CDKN1A and α-SMA	[36]
2012	Horie. M et al.	Tumour and non-tumour resected from NSCLC patient	Characterization of human lung CAFs in 3D *in vitro* co-culture model	1) CAFs showed higher α-SMA expression than NFs 2) CAFs enhanced and were more potent in inducing collagen gel contraction compared to NFs 3) CAFs had more potential to increase invasion of A549 cells compared to NFs.	[11]
2012	Ruiz P et al.	NHLFs	To investigate role of Discoidin Domain Receptor 2 (DDR) in primary human lung fibroblasts migration	1) DDR2 activation and associated signalling kinases JAK2 and ERK1/2 expression mediates fibroblast migration 2) Collagen I-induced expression of MMP-10 and MMP-2 is DDR2 but not DDR1 dependent 3) DDR2 is involved in fibroblast proliferation	[37]
2012	Mishra D et al.	Murine lung matrices	To compare the growth of human lung cancer cells in an *ex vivo* 3D lung model and 2D culture	3D lung model produced MMP which was not observed in 2D culture	[38]
2011	Shieh A et al.	IMR-90	To explore effect of interstitial fluid flow on fibroblast–tumour cell interactions	1) Interstitial flow stimulates fibroblast and concomitant tumour cell invasion 2) Flow-enhanced fibroblast invasion involved TGF-β1 activation 3) Interstitial flow increased collagen degradation	[39]
2011	Li Y et al.	Primary lung fibroblasts from patients with IPF	To determine regulatory effect of human hyaluronan synthase 2 (HAS2) and CD44 on IPF fibroblast invasion	HAS2 regulates IPF fibroblast invasion by modulating CD44 and MMP expression levels	[40]
2011	Asaithamby A et al.	IMR90	To investigate the biological significance of unrepaired	1) Double Strand Breaks (DSB) were repaired with slower kinetics in 3D culture than in 2D culture	[41]

			DNA lesions in differentiated lung epithelial cells in 3D and 2D co-culture	2) Defective DNA damage repair in 3D due to downregulation of multiple DNA repair pathway genes 3) Irreparable, complex DNA DSBs resulted in generation of chromosome aberrations	
2011	Gaud G et al.	CCD19-Lu	To investigated the impact of stable Tissue Factor Pathway Inhibitor-2 (TFPI2) inactivation in NCI-H460 NSCLC cells on their behaviour toward lung fibroblasts	1) TFPI-2 down-regulation promotes lung cancer cell migration and invasion without impact on cell proliferation 2) Down-regulation of TFPI-2 increases lung cancer cell adhesion to extracellular matrix proteins 3) TFPI-2 down-regulation enhanced cell adhesion to collagen IV and laminin and increased MMP expression	[42]
2010	Kamio K et al.	CCL-153 CCL-121	To evaluate role of statins in release of MMPs from human lung fibroblasts	1) Cytokines stimulated MMP-9 release in fibroblasts 2) Atorvastatin inhibited MMP-9 release in fibroblasts 3) Cytokines together with neutrophil elastase (NE) induced collagen degradation which was inhibited by atorvastatin	[43]
2010	Saito R et al.	Primary murine lung fibroblasts IMR-90	To investigate the role of FoxF1 in lung CAF	1) FoxF1 is expressed in CAFs of human lung cancer and is associated with activation of hedgehog signalling 2) FoxF1 controls the expression of HGF and FGF-2 3) FoxF1 controls the ability of fibroblasts to stimulate lung cancer cell migration 4) FoxF1 status of fibroblasts determines their ability to support subcutaneous tumour growth	[21]
2010	Liu T et al.	HFL1	1) To develop a microfluidic-based 3D co-culture device to reconstruct an in vitro tumor microenvironment 2) To investigate the effect of CAFs on cancer cell invasion in 3D matrix	1) Co-culture device reproducibly reflected the *in vivo* growth and invasion pattern of ACC 2) CAFs promoted ACC cell invasion in 3D matrix in a spheroid fashion, indicating that CAFs play a critical role in cancer invasion	[44]
2010	Navab R et al.	Tissues from NSCLC patients	To gain greater insight into the gene-expression characteristics in CAFs and tumor stroma of NSCLC	1) CAFs have greater ability than NFs to enhance the invasiveness and tumourigenicity of lung cancer cell lines 2) Genes differentially expressed between CAFs and NFs were also commonly differentially expressed in NSCLC	[45]

				tumour stroma compared with normal lung parenchyma	
2009	Vaira V et al.,	Lung tissue after surgical resection	Development of an organotypic model to investigate anti-tumoural and pharmacological properties that preserves the original cancer microenvironment	Model preserves tissue 3D architecture, morphology, cell viability, proliferative activity, PI3K/Akt pathway activity, and global gene expression profiles up to 5 days *ex vivo*	[46]
2009	Fujita H et al.	MRC5	To establish a co-culture system that could be used to quantify populations of cancer cells in co-culture with pancreatic stellate cells (PSC)s	1) MRC5 enhanced proliferation of pancreatic cancer cells, induced EMT-like morphological change and activated the Notch signalling pathway 2) The co-culture system can be used to quantitatively and reproducibly to evaluate GFP-expressing cell populations	[47]
2009	Zhu S et al.	NHLFs	To define urokinase-plasminogen activator receptor (u PAR) -integrin interactions and to determine the functional consequences of such interactions on NHLF	1) NHLFs express u-PAR and multiple integrin receptors 2) u-PAR and the integrins α_v-, α_3-, and α_5 and β_1-subunits co-localize during the initial phase of cell spreading 3) u-PAR/integrin interaction in NHLFs promotes NHLF's attachment, spreading, and migration	[48]
2009	Wang W et al.	Fibroblasts from patient lung cancer tissues	To assess the effect of crosstalk on the susceptibility to EGFR-TKI	1) CAFs from lung cancer tissue when co-cultured with EGFR mutated lung cancer cell line produced HGF and activated the c-Met pathway 2) EGFR sensitive cells became resistant to EGFR-TKI when co-cultured with activated HGF-producing CAFs by activating the MET/PI3K/Akt axis	[49]
2008	Nakao M et al.	Surgically resected lungs of lung cancer patients	To assess the significance of Carbonic Anhydrase (CA) IX expression by CAFs in adenocarcinoma of the lung	1) CAFs expressed CA IX. Noncancerous lung tissue expressed CA IX only when cultured under hypoxic conditions 2) Significant up-regulation of CA IX in response to hypoxia observed in the A549 cells 3) CA IX expression by CAFs was associated with smoking history 4) CA IX expression by CAFs was a better prognostic marker	[50]

				than CA IX expression by cancer cells	
2008	Martin M et al.	Primary murine lung fibroblasts	Development of a novel 3D in vitro organotypic model of breast cancer metastasis to lung	1) Better understanding of the tumour/host interaction 2) Novel model for studying metastatic breast cancer	[51]
2008	Maneva-Radicheva L et al.	NHLF	To provide new morphological insights into remodelling of collagen IV matrix by tumour/stromal cells	1) Fibroblasts alone were able to remodel collagen IV in a specific linear pattern 2) H460 carcinoma cells also tended to rearrange collagen IV 3) Fibroblasts co-cultured with H460 induced expression and activation of MMP-2	[52]
2006	Cekanova M et al.	CCD-19Lu HLF-A	To test whether fibroblasts stimulate growth of tumour cells	1) Fibroblast significantly increased proliferation of pulmonary adenocarcinoma cells via stimulation of EGF, Androgen receptor (AR) and TGF-α from pulmonary fibroblasts 2) ERK1/2 and Akt kinases were activated after culturing cells in fibroblast conditioned media 3) Expression of cell cyclin proteins cyclin D1, cyclin E and p21increased in cancer cells after co-culture with fibroblasts	[53]
2005	Vancheri C et al.	Surgically derived NHLFs	To study the interactions between NHLFs and T-cells	1) Co-culture increased the expression of COX-2 and ICAM-1 in NHLFs 3) Co-culture significantly reduced the expression of LFA-1, CD28 and CD69 4) Co-cultured cells showed significant reduction in production of TNFα. No effect on IL-10 was observed	[54]
2005	Bartling B et al.	WI-38	To study the role of receptor for advanced glycation endproducts (RAGE) in lung cancer progression	1) RAGE expression in cancer cells resulted in diminished proliferation and growth mediated by fibroblasts 2) Blockade of RAGE improved the proliferation of RAGE-expressing cells 3) Less activation of p42/44-MAPK in RAGE expressing cells 4) RAGE expression impaired growth stimulation mediated by IGF-1 and bFGF	[55]
2005	Pechkovsky DV et al.	NHLFs	To investigate the interaction of NHLFs and alveolar	1) CCL18 production by alveolar macrophages was significantly higher in co-culture than in alveolar	[56]

			macrophages	macrophages alone 2) NHLFs strongly enhanced the up-regulatory effect of IL-4 and IL-10 on CCL18 expression and production by alveolar macrophages *in vitro*	
2003	Fromigue O et al.	CCL-210	Gene expression profiling of NHLFs following co-culture with NSCLC cells	A network of early genes were identified which were induced in response to heterotypic interactions between epithelial tumour cells and normal fibroblasts.	[57]
2001	Pan T et al.	AG02262	To investigate the effect of adult rat type II cells on proliferation of adult human lung fibroblasts	1) Type II cells inhibit fibroblast proliferation by secreting factor(s) that stimulates PGE_2 production by fibroblasts 2) PGE_2 directly inhibits fibroblast proliferation	[58]
2000	Anderson I et al.	CCL-153 CCL-210	To elucidate the role of stromal elements in production of IL 8 in NSCLC	IL-8 transcripts and protein were consistently induced in fibroblasts and a subset of NSCLCs as a consequence of tumour/stromal coculture.	[59]
1998	Mari B et al.	CCL-153 CCL-210	To study role of stromelysin-3 (STR-3), a stromal cell product in tumour development and invasion	1) NSCLC cells stimulate normal pulmonary fibroblasts to release STR-3 and bFGF 2) STR-3 protein detected only when normal pulmonary fibroblasts are cultured with malignant bronchial epithelial cells	[60]

Abbreviations: ACC, adenoid cystic carcinoma; bFGF, basic fibroblast growth factor; CA, carbonic anhydrase; CAF, cancer associated fibroblasts; CCL, Chemokine ligand; CDKN1A, cyclin-dependent kinase inhibitor 1A; CTLA, cytotoxic T-lymphocyte-associated protein; DDR, discoidin domain receptor; DSB, double stranded break; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; ERK, extracellular signal-regulated kinases; FAP, fibrinogen activating protein; FGF, fibroblast growth factor; FOX, forkhead box; GFP, green fluorescent protein; HAS2, human hyaluronan synthase 2; HFL1, human foetal lung 1; HGF, hepatocyte growth factor; ICAM, intercellular adhesion molecule; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; IPF, idiopathic pulmonary fibrosis; JAK, janus kinase; LFA, lymphocyte function-associated antigen; MAPK, mitogen activated protein kinase; MMP, matrix metalloproteinase; NE, neutrophil elastase; NF, normal fibroblasts; NHLF, normal human lung fibroblasts; NSCLC, non-small cell lung cancer; PDGFR, platelet-derived growth factor receptor; PGE, prostaglandin E; PIK3, phosphoinositide 3-kinase; PSC, pancreatic stellate cells; RAGE, receptor for advanced glycation

endproducts; ROR, RAR-related orphan receptor; RAR, retinoic acid receptor; STR-3, stromelysin-3.TAF, tumour associated fibroblasts; TAT, tumor associated T cells; TCR, T cell receptor; TFPI, tissue factor pathway inhibitor; TGF, transforming growth factor; TKI, tyrosine kinase receptor; u-PAR, urokinase-plasminogen activator receptor; α-SMA, alpha-smooth muscle actin

Table 2. Drug treatments that may be used to specifically target fibroblasts.

Year	Author	Drug	Category	Model	Effect on CAF related proteins/pathways	Ref
2011	Diop-Frimpong, B., et al	Losartan	Anti-hypertensive	CAFs isolated from human breast cancer biopsies	1) No effect on levels of TGF-β1 2) Reduced levels of activated TGF-β1 following losartan treatment 3) Inhibits collagen I synthesis in CAFs 4) Losartan decreases thrombospondin (TSP)-1 expression	[77]
2010	Kinoshita, K., et al	Imatinib mesylate	Tyrosine kinase inhibitor	Primary cultured fibroblasts from human lung cancer tissues	1) Imatinib inhibited the PDGF-BB induced tyrosine kinase activity of PDGFRß in fibroblasts 2) Significant reduction in the levels of pAkt and pErk1/2 3) Inhibition of the PDGF-induced proliferation of fibroblasts 3) Imatinib reduced the proliferation-stimulating effect of fibroblasts on cancer cells 4) Possible direct inhibition of PDGF signalling by Imatinib	[78]
2010	Haubeiss, S., et al	Dasatinib Imatinib Nilotinib Sorafenib	Tyrosine kinase inhibitor	CAFs isolated from primary lung cancer specimens	1) All four drugs inhibit PDGFR and block growth in fibroblasts 2) Dasatinib and Imatinib inhibited DNA synthesis 3) Dasatinib inhibited tumour promoting activity of conditioned media in CAFs 4) Dasatinib treatment partially reverses CAF phenotype in fibroblasts from lung cancer tissues	[79]
2014	Song, L et al	Siltuximab	IL-6 neutralizing antibody	*In vivo* xenograft model with tumour cells co-administered with or without CAFs	1) Siltuximab had a more potent effect on tumour inhibition in models were tumour cells were co-administered with CAFs 2) No significant effect on *in vitro* cell viability 3) Siltuximab suppressed IL-6-induced STAT phosphorylation	[80]

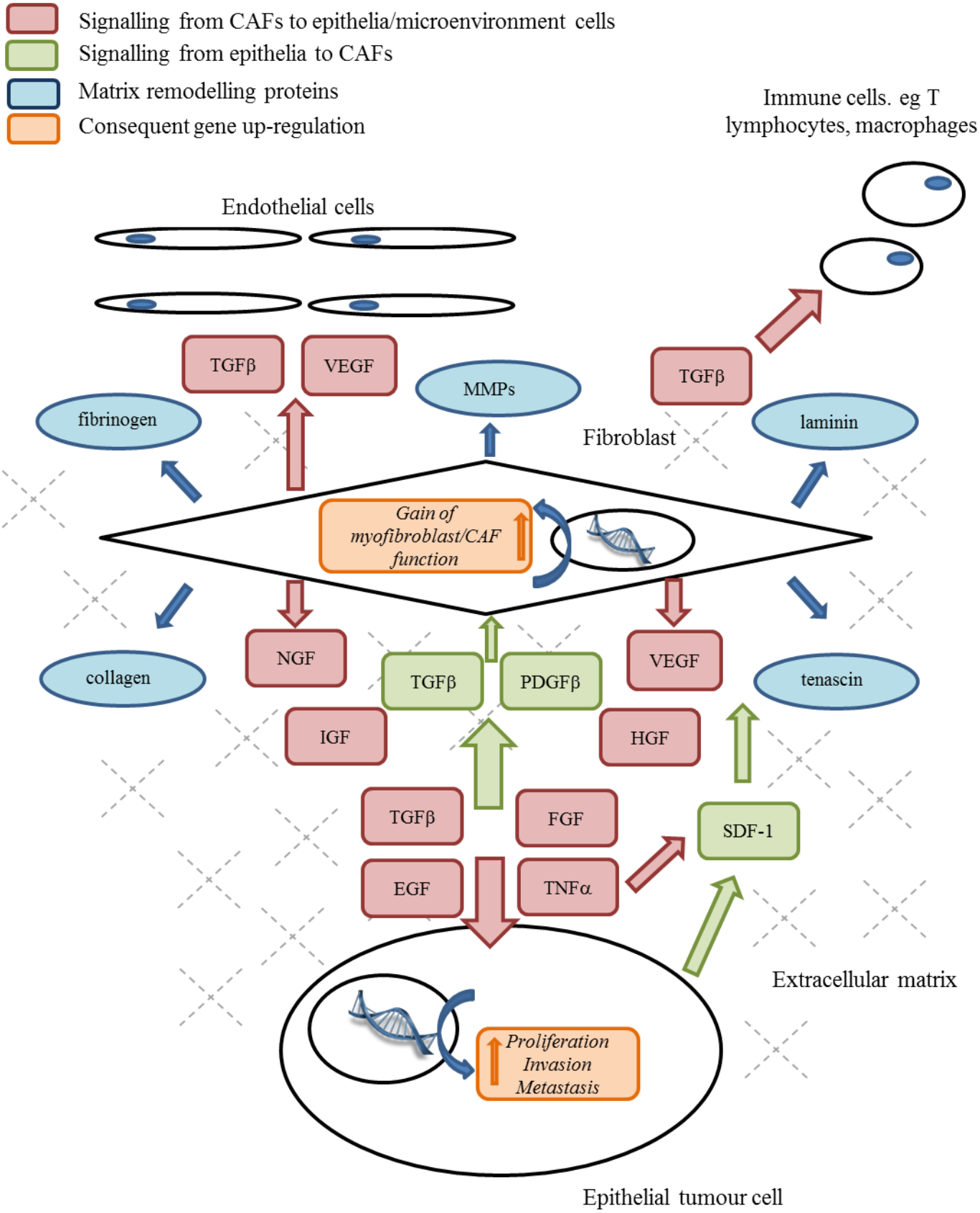
2003	Scott, A.M., et al	Sibrotuximab	FAP inhibitor	Phase 2 clinical study	Sibrotuximab found to be safe but ineffective in treating metastatic CRC	[81]
2014	Iveson, T., et al	Rilotumumab	Anti- HGF	Phase IIb study	1) Rilotumumab acts as Met inactivator by HGF blockade 2) Greater efficacy in treatment of oesopho-gastric cancers in combination with epirubicin, cisplatin and capecitebine	[82]
2012	Mercier, I., et al	Rapamycin	m-TOR inhibitor	Cav-1–KO mice xenograft model	1) Rapamycin effectively reduced the stromal content of tumours 2) Significant inhibition of growth of mammary tumours 3) Vimentin and phospho-S6 significantly decreased in Cav-1– deficient CAFs 4) Rapamycin treatment inhibited mTOR/pS6 signalling pathway 5) Decreased CD31-positive vessels after treatment 6) mTOR/S6-Kinase signalling in the tumour microenvironment increased in human breast cancer patients	[83]
2010	Hsu, Y.C., et al	Curcuminoids	Affects multiple targets	Dermis tissues from keloid patients	1) Curcuminoids inhibited bleomycin-induced ECM expression in keloid fibroblast (KF) cells 2) Curcuminoids inhibited bleomycin-induced elevation of TGF-β1 expression in KF cells 3) Curcuminoids inhibited bleomycin-activated TGFβ1/ SMAD-2 signal pathway in KF cells.	[84]
2011	Zhang, D., et al	Curcumin	Affects multiple targets	Bleomycin stimulated C57BL/6 mice and fibroblasts	1) Collagen deposition in lungs decreased after curcumin treatment 2) Increased expression levels of cathepsins L and K 3) Decrease in TGF-β1 expression 4) Caspase-3 expression and the ratio of Bax/Bcl-2 in HFL-1 cells were dose-dependently increased after curcumin treatment	[85]

2013	Chang, J.Z., et al	Epigallocatechin Gallate (EGCG)	Anti-oxidant	primary human BMF	EGCG dose-dependently inhibited TGFβ1-induced connective tissue growth factor (CCN2) expression by inhibiting the phosphorylation of JNK and p38 MAPK.	[86]

Abbreviations: BMF, buccal mucosal fibroblasts; CAF, cancer associated fibroblasts; Cav, caveolin; CCN, connective tissue growth factor; CRC, colorectal cancer; ERK, extracellular signal-regulated kinases; HFL1, human foetal lung 1; HGF, hepatocyte growth factor; IL, interleukin; JNK, c-jun N-terminal kinase; KF, keloid fibroblasts; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; SMAD-2, Mothers against decapentaplegic homolog 2**;** STAT, signal transducer and activator of transcription; TGF, transforming growth factor; TSP, thrombospondin

Figure Legends

Figure 1. Overview of some of the interactions between lung cancer cells and fibroblasts. Abbreviations: EGF – Epidermal Growth factor; FGF – Fibroblast Growth Factor; HGF – Hepatocyte Growth Factor; IGF – Insulin-like Growth Factor; MMP - Matrix Metallo Proteases; NGF- Nerve Growth Factor; PDGF – Platelet-derived Growth Factor; SDF – Stromal Derived Factor; TGF – Transforming Growth Factor; TNF – Tumour Necrosis Factor; VEGF – Vascular Endothelial Growth Factor.



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