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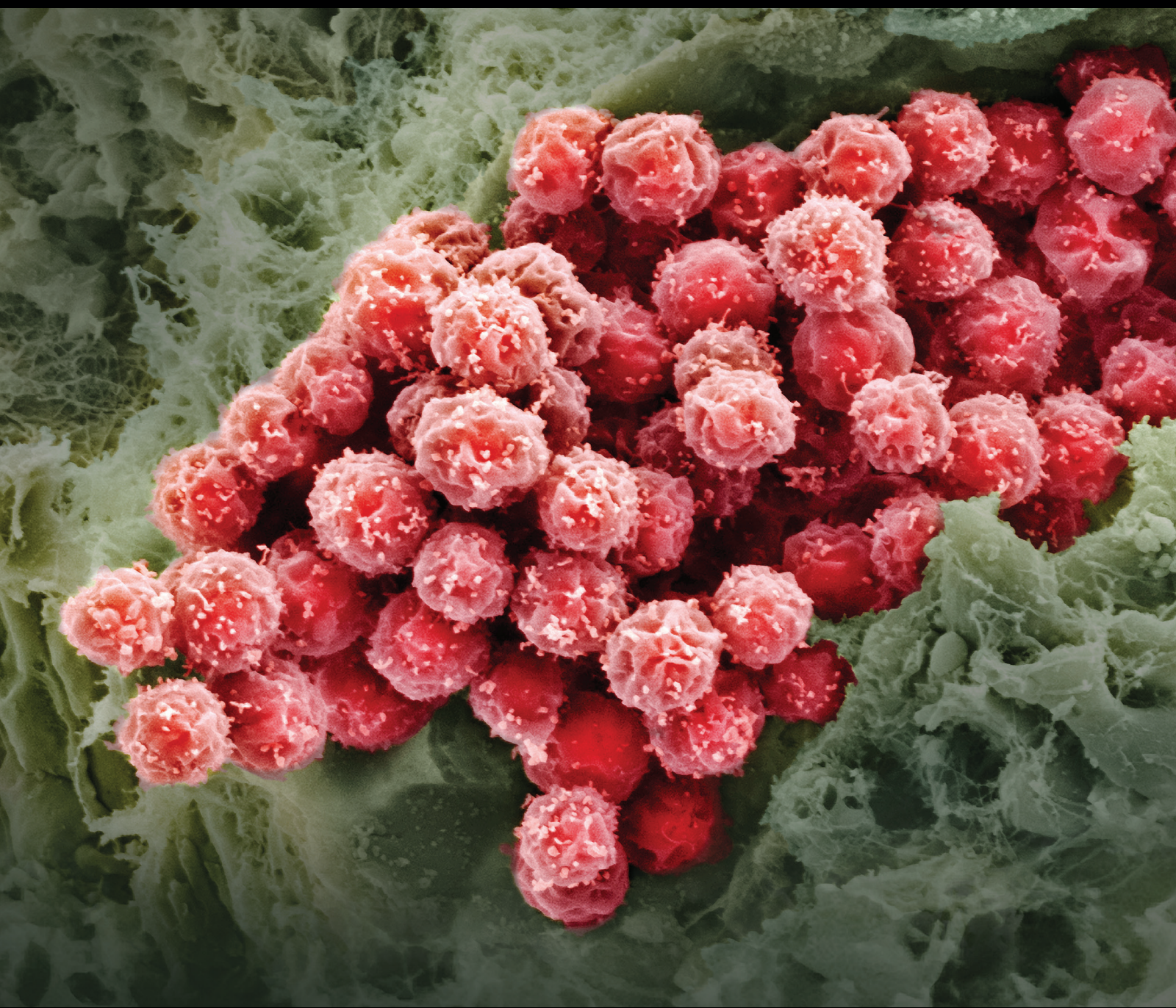
Mesenchymal Transitions in Development and Disease

Guest Editors: Damian Medici, Pura Muñoz-Cánoves, Pan-Chyr Yang, and Silvia Brunelli



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Editorial Board

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Editorial

Mesenchymal Transitions in Development and Disease

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The ability of epithelial cells and endothelial cells to transform into mesenchymal cells is one of the most basic cellular mechanisms in biology. This process, referred to as epithelial-mesenchymal transition (EMT) or endothelial-mesenchymal transition (EndMT), regulates various stages of embryonic development and contributes to the progression of a wide array of diseases and in tissue repair [1, 2].

During embryogenesis, EMT is essential for gastrulation, primitive streak formation, somite dissociation, neural crest development, and palate and lip fusion [3]. EndMT is critical for cardiac development, particularly in the formation of the valves and septa of the heart [4] and the generation of mesodermal cells and multipotent progenitors [5].

In the adult organism, EMT and EndMT are usually dormant until pathological stimuli awaken this embryonic mechanism. For example, EMT is the primary mechanism of cancer metastasis [6, 7], whereas EndMT forms cancer-associated fibroblasts in the tumor microenvironment [8]. Also, both EMT and EndMT have been shown to generate fibroblasts that cause the formation of scar tissue after tissue injury or in association with inflammatory and fibrotic diseases [9–11].

Mesenchymal transitions have traditionally been considered to have a positive effect in development and a negative effect in disease. However, novel findings regarding the stem cell phenotype generated by EMT and EndMT [12, 13] suggest that they may have therapeutic potential for the treatment of various degenerative diseases. This marks an exciting period in this field of research, which may provide new methods for tissue engineering and regeneration by harnessing the power of this embryonic mechanism.

In this special issue, the articles focus on the cutting-edge research on EMT/EndMT, including the role of this mechanism in regenerative medicine, peritoneal fibrosis, liver fibrosis, systemic sclerosis, and angiogenesis. This issue also explores how factors such as mechanical force, vitamin D signaling, and noncoding RNAs regulate mesenchymal transitions, which may provide novel insight into future avenues of research and therapeutic development.

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Review Article

Lymphoid Tissue Mesenchymal Stromal Cells in Development and Tissue Remodeling

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Secondary lymphoid organs (SLOs) are sites that facilitate cell-cell interactions required for generating adaptive immune responses. Nonhematopoietic mesenchymal stromal cells have been shown to play a critical role in SLO function, organization, and tissue homeostasis. The stromal microenvironment undergoes profound remodeling to support immune responses. However, chronic inflammatory conditions can promote uncontrolled stromal cell activation and aberrant tissue remodeling including fibrosis, thus leading to tissue damage. Despite recent advancements, the origin and role of mesenchymal stromal cells involved in SLO development and remodeling remain unclear.

1. Introduction

Secondary lymphoid organs (SLOs) such as spleen and lymph nodes (LNs) play a critical role in host defense. This function is ensured by the unique cellular composition of lymphoid tissues characterized by the presence of stationary mesenchymal stromal cells and highly motile hematopoietic cells. Although most of the attention has been concentrated on hematopoietic cells and their functions, the stromal counterpart has recently emerged as an important player in regulating immune responses and tissue homeostasis [1]. Alterations in stromal cell composition and function have been associated with different pathological conditions such as autoimmunity, infections, and cancer. Despite recent advances in the field, little is known about the origin and nature of the different mesenchymal stromal cells involved in tissue remodeling during homeostasis and disease. Indeed, a better understanding of the cells and signals contributing to tissue remodeling will provide basic knowledge for designing strategies aiming to promote tissue repair during pathological conditions such as chronic inflammation. Here we discuss the different steps involved in the maturation of lymphoid tissue mesenchymal stromal cells and how these cells contribute to tissue remodeling during normal and pathological conditions.

2. Development of Secondary Lymphoid Tissues and Origin of Stromal Diversity

Development of SLOs is spatiotemporally regulated during embryogenesis and requires interaction between lymphoid tissue stromal organizer (LTo) cells of mesenchymal origin and lymphoid tissue inducer (LTi) cells derived from the hematopoietic lineage [2–4]. The interaction between these two-cell types occurs through engagement of several molecules including the lymphotoxin β receptor (LTβR) expressed on mesenchymal cells by lymphotoxin $\alpha 1\beta 2$ (LT$\alpha\beta$) expressed on hematopoietic cells. LTi cells, which belong to the family of type 3 innate lymphoid cells, are also characterized by expression of CD45, CD4, interleukin-7 receptor α (IL-7Rα), integrin $\alpha 4\beta 7$, receptor-activator of NF-κB (RANK/TRANCE-R), and the chemokine receptor CXCR5. Conversely, mesenchymal stromal cells express, in addition to LTβR, platelet-derived grow factor-receptor α (PDGFRα) and the chemokine CXCL13 [5]. The latter is a critical signal for attracting LTi cells expressing the CXCL13-receptor CXCR5 to the site of organ formation [3]. Although differences exist in the initial steps of spleen and lymph node development, lymphomesenchymal interactions are critical to promote the differentiation of mesenchymal progenitors

to mature stromal cells and the establishment of distinct tissue compartments. Studies on mice deficient for molecules expressed by LTi (e.g., CXCR5) or by LTo cells (e.g., CXCL13, LTβR) have shown defects ranging from organ agenesis to disrupted tissue architecture [3]. Although the developmental relationship between embryonic mesenchymal cells of the lymphoid tissue anlage and the adult stromal compartment is not fully elucidated, recent findings demonstrated that spleen stromal cells arise from multipotent embryonic mesenchymal cells of the Nkx2-5$^+$Isl1$^+$ lineage [6]. It was shown that nearly all mature mesenchymal stromal cells, namely, follicular dendritic cells (FDCs) of the B-cell follicle, marginal reticular cells (MRCs) localized underneath the marginal sinus, fibroblastic reticular cells (FRCs) in the T-cell zone, and NG2+ perivascular cells, originate from embryonic mesenchymal descendants [6]. While this mechanism for generating stromal diversity applies to the spleen, the embryonic lineages contributing to the different stromal cells of the LN remain unclear. Furthermore, Nkx2-5$^+$Isl1$^+$ mesodermal precursors do not contribute to spleen or lymph node endothelial cells, thus indicating that different mesodermal lineages are involved in generating SLO stromal diversity including lymphatic and endothelial cells. Interestingly, endothelial cells have been shown to undergo endothelial-mesenchymal transition (EndMT) during cardiac development [7, 8]. Whether this mechanism also contributes to the generation of stromal diversity during SLO development remains an open question.

Stromal cells express several receptors of TNF superfamily of proteins including LTβR, RANK, and Tumor Necrosis Factor Receptors [9]. By engaging with their ligands, LT$\alpha\beta$ and Tumor Necrosis Factor (TNF) expressed by hematopoietic cells, these receptors trigger the secretion of homeostatic chemokines such as CCL19/CCL21 and CXCL13 that play a critical role in attracting and positioning T- and B-cells within SLOs [9]. Indeed, mice deficient for LTβR or genes encoding chemokines secreted by stromal cells have profound disorganization of the white pulp area and defective immune functions, demonstrating the critical role played by mesenchymal stromal cells as "organizers" of the lymphoid compartments [10]. Stromal cells also produce the extracellular matrix (ECM), a tridimensional framework of reticular fibers composed of basement membrane and interstitial matrix components that provide structural support [11]. In the T-cell zone, FRCs form the so-called conduit system, a reticular collagenous network that allows the transport and distribution of small molecules or particles from the periphery to T-cell zone [12].

In the B-cell follicle, FDCs play a crucial role in promoting B-cell immunity [13]. FDCs promote recruitment of B-lymphocytes into the follicles through secretion of CXCL13 that binds CXCR5 expressed on B-cells. This stromal cell-type presents antigens in the form of immune complexes that are bound via Fc and complement receptors, thus stimulating B-cells through the B-cell receptor (BCR) and promoting germinal center formation. Generation of FDC networks relies on TNFR and LTβR signaling; however, only signals through LTβR were shown to be required for FDC maintenance [14]. MRCs are stromal cells that localize underneath the marginal sinus and in the outermost region of the follicle and express CXCL13 and MAdCAM-1 [15]. Although the exact function of MRCs remains elusive, recent work showed that MRCs contribute to the accumulation of FDC during germinal center formation [16]. In addition, the expression of B-cell chemokines and the close association of this cell-type with CD169$^+$ marginal metallophilic macrophages suggest their possible involvement in supporting local niches.

3. The Extracellular Matrix of Secondary Lymphoid Organs

The stroma is defined as the connective and functionally supportive structure of a tissue or organ. It consists of fibroblasts and vascular cells and their associated extracellular matrix (ECM) proteins such as collagens, fibronectin, glycosaminoglycans, and proteoglycans [17]. The ECM has been viewed only as a tridimensional framework to which cells adhere. However, work over the past years has demonstrated that the ECM is not merely an inactive player in tissue homeostasis, but, instead, a structure with define physical and biochemical properties able to affect cell behavior [11]. Indeed, the continuous cell-ECM cross talk allows cells to sense the surrounding environment, resulting in changes in gene expression. For instance, the ECM affects cell behavior by different mechanisms: (i) by regulating cell-accessibility to growth factors; (ii) by providing cells with ligands for cell-surface receptors; (iii) and by affecting migration and proliferation through ECM-stiffness and composition [18, 19]. Deregulation in ECM structure and composition has been associated with different pathological conditions including tissue fibrosis and cancer by promoting apoptotic evasion, cell survival, proliferation, and invasion [18, 20, 21].

