Cancer Microenvironment (2012) 5:5–18
DOI 10.1007/s12307-011-0089-0

ORIGINAL PAPER

The Inflammatory Tumor Microenvironment, Epithelial Mesenchymal Transition and Lung Carcinogenesis

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Received: 6 June 2011 / Accepted: 30 August 2011 / Published online: 16 September 2011

Abstract The inflammatory tumor microenvironment (TME) has many roles in tumor progression and metastasis, including creation of a hypoxic environment, increased angiogenesis and invasion, changes in expression of micro-RNAs (miRNAs) and an increase in a stem cell phenotype. Each of these has an impact on epithelial mesenchymal transition (EMT), particularly through the downregulation of E-cadherin. Here we review seminal work and recent findings linking the role of inflammation in the TME, EMT and lung cancer initiation, progression and metastasis. Finally, we discuss the potential of targeting aspects of inflammation and EMT in cancer prevention and treatment.

Keywords Inflammation · NSCLC · E-cadherin

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Introduction

Chronic overexpression of inflammatory mediators in the TME, as seen in smokers [1] and patients with non-small cell lung cancer (NSCLC) [2], can lead to increased tumor initiation, progression, invasion and metastasis [3]. The link between unresolved inflammation and cancer has been well established with estimates that 15% of cancer deaths are inflammation-related [4]. Evidence for this link includes the following: a) some inflammatory diseases are associated with increased risk of cancer development; b) inflammatory mediators are present surrounding and within most tumors [5]; c) overexpression of inflammatory cytokines increases cancer development and progression in murine studies; d) inhibition of inflammatory mediators decreases cancer development and progression; and e) the use of non-steroidal anti-inflammatory drugs (NSAIDs) has been found to decrease cancer incidence and delay progression in patients with breast, prostate, lung and colorectal cancers [6, 7].

Although the origin of the inflammatory TME is currently unclear, two pathways have been postulated. In the intrinsic pathway the inflammatory microenvironment is caused by genetic alterations within neoplastic cells that lead to increased production of inflammatory mediators. Conversely, in the extrinsic pathway, the inflammatory environment is accommodating to cancer development. This inflammation could be present due to an unresolved infection, an autoimmune disease or chronic exposure to an

irritant [3, 8]. It is likely that a convergence of these two pathways is ultimately involved in inflammation-mediated cancer development and progression, although the molecular pathways explicitly linking these two have not yet been fully revealed.

Carcinoma-associated fibroblasts (CAFs) are major components of the tumor stroma and coordinate events responsible for cancer cell growth and survival, including angiogenesis and invasion. The replacement of fibroblasts with CAFs in the stromal compartment occurs at the invasive front of the tumor. CAFs are most frequently derived from local fibroblasts but may also be derived from pericytes or smooth muscle cells in the microvasculature or from tumor cells that have undergone EMT. The conversion of fibroblasts into CAFs is driven by cancer cell-derived cytokines, such as transforming growth factor-β (TGF-β) [9]. CAFs over-produce extra-cellular matrix (ECM) molecules and growth factors, including TGF-β, fibroblast growth factor 2 and vascular endothelial growth factor (VEGF), leading to a conversion from a normal to a cancer-supporting microenvironment, a process known as tumor stromatogenesis [10]. Tumor stromatogenesis leads to degradation of the basement membrane resulting in modified cytokine production by both tumor cells and CAFs [11]. Stromatogenesis begins early in tumor development as a result of cross-talk between the tumor and the stroma. The continuous and bilateral cross-talk, which occurs in normal tissue homeostasis as well as in the TME, is mediated through direct cell-cell contact or by molecules secreted by either tumor or stromal cells [9]. This cross-talk between tumors and their modified stroma results in inflammatory mediator production, tumor cell invasion, angiogenesis and ultimately metastasis [12]. While tumor cells may secrete VEGF minimally, it is secreted at high levels by activated CAFs and the inflammatory cells they recruit [13]. VEGF production leads to neovascularization and microvascular permeability, contributing to a highly permissive TME.

The main focus of our present review is chronic inflammation and EMT in the TME. EMT is an important process during embryogenesis, fetal development, wound-healing and fibrosis enabling cell motility as a part of tissue remodeling [14]. During EMT, in response to signals from the TME, cells change from a highly polarized epithelial phenotype with intact cell-cell junctions to a migratory, mesenchymal phenotype. As part of embryonic development and wound healing, EMT is tightly regulated. However, in chronic inflammation and in tumor progression, this regulation is lost. During EMT cell-cell junctions dissolve, the cytoskeleton reorganizes, proliferation increases and a switch from E-cadherin to integrin-mediated adhesion and degradation of the basement membrane occurs [15]. Normally the intact basement membrane prevents epithelial cells from contact with the interstitial space; exposure to the extracellular matrix and the growth factors contained there could promote and/or enhance EMT and tumor progression [16]. There is also increasing evidence that inflammation and EMT may be influential in tumorigenesis.

Inflammatory Mediators in the Developing TME

Chronic or deregulated inflammation in the pulmonary microenvironment is characteristic of pulmonary diseases that have the greatest risk for developing lung cancer, such as emphysema, chronic obstructive pulmonary disease (COPD) and pulmonary fibrosis [17–19]. These diseases are driven by common inflammatory mediators. Here, we will discuss the functions of TGF-β, tumor necrosis factor-α (TNF-α), prostaglandin E2 (PGE2), hepatocyte growth factor (HGF) and interleukin-1β (IL-1β) and their roles in EMT and carcinogenesis.

TGF-β is a member of a larger superfamily of 30 cytokines, which includes activin, myostatin and bone morphogenetic proteins. TGF-β exists in TGF-β1, TGF-β2 and TGF-β3 variant forms. They are expressed as precursors, requiring enzymatic cleavage to form biologically active dimers. The activation of TGF-β can also be initiated by integrins, particularly αvβ5, αvβ6 and αvβ8. These bind to an RDG sequence on the latent TGF-β complex and recruit proteases. The integrins form a docking station bringing proteases and latent TGF-β in close proximity thus allowing for activation [20]. Activated TGF-β is capable of binding its receptors, the TGF-β type I and type II receptors (TβRI and TβRII). Upon ligand binding, TβRII dimerizes with TβRI, activating its serine/threonine kinase and allowing for phosphorylation of Smad2 and Smad3. Activated Smad2/Smad3 can then bind with Smad4, enter the nucleus and act with other transcription factors to initiate downstream TGF-β target genes [21, 22]. In addition to this canonical Smad-mediated signaling, TGF-β can also signal via Smad-independent pathways including various MAP kinase, Rho-like GTPase and phosphatidylinositol-3-kinase/AKT signaling pathways [23]. TGF-β1, TβRI and TβRII are expressed in most tissues; thus, a cell's response to TGF-β is largely dependent upon cellular context.

Chronic overexpression of TGF-β1 in the pulmonary microenvironment often leads to fibrosis, a common precursor to lung cancer [19, 22]. Adamson et al. established that epithelial injury is sufficient to promote fibrosis in explanted mouse lung in the absence of inflammation, and that TGF-β1 is expressed at these sites following injury [24]. Alveolar epithelial type II cells, which are believed to be the progenitor cells of the lung

epithelium following injury, as well as differentiated alveolar epithelial cells, can respond to mediators of fibrosis. Both cell types have been shown to undergo EMT following chronic exposure to TGF-β1 in vitro and in vivo, leading to the loss of epithelial proteins, such as cytokeratins and zonula occludens-1, and the gain of expression of mesenchymal proteins, including vimentin and collagen type I [22].

Under normal conditions, TGF-β1 regulates tissue homeostasis, including proliferation, differentiation, survival and adhesion. In normal epithelial cells, TGF-β1 can induce growth arrest or apoptosis through its ability to increase expression of cyclin-dependent kinase inhibitors, such as $p15^{INK4b}$ and $p21^{CIP1}$, as well as repress c-myc [21]. In tumors, the TGF-β1 signaling cascade is often dysregulated, such that TGF-β contributes to evasion of immune surveillance, apoptosis resistance, increased proliferation, increased invasion and EMT [21, 25]. Hence, TGF-β has both anti-tumor and pro-tumor functions. Seigel et al. demonstrated the duality of TGF-β signaling in a study of breast cancer cells engineered to overexpress either dominant negative TβRII receptor or activated TβRI receptor. They showed that during early tumor development, TβRI-activated mice displayed delayed tumor development and decreased cellular proliferation, while the dominant negative TβRII mice showed no difference in proliferation. Yet, during metastasis to the lung, inhibition of TGF-β1 signaling via dominant negative TβRII receptor decreased metastases by reducing the number of cells that were able to extravasate from the blood vessels and enter the lung tissue. TβRI activation, however, increased extravasation and metastases [26]. Studies have demonstrated that throughout the progression of NSCLC TGF-β participates in EMT [21, 27] by HMGA2 induction, which leads to the upregulation of the transcriptional repressors Snail and Twist [28].

Another inflammatory mediator capable of acting in both an anti-tumor and pro-tumor manner is TNF-α. TNF-α can be secreted by a number of cell types, including macrophages, T- and B-lymphocytes. Originally described as reducing tumor burden, it is a potent pro-inflammatory mediator that can induce cytotoxicity [29]. However, in an inflammatory environment, TNF-α is often overexpressed and its control systems are frequently dysregulated. Studies associated TNF-α with a number of pulmonary diseases, such as asthma, chronic bronchitis and smoking-induced emphysema [18]. TNF-α overexpression in the TME can lead to EMT, the recruitment of leukocytes and the depletion of antioxidants from the microenvironment, which can lead to cellular oxidative stress [30]. Wu et al. provide an example of TNF-α-mediated EMT via their demonstration of TNF-α stabilization of the Snail protein in an NF-κB-dependent manner. This stabilization of Snail facilitates E-cadherin downregulation and EMT induction [31].

A number of tumorigenic properties of TNF-α are mediated via signaling through the NF-κB transcription factors [29]. The NF-κB transcription factor family members are ubiquitously expressed, but normally the pathway is not constitutively active, except for in a few cell types, such as mature B lymphocytes [32]. In all other cell types it is transiently activated by growth factors and cytokines, including HGF and TGF-β, and the downstream effects are context-specific. Aberrant NF-κB activation has been reported in a number of tumor types, including lung. TNF-α-induced NF-κB activation increases proliferation, angiogenesis, invasion, metastasis and cell survival [29].

Cyclooxygenase (COX) enzymes also play a role in inflammation and cancer progression in the pulmonary microenvironment. There are two isotypes of COX enzymes, COX-1 and COX-2. COX-1 is constitutively expressed in most tissues and cell types, while COX-2 is inducible by stimuli such as cytokines, growth factors and inflammatory mediators such as IL-1β, TGF-β and epidermal growth factor (EGF), as well as by cigarette smoke [33]. COX-2 is a rate-limiting enzyme in the conversion of free arachidonic acid into prostaglandin H2, a substrate for specific prostaglandin and thromboxane synthases. Prostaglandins mediate multiple biological effects, such as immune response, wound healing and blood vessel tone in both autocrine and paracrine fashion [34]. In particular, the major COX-2 metabolite, PGE2, signals through four G protein coupled receptors (EP1, EP2, EP3 and EP4) triggering downstream signaling cascades, such as mitogen activated protein kinase (MAPK)/Erk, [34, 35] and in a receptor-independent manner through a family of ligand-dependent transcription factors (PPARα, PPARγ and PPARδ), leading to the activation of downstream gene expression. Elevated PGE2 levels can be found throughout NSCLC progression, including in some pre-malignant lesions [36], and are associated with proliferation, invasion, apoptosis resistance, suppression of immune response and EMT [35, 37–39]. High tumor COX-2 and PGE2 expression is associated with poor patient prognosis independent of cancer stage. Overexpression of COX-2 in NSCLC has been shown to induce EMT through the downregulation of E-cadherin via the transcriptional repressors Snail and Zeb1 [40].

First identified as scatter factor due to its ability to induce epithelial cell scattering in canine kidney cells in culture, HGF is a potent growth factor that is secreted by mesenchymal cells. It begins as a pro-form that is activated through cleavage by serine proteases, such as the plasminogen activators [41], which allows HGF to form a fully activated heterodimer capable of signaling via its receptor tyrosine kinase, Met. HGF has a limited capacity to diffuse

in vivo such that the mesenchymal cells expressing HGF normally reside within close proximity to Met-expressing cells. In addition to paracrine signaling, HGF can also be sequestered within the ECM and, in the case of some tumors, signal in an autocrine fashion [42].

HGF/Met signaling acts on epithelial cells to increase motility and proliferation and is functional in both embryonic and adult tissues. During cancer progression, HGF can be induced by several inflammatory mediators, including TGF-β and TNF-α, as well as released from the ECM and activated by serine proteases secreted by infiltrating inflammatory cells and the tumor stroma [43]. HGF/Met signaling activates a number of downstream pathways that are implicated in cancer progression; the most prominent include MAPK, PI3K/Akt and NF-κB [41]. During development, the HGF/Met axis is a potent inducer of EMT, signaling epithelial cells to become mesenchymal in order to migrate to distant sites to form organs and organ structures [44, 45]. In normal adult tissues, HGF is increased during tissue injury in the liver, kidney, heart and lung. It is often elevated in the serum of lung cancer patients and serves as an indicator of poor prognosis independent of tumor stage [46, 47]. HGF/Met signaling induces angiogenesis, proliferation, motility, cell survival and EMT [41]. Recent studies demonstrated that HGF contributes to EMT via upregulation of Snail and subsequent downregulation of E-cadherin through the MAPK pathway [48]. Similarly, HGF/Met signaling through the MAPK and PI3K pathways is known to stimulate the disassembly of epithelial junction complexes by downregulating desmoglein 1 in melanoma, as well as mediating methylation of the claudin-7 gene in human breast cancer cells [49, 50].

COX-2 and Met inhibitors have been tested as possible treatments for NSCLC both as single agents as well as in combination with chemotherapy [33, 51]. The treatment of lung cancer patients with COX-2- or Met-specific inhibitors may sensitize NSCLC to other treatments, such as epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs). EGFR is commonly activated in NSCLC via mutation, amplification and overexpression of EGFR ligands [52]. Although a subset of patients whose tumors have activating EGFR mutations respond to treatment with EGFR TKIs, most NSCLC tumors do not respond, and the majority that do respond develop drug resistance. Interestingly, EMT has been implicated as a predictor of sensitivity to EGFR TKIs [52, 53]. As stated above, both PGE2 and HGF/Met signaling contribute to EMT. PGE2 signaling via EP receptors is able to activate MAPK/Erk, while MET amplification is able to activate ERBB3 allowing downstream PI3K/Akt signaling [35, 54]. Both MAPK/Erk and PI3K/Akt are important downstream pathways of EGFR signaling, and their activation by PGE2 and/or MET amplification bypasses EGFR TKI inhibition, conferring drug resistance. Therefore, PGE2 and Met inhibitors, in combination with EGFR TKIs, have been suggested for the treatment of NSCLC [54, 55].

IL-1β is an important mediator of inflammation and can be secreted by immune, stromal and malignant cells [56]. It is one of the first responders following injury and induces a number of other inflammatory cells. While IL-1β mRNA expression is not normally seen in healthy tissues, it is increased in a number of malignancies, including melanoma, colon cancer and lung cancer [56]. In a study by Colasante et al., IL-1β expression was measured in five NSCLC cell lines and in 26 lung tumor samples. None of the cell lines constitutively secreted IL-1β. In the patient samples, only 3 of 18 normal tissue samples expressed IL-1β, whereas almost all tumor specimens did [57].

It has been shown that elevated IL-1β levels are associated with increased tumor aggressiveness and invasion; however, the mechanisms underlying this relationship have not been fully explored. High levels of IL-1β expression have been implicated in tumor development and progression, a more invasive phenotype and poor patient prognosis [57].

The role of IL-1β in early tumorigenesis has been established in murine experiments. Overexpression of IL-1β in parietal cells of the stomach led to the development of chronic gastritis, metaplasia and high-grade dysplasia/carcinoma [58]. IL-1β can also participate in chemically induced carcinogenesis. In studies utilizing 3-methylcholanthrene, a known initiator and promoter of tumorigenesis, mice deficient in IL-1β had delayed and diminished tumor development compared to wild type. Conversely, when IL-1 receptor antagonist expression was depleted, the mice had a greater number of tumors and faster tumor development than wild type [59].

Murine studies have also shed light on the mechanisms of increased tumor aggressiveness that accompanies IL-1β expression in the TME. Experiments performed using fibrosarcoma cells transfected with the cDNA of activated IL-1β and transplanted into mice showed that tumor invasiveness positively correlated with the amount of IL-1β secreted [7]. In another study, mice were either treated with cells engineered to secrete IL-1β or exposed to lipopolysaccharide to induce IL-1β expression, prior to the injection of metastatic melanoma cells. Mice with high levels of IL-1β had significantly more lung metastases than control mice [60].

Studies in human NSCLC cell lines have demonstrated that IL-1β expression leads to downregulation of E-cadherin, increased tumor aggressiveness and invasion [61]. Similarly, IL-1β induces the production of factors involved in invasion and growth, such as matrix metalloproteinases (MMP) [62].

Thus, inflammation in the lung microenvironment contributes to tumor initiation and malignancy, promoting EMT through its ability to induce the downregulation of epithelial cell proteins and subsequent upregulation of mesenchymal cell proteins [22, 31]. As stated above, there are numerous examples of inflammation-mediated E-cadherin downregulation, which can be attributed to both the increased expression of the transcriptional repressors of E-cadherin (Snail, Slug, Twist and Zeb1) and the post-translational stabilization of Snail. This switch from an epithelial to mesenchymal phenotype highlights the importance of inflammation in the progression of lung cancer.

Inflammation Creates a Hypoxic Microenvironment

Hypoxia, a reduction in tissue oxygen tension, is an essential characteristic of the cancer microenvironment. Indeed, hypoxic areas and increased expression of hypoxia-related proteins are found in central necrotic regions of solid tumor masses and the invading front, respectively [63, 64]. Tumors invariably become hypoxic as uncontrolled proliferation causes tumors to outgrow the pre-existing vasculature, thereby depleting their oxygen and nutrient supply. Moreover, the new blood vessels the tumors develop are aberrant and have poor blood flow, compounding oxygen deprivation. Chronic inflammation also contributes to the local deficiency of oxygen due to the combination of reduced circulation at inflammatory sites and increased metabolic demand from infiltrating immune cells. In a feed-forward manner, hypoxia can promote chronic inflammation and thus, itself, in the developing TME through activation of NF-κB signaling in macrophages, neutrophils and non-immune cells, a finding recently confirmed in vivo in the lungs of mice [65, 66].

Although hypoxia has traditionally been viewed as a consequence of malignant tumor growth, it is now widely appreciated to play a critical role in the development and progression of tumors [63, 64, 67]. Hypoxia is associated with increased resistance to conventional radiation and chemotherapy, and not surprisingly, correlates with poor clinical prognosis in many cancer types, including breast, pancreatic and NSCLC [63, 64, 67]. The central mediator of the canonical cellular response to reduced oxygen levels is the basic helix-loop-helix heterodimeric transcription factor hypoxia-inducible factor (HIF)-1, which consists of a constitutively expressed β subunit (also known as aryl hydrocarbon receptor nuclear translocator) and an oxygen-regulated α subunit. In normoxic conditions, oxygen-dependent prolyl hydroxylase domain enzymes hydroxylate HIF-1α on two conserved proline residues. The von Hippel–Lindau (VHL) E3 ubiquitin ligase complex recognizes the hydroxylated proline and targets HIF-1α for ubiquitin-mediated proteasomal degradation. When oxygen is limited, hydroxylation of HIF-1α is inhibited, resulting in stabilization and translocation of HIF-1α into the nucleus, where it heterodimerizes with HIF-1β to form active HIF-1 transcription factor. Following recruitment of numerous co-activators, the HIF complex binds to hypoxia-response elements (HREs) in target genes to regulate a number of pathways including cell survival, proliferation, extracellular matrix remodeling, angiogenesis and apoptosis, consequently promoting tumor invasion and metastasis [63, 64, 67].

The hypoxic microenvironment has emerged as an important factor in the induction of EMT. Since the early observation of HIF-induced EMT in models of renal fibrosis [68], considerable evidence has further implicated the HIF pathway in EMT and metastasis. In this regard, HIF activation is associated with loss of E-cadherin and induction of mesenchymal gene expression. Interestingly, the transcriptional repressors of E-cadherin, namely, Snail, Slug, Twist, Zeb1 and Zeb2, can be regulated by hypoxia in human cancer. In VHL-deficient renal cell carcinoma, stable expression of the HIF-1α and HIF-2α isoforms is associated with increased expression of Snail, Zeb1 and Zeb2, loss of E-cadherin and an increase in invasion [69, 70]. Twist, a master regulator of gastrulation and mesoderm specification, is also essential in mediating cancer metastasis and can be directly regulated by both HIF-1α and HIF-2α [71, 72]. In NSCLC and breast cancer cell lines, hypoxia or overexpression of HIF-1α reduced E-cadherin expression and increased cell migration, invasion and metastasis, effects that were reversed by knockdown of Twist by siRNA [71]. Moreover, co-expression of HIF-1α, Twist and Snail in primary tumors of head and neck squamous cell carcinoma (HNSCC) patients correlated with the highest probability of metastasis and the worst prognosis. In accord with these findings, a study by Hung et al. reported that overexpression of HIF-1α, Twist or Snail correlated with poor overall survival in patients with NSCLC [73]. Furthermore, co-expression of any two or all markers correlated with a significantly worse prognosis, demonstrating the value of HIF-1α, Twist and Snail to predict the overall and recurrence-free survival in patients with resectable NSCLC. Although hypoxic induction of Snail transcription has been reported in multiple cancer types, an HRE in the minimal promoter of its gene *SNAI1* has only recently been identified [74]. In addition to HIF-1α and HIF-2α binding to the HRE in its promoter region, expression of *SNAI1* can be upregulated during hypoxia via other mechanisms, namely, Notch signaling. In a range of tumor cell lines, hypoxic activation of Notch signaling induced EMT and promoted cell migration, invasion and survival, effects that were attributed to direct upregulation of Snail and Slug expression, as well as the lysyl oxidase (LOX)-dependent stabilization of Snail protein [75, 76].

In addition to E-cadherin downregulation, hypoxia can increase the metastatic potential of tumor cells via other mechanisms. In NSCLC cell lines, HIF-1α activation of c-Met sensitized tumor cells to HGF stimulation, leading to ECM degradation, cell dissociation and increased cell migration through tissue parenchyma. Activation of the Wnt/β-catenin signaling pathway through HIF-1α can induce prostate cancer cells to be more motile and invasive [77, 78]. HIF-1α-dependent activation of TGF-β1 signaling and upregulation of survivin also contributes to EMT and resistance to apoptosis, respectively, in human NSCLC cells [79, 80]. Recently, hypoxia-induced metastasis has been linked to activation of the c-Myc pathway, upregulation of membrane-type 4 MMP via Slug and induction of LOX, which acts both extracellularly to stabilize collagen deposition and intracellularly to foster EMT thru stabilization of Snail [75, 81–83].

Thus, it is evident that hypoxia plays a critical role in the lung TME to promote malignant progression. In addition to its role as a transcription factor, which mediates the canonical hypoxia response via binding to HRE, HIF-α subunits can also regulate cellular functions through molecular interactions with other signaling pathways to induce EMT, invasion and metastasis.

The Inflammatory TME Contributes to Angiogenesis

One of the major processes that allow solid tumors to grow in size and become metastatic is the formation of their own blood supply through neovascularization or angiogenesis. Angiogenesis facilitates growth and metastasis by supplying nutrients to the tumor through a vascular system that is typically incompletely constructed [13]. The recruitment of endothelial cells and other blood vessel components is promoted by a number of cytokines and secreted signals present in the TME, including the prototypical pro-angiogenic mediator VEGF [84]. While tumor cells may secrete VEGF minimally, it is secreted at high levels by activated CAFs and the inflammatory cells they recruit [13]. VEGF production leads to endothelial cell recruitment, neovascularization and microvascular permeability, contributing to a highly permissive TME. Other cells present in the inflammatory TME are capable of promoting angiogenesis as well. Inflammatory leukocytes release endothelial cell stimulating molecules, including VEGF, HGF and IL-8 [84]. Eosinophils are recruited to the TME by cytokines secreted by the tumor cells. Because they express Flt-1 and Tie-2, the receptors for VEGF and angiopoietins respectively, they are also recruited by the pro-angiogenic stimuli secreted by tumor cells. Recruited eosinophils release VEGF stored in their granules and, under stimulation, produce additional VEGF [84].

The inflammatory TME developed during stromatogenesis leads to dysregulated cytokine and growth factor expression. Overexpression of the inflammatory mediator COX-2 in tumors is associated with angiogenesis, enhanced invasion and metastasis [85–88]. Exposure to PGE2 has been shown to upregulate the transcription factor Snail in NSCLC, as have the cytokines TGF-β and interleukin-6 in other tumor types [40, 89]. Snail overexpressing tumors have increased vascularity in part due to increased production of pro-angiogenic chemokines CXCL8 and CXCL5 and their receptor CXCR2 [90]. Both CXCL8 and CXCL5 are elevated in NSCLC tumor specimens, and their presence correlates with poor survival [91].

CXCL8 and CXCL5 are members of the CXC family of chemokines and play an important role in the induction of angiogenesis, even in the absence of VEGF [92]. Induction of CXCR2 by inflammatory mediators in immortalized human bronchial epithelial cells leads to malignant progression, indicating that inflammation may play a role in early as well as late stage lung tumor development and metastatic progression [93]. In vivo studies, which show that neutralization of CXCR2 in various models of lung tumorigenesis leads to inhibition of tumor progression and angiogenesis, highlight the importance of the TME in this process [90, 93, 94]. Also, stimulation with CXCL5 and CXCL8 induces release of VEGF into the TME by neutrophils [84]. Recent studies have shown that CXCL8, a transcriptional target of Ras signaling, along with CXCR2, is upregulated in cancers harboring oncogenic K-Ras mutations, further supporting the role of pro-angiogenic signaling at all stages of tumorigenesis [94].

Inflammation in the TME Drives Invasion

Inflammatory factors present in the TME, including TGF-β and IL-1β, lead to increased invasion and metastasis in melanoma, glioma and NSCLC [95–97]. These inflammatory mediators are present early in the TME. They may be capable of inducing a subset of pre-malignant and malignant tumor cells, ultimately leading to metastasis. This is consistent with the parallel progression model of metastasis [98]. Alternatively, sustained inflammation may also contribute to a more classical model of metastatic progression from an established tumor [98].

In addition to growth factors and pro-angiogenic factors, CAFs secrete ECM remodeling factors, including collagen type I & IV, secreted protein acidic and rich in cysteine (SPARC) and MMPs [99, 100]. Secretion of these factors by CAFs is likely induced by the signals derived from the tumor cells or other stromal components and can lead to development of an invasive phenotype [12]. For tumor cells to develop the ability to migrate through the basement

membrane and become invasive, the expression of membrane-degrading proteins, such as members of the MMP family, is required [101]. Induction of this phenotype is mediated by the same transcriptional repressors that are responsible for EMT initiation, including Snail and Slug [102, 103].

Snail-mediated morphologic changes include a spindle-like shape, larger cellular size and loss of cell-cell contacts due to altered expression of a variety of proteins [104]. Aberrant Snail expression has been noted in lung adenocarcinomas, squamous cell carcinomas and in lymphocytes present in the TME [90]. Loss of E-cadherin expression is seen especially at the leading edges of squamous cell carcinomas with strong Snail expression, suggesting a role for Snail in migration and invasion [90].

As discussed in the previous sections, the elevated expression of COX-2 is commonly found in the TME and leads to upregulation of Snail and therefore increased invasion [86]. However, COX-2 overexpression also has a Snail-independent pro-invasive effect. Recent studies have demonstrated that CD44, the cell surface receptor for hyaluronate that mediates cellular adhesion to ECM, is positively regulated by PGE2 in NSCLC cell lines, and its blockage in COX-2 overexpressing cells effectively inhibits invasion [39, 40].

The role of Snail in tumor progression and metastasis was further evaluated utilizing the murine tumor xenograft model of NSCLC [90]. These experiments clearly demonstrated that Snail overexpression contributed to increased primary tumor size and an increase in both proximal and distal metastasis. Furthermore, studies utilizing the chick embryo chorioallantoic membrane model to investigate the interactions between the basement membrane and the cancer cell in the earliest stages of progression have shown a critical role for Snail in these processes. In these studies, Snail-induced mobilization of membrane type-MMPs allowed cancer cells to migrate through the basement membrane barrier [105, 106]. Also, the gene expression analysis of lung cancer cell lines ectopically expressing the *SNAI1* gene revealed that a number of MMPs were upregulated by Snail overexpression [90].

Additional ECM-remodeling proteins are also upregulated by Snail, including the matricellular protein SPARC [90, 107]. SPARC is involved in cell-matrix interactions during tissue remodeling, wound healing and embryonic development [108, 109]. Expression of SPARC in tumor cells, CAFs and stromal endothelial cells can lead to an EMT-like phenotype, including loss of cell-cell adhesion and induction of MMPs and has been shown to increase tumorigenicity [110–112]. Expression of SPARC in the tumor stroma is correlated independently with poor prognosis and increased mortality in NSCLC and pancreatic cancers [113, 114] and leads to increased invasion and metastasis in melanoma, glioma and NSCLC [95–97, 115]. Tumor-associated macrophages recruited to the inflammatory microenvironment also secrete SPARC [116]. In a mammary carcinoma model, this macrophage-derived SPARC is required for in vivo metastasis. This metastasis requires deposition of fibronectin by SPARC, leading to enhanced tumor cell migration [116]. The relationship between inflammatory factors, EMT and invasion may play a critical role at all stages of cancer progression.

EMT and miRNA regulation

MiRNAs are a class of small non-coding RNAs that negatively regulate gene expression at the post-transcriptional level by binding to homologous regions in the target mRNAs blocking translation and/or inducing mRNA degradation [117, 118]. MiRNAs play important roles in essential cellular processes, including cell growth and differentiation, apoptosis and immune response.

Recent studies have implicated deregulated miRNA expression in EMT [119], as well as in the development and progression of various cancers [120, 121]. One of the first characterized miRNA families relevant in carcinogenesis was the miR-17-92 cluster, also known as onco-miR-17-92 [122]. Of interest here, miR-17-92 is involved in immune system regulation and is upregulated in lung cancer [123]. Early studies have demonstrated that miR-17-92 cluster expression is upregulated by the proto-oncogene c-myc, which itself is commonly dysregulated in human malignancies [124]. In addition to being highly expressed and promoting cell proliferation and apoptosis resistance in cancer cells [125], high levels of the miR-17-92 cluster are found in embryonic stem cells. This suggests that miR-17-92 promotes the proliferation of progenitor cells and inhibits their differentiation [126]. Similarly, ectopic expression of miR-17-92 promotes cell proliferation and inhibits differentiation of lung epithelial progenitor cells [127]. Among other c-myc-induced miRNAs are miR-221 and miR-222, which target proteins involved in cell cycle arrest [128], and miR-9, which suppresses E-cadherin expression and promotes metastasis. MiR-9 has also been found to sensitize cells to EMT-inducing signals arising from the TME [129]. Overall, the c-myc-induced miRNA network is reported to be directly related to tumor aggressiveness in various types of cancers [130].

The role of EMT mediators in the regulation of miRNAs is just beginning to be revealed. Transcriptional repressors, such as Snail, Slug and Twist that are induced by inflammation are also involved in this regulation. For example, Snail is able to upregulate miR-661, which in turn suppresses Nectin-1 and StarD10 expression, increasing the metastatic potential of breast cancer cells [131].

Twist upregulates a positive regulator of cancer cell migration and invasion, miR-10b, and thus initiates the development of distant metastases [132]. The latter study also described abundant expression of miR-10b in metastatic breast cancer specimens. Mir-21 and miR-31 have been identified as TGF-β-dependent positive regulators of tumor cells migration and invasion that act through suppression of T-cell lymphoma invasion and metastasis-inducing protein, a guanidine exchange factor of the Rac GTPase [133]. High levels of miR-21 have been found in many cancers, including lung cancer, and its target network includes tumor suppressive components of the p53, TGF-β and mitochondrial apoptosis pathways, as well as several tumor suppressors themselves [134].

In the TME, exosomes and other microvesicles generated by budding of the plasma membrane and subsequent release into the cellular microenvironment carry proteins as well as nucleic acids [135]. Importantly, aberrant release of microvesicles by malignant cells has been shown to be indicative of the extent of tumor invasiveness [136]. MiRNA-loaded exosomes shed from malignant cells can be transferred within the TME and are able to fuse with non-involved cells, introducing the functional material they contain [137]. Similarly, microvesicles derived from immune cells can transfer pro-inflammatory mediators and produce local sites of inflammation that may contribute to tumorigenesis [138]. This cell-to-cell communication mediated via the transfer of miRNAs from tumor cells may facilitate the pre-malignant transformation of non-involved cells in the TME. This effect is exacerbated by the fact that microvesicles are predominantly produced by the tumor cells. Studies have indicated that cancer patients have a significantly higher overall content of systemic miRNA in their blood compared to healthy subjects [139]. In recent years, advances in circulating miRNA studies have demonstrated that miRNAs implicated in EMT could serve as diagnostic and prognostic markers for various types of cancer [140]. In summary, the contribution of horizontal miRNA transfer in the inflammatory TME to cancer progression is only beginning to be realized. Further studies are needed to define the detailed mechanisms that govern this process and to validate the role of individual miRNAs in the promotion of malignancy. This will allow the development of novel therapeutic strategies to prevent transfer of circulating oncogenic miRNAs between cells in the TME.

Inflammation-Inducible EMT Drives Stemness and Contributes to Lung Carcinogenesis

Tumors harbor cancer stem cells (CSCs) or tumor-initiating cells capable of giving rise to new tumors with all the cellular and molecular heterogeneity characteristic of the original tumor [141–143]. The identities and origins of these CSCs within adult tissues and the mechanisms by which they drive carcinogenesis are areas of intense investigation. As previously described, many components of the developing TME, including inflammatory mediators, hypoxia and miRNAs, are able to induce EMT. Many of these same factors have also been associated with CSCs and carcinogenesis, suggesting that the developing TME drives expansion and possible malignant conversion of stem cells into CSCs via induction of EMT. Of note, the preponderance of research related to CSCs, in particular, inflammation-driven and EMT-mediated stemness, has come from the breast cancer research community, and the dearth of similar studies pertaining to lung carcinogenesis is striking.

In the seminal publication linking EMT, stemness and carcinogenesis, Mani et al. demonstrated that immortalized human mammary epithelial cells induced to undergo EMT also acquired expression of stem cell markers [144]. Differentiated mammary epithelial cells that had undergone EMT via TGF-β treatment or ectopic overexpression of Snail or Twist gave rise to CD44+CD24- cells with tumor-initiating capacity. An observational study utilizing human breast cancer specimens quickly followed and indicated that CSCs isolated from breast cancer display a distinct EMT signature [145]. More recently, LBX1, which directs expression of Snail, Zeb1 and Zeb2, was also noted to expand the CD44+CD24- CSC subpopulation and to morphologically transform mammary epithelial cells [146]. Finally, in the setting of head and neck cancer, Yang et al. provided convincing evidence that Twist1 directly regulates the stemness factor Bmi1 via cooperative repression of E-cadherin and $p16^{INK4a}$. Both Twist1 and Bmi1 were required for the observation of EMT and tumor-initiating capacity, and both were associated with reduced patient survival [147]. While the role of EMT in acquisition of stem cell characteristics and malignant conversion of the otherwise normal pulmonary airway epithelium is only beginning to be evaluated, there is abundant evidence of CSC induction by EMT in other solid tumors [141–143].

In addition to CSCs emerging directly from inflammation and transcriptional repressor-induced EMT, a hypoxic microenvironment is increasingly implicated as a driver of the EMT-stemness-carcinogenesis continuum. For example, CAFs have been shown to induce EMT and stemness through pro-inflammatory signaling which elicits COX-2/Rac1b-mediated release of reactive oxygen species (ROS) that ultimately drive a migratory and aggressive phenotype of prostate carcinoma cells [148]. This ROS-mediated induction of EMT and stemness by hypoxia is dependent on both NF-κB and HIF-1. Using repetitive cycles of hypoxia and reoxygenation, Louie et al. identified a CSC-

like subpopulation of metastatic breast cancer cells with enhanced EMT and stemness phenotypes, as well as increased tumorigenic potential both in vitro and in vivo [149].

Inflammation-induced dysregulation of oncogene and tumor suppressor gene expression is also beginning to be investigated as a critical mediator of the EMT-stemness-carcinogenesis axis. In one of the first accounts, Kurrey et al. described Snail and Slug activation of EMT, inactivation of p53-mediated apoptosis and de-repression of stemness-associated genes under conditions of radiation- and drug-induced stress. The authors proposed that the resulting CSCs were capable of escaping the unfavorable primary tumor niche, traveling to distant sites, and surviving/colonizing the metastatic niche, leading to their characterization of Snail and Slug as critical determinants of ovarian cancer progression and resistance to therapy [150]. Using pancreatic epithelial cells derived from p53−/− mice that were also cultured under stress conditions, Pinho et al. described similar EMT and stemness features mediated by the self-renewal factor Bmi1 [151]. Finally, Chang et al. extended this observation in breast cancer by demonstrating that p53 is a transcriptional activator of the *MIR200C* gene [152].

Similarly, in their initial investigation of the putative intestinal stem cell marker DCAMKL-1 [153], Sureban et al. reported expression in both normal intestinal stem cells and colorectal cancer cells, evidencing promotion of tumorigenesis via miRNA- and c-myc-dependent mechanisms [154]. In a subsequent investigation by the group, DCAMKL-1 staining was also observed in both human pancreatic intraepithelial neoplasia lesions and pancreatic adenocarcinomas [155]. Knockdown of DCAMKL-1 in human pancreatic cell lines resulted in reduced expression of Snail, Slug and Twist, elevated expression of miR-200a, downregulation of the protooncogenes c-myc and KRAS and inhibiton of Notch-1 via miRNA-dependent mechanisms. These new connections between EMT-induced stemness and critical genetic alterations that are widespread in human cancers (e.g., p53, c-myc and KRAS) suggest that the EMT-stemness-carcinogenesis continuum may be widely applicable across cancer types. We anticipate identification of novel and abundant targets for prevention and therapy as the relationships between inflammation, EMT and stemness in lung carcinogenesis are further explored.

As previously described, miRNAs are both induced and regulated by chronic inflammation and numerous components of the developing TME. A body of literature now substantiates regulation of EMT and stemness by miRNA. In one of the earliest studies of pancreatic and colon cancers, Wellner et al. discovered that Zeb1 promotes tumorigenicity by repressing stemness-inhibiting miRNAs [156]. In follow up investigations, they determined that the miR-200 family targets Notch pathway components, such as Jagged1, to mediate enhanced Notch activation of Zeb1 in two aggressive types of human solid tumors [157]. MiR-200 essentially counteracts classical EMT properties such as cell motility, and suppresses translation of stem cells factors, including Bmi1. Similarly, Tellez et al. used tobacco carcinogen exposure of immortalized human bronchial epithelial cells to induce EMT and stemness phenotypes [158]. The observed induction of EMT was driven initially by epigenetic silencing of miR-200 and miR-205 that included chromatin remodeling with subsequent promoter methylation.

Translating these findings into therapies and diagnostic tools for lung cancer will only be accomplished if human lung cancer stem cells can be accurately identified and characterized. Though the utility of each is still hotly debated, the markers most used at present to isolate lung CSCs are CD44, CD24, CD133 and ALDH [142, 159–161]. For example, Sullivan et al. recently discovered that ALDH selects for a subpopulation of self-renewing NSCLC stem-like cells with increased tumorigenic potential, and NSCLC patients with ALDH1A1+ tumor cells were found to have a worse prognosis. Utilizing both shRNAs against NOTCH3 and gamma-secretase inhibitors, the Notch signaling pathway was implicated as the mediator of the malignant potential of ALDH+ cells [161]. While the lung cancer research community must still identify the exact population of CSCs and the aberrant signaling pathways responsible for lung carcinogenesis, it is now clear that pathways associated with EMT (e.g., PGE2 or TGF-β) and stem cell maintenance (eg. Wnt or Notch) are points of intervention that should be interrogated as a high priority.

Future Perspectives

Pulmonary diseases associated with a heightened risk of lung cancer, such as COPD and pulmonary fibrosis, show both increased and dysregulated inflammation [162, 163]. Tobacco smoke exposure is associated with chronic airway inflammation and is the strongest risk factor for the development of lung cancer [17]. The inflammatory TME has many roles in tumor progression and metastasis, including the creation of a hypoxic environment, increased angiogenesis and invasion, changes in expression of miRNAs and an increase in a stem cell phenotype. All of these can affect expression and activity of transcriptional repressors of E-cadherin and subsequent EMT. Delineating signaling pathways linking inflammation and EMT may provide novel targets for prevention and/or treatment of lung cancer. Modulation of the arachidonic acid pathway, the goal of several ongoing clinical trials, is one approach to accomplish this goal. We believe that novel combined

approaches to simultaneously target chronic inflammation and EMT would also benefit by targeting CSCs.

Recently, investigations into the involvement of inflammation in tumor initiation have been undertaken. Polymorphisms in genes coding for inflammatory mediators found to be important in EMT, such as IL-1β, are associated with a heightened risk of gastric and lung cancer development [2, 58]. Along with widespread inflammation, Snail is reported to be increased in pre-malignant lung lesions and in the lungs of patients with COPD [141]. These findings implicate inflammation and EMT in early events in tumorigenesis and should be further explored. This is particularly true in lung cancer where disease is not often detected until metastasis has occurred.

Many strategies are being explored to develop non-invasive methods to detect cancer. These include investigations into the detection of circulating cancer cells, as well as identifying biomarkers or gene signatures in bronchial, oral or nasal samples that could distinguish between patients with and without cancer. Profiling serum-based miRNA is also under investigation and shows promise in identifying patients with cancer versus non-cancer [126, 127]. Once researchers can consistently identify and separate CSCs from normal stem cells and other cancer cells, molecular profiling of CSCs (mRNA, miRNA and protein) will also allow development of signatures enriched for EMT and stemness markers. These will provide a rich source of prevention and therapy targets as well as biomarkers of disease progression. Breast cancer researchers are already utilizing markers of EMT and stemness to identify metastatic circulating tumor cells from those with less metastatic potential [160], and this approach holds potential as a powerful diagnostic tool for lung cancer as well. This avenue has the potential to improve patient stratification, early identification of therapy failure and resistance risk assessment. These priorities and strategies should be part of a future discussion of new approaches to personalized medicine for lung cancer.

Acknowledgements This work was supported in part by grants from The Tobacco Related Disease Research Program: 18DT-0005 (ELH) and 18FT-0060 (TCW), American Thoracic Society (KK), LUNGevity Foundation (KK).

Conflict of interest The authors declare that they have no conflicts of interest.

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