In peripheral lymphoid tissues, two biochemically and morphologically different ECMs exist: the interstitial matrix (IM) and the basement membrane (BM). The IM represents the ECM that connect fibroblastic reticular cells and is composed of interstitial collagens (types I, III, V, and XI) that confer high flexibility and tensile strength, as well as proteoglycans and glycoproteins, such as fibronectin, tenascin, and vitronectin, able to recognize and bind several cytokines, chemokine, and growth factors [17, 19]. The BM is a sheet of ECM that acts primarily to separate the different functional compartments of the organ. It is mainly composed of four molecules: type IV collagen, noncollagenous glycoproteins belonging to the family of laminins, heparan sulphate proteoglycans, and glycoproteins [17, 19, 22]. One of the peculiar three-dimensional structures of SLOs is the conduit system, a complex structure of FRCs and reticular fibers that promotes the rapid transport of small molecules, such as chemokines, cytokines, and small molecular weight antigens, from peripheral sites to the lymphoid compartments [23]. The conduit also acts as a scaffold for lymphocyte locomotion within SLOs, thus facilitating cell distribution and interactions [24]. The reticular fibers of the conduits show a highly organized core of collagens, mostly type I and type III, and associated with fibrils ensheathed by the BM. The latter is composed of laminin isoforms 511, 411, and 332,

heparan sulphate proteoglycan, perlecan, collagen type IV, and nidogen to which FRCs adhere [25–28]. Collagen IV can bind several chemokines and cytokines such as CCL21 and IL7 produced by FRC, thus facilitating the positioning of T lymphocytes within SLOs. FRCs are interconnected and ensheathing the conduit system in which dendritic cells (DCs) fill the free space and pick up antigens directly from the conduit [29, 30]. This means that lymphocytes are not in direct contact with the basal membrane and the fluid present in the conduit, though antigens and small molecules are accessible through FRCs or DCs present in the gaps of the conduit. Recently, it has been demonstrated that the specific expression of perifollicular laminin $\alpha 5$ in the marginal zone (MZ) of the spleen drives the localization of a specialized B-cell population expressing integrin $\alpha 6\beta 1$ to this area. Moreover, laminin $\alpha 5$ was found to regulate not only the localization but also the fate and long-term survival including the antibody responses of MZ B-cells. These findings indicate that stromal-derived ECM actively influences immune cell behavior through several mechanisms [18, 19].

4. Remodeling of the Stromal Microenvironment in Acute Inflammation

The acute phase of an adaptive immune response is characterized by lymph node expansion in order to host the incoming wave of naïve lymphocytes and the proliferation of antigen-specific lymphocytes prior to returning to its physiological size during the resolution phase [31–33]. In this process, the distribution of stromal cells and their associated ECM undergoes transient changes to support immune responses. These include the expansion of fibroblastic reticular and lymphatic networks and the increase in size and permeability of high endothelial venues (HEVs) and lymphatic vessels in order to facilitate the extensive accumulation of naïve lymphocytes and fluid from the periphery [34–37]. Although the origin and nature of stromal cells that participate in LN hypertrophy and remodeling remain elusive, recent studies have identified FRCs as key players in the process [38]. Stretch of preexisting FRC networks and FRC proliferation are involved in LN enlargement [33, 36, 39]. Dendritic cells (DCs) have been shown to regulate the stretch of FRCs, via CLEC-2 on DC binding to podoplanin (PDPN) on FRC and resulting in the inhibition of PDPN-mediated FRC contractility, and relaxation of the stromal networks [31, 32]. Changes in FRC contractility could directly influence FRC proliferation through mechanotransduction, a process known to convert mechanical forces into chemical or genetic changes at cellular level [32, 40]. The nature of inflammatory stimuli affects the timing at which the proliferation of FRC occurs. Indeed, whereas LPS stimulates FRC proliferation as early as 24 hrs after injection, immunization with ovalbumin (OVA) in complete Freund's adjuvant (CFA) or Montanide causes stromal cells to proliferate modestly within 2 days and more vigorously until day 5 after injection [31, 33, 41]. The initial phase of proliferation is dependent on $CD11c^+$ DC, whereas T- and B-cells contribute to the subsequent expansion phase [33, 41]. The findings that ablation of LTβR signaling in stromal cells abrogated FRC proliferation indicate that LT$\alpha\beta$ from lymphocytes plays, at least in part, a role in remodeling of the FRC network [41, 42]. Interestingly, inflammation following CFA immunization causes changes in stromal composition and gene expression within T-cell zone stromal cells. It was reported that inflamed B-cell follicles extend towards the T-cell zone and induce the expression of CXCL13, a chemokine normally produced by FDCs, in stromal cells. Induction of CXCL13 was shown to depend on LT$\alpha\beta$ from B-cells and the cells induced to express CXCL13 were called versatile stromal cells (VSC) [43]. Interestingly, during the contraction phase of B-cell follicles, VSCs downregulate CXCL13 expression, thus indicating a degree of plasticity of this mesenchymal cell type. From a developmental perspective, the origin and nature of VSCs remain unknown as the signaling underlying their plasticity [43].

Many viral infections induce a generalized immunosuppression that could be transient, during the acute phase, or prolonged, in chronic viral infections. In the case of lymphocytic choriomeningitis virus (LCMV), it was shown that infected FRCs are killed by LCMV-specific $CD8^+$ T-cells during the acute phase of infection. Loss of the FRCs appears to be mediated by perforin-dependent and perforin-independent mechanisms and strongly correlates with the impairment of CCL19 and CCL21 expression, two chemokines important for positioning T-cells within the FRC zone [1, 44]. Interestingly, remodeling and restoration of stromal network integrity occurs approximately four weeks after LCMV infection and depends, at least in part, on LTi-stroma interactions via LTβR signaling [44]. The survival and proliferation of adult LTi cells are induced by IL-7. Stromal and lymphatic endothelial cells expressing IL-7 are critical during LN remodeling after LCMV infection, as demonstrated by the findings that ablation of IL-7 expressing stromal cells strongly impairs restoration of tissue integrity [45]. In the spleen, regeneration of the stromal network was shown to depend on local $Nkx2\text{-}5^+Islet1^+$ mesenchymal descendants, possibly possessing stem cell activity. In this setting, local expansion of mesenchymal stromal cells and not migration of peripheral cells appeared to be the underlying mechanism of tissue regeneration [6]. Nevertheless, the exact nature of mesenchymal stromal cells involved in tissue repair after LCMV infection remains unclear. Perivascular cells have been proposed to act as mesenchymal stem cells during tissue repair and thus could represent good candidates in SLO remodeling after loss of tissue integrity.

5. Persistent Stromal Remodeling and Tissue Fibrosis during Chronic Inflammation

Chronic inflammation is characterized by persistent inflammatory stimuli or by deregulation of the mechanisms involved in the resolution phase [9]. This condition stimulates uncontrolled stromal cell activation and consequent aberrant tissue remodeling including fibrosis [9, 46]. One example is the chronic infection caused by human immunodeficiency virus-1 (HIV-1). Indeed, a large number of patients infected

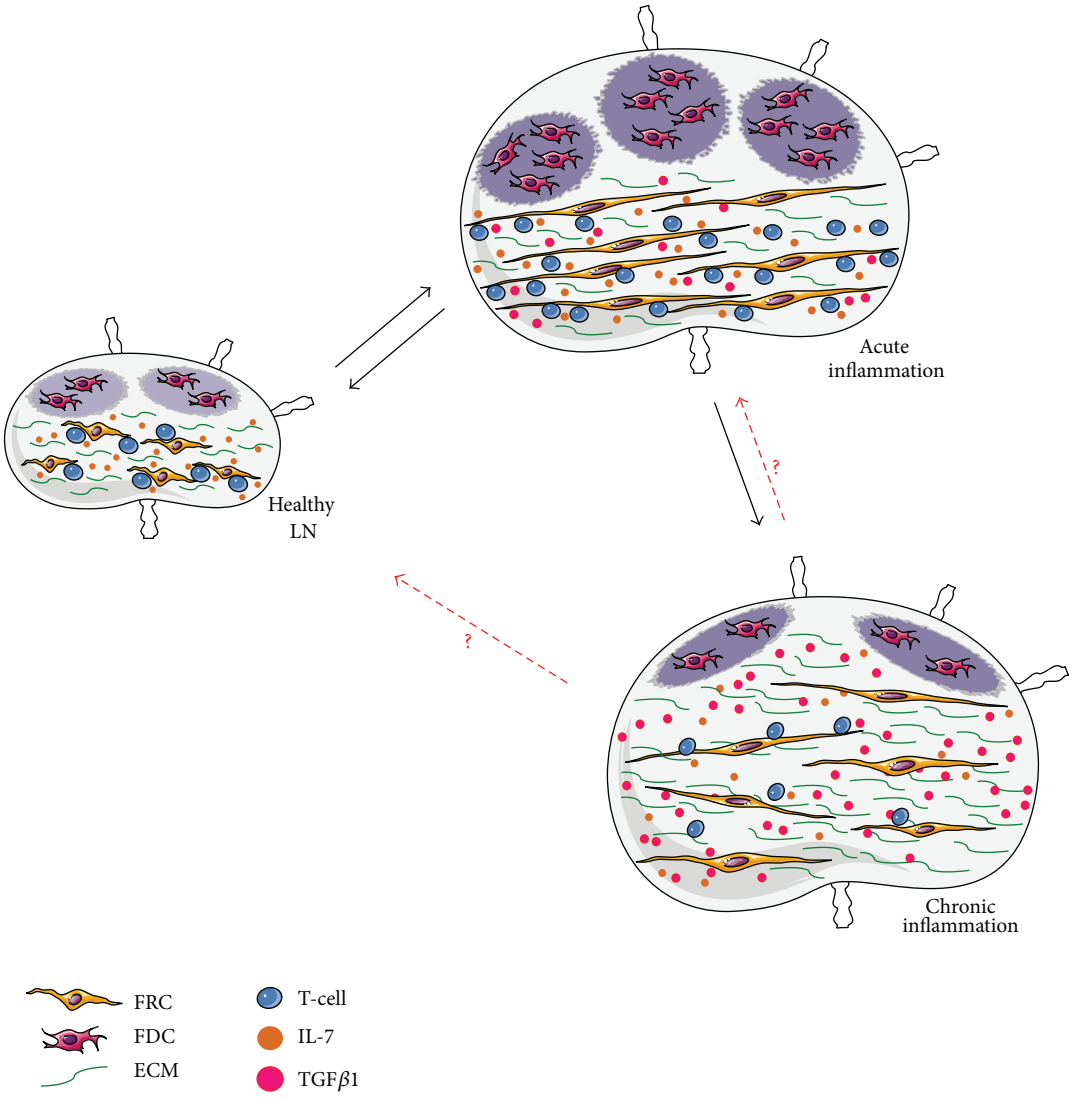

Figure 1

with HIV-1 have profound lymphoid tissue disorganization and show limited or absent immune reconstitution despite suppression of replicating virus in plasma [47–49].

Immunohistological studies have demonstrated that acute HIV-1 infection is associated with generalized lymph node enlargement. Moreover, abnormal LN architecture is associated with progressive loss of immune responses and correlates with disease progression, culminating in end-stage AIDS [50–54]. Furthermore, several observations describe an inverse correlation between the number of $CD4^+$ T-cells in the LN paracortical region and tissue fibrosis in HIV-1 infected patients [55, 56]. In the case of nonhuman primates (NHP) infected with simian immunodeficiency virus (SIV), the accumulation of T_{reg} cells expressing transforming growth factor β1 (TGFβ1) correlates with the pathological deposition of fibrotic collagen by T-cell zone mesenchymal stromal cells. Indeed, T_{reg} cells were shown to secrete TGFβ1 and stimulate resident fibroblasts to produce procollagen and chitinase 3-like-1 (CHI3L1), an enzyme involved in the maturation of procollagen and fibrosis [42, 57, 58]. This increased and uncontrolled deposition of fibrotic ECM strongly affects the capacity of the T-cells to recognize the prosurvival factor IL-7 produced by FRCs. This mechanism seems to explain the high degree of apoptosis and the depletion of both naïve $CD4^+$ and $CD8^+$ T-cells (the latter are not usually infected by HIV-1) occurring in infected patients [42, 59]. On the other hand, the survival of FRCs depends on LT$\alpha\beta$ from T-cells [42], and the increase in T-cell apoptosis causes a reduction of LT$\alpha\beta$ that ultimately results in loss of FRC networks and, consequently, the prosurvival signal IL-7 [42, 60, 61]. The reciprocal interactions between the FRCs and T-cells have been recently demonstrated in mice upon LTβR-Ig treatment, to deplete the FRC networks, or anti-CD3 administration, to induce T-cell apoptosis. Indeed, mice with a depleted FRC network have reduced T-cells, and vice versa mice depleted of T-cells have lost FRC networks [42, 62]. In addition to

LTβR, TNF is also involved in the maintenance of FRC, as demonstrated by reduced lymphoid tissue fibrosis in NHP treated with anti-TNF antibody [63].

Given the important role of the FRC network in lymphocytes locomotion, loss of it has an effect on T-cell migration within the LNs. Thus, a vicious cycle of progressive destruction of the LN architecture ultimately limits the possibility of restoring normal immune responses, despite suppression of replicating virus in the plasma [47–49]. It remains unclear whether stromal cell subsets other than FRC contribute to fibrosis and if this process could be reverted by pharmacological means. Endothelial cells have been implicated in tissue fibrosis, though it is unknown if this lineage is involved in remodeling the lymphoid stromal microenvironment through endomesenchymal transition during chronic inflammation [64].

In addition to FRCs, progressive loss of the FDC networks has been also described in HIV-1 infection. As a consequence, B-cell specific immune responses to HIV-1 and other pathogens are compromised [65]. The finding that FDC networks are present in HIV-1 infected patients after 2.5 years of antiretroviral therapy, with a pattern similar to the one shown in SLO from healthy volunteers, indicates that tissue remodeling and repair of follicular stromal cell are reversible. However, it is not clear whether changes in FDCs correlate with fibrosis or are directly linked to the viral load [66]. Nevertheless, the cellular mechanism involved in restoration of FDC networks upon treatment remains unclear.

6. Conclusion

Secondary lymphoid organs represent the primary site for initiating and developing adaptive immune responses, as well as for maintenance of lymphocyte homeostasis. During inflammation, the stromal microenvironment undergoes profound remodeling to support immune responses and mesenchymal stromal cells are emerging as important players (Figure 1). A better understanding of the nature of mesenchymal stromal cells involved in lymphoid tissue remodeling together with knowledge on the signaling networks contributing to stromal cell activation and proliferation will help to identify novel targets and design new strategies in order to prevent tissue damage and to restore integrity upon injury.

Competing Interests

The authors declare no potential competing interests.

Authors' Contributions

All authors contributed in intellectual discussions and critical review of the paper.

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Review Article

Endothelial-Mesenchymal Transition in Regenerative Medicine

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Endothelial-mesenchymal transition (EndMT) is a fundamental cellular mechanism that regulates embryonic development and diseases such as cancer and fibrosis. Recent developments in biomedical research have shown remarkable potential to harness the EndMT process for tissue engineering and regeneration. As an alternative to traditional or artificial stem cell therapies, EndMT may represent a safe method for engineering new tissues to treat degenerative diseases by mimicking a process that occurs in nature. This review discusses the signaling mechanisms and therapeutic inhibitors of EndMT, as well as the role of EndMT in development, disease, acquiring stem cell properties and generating connective tissues, and its potential as a novel mechanism for tissue regeneration.

1. Introduction

Endothelial cells line the interior of blood vessels and lymphatic vessels [1]. Endothelial cell plasticity plays a critical role in various developmental and pathological processes [2]. EndMT is defined by the loss of cellular adhesion and cytoskeletal reorganization of actin and intermediate filaments that convert apical-basal polarity to front end-back end polarity to form spindle-shaped cells. During this transformation, there is a marked decrease in endothelial biomarkers such as VE-cadherin, CD31, TIE1, and vWF, as well as increased expression of mesenchymal biomarkers such as CD44, vimentin, FSP1, and α-SMA [3]. The basal lamina, primarily composed of type IV collagen and laminin, is cleaved by secreted matrix metalloproteinases (MMPs) and replaced by extracellular matrix composed of type I and type III collagen and fibronectin, which promotes cell motility [4]. These cells also acquire stem cell properties by expressing mesenchymal stem cell biomarkers and gaining multipotency [5]. This transformation is reversible through a process known as mesenchymal-endothelial transition, which is an important mechanism that regulates cardiac neovascularization [6].

Signaling Mechanisms of EndMT. A number of autocrine or paracrine signaling molecules can induce EndMT. These may be produced by tissue injury or immune cells recruited to the sight of injury in response to inflammation [7]. The most common cytokines that stimulate EndMT are the Transforming Growth Factor-Beta (TGF-β) superfamily of proteins, which include isoforms TGF-β1 and TGF-β2 as well as Bone Morphogenetic Proteins (BMPs) BMP2, BMP4, BMP6, BMP9, and BMP10 [8–14]. Other signaling pathways such as Wnt/β-catenin [15], Notch [16], and various receptor tyrosine kinases [17] have also been shown to activate EndMT. All of these pathways induce expression of transcription factors such as Snail, Slug, Twist, LEF-1, ZEB1, and ZEB2 that cause the repression of endothelial genes and/or expression of mesenchymal genes [17, 18]. These identified pathways allow for therapeutic targeting with the potential to inhibit this process for the treatment of EndMT-related pathologies.

Several microRNAs have been described to regulate endothelial plasticity. miR-9, a microRNA regulated by Tumor Necrosis Factor-α (TNF-α) signaling, induces EndMT in lymphatic endothelial cells [19]. miR-21 targets PTEN and mediates EndMT induced by TGF-β signaling [20]. miR-31

targets VAV3 to control actin remodeling and promotes the secretion of various inflammatory cytokines that promote EndMT [21].

Other positive regulators of EndMT include bleomycin, which promotes EndMT through activation of the mTOR signaling pathway [22]. Safrole oxide induces EndMT by initiating the ATF4/p75NTR/IL-8 pathway [23]. Parathyroid hormone (PTH) stimulates EndMT by enhancing nuclear localization of β-catenin [24]. The Kaposi sarcoma herpesvirus has been shown to induce EndMT by enhancing Notch signaling [16].

Physiological processes such as endothelial cell apoptosis can also cause EndMT through the upregulation of TGF-β1 in both apoptotic cells and in the adjacent viable cells [25]. Fluid shear stress studies have shown no EndMT with laminar fluid shear stress but induction of EndMT with disturbed flow shear stress [26]. Ventricular mechanical stretching causes EndMT associated with dyssynchronous heart failure [27]. High glucose levels can cause endothelial cell damage and subsequent stimulation of EndMT [28]. Hypoxia associated with tissue damage, ischemia, and/or inflammation most commonly promotes angiogenesis but can also contribute to EndMT [29, 30].

EndMT Inhibitors. While most BMPs promote EndMT, BMP7 appears to be a negative regulator of EndMT [31], although the distinct differences between the downstream signals of the individual BMP isoforms remain elusive. Vascular Endothelial Growth Factor-A (VEGF-A) is known to inhibit EndMT through VEGFR2 signaling [32]. Inversely, VEGR1 can have a positive effect on EndMT by sequestering VEGF-A and preventing its interaction with VEGFR2 [33]. Recent evidence has shown that BMP signaling can also repress VEGF-A to help promote EndMT [34]. Fibroblast Growth Factor Receptor 1 (FGFR1) signaling can inhibit TGF-β-induced EndMT [35]. FGF-2, although found to be an inducer of EndMT in some types of endothelial cells [36], has also been shown to inhibit EndMT in others through miR-20a-mediated inhibition of TGF-β signaling [37].

MicroRNAs miR-15a, miR-23b, and miR-199a impair EndMT during heart development, although the miR-15a-dependent inhibition is only partial [38]. miR-126 blocks TGF-β1-induced EndMT of bone-marrow derived endothelial progenitor cells through direct targeting of the PI3K subunit p85 [39]. miR-155 impairs TGF-β-induced EndMT by inhibiting RhoA expression [40]. miR-302c negatively regulates expression of metadherin (MTDH) to impair EndMT associated with hepatocellular carcinoma [41]. N-acetyl-seryl-aspartyl-lysyl-proline (AcSDKP), a peptide substrate of angiotensin-converting enzyme (ACE), inhibits EndMT through the upregulation of microRNA let-7 and restoration of the FGF receptor [42].

Hydrogen sulfide can ameliorate EndMT caused by endoplasmic reticulum stress by activating the Src signaling pathway [43]. Aqueous extracts of *Psoralea corylifolia* L. have been shown to inhibit lipopolysaccharide-induced EndMT by inhibiting NF-κB-dependent expression of Snail [44]. Glucagon-like peptide-1 (GLP-1) blocks high glucose-induced EndMT by reducing expression of reactive oxygen species (ROS) and inhibiting poly(ADP-ribose) polymerase 1 (PARP-1) [45]. The extracellular matrix protein fibulin-1 can suppress EndMT by reducing expression TGF-β2 [46]. High-density lipoproteins (HDL) have been shown to inhibit EndMT induced by TGF-β1 signaling [47].

Several drugs have been proposed as EndMT inhibitors. Linagliptin, a DPP-4 inhibitor that impairs its interaction with integrin β1, has been shown to block TGF-β2-induced EndMT [48]. Rapamycin blocks EndMT by suppressing the mTOR signaling pathway [49]. Relaxin (RLX) has been shown to inhibit isoproterenol-induced EndMT in a cardiac fibrosis model in rats through notch-mediated signaling [50]. Macitentan, an endothelin-1 receptor inhibitor, was shown to impair EndMT induced by either endothelin-1 or TGF-β1 [51]. Marimastat, a broad-spectrum MMP inhibitor, prevents FGF-2-dependent EndMT of corneal endothelial cells [52]. Kallistatin blocks TGF-β-induced EndMT through upregulation of endothelial nitric oxide synthase (eNOS) and by differential regulation of miR-21 [53]. Spironolactone, an aldosterone receptor blocker, can also inhibit TGF-β-induced EndMT by controlling Notch1 expression [54]. Scutellarin can also regulate Notch1 and Jagged1 expression to prevent isoprenaline-induced EndMT [55]. Losartan, an inhibitor of angiotensin II type 1 receptor, impairs EndMT by blocking TGF-β signaling [56]. Cinacalcet attenuates EndMT in cardiac fibrosis associated with elevated serum levels of parathyroid hormone (PTH) by suppressing the hormone levels [57]. Interestingly, hydrocortisone has been proposed to reverse EndMT through mesenchymal-endothelial transition by enhancing endothelial cell adhesion [58]. These functional inhibitors may be used as potential therapeutic agents to perturb the pathological effects of EndMT.

EndMT in Development and Disease. EndMT has been shown to regulate angiogenesis [59], as well as cardiac development [60]. EndMT causes formation of the valves and septa of the heart during embryogenesis [60, 61]. In the postnatal organism, tissue damage and/or inflammation can stimulate this embryonic mechanism to give rise to fibroblasts and myofibroblasts that form scar tissue during wound healing or fibrotic diseases [2].

EndMT has a critical role in the generation of fibroblasts in kidney [62], lung [29], intestinal [63], and cardiac fibrosis [64]. This EndMT-dependent fibrotic phenotype contributes to diseases such as systemic sclerosis [65], atherosclerosis [66], pulmonary hypertension [67], diabetic nephropathy [68], diabetic retinopathy [69], sepsis [70], and cerebral cavernous malformations [71]. It also plays a central role in vein graft remodeling [72].

Further, while the epithelial-mesenchymal transition (EMT) has been shown to be the primary mechanism of cancer metastasis [73] and for the formation of cancer stem cells [74], EndMT occurs to form cancer-associated fibroblasts in the tumor microenvironment that help regulate the progression of the disease [75]. EndMT has also been proposed to have a role in the metastatic extravasation of cancer cells [76]. It may also have a part in central nervous system diseases associated with dysfunction of the blood-brain barrier [77].

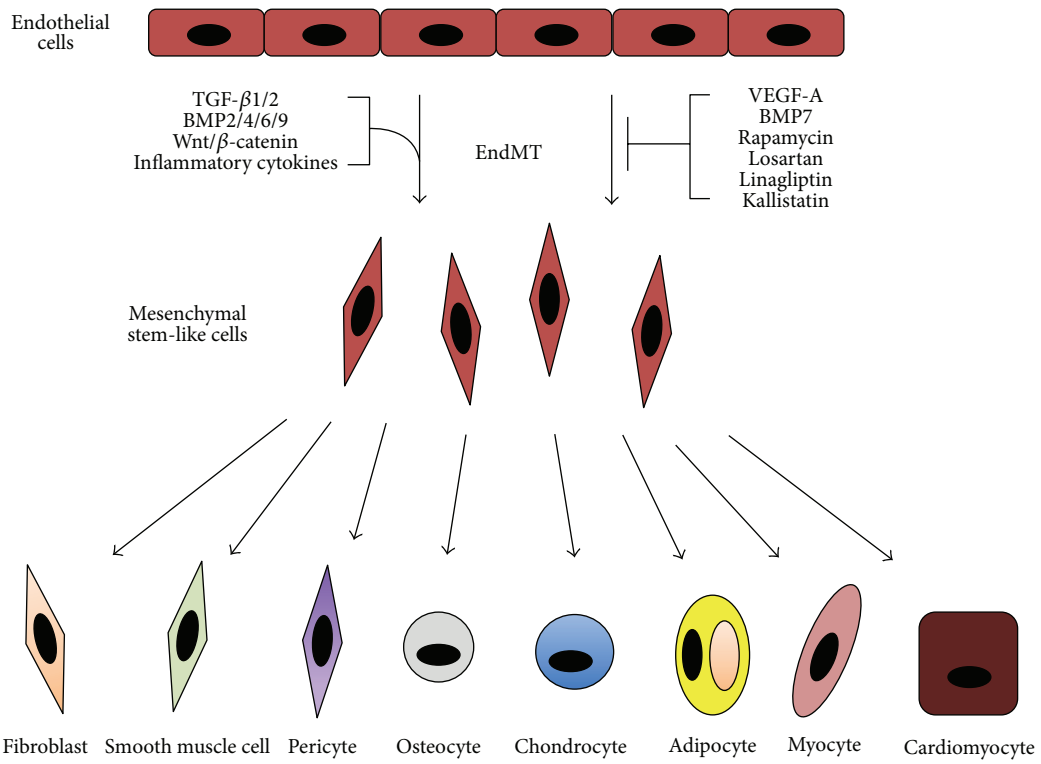

Figure 1: The multipotency of EndMT. Vascular endothelial cells are stimulated to undergo EndMT by various growth factors and inflammatory cytokines such as TGF-βs, BMPs, and Wnt. Proteins such as VEGF-A and BMP7, as well as drugs such as rapamycin, losartan, linagliptin, and kallistatin, can inhibit this cellular transformation. Endothelial-derived mesenchymal cells take on the properties of multipotent stem cells and can differentiate into fibroblasts, pericytes, smooth muscle, skeletal muscle, cardiac muscle, bone, cartilage, and fat cells.

EndMT in the Generation of Connective Tissues. Other than fibroblasts, recent studies have shown the ability of EndMT to generate various different types of connective tissues. Lineage tracing and biomarker studies have suggested an endothelial origin of heterotopic cartilage and bone that forms in a rare disease called fibrodysplasia ossificans progressiva (FOP) [5, 78, 79]. Patients with this disease carry a gain-of-function mutation in the gene encoding activin-like kinase 2 (ALK2) receptor [80]. Upon expressing this mutated gene in endothelial cells, they undergo EndMT and acquire properties of mesenchymal stem cells with the ability to transform into bone, cartilage, or fat cells [5]. A recent study has shown that kidney cells isolated from FOP patients can be transformed into induced pluripotent stem cells (iPSC) and subsequently differentiated into endothelial cells, which spontaneously underwent EndMT in culture [81].

The ability of EndMT to generate osteoprogenitor cells has also been observed in vascular calcifications [82, 83], valvular calcifications [84], and tumor calcifications [85]. Another recent study has shown that BMP6 has the ability to stimulate EndMT and subsequent differentiation to osteoblasts both independently and synergistically with oxidized low-density lipoprotein [86]. Tang et al. showed that high glucose levels mediate endothelial differentiation to chondrocytes through EndMT [87].

Lineage tracing studies using VE-cadherin-Cre reporter mice have demonstrated an endothelial origin of white and brown fat cells [88]. A recent study that isolated endothelium from vascular tumors showed that these cells spontaneously undergo EndMT in culture and have the ability to form adipocytes and mural cells such as pericytes and smooth muscle cells [89]. Endothelial progenitor cells (EPCs) have also been induced to undergo EndMT and transform into smooth muscle cells [90].

Endothelial plasticity has also been linked to generation of skeletal myocytes for muscle repair [91]. Furthermore, lineage tracing in Tie1-Cre and VE-cadherin-Cre reporter mice has demonstrated an endothelial origin of cardiomyocytes during cardiac homeostasis, which are proposed to arise by EndMT [92].

EndMT for Tissue Engineering and Regeneration. The ability of EndMT to generate various different types of connective tissue (Figure 1) provides hope for using it as a potential method for tissue regeneration. For example, EndMT-dependent osteogenesis could be used to treat disorders such as osteoporosis or osteonecrosis. EndMT-induced chondrogenesis could be utilized for the treatment of osteoarthritis or temporal mandibular joint disorder (TMJD). Using EndMT to induce myogenesis could prove beneficial for muscular

dystrophy, while cardiomyogenesis might be helpful for regenerating heart muscle after myocardial infarction. The process may also aid in vascular tissue regeneration, particularly in vasculogenesis through its ability to generate smooth muscle cells and pericytes. EndMT has already been found to be important in engineering cardiovascular tissue grafts through its ability to increase the production and remodeling of the extracellular matrix [93].

Tissue engineering *ex vivo* may be achieved through EndMT for the replacement of degenerated tissues. For personalized medicine, to avoid any potential host rejection, vascular endothelial cells can be easily obtained from patients from a skin sample. The tissue can be enzymatically digested and endothelial cells can be isolated using magnetic beads conjugated with endothelial-specific antibodies. These isolated endothelial cells can then be grown and expanded in culture and then loaded onto three-dimensional scaffolds composed of collagen, polylactic acid, hydrogel, and so forth. The endothelial cells can then be induced to undergo EndMT using any of the known cytokines that stimulate the transformation, followed by addition of differentiation medium to change the newly formed mesenchymal cells into the desired tissue type [94]. The engineered tissue may then be surgically transplanted into the patient.

For tissue regeneration *in vivo*, the potential use of EndMT is virtually endless since almost every tissue in the body is highly vascularized, so an abundant source of vascular endothelial cells should be present in damaged or degenerated tissues in need of repair. Drugs can be developed and locally applied to degenerated tissue to convert the vascular endothelium into the cell type of need. If some capillary blood vessels are lost during this cellular transformation, they should be naturally replenished through hypoxia-induced angiogenesis [95]. Therefore, EndMT should provide a natural and effective method for building new connective tissues from blood vessels.

2. Discussion

Although EndMT has positive effects in embryonic development and wound healing, it has traditionally been considered to have negative effects in disease. While most therapeutic studies attempt to inhibit the harmful effects of EndMT in progressive diseases such as cancer and fibrosis, it is now proposed that researchers harness this natural mechanism by inducing it for tissue regeneration for treatment of degenerative diseases. Although there may be potential risks of converting the vascular endothelium into other cell types for tissue regeneration, such as blood vessel leakage or cell death associated with hypoxia, the target tissue would already be degenerated and the natural mechanism of angiogenesis should replenish the blood vessels. Therefore, the potential benefits of restoring degenerated tissue using EndMT far outweigh the risks for regenerative medicine.

Competing Interests

The author declares that he has no competing interests.

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Review Article

Vascular Remodelling and Mesenchymal Transition in Systemic Sclerosis

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Fibrosis of the skin and of internal organs, autoimmunity, and vascular inflammation are hallmarks of Systemic Sclerosis (SSc). The injury and activation of endothelial cells, with hyperplasia of the intima and eventual obliteration of the vascular lumen, are early features of SSc. Reduced capillary blood flow coupled with deficient angiogenesis leads to chronic hypoxia and tissue ischemia, enforcing a positive feed-forward loop sustaining vascular remodelling, further exacerbated by extracellular matrix accumulation due to fibrosis. Despite numerous developments and a growing number of controlled clinical trials no treatment has been shown so far to alter SSc natural history, outlining the need of further investigation in the molecular pathways involved in the pathogenesis of the disease. We review some processes potentially involved in SSc vasculopathy, with attention to the possible effect of sustained vascular inflammation on the plasticity of vascular cells. Specifically we focus on mesenchymal transition, a key phenomenon in the cardiac and vascular development as well as in the remodelling of injured vessels. Recent work supports the role of transforming growth factor-beta, Wnt, and Notch signaling in these processes. Importantly, endothelial-mesenchymal transition may be reversible, possibly offering novel cues for treatment.

1. The Scenario: Damage and Remodelling of the Microvasculature in Systemic Sclerosis

Systemic Sclerosis (SSc) is a multisystem disease, characterized by autoimmunity, a broad microvasculopathy, and fibrosis of the skin and of visceral organs. Events still poorly characterized support the activation of myofibroblasts and self-amplifying circles lead to aberrant and sustained fibrogenesis [1]. Injury and activation of endothelial cell linings are early events in the natural history of SSc [2, 3] and excessive/deregulated innate immune responses in response to vessel and tissue injuries are hallmarks of SSc [4–8]. Vascular inflammation and remodelling characterize diverse districts, including the lung, the heart, the skin, and the kidney. Small- and medium-size arteries are usually involved, with the frequent intimal hyperplasia, medial thickening, obliteration of the lumen, perivascular inflammation, and occasionally microthrombi [9, 10] (see also below). SSc also affects capillaries. Nailfold capillaroscopy, which is routinely used in the clinical settings, often reveals dilatation of capillaries in early stages and loss in later phases, an event that possibly represents the counterpart of the lumen obliteration of small arteries in other tissues [11], a process that involves the proliferation of the intimal layer, with accumulation of constituents of the extracellular matrix [3, 12].

Of importance, the occlusion of the microvasculature results in persistent hypoxia of peripheral tissues, which in turn is not repaired by the physiologic mechanisms of vasculogenesis or angiogenesis [13]. Hypoxia represents a massive stimulus for the generation of various growth factors that influence the fate of vascular cells, prompting mesenchymal transition and fibrosis [14, 15]. On the other hand hypoxia is a key element prompting oxidative stress, another hallmark of SSc [12].

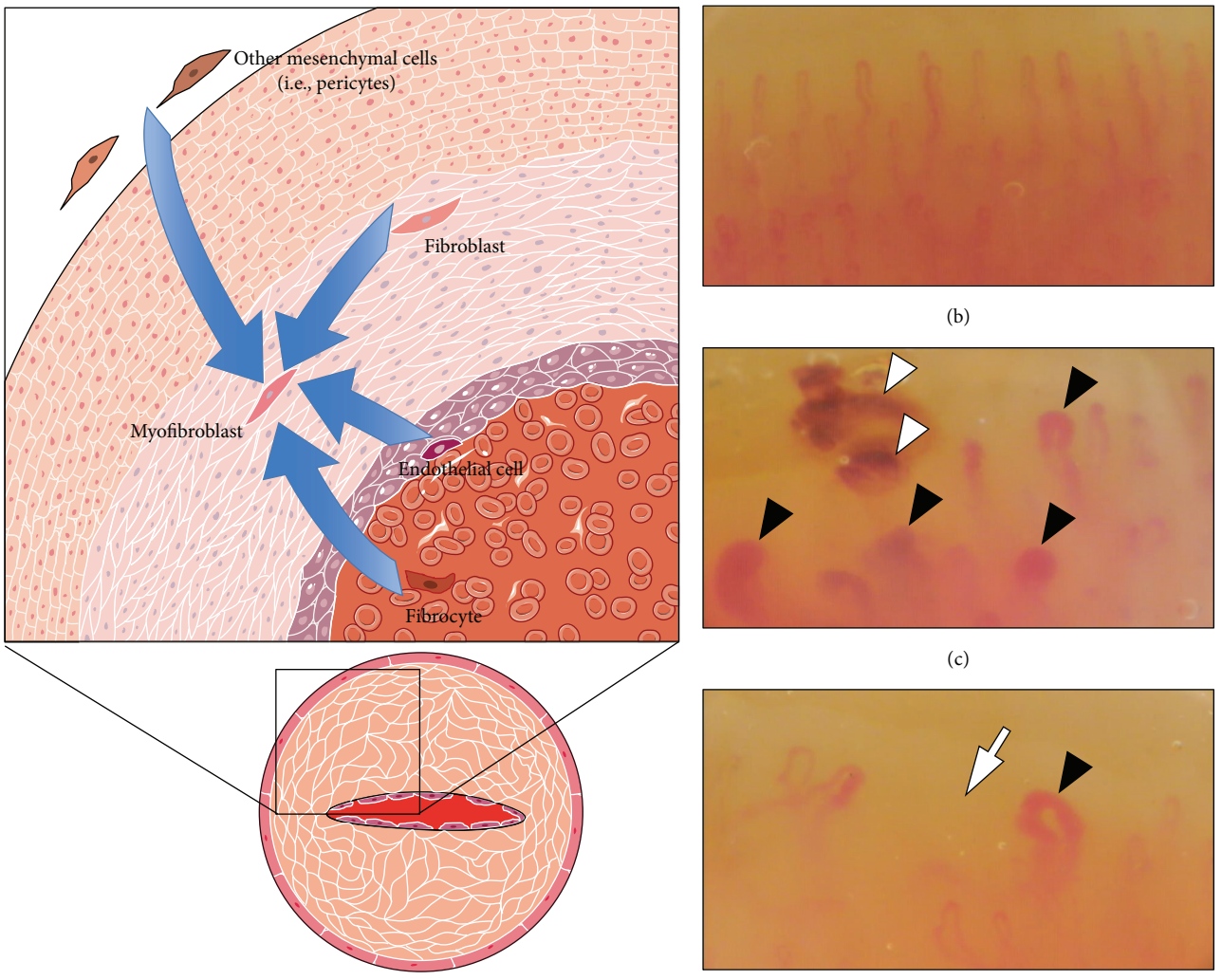

FIGURE 1: Vascular remodelling and capillaroscopic pattern in Systemic Sclerosis (SSc). (a) Stenoocclusive remodelling in SSc microvasculature (bottom right) is believed to result from an abnormal reparative attempt triggered by chronic endothelial damage, which drives intima-media hyperplasia and increased ECM production within the vessel wall. Mesenchymal cells, specifically myofibroblasts with a highly secretory phenotype, are the main final effectors responsible for these structural changes. Myofibroblasts in SSc vessels can originate from multiple cellular sources (upper left), either of mesenchymal origin, such as pericytes or fibroblast, or of nonmesenchymal origin, such as endothelial cells. (b)–(d) Capillaroscopic pattern in normal subjects (b) and scleroderma patients at magnification 200x ((c): "active" SSc pattern; (d): "late" SSc pattern). Note the heterogeneity in the architecture and morphology of SSc capillaries with frequent ectasias (black arrowheads). In the "active" scleroderma pattern there are plenty of giant capillaries (i.e., more than 50 μm of diameter) and microhaemorrhages (white arrowheads), with mild loss of capillaries. In the "late" scleroderma pattern giant capillaries and microhaemorrhages are less frequent, but a severe loss of capillaries is evident, with extensive avascular areas (white arrows).

Soluble moieties present in the blood of SSc patients activate and induce in the presence of neutrophils the programmed death via apoptosis of endothelial cells [16], suggesting that inflammatory leukocytes directly contribute to the endothelial injury [17]. The apoptosis of endothelial cells [3], the aberrant expression of transcription factors [18–20], of cytokines and of growth factors, specifically including the production of the antiangiogenic VEGF165b isoform of the vascular endothelial growth factor (VEGF) [21], alterations of pathways activated by the interaction of components of the class III semaphorin family and of their receptors, Plexin-D1 and Neuropilin-1 [22, 23], and the defects of sprouting angiogenesis and vasculogenesis participate in the remodelling of the vasculature [24]. The events occurring at the cellular levels are poorly characterized. The recent insight on the relative plasticity of vascular cells, including ECs and pericytes, raises the possibility that transdifferentiation programs are activated and contribute to the maladaptive remodelling characteristic of the SSc vasculature (Figure 1).

Platelets are critical players in vascular remodelling. As guardians of the integrity of the vessels platelets respond to the early changes of the endothelial lining undergoing a burst of activation, which become persistent and sustained activation [25–27]. They represent a source of VEGF, which acts

on endothelial cells [28]. Moreover they generate and release an array of profibrotic signals, including transforming growth factor-β (TGF-β), platelet-derived growth factor (PDGF), and serotonin [15]. Thrombosis of microvessels is frequent in SSc and could be facilitated by the release from damaged and activated endothelial cells of extralarge multimers of von Willebrand Factor (vWF) [29]. Platelets also contain a substantial amount of the High Mobility Group Box-1 (HMGB1) protein [30], a prototypic Damage Associated Molecular Pattern (DAMP) [31].

HMGB1 is a key signal shaping the characteristics of the inflammatory response elicited in response to sterile and microbial insults [32, 33]. It mediates the homeostatic response to injury [34–37], prompting fibrogenesis in response to endothelial damage [38–41] and playing a nonredundant role in the remodelling of vessels that takes place in injured tissues [42, 43]. Blood levels of HMGB1 are elevated in patients with SSc [44]. Conversely platelets of SSc patients undergo the depletion of the intracellular HMGB1 content [45]. The two events possibly reflect the generation of $HMGB1^+$ microparticles (μPs), an event that seems to dominate the release of the molecule from activated platelets [46, 47].

Platelets are an established source of μPs and platelet-derived μPs in the plasma of patients with SSc are abundant [48, 49]. μPs have various actions that might be involved in the natural history of the disease, including the regulation of the survival and of the activation state of endothelial cells and, importantly, of endothelial cell precursors [50, 51]. Moreover, subpopulations of μPs might be associated with specific features of SSc, including lung involvement and the extent of fibrosis [52]. $HMGB1^+$ μPs purified from SSc patients, but not $HMGB1^-$ μPs purified from control subjects, activate human leukocytes while HMGB1 inhibitors reverse the effects *in vitro*, suggesting that the moiety might be important in the maintenance of the SSc vascular inflammation [46].

Of importance, HMGB1 is a redox-sensitive moiety [53]. HMGB1 contains cysteine residues in positions 23, 45, and 106 and resides in a predominantly reduced state in the nucleus and the cytosol [53–55]. Reduced HMGB1 in the extracellular environment forms bioactive complexes with the CXCL12/SDF1 chemokine and effectively triggers *in vitro* cell migration [53, 56–59]. An oxidizing environment in contrast enhances the ability of the molecule to prompt the secretion of inflammatory cytokines from macrophages and to promote autoimmunity [59–65]. Oxidative stress is a critical player in SSc, which contributes to the persistent activation of fibroblasts and of vascular cells [12, 66]. Indeed oxidation is critical for HMGB1 ability to support the activation of blood leukocytes in response to platelets- or μPs-derived signals [46] and possibly for their action on vascular cells, including pericytes [67, 68]. Platelet-derived HMGB1 is gaining increasing attention as a key moiety in intravascular immunity and in the activation/regulation of the coagulation cascade [45–47, 69, 70]. Further studies are necessary to validate the involvement of this pathway in SSc and specifically to reveal whether it might contribute to the remodelling of the microcirculation in particular. Of interest, HMGB1 has a well-characterized fibrogenic action and is an established inducer of epithelial-to-mesenchymal transition (EMT), a process that is associated with the origin of myofibroblasts from various precursors, including those associated with the vessel wall [40] (see below).

Other nonmural cells, such as fibrocytes and macrophages, might play a role in the development of the fibroproliferative vasculopathy in SSc. Circulating fibrocytes comprise bone marrow-derived cells that have both hematopoietic and mesenchymal features, endowed with a physiologic role in the physiologic wound healing [71]. Fibrocytes are increased in autoimmune conditions, including SSc [72, 73], and might play a part in tissue and vessel remodelling via multiple mechanisms, including the differentiation into activated myofibroblasts [71, 74].

Macrophages are attracting increasing attention for their role in the SSc (for recent excellent reviews, see [75, 76]). A detailed description of the role of macrophages in promoting and sustaining SSc vasculopathy is outside the scope of this work. However, several evidences support the contention that the recognition of endogenous ligands in peripheral tissues of SSc patients by macrophages might be involved in feed-forward self-sustaining amplificatory circuits of vascular inflammation and fibrosis [6, 37, 77].

2. Clinical Impact of SSc Vasculopathy

Although vasculopathy is present early and almost invariably during the course of SSc, clinical complications are traditionally classified mainly within either the fibrotic or the vascular components of the disease (Table 1). This classification is mainly based on histology and does not take into account the possible role of vascular inflammation and of vasculopathy in driving the fibrotic component of the disease.

Despite the fact that therapeutic improvements have changed the relative impact of SSc complications on patients' prognosis [78] SSc vasculopathy, in terms of pulmonary arterial hypertension (PAH), heart involvement, and scleroderma renal crisis (SRC), still represents the first cause of disease-related mortality. Therapeutic targets are different in the vascular complications of SSc, suggesting that the pathogenesis of these conditions only partially overlaps. SRC prognosis has fortunately much improved since the recognition of the therapeutic role of ACE inhibitors [79]. SRC is typically characterized by malignant hypertension and rapidly progressive renal failure. Organ dysfunction (hearth failure, encephalopathy, and microangiopathic haemolytic anaemia) frequently coexist [80]. Histology shows onionskin-like lesions and fibrotic intimal sclerosis, with possible adventitial fibrosis and intravascular thrombosis [81]. Pathophysiology of SSc hearth involvement is complex and heterogeneous, but vasculopathy is believed to be the most frequent mechanism, resulting in focal and patchy myocardial ischemia and consequent fibrosis with either systolic or diastolic dysfunction [82].

3. Pulmonary Arterial Hypertension

Pulmonary hypertension (PH) is defined as an elevated mean pulmonary arterial pressure (mPAP) $\geq$ 25 mmHg at rest [83]. PH is frequent in SSc and can be associated with

Table 1: Most prominent fibrotic and vascular complications of SSc.

Fibrotic complications	Vascular complications
Skin fibrosis	Raynaud phenomenon
Lung fibrosis	Ischemic ulcers
Gastrointestinal involvement	Acral ischaemia/necrosis
	Gastral antral vascular ectasia (GAVE) and gastrointestinal telangiectasias
	Scleroderma renal crisis
	Heart involvement
	Pulmonary arterial hypertension

lung and hearth involvement or thromboembolic disease. Pulmonary arterial hypertension (PAH) is a disease characterized by progressive obliterative vasculopathy involving the distal pulmonary circulation, the distal pulmonary arteries in particular [83]. Progressive precapillary PH (i.e., PH with a pulmonary capillary wedge pressure ≤ 15 mmHg and pulmonary vascular resistance > 3 Wood Units) defines PAH. This results in progressive right heart failure [84], with a median survival without therapy of about 2.8 years from diagnosis [85]. SSc is one of the main causes of PAH [83]. Currently, PAH and interstitial lung disease represent the first causes of disease-related mortality in SSc patients. SSc-associated PAH (SSc-PAH) has a prevalence between 10 and 12% of SSc patients and may occur even many years after the diagnosis [86]. SSc-PAH is associated with limited scleroderma, presence of anti-U3RNP autoantibodies, late-onset disease, multiple telangiectasias, digital ulcers, and worsening lung diffusion [86].

Mortality of SSc-PAH is worse than mortality of idiopathic PAH [87, 88]. Early detection is therefore fundamental but remains challenging. Symptoms are caused by heart failure or worsening respiratory function and occur late during disease course. The diagnosis is not based on the direct identification of the lung vasculopathy but on the indirect evaluation of its hemodynamic impact, which can be definitively assessed with right heart catheterisation only, when the lung vascular reserve is already substantially compromised [89].

With the exception of a small group of patients with hereditary or idiopathic PAH responding to calcium channel blocker vasodilators, structural remodelling of the lung microcirculation is substantial. Currently available therapies for PAH antagonise endothelin-1 (ET-1) receptors increase concentrations of prostacyclin or its analogues or increase cyclic GMP in the lung vasculature antagonising phosphodiesterase-5. All these agents are believed to target both the vasoconstriction and the remodelling observed in the lung vasculature. However, patients with SSc-PAH have a poorer response to therapies, in comparison with other PAH subgroups [88], and up-front combination regimens of oral agents antagonising ET-1 receptors and phosphodiesterase-5 may provide a more effective intervention [90, 91]. Autoimmunity with unrelenting inflammatory responses and more severe vessel and cardiac involvement might account for the poorer response to therapy of patients with SSc-PAH as compared to those with idiopathic PAH [88].

Histology of PAH is reminiscent of other small vessel vasculopathies, such as SSc. An obliterative and onionskin-like intimal and medial thickening is the pivotal finding. Intravascular thrombosis is frequent, and perivascular inflammation is observed. Muscularisation of small arteries as well as perivascular inflammation is typical. Endothelial cells may have a disorganised growth within the lumen of remodelled vessels, to form the so-called plexiform lesions [92]. SSc-PAH pathology is similar, with more abundant inflammatory infiltrates and more frequent concomitant involvement of the venous compartment of the lung circulation [93].

Mechanisms underlying these changes are poorly understood. Similar histologic features of remodelled arteries and intimal hyperplasia are not exclusive of SSc but are believed to be a stereotyped vascular response to many types of injuries. Large-vessel vasculitides such as Takayasu arteritis [94] and giant cell arteritis [95] are inflammatory conditions in which arterial remodelling and intimal hyperplasia play a central role. Similarly to SSc, in Takayasu arteritis the progression in vascular stenoocclusions and the intensity of systemic inflammation poorly correlate [96–98]. Further studies are required to verify whether molecular events regulating cell plasticity in the SSc vessel walls might have a role in macrovascular diseases.

Increased numbers of cells expressing alpha-smooth muscle actin (alpha-SMA) are a nearly universal finding in the remodelled artery. Resident smooth muscle cells have been traditionally regarded as the predominant source of the newly appearing alpha-SMA-expressing cells. However, rapidly emerging experimental evidence suggests that other sources might play a role. We will briefly discuss below the possible contribution of the Endothelial to Mesenchymal Transition (EndoMT) and the evidence supporting a role of transforming growth factor-beta, Wnt, and Notch signaling in this process.

4. EndoMT and TGF-β

EndoMT refers to a transdifferentiation process in which endothelial cells downregulate the expression of endothelial markers, such as CD31 and vascular endothelial cadherin (VE-cadherin), acquiring a mesenchymal/myofibroblast phenotype, which is characterized by the expression of SMA, collagen type I (Col I), together with Twist 1, a specific marker of mesenchymal transition [74, 99].

EndoMT has emerged as a player in the pathogenesis of tissue fibrosis in various diseases, including diabetic nephropathy, cardiac fibrosis, intestinal fibrosis, portal hypertension, and PAH [100]. Experimental evidence supports a role of EndoMT in SSc as well [100–102]. Of importance, lung tissues of patients with interstitial lung disease associated with SSc have been elegantly shown to contain cells that simultaneously express EC-specific and mesenchymal proteins and transcripts, demonstrating that EndoMT actually occurs in target organs of the disease [103]. EndoMT could contribute, under the action of signals generated by inflammatory leukocytes recruited and activated into the

perivascular tissues, to the conversion of endothelial cells into activated myofibroblasts, that is, cells responsible for the formation of scar tissue and for fibrosis [74, 99]. Thus, EndoMT would causally connect two hallmarks of SSc, the aberrant fibrogenesis and the persistent endothelial injury. TGF-β, a cytokine involved in embryogenesis, cellular differentiation, development, and inflammatory response, plays a role in fibrotic diseases by stimulating the production of collagens and other ECM components and by inhibiting the expression of various relevant metalloproteinases. TGF-β is in particular a central cytokine in SSc [10]. TGF-β-regulated genes are expressed in the skin and the lung of patients with SSc and the extent of the cytokine expression correlates with the disease activity [10]. Moreover, mutations in the TGF-β-sensing ALK-1 signaling pathway cause familial PAH and hereditary haemorrhagic telangiectasia, indicating a role of TGF-β signaling in both SSc vasculopathy and fibrosis [10].

TGF-β is able to induce plasticity in endothelial cells, committing them toward a fibrogenic fate. The process involves the acquisition of a mesenchymal progenitor multipotent status and is characterized by the transient expression of PDGFRα mRNA, by the increase of the mesenchymal markers expression (such as α-SMA and Col I), and by the reduction of endothelial markers expression, CD31 and Tie 1 [104]. Li and Jimenez in 2011 observed in primary mouse pulmonary ECs the ability of TGF-β to induce α-SMA and type I collagen expression together with an inhibition of VE-cadherin. These effects were associated with an increased Snail-1 expression, involving the c-Abl tyrosine kinase and protein kinase Cδ (PKCδ) activity [105].

5. Wnt

The Wnt proteins comprise a family of glycoproteins that via canonical and noncanonical intracellular signaling pathways play crucial roles during embryonic development. Wnt proteins and pathways have been also implicated in the pathogenesis of fibrotic diseases, including SSc [106–108]. TGF-β activates the canonical Wnt pathway, and multiple genes involved in tissue repair and in fibrosis are transcriptional targets of Wnt/β-catenin [109]. Transcriptional analysis of primary alveolar epithelial type II (ATII) cells from patients with idiopathic pulmonary fibrosis (IPF) revealed an elevated expression of genes coding for Wnt ligands, receptors, regulators, and targets [110, 111]. Other studies provided evidence of an increased Wnt expression and activity in the skin and the blood of patients with SSc [112]. Nuclear β-catenin, a marker of active canonical Wnt signaling, was strongly upregulated in the lung of patients with SSc-associated fibrosis [106]. Wnt3a could be implicated in the modulation of EndoMT in human dermal microvascular endothelial cells via the reduction of vascular endothelial cadherin mRNA expression and induction of vimentin and slug mRNA expression [113].

6. Notch-Jagged

The Notch signaling is also a fundamental pathway governing development. Notch receptors and their ligands have been located in the vascular system. Notch activation in endothelial cells results in morphological, phenotypic, and functional changes consistent with mesenchymal transformation. These changes are correlated with EndoMT, including downregulation of endothelial markers, upregulation of mesenchymal markers, and migration toward platelet-derived growth factor-BB. Notch and TGF-β signaling synergistically induce the Snail expression in endothelial cells. Notch activation inhibits TGF-β/Smad1 and TGF-β/Smad2 signaling pathways by decreasing the expression of Smad1 and Smad2 and their target genes. In contrast, Notch increases Smad3 mRNA expression and protein half-life and regulates the expression of TGF-β/Smad3 target genes in a gene-specific manner [114].

Notch signaling appears to be activated in the skin of patients with SSc, with overexpression of the ligand, jagged-1. This appears to be a nonredundant event in fibrogenesis, since genetic or pharmacological interference with this pathway inhibited the development of fibrosis in experimental animals, interfering with the generation of autoantibodies as well [115]. Thus, data in the literature suggest that the Notch pathway is correlated with EndoMT and that the same pathway is deregulated in SSc. However direct experimental evidence of Notch involvement in the modulation of EndoMT in SSc is so far missing.

7. Endothelin 1

Endothelin-1 (ET-1), a 21-residue peptide, is a potent vasoconstrictor. ET-1 regulates the vascular tone through interaction with endothelin receptors A (ETRA) and B (ETRB), prompts fibrogenesis, and possibly contributes to the vessel's instability and capillary rarefaction during SSc. Some *in vitro* evidence suggests that ET-1 might promote EndoMT on ECs isolated from SSc patients and macitentan, a dual endothelin-1 receptor antagonist, blocks the EndoMT induced *in vitro* by the combination of TGF-β and ET-1 [116]. The actual relevance of *these in vitro* observations for the SSc vasculopathy remains to be established. For example, *in vitro* studies supported an antifibrotic effect of the ET-1 receptor antagonist, bosentan, which however was not consistent upon treatment of SSc patients with PAH (e.g., see [117]).

8. Interferon

Interferon has also been studied in the setting of EndoMT. IFN-α appears to downregulate while IFN-γ appears to upregulate α-SMA, CTGF, ET-1, and TGFβ2 expression in human dermal microvascular endothelial cells. In this *in vitro* experimental setting, the blockade of TGFβ signaling normalized IFN-γ-mediated changes in Fli-1, VE-cadherin, CTGF, and ET-1 levels, whereas the upregulation of α-SMA and TGFβ2 was not affected. IFN-γ also induced the expression of selected genes related to EndoMT, including Snail-1, FN1, PAI1, TWIST1, STAT3, RGS2, and components of the Wnt pathway [118].

9. MicroRNAs

MicroRNAs (miRNAs) consist of a class of small endogenous noncoding RNAs, approximately 22 nucleotides long, able to regulate posttranscriptionally gene expression. A single miRNA can modulate hundreds of target genes by suppressing translation, mediating mRNA segmentation, or causing RNA destabilization. On the other hand, multiple miRNAs can cooperate to regulate the expression of a single target gene. miRNAs might be involved in the natural history of SSc. A downregulation of miRNAs involved in the suppression of fibrosis (such as miR-29a, miR-196a, and miR-150) has been reported in SSc patients [119]. Upregulation of miRNAs able to induce the Col1A1 expression (such as miR-21b) or other ECM molecules (miR-92a) has also been reported [120]. Conversely, miR-7, a miRNA with a role in the suppression of fibrosis, is upregulated in SSc fibroblasts possibly because of a negative feedback loop, associated with thrombospondin-2 upregulation [121].

TGF-β significantly increased miR-21 expression in endothelial cells and induced EndoMT. Mechanistically, miR-21 acts on phosphatase and tensin homolog in endothelial cells, favoring the activation of the Akt pathway [122]. A miRNA array on mouse cardiac endothelial cells and EndoMT-derived fibroblast-like cells revealed that miR-125b, Let-7c, Let-7g, miR-21, miR-30b, and miR-195 were significantly elevated during EndoMT, while levels of several miRNAs including miR-122a, miR-127, miR-196, and miR-375 were significantly downregulated [123]. Some of these signals, such as the miR-125b, might be directly implicated in the fibroblast-to-myofibroblast transition [124]. Although miRNA modulation appears to be an interesting field that might shed light on biological events occurring in SSc, little experimental evidence supports so far the contention that miRNA modulation actually occurs in endothelial cells of SSc patients. Moreover, like for other epigenetic regulations that have been implicated in the pathogenesis of SSc, their causal role in the various disease features remains elusive [125]. Specifically, it remains to be seen whether miRNA modulation reflects ongoing EndoMT, or it is a necessary condition to begin or effectively conclude the process.

10. Oxidative Stress and EndoMT

Oxidative stress mediated by reactive oxygen species (ROS) plays a role in various features of SSc [12, 66, 126, 127], possibly including senescence-correlated changes of SSc fibroblasts [128] and of bone marrow-derived mesenchymal stem cells of SSc patients, which express markers of early senescence and have an impaired ability to differentiate into endothelial cells [129]. Defective function of endothelial progenitor cells might contribute to the defective angiogenesis typical of the disease [13]. The NADPH oxidase (NOX) family of membrane-associated enzymes catalyzes the reduction of oxygen to form ROS. NOX4 in particular has a key role in the establishment and maintenance of tissue fibrosis. Several signals involved in SSc pathogenesis, including TGF-β, PDGF, and ET-1, modulate the expression of NOX and of NOX4 in particular [130]. Oxidative stress also induces the conversion of ECs into myofibroblasts via a mechanism possibly depending on ALK5/Smad3/NF-κB pathway [126].

11. Shear Stress

Uniform laminar shear stress (LSS) has anti-inflammatory and anticoagulant effects on ECs [131]. Conversely, EndoMT might be implicated in the fibroproliferative vascular disease and might be modulated by shear stress in a ERK5-dependent manner [131]. Prolonged exposure of EC to LSS results in sustained activation of p53 and in growth arrest [132]. KLF4 physically interacts with p53 in synergistic activation of p21, indicating interaction between p53 and ERK5 signaling pathways. Activation of ERK5 thus not only inhibits mesenchymal transition of EC, but also might be the key to reversal of the transition [131].

12. EMT in SSc

EMT is a process in which adhesive properties and polarity of epithelial cells are modified, with decreased expression of epithelial markers, including E-cadherin and Zo-1. In contrast expression of mesenchymal markers, such as vimentin and fibronectin, is upregulated [133] and matrix metalloproteinases (MMPs) are generated including MMP-2 and MMP-9, which degrade collagen IV, the main component of the basement membrane, and aid the development of a migratory phenotype. The retained plasticity of pulmonary and renal epithelial cells and their ability to contribute directly to human fibrotic disease via EMT are well defined [134, 135]. *In vitro* data suggest the involvement of TGF-β and TNF-α synergic activity in driving EMT of primary keratinocytes, in a Smad-dependent manner. The use of specific Smad inhibitors could prevent EMT but more importantly can also reverse established EMT open to a new potential therapeutic intervention [133]. SSc keratinocytes exhibit a phenotype normally associated with tissue repair, including phosphorylation profiles indicative of TGF-β signaling, with increased phosphorylated Smad2/3 nuclear translocation [136].

An important role in the EMT during SSc is also played by the lacking activity of Fli-1. The transcription factor Fli-1, a member of the Ets transcription factor family, is epigenetically suppressed in SSc skin and SSc dermal fibroblasts and may represent such a predisposing factor for SSc [137]. Fli-1 expression is decreased in nonlesional SSc skin in various cell types, including dermal fibroblasts, endothelial cells, and perivascular inflammatory cells, suggesting that downregulation of Fli-1 is an early event preceding the development of fibrosis. The factors that might be involved in the downregulation of Fli-1 include TGF-β and interferon-γ, in addition to epigenetic mechanisms, and recent data suggest a new *in vivo* model to study the SSc phenotype in various cell types [138]. Indeed, bleomycin-induced skin fibrosis in Fli-$1^{+/-}$ mice highlights alterations of dermal fibroblasts, endothelial cells, and macrophages reminiscent of the human disease, suggesting a new promising tool for the *in vivo* study of SSc [138].

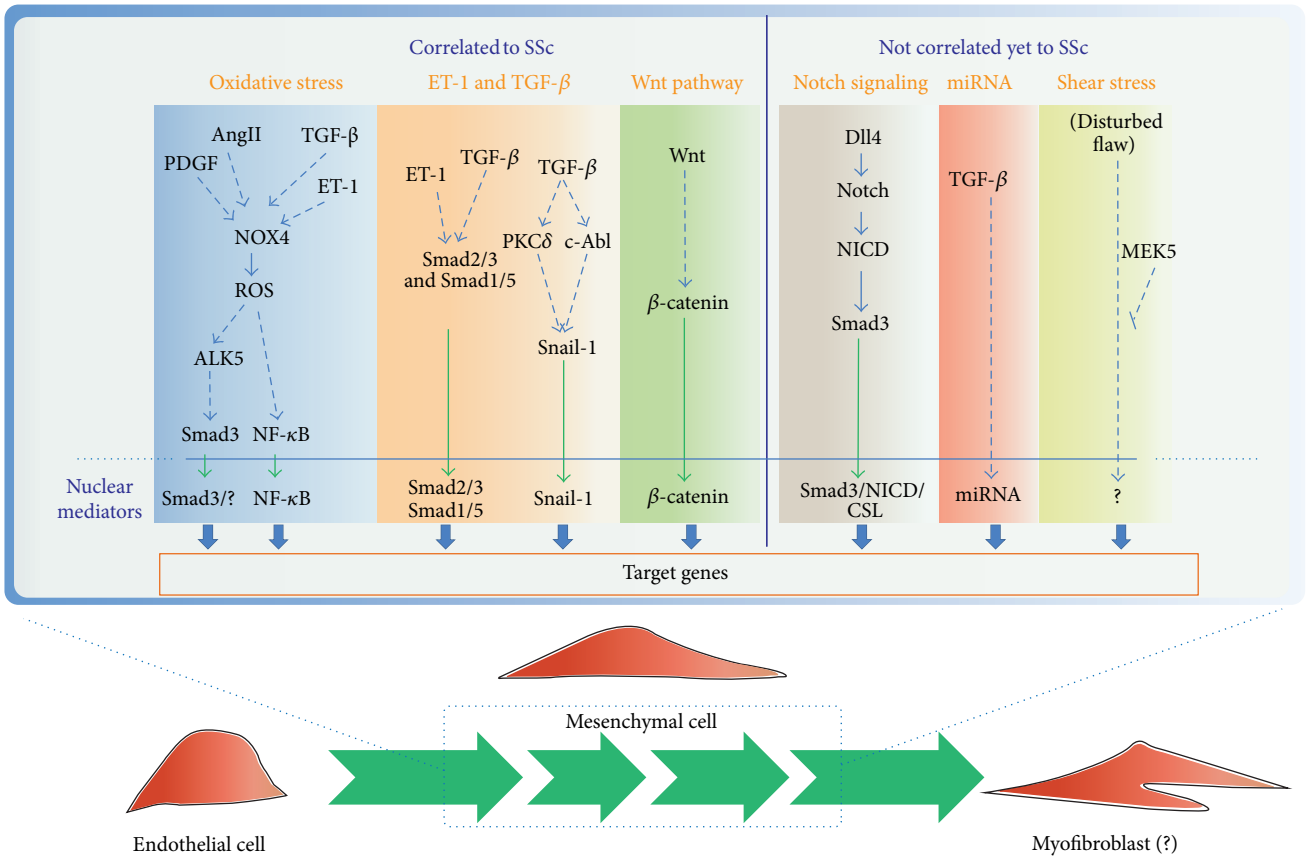

FIGURE 2: Pathways involved in the EndoMT. The scheme summarized the putative pathways involved in the EndoMT highlighting which are already correlated or not with SSc. The activation of specific nuclear mediator leads to activation of target genes that are correlated with the increase of mesenchymal markers (such as Col I, α-SMA, and Twist 1) and/or decrease of endothelial markers (such as CD31, VE-Cad, and Fli-1). The activation of these pathways could lead endothelial cells to acquire initially mesenchymal characteristics and later on to acquire myofibroblastic features.

13. Conclusions

A failure of various intermingled homeostatic programs accounts for the complex phenotype of SSc patients. Defective homeostatic processes are governed by interacting signaling pathways, most of which are involved in the regulation of the vascular cell plasticity (Figure 2). Although much new information has been obtained on the mesenchymal transition of endothelial and epithelial cells and on the transition from fibroblasts to myofibroblasts over the past few years, many issues still require characterization, including the actual extent to which mesenchymal transition occurs in SSc patients. The contention that cell plasticity causally links the generalized vascular inflammation and remodelling with the fibrosis associated with SSc has not been formally demonstrated. In case it was, the molecular regulation underlying the substantially variable fibrosis (generalized versus limited) which characterizes each single patient would remain to be established. Moreover the contribution of autoimmunity in the process, which is felt to be important, remains elusive. New targets for molecular treatments are being identified. These discoveries may lead to profound advances in therapies for SSc and possibly for other persistent fibrotic and inflammatory diseases.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Pier Andrea Nicolosi and Enrico Tombetti equally contributed to the paper.

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Review Article

Noncoding RNAs in Tumor Epithelial-to-Mesenchymal Transition

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Epithelial-derived tumor cells acquire the capacity for epithelial-to-mesenchymal transition (EMT), which enables them to invade adjacent tissues and/or metastasize to distant organs. Cancer metastasis is the main cause of cancer-related death. Molecular mechanisms involved in the switch from an epithelial phenotype to mesenchymal status are complicated and are controlled by a variety of signaling pathways. Recently, a set of noncoding RNAs (ncRNAs), including miRNAs and long noncoding RNAs (lncRNAs), were found to modulate gene expressions at either transcriptional or posttranscriptional levels. These ncRNAs are involved in EMT through their interplay with EMT-related transcription factors (EMT-TFs) and EMT-associated signaling. Reciprocal regulatory interactions between lncRNAs and miRNAs further increase the complexity of the regulation of gene expression and protein translation. In this review, we discuss recent findings regarding EMT-regulating ncRNAs and their associated signaling pathways involved in cancer progression.

1. Introduction

Epithelial-to-mesenchymal transition (EMT) is a critical step in both embryonic development and tumor metastasis. EMT is composed of serial phenotypic changes through which epithelial cells lose their apical-basal polarity and tight cellular adhesions, while acquiring protease-producing properties that increase cell motility [1]. EMT is a well-recognized process in tumor metastasis through which tumor cells seed and colonize areas distant from their primary sites. The process of EMT is sophisticatedly regulated and requires the acquisition of variable genetic alterations among tumor cells and their microenvironment [2, 3]. Important cellular components of the tumor microenvironment (TME) include tumor-infiltrating immune cells, cancer-associated fibroblasts, and endothelial cells. In addition, hypoxic conditions, which alter the composition of extracellular matrix (ECM), cytokines, chemokines, and growth factors, are critical in the development of EMT [4, 5].

Among important TME-associated cytokines are members of the transforming growth factor-β (TGF-β) family, which paradoxically suppress tumor metastasis in early-stage cancers but drive the metastatic process in advanced disease. TGF-β signaling initiates EMT by activating EMT-inducing transcription factors (EMT-TFs), such as Snail/Slug, zinc-finger E-box-binding homeobox 1/2 (ZEB1/2), basic helix-loop-helix (bHLH) protein, E47, and Twist, or by transcriptionally repressing epithelial-specific genes via members of the histone deacetylase (HDAC) family [6–10]. Epithelial-specific genes, such as E-cadherin (*CDH1*), zona occludens 1 (*ZO-1*), and occludin (*OCLN*), are substantially downregulated at the transcriptional level during the EMT process [11–13]. Notably, promoter regions of *CDH1* and *OCLN* genes contain EMT-TF binding sites, termed E-boxes. *CDH1* and *OCLN* are frequently downregulated in high-grade malignancies with poor clinical outcomes [14–17], whereas mesenchymal markers, such as N-cadherin, vimentin, fibronectin, and α-smooth muscle actin (α-SMA),

Table 1: miRNAs and other molecules involved in EMT.

miRNA	Expression levels in cancer	Upstream regulator	Known targets	References
miR-200 family	Breast cancer; prostate cancer	ZEB1/2, miR-22, Slug, GATA3, TGF-β, Foxf2	ZEB1/2, Slug, GATA3, Maml2/3, Foxf2	[69–72]
miR-1	Breast cancer; prostate cancer	ZEB1/2, miR-22, Slug, GATA3, TGF-β	ZEB1/2, Slug, GATA3, Maml2/3	[69–72]
miR-203	Breast cancer, pancreatic cancer	Slug, Snail, TGF-β	Bmi-1, Snail, ZEB1/2	[73, 74]
miR-34 family	Pancreatic stem cell, neuroblastoma	p53, epigenetic regulation	Snail, ZEB1	[75–77]
miR-9	Breast cancer	c-myc	E-cadherin, LIFR	[78, 79]
miR-135b	Colon cancer, NSCLC, HNSCC	Epigenetic regulation, NF-κB, hypoxia	APC, LATS2, β-TrCP, NDR2, MOB1B	[80–82]
miR-210	Breast cancer	Hypoxia	E2F3, HOXA1, FGFLR1, EFNA3, PTP1B, VMP1	[83–85]
miR-103/107	CRC, breast cancer	Hypoxia	DAPK, KLF4, Dicer	[78, 86]
miR-10b	Breast cancer	Twist	HOXD-10	[87]
miR-21	NSCLC, CRC, breast cancer	TGF-β/BMP, HER2/neu, hypoxia	Pdcd4, TGFBR2, PTEN, TAp63	[88–92]
miR-205	Breast cancer	ΔNp63α	ZEB1/2, Jagged1	[69, 93, 94]
miR-23b	Colon cancer; bladder cancer	n/a	Src, ZEB1	[95–97]
miR-138	Ovarian cancer; HNSCC	n/a	SOX4, HIF-1α, vimentin	[98–100]
miR-7	Gastric cancer; breast cancer	WISP	IGF1R, Snail, SETDB1	[27, 101, 102]

HNSCC: head and neck squamous cell carcinoma; NSCLC: non-small-cell lung carcinoma; CRC: colorectal cancer; ZEB1/2: zinc-finger E-box binding homeobox 1/2; LIFR: leukemia inhibitory factor receptor alpha; APC: adenomatous polyposis coli; LATS2: large tumor-suppressor kinase 2; β-TrCP: beta-transducin repeat-containing protein; NDR2: nuclear-Dbf2-related 2; MOB1B: Mps one binder 1b; E2F3: E2F transcription factor 3; HOXA1: homeobox A1; FGFLR1: fibroblast growth factor receptor like-1; EFNA3: ephrin-A3; PTP1B: protein-tyrosine phosphatase 1B; VMP1: vacuole membrane protein 1; DAPK: death-associated protein kinase; KLF4: Krüppel-like factor 4; HOXD10: homeobox D10; Pdcd4: programmed cell death protein 4; TGFBR2: TGF beta receptor 2; PTEN: phosphatase and tensin homolog; WISP: WNT1-inducible signaling pathway protein 2; IGF1R: insulin-like growth factor 1 receptor; SETDB1: SET domain, bifurcated 1; n/a: not available.

are upregulated [18]. Dynamic expression of these proteins results in alterations in cytoskeleton arrangements and cellular polarity, as well as changes in the ability of cells to degrade ECM.

Recent cancer genomic studies have identified numerous RNAs that do not encode proteins. These noncoding RNAs (ncRNAs), including snRNAs, snoRNAs, rRNAs, tRNAs, piRNAs, microRNAs (miRNAs), and long noncoding RNAs (lncRNAs), regulate biological functions through interactions between their specific structural domains and DNA, RNA, or proteins [19]. Of these ncRNAs, miRNA and lncRNAs have been found to serve as important gene expression regulators that fine-tune cell transcriptomes and adjust proteomes in response to extracellular stimulation [20]. In addition, these noncoding RNAs could be transported from primary site to another cell or distant organ through extracellular vesicles and alter the gene expression profile as well as their morphology and functions within the target sites [21, 22]. Furthermore, mutations and dysregulations of miRNAs and/or lncRNAs are associated with a diverse array of human diseases, including cancer [23–27].

In this review, we discuss recent findings on the roles of miRNAs and lncRNAs in regulating EMT-TFs (Tables 1 and 2). We also discuss the multilayered regulatory circuits among miRNAs, lncRNAs, and protein-coding genes that are associated with cancer EMT (Figure 1).

2. EMT-Related Signaling Pathways and the Tumor Microenvironment

The TME is a niche composed of various growth factors secreted by tumor cells or adjacent tissues, cytokines released by lymphoid cells, molecular components of the ECM, and intratumor hypoxia. The expression of EMT-TFs in cancer cells can be turned on in response to changes in the extracellular microenvironment. Signaling pathways, including those mediated by TGF-β, bone morphogenetic protein (BMP), Wnt, Notch, integrin, epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), and sonic hedgehog (SHH), are overactivated during carcinogenesis [28, 29]. In addition, tumor hypoxia is responsible for the expression of a subset of EMT-TFs and

Table 2: lncRNAs and EMT.

lncRNAs	Expression levels in cancer	Upstream regulator	Targets	References
ZEB1-AS1	HCC	n/a	ZEB1↑	[103]
lncRNA-ATB	HCC	TGF-β	ZEB1/2↑, IL-11↑, miR-200↓	[104]
lncRNA-HIT	Breast cancer	TGF-β	E-cadherin↓	[105]
MEG3	HCC		TGFBR1↑, TGFB2↑, SMAD2↑	[106]
lncRNA-Hh	Breast cancer	Twist	GAS1↑	[107]
lncTCF7	Liver cancer	IL-6	TCF↑ (Wnt signaling)	[108, 109]
treRNA	Breast cancer		E-cadherin↓	[110]
H19	n/a	CTCF	IGF1R↓, NOMO1↓, Twist↓, TGF-β1/SMAD↓, miR-138↓, miR-200↓, Let-7↓,	[111–117]
MALAT1	Lung cancer, breast cancer, liver cancer, prostate cancer, renal cell carcinoma	TGF-β1, EZH2↑	miR-205↓	[118–120]
Hotair	n/a	TGF-β1, miR-141	miR-34↓, miR-141↓, miR-7	[27, 121–124]

HCC: hepatocellular carcinoma; TGFBR1: transforming growth factor beta receptor 1; TGFB2: transforming growth factor beta 2; GAS1: growth arrest-specific 1; TCF: transcription factor; CTCF: CCCTC-binding factor; IGF1R: insulin-like growth factor 1 receptor; NOMO1: NODAL modulator 1; n/a: not available.

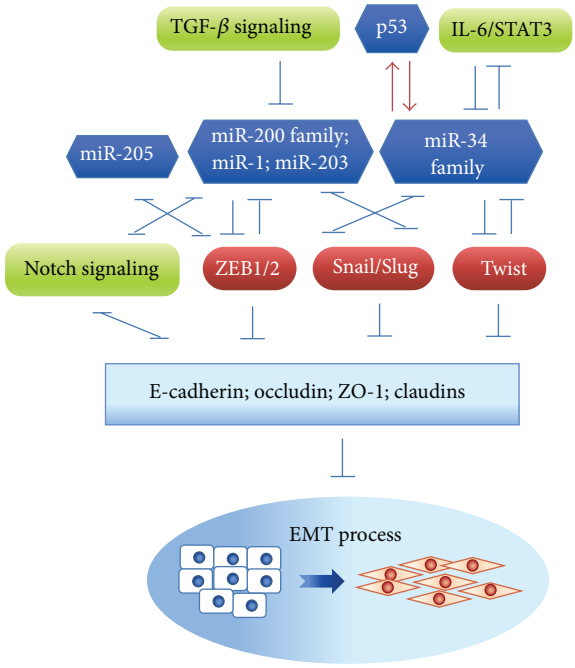

Figure 1: The reciprocally regulatory feedback loop between miRNAs and EMT-TFs that are involved in EMT. miRNAs form regulatory networks with EMT-TFs and EMT-associated signaling pathways that individually or cooperatively modulate EMT. EMT-suppressing miRNAs, such as the miR-200 family, miR-1, miR-203, and the miR-34 family (in blue), reciprocally suppress EMT-TFs (ZEB1/2, Snail/Slug, and Twist) and consequently downregulate the expression of epithelial markers (E-cadherin, occludin, ZO-1, and claudins). This negative feedback loop can be broken by TGF-β or IL-6/STAT3 signaling, and p53.

activation of a category of EMT-related signaling pathways. Here, we discuss several signaling pathways that participate in the initiation of cancer EMT.

2.1. TGF-β Signaling Pathway. TGF-β signaling is a core pathway that tightly controls the process of cell proliferation and EMT during organ development, tissue fibrosis, and cancer progression [30]. This signaling pathway is typically initiated by ligands belonging to the TGF-β superfamily, which includes three isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) and six isoforms of BMP (BMP2–7). These ligands are expressed and secreted in different cellular contexts and in response to various stimuli [31]. TGF-β receptors are single-pass serine/threonine kinases that exist in different isoforms, including seven Type I (TGF-βRI) and five Type II (TGF-βRII) receptors that can form homo- or heterodimers. Combinatorial dimerization enables TGF-β receptors to differentially activate intracellular signaling pathways that are broadly distinguished by their SMAD dependence or independence. In response to phosphorylation of TGF-β receptors, a SMADs ternary complex, composed of SMAD2/3, R-SMAD and SMAD4, forms and translocates from the cytoplasm to the nucleus [32].

Several EMT-TFs, including members of the ZEB family, Snail/Slug and Twist, are transcriptionally upregulated in cancer cells by TGF-β signaling through conserved response elements on the promoters of the corresponding genes [10, 33–36]. TGF-β-SMADs was also shown to indirectly induce expression of EMT-TFs by enhancing the expression of its downstream effector, high mobility group A2 (HMGA2) [37, 38]. Activated TGF-β signaling is sustained by an autocrine

loop, which in turn reinforces the EMT process [39–41]. Furthermore, Snail and SMAD3/4 form a transcriptional repressor complex, which synergistically suppresses the expression of coxsackie and adenovirus receptor (*CAR*), *OCLN*, and *CDH1*, and thus promotes EMT [42]. These results suggest the importance of TGF-β signaling in cancer EMT and its potential to serve as a therapeutic target.

2.2. Wnt, Notch, and MAPK Signaling Pathways. The Wnt signaling pathway is an important regulator of EMT-TF expression and the EMT process. WNT couples with the membrane protein Frizzled and low-density lipoprotein receptor (LRP), promoting translocation of β-catenin from the cytoplasm to the nucleus. In the nucleus, β-catenin acts as a coactivator of TCF/LEF1 (T cell factor/lymphoid-enhancing factor-1) and upregulates the transcription of *SNAIL1/2* and *TWIST*, which in turn repress E-cadherin [43–45].

Notch signaling is activated by cell-cell contact. Interactions between JAG1/2 (Jagged-1/2), Notch ligand, and Notch receptors facilitate nuclear translocation of the Notch intracellular domain (NICD), which subsequently activates Notch effector genes [46]. Notch signaling not only enhances *SNAIL* transcription but also enhances SNAIL1/2 function through upregulation of hypoxia-inducible factor 1α (HIF-1α), thereby promoting tumor invasion and/or metastasis [47, 48].

Additional pathways are also involved in cancer EMT. For example, hepatocyte growth factors (HGFs) and insulin-like growth factor-1 (IGF-1) upregulate expression of *SNAIL* and *ZEB1*, respectively, through the mitogen-activated protein kinase (MAPK) pathway [49–51]. Collectively, these observations suggest that EMT-TF regulatory circuits are tightly controlled.

2.3. Tumor Microenvironment and Hypoxia. Hypoxic microenvironments, defined as those with a pO_2 level less than 10 mmHg, trigger signaling cascades and immune responses that drive cancer progression [52, 53]. The hypoxic microenvironment contributes to the immune escape of tumors as well as tumor neovascularization and also promotes EMT [4]. Tumor hypoxia-dependent signaling is predominantly mediated by hypoxia-inducible factors (HIFs)—important protein complexes that regulate tumor progression and metastasis [52]. HIFs, which consist of an unstable α-subunit and a stable β-subunit [54], bind to promoters of target genes that contain the hypoxia response element (HRE) and promote the recruitment of transcriptional coactivators. In HIF-1α-mediated canonical hypoxia signaling, expression levels of Twist, Snail, ZEB1, and E12/E47 are upregulated [55, 56].

Studies have shown that a hypoxic TME contributes to the stabilization of HIF-1α, which functions to activate TGF-β signaling [57, 58]. TGF-β, in turn, assists in the maintenance of HIF-regulated vascular homeostasis and angiogenesis [59, 60]. The promoter region of *VEGF* (vascular endothelial growth factor), encoding a secretory factor involved in vasculogenesis and angiogenesis, harbors both HIF-1α and SMAD binding sites, suggesting the possibility that both hypoxia and TGF-β signaling pathways regulate *VEGF* expression [61]. The positive feedback loop between HIF-1α and TGF-β functions in the regulation of cancer EMT and angiogenesis [62].

The TME-associated HIF-1α-mediated hypoxia pathway also regulates cancer EMT through Notch signaling [48, 63, 64]. It has been shown that interaction between NICD and HIF-1α increases the expression of Snail and Slug, which enhance cancer invasion and migration [48, 65]. In addition, a hypoxic TME augments the nuclear translocation of β-catenin, which promotes activation of Wnt signaling [66]. Collectively, these findings demonstrate that a hypoxic TME acts as a driving force for cancer EMT, both directly, through stabilization of HIFs, and indirectly, through paracrine/autocrine stimulation.

2.4. Chemoresistance. Recent findings by *in vivo* mesenchymal lineage tracing showed that EMT might not be essential for tumor metastasis, and interestingly the phenotype of EMT in tumor cells was resistant to CTX (cyclophosphamide) and gemcitabine treatment [67, 68]. Despite the fact that there may be other EMT-inducing factors function to compensate for the genes that were manipulated in these studies, the discovery of EMT tumors displayed chemoresistance may provide a new insight for developing novel therapy targeting tumor metastasis.

3. miRNAs and EMT

miRNAs are a group of small (~22 nucleotides) ncRNAs that mediate destabilization and translational suppression of downstream RNAs at the posttranscriptional level. The expression of miRNAs can be ubiquitous or context-specific (e.g., during development or within certain tissues). miRNAs participate in a broad range of physiological functions. Therefore, miRNA dysregulation may break the harmony of normal genetic activity and result in a diverse array of diseases, including cancer. Recent studies have revealed that more than 50% of miRNAs are dysregulated in human cancer. Moreover, prognostic and predictive miRNA signatures have been reported in different types of cancer [125–127].

miRNAs serve both positive and negative roles in regulating cancer EMT (Figure 1 and Table 1) [128]. The regulation of miRNAs is complicated, as highlighted by the fact that some miRNA targets can, in turn, regulate the expression of miRNAs, forming regulatory loops. Two EMT-related regulatory feedback loops formed by miRNAs and EMT-TFs will be discussed here: the miR-200 family and ZEB1/2, and miR-203/miR-34 and Snail/Slug.

3.1. Reciprocal Regulation between miRNAs and EMT-TFs: The miR-200 Family and ZEB1/2. The miR-200 family consists of five members, including miR-200a/b/c, miR-429, and miR-141, all of which contain a similar seed sequence that targets a large common subset of genes [129]. The miR-200 family directly suppresses ZEB1/2 translation, consequently upregulating the expression of E-cadherin and maintaining an epithelial cellular morphology [69, 129, 130]. Conversely, ZEB1 has been shown to strongly promote tumorigenesis and cancer metastasis by inhibiting miR-200c and miR-203

transcription. These data suggest that the miR-200 family and ZEB1/2 form a negative regulatory feedback loop [73].

In pancreatic and breast cancer models, the miR-200 family is reported to suppress Notch-mediated ZEB1 activation by directly targeting the Notch coactivators MAML2 and MAML3 and the Notch ligand, JAG1 [70]. In lung cancer, miR-200 and GATA binding protein 3 (GATA3), a direct downstream target of Notch involved in lung cancer metastasis, have been shown to mutually inhibit each other. This inhibitory loop between miR-200 and GATA3 is perturbed by the Notch ligand, JAG2 [131]. These results suggest that Notch signaling broadens the spectrum of the miR-200/ZEB1 negative feedback loop in regulating cancer EMT and metastasis.

It has also been reported that miR-200 is associated with the reverse EMT process—mesenchymal-epithelial transition (MET)—in prostate cancer. miR-200 and miR-1 directly target the *SLUG* 3′-UTR (untranslated region), and Slug in turn inhibits miR-200/miR-1 expression [71]. In addition, prolonged TGF-β signaling increases miR-200 promoter methylation and leads to miR-200 suppression. This suggests that the induction and maintenance of a mesenchymal state require autocrine TGF-β signaling to sustain expression of EMT-TFs and inhibition of the miR-200 family [132, 133].

3.2. Reciprocal Regulation between miRNAs and EMT-TFs: miR-203/miR-34 and Snail/Slug. Similar to the ZEB/miR-200 negative feedback loop, Snail together with the miR-34 family (miR-34a, miR-34b, and miR-34c) and miR-203 constitutes another negative feedback loop. This negative feedback loop regulates epithelial plasticity [75]. Snail and miR-34a/b/c control ZNF281/ZBP-99, a Krüppel-type zinc-finger domain-containing transcription factor, acting as an integral component of an EMT-related feed-forward loop [134]. A negative feedback loop between miR-203 and Snail controls the dynamic transition between epithelial and mesenchymal phenotypes [135, 136]. miR-203 has been shown to suppress Slug expression in breast cancer cells, whereas TGF-β-mediated Slug activation reciprocally downregulates miR-203 expression [136, 137].

A feedback loop also exists between p53 and miR34. The tumor-suppressor p53 upregulates the expression of miR-34, which subsequently suppresses EMT. Mutated p53 proteins, in contrast, are unable to induce miR-34 expression, thus shifting the equilibrium toward a mesenchymal phenotype [76, 138–140]. In addition, the p53/miR-34 axis suppresses Wnt signaling, both in development and during cancer progression [138].

These results illustrate how miRNAs create networks that connect different EMT-associated signaling pathways. EMT-related signaling not only upregulates EMT-TFs but also suppresses miRNAs; this, in turn, breaks down miR-200/ZEB and/or miR-203/Snail/Slug feedback loops and facilitates cancer EMT (Figure 1).

3.3. Other EMT-Related miRNAs. Several other miRNAs are reported to be involved in EMT (Table 1). For example, miR-10b is transcriptionally upregulated by Twist and induces tumor invasion and metastasis in breast cancers by targeting homeobox D10 (HOXD10) [87]. miR-9 directly targets E-cadherin mRNA, resulting in activation of β-catenin signaling, which promotes EMT and metastasis [78]. miR-9 upregulation has also been observed in *c-myc*-induced mouse mammary tumors [141]. In addition, miR-9 has been shown to downregulate leukemia inhibitory factor receptor (LIFR). LIFR suppresses breast cancer metastasis by activating the Hippo kinase cascade, which in turn results in YAP (YES-associated protein) inactivation [79].

A recent study revealed that miR-9-3p negatively regulates the expression of TAZ, a YAP homolog [142]. A recent study by our laboratory also showed that miR-135b increases the levels of nuclear TAZ by directly suppressing multiple components of the Hippo pathway, including LATS2 (large tumor-suppressor kinase 2), MOB1B (Mps one binder 1b), NDR2 (nuclear-Dbf2-related 2), and β-TrCP (β-transducin repeat-containing protein) [80]. Notably, miR-135b expression level, LATS2 protein, and nuclear TAZ protein levels correlate with disease prognosis in non-small-cell lung cancer patients [80]. Furthermore, YAP was found to physically interact with p72, a RNA helicase that plays roles in miRNA processing [143]. These results may suggest how the Hippo pathway suppresses proliferation in response to contact inhibition.

3.4. miRNAs Involved in Hypoxia-Induced EMT. Some miRNAs are involved in hypoxia-induced EMT. Expression of miR-205 and miR-124, which regulate EMT by targeting ZEB1/2 and MMP2 (matrix metallopeptidase 2), respectively, is suppressed by hypoxia [69, 144, 145]. Hypoxia also downregulates the expression of miR-34a, which acts as a suppressor of Snail and ZEB1. miR-34a suppression results in upregulation of Notch1 and JAG1, and activated Notch signaling promotes EMT [146]. Forced expression of miR-34a under conditions of hypoxia not only reduces Notch1 and JAG1 expression but also abolishes Snail expression, suggesting that the interplay between hypoxia and Notch signaling is important in EMT modulation (Figure 1).

4. Long Noncoding RNAs and EMT

lncRNAs are RNA transcripts longer than 200 nucleotides that do not encode proteins [147]. The FANTOM project revealed that, in mammals, the number of lncRNAs is at least four times that of protein-coding RNAs [148, 149]. Although the functions of lncRNAs are largely unknown, accumulating evidence suggests that they are key regulators of a number of important biological processes, possibly exerting tissue-specific imprinting patterns and functioning in embryogenesis, development, lineage differentiation, tumorigenesis, and EMT regulation [150–152].

Recent studies have shown that lncRNA dysregulation is associated with cancer progression [153]. lncRNAs may interact with their nearby protein-coding genes, for example, *TAL1, SNAIL, SLUG,* and the master regulator of hematopoiesis, SCL/TAL1 (T Cell Acute Lymphocytic Leukemia 1), as evidenced by the fact that depletion of certain lncRNAs results in upregulation of these genes [154]. In addition, some lncRNAs encompass miRNA binding sites.

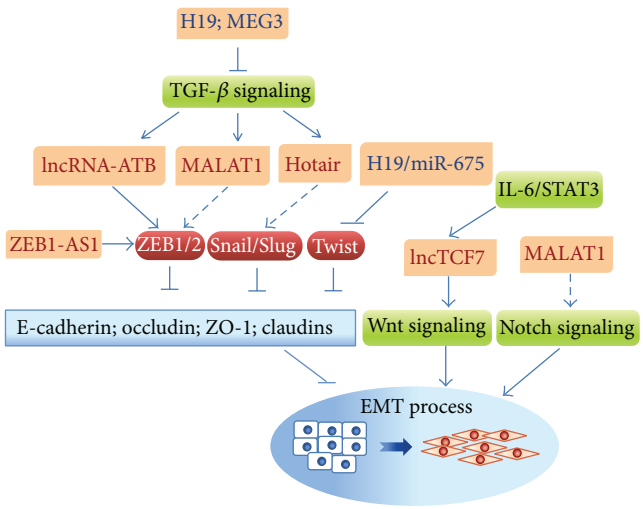

FIGURE 2: The reciprocally regulatory feedback loop between lncRNAs and EMT-TFs that are involved in EMT. lncRNAs form regulatory networks with EMT-TFs and EMT-associated signaling pathways that individually or cooperatively modulate EMT. The EMT-suppressive lncRNAs, H19 and MEG3, can downregulate TGF-β signaling. H19, lncRNA-ATB, and ZEB-AS1 promote EMT-TFs through direct or indirect regulation. In addition, H19 is reported to possess a controversial ability to downregulate Twist though its intergenic miRNA, miR-675. TGF-β and IL-6/STAT3 signaling pathways also promote activity of the lncRNAs, Hotair, lncTCF7, and MALAT1, and thus crosstalk with Notch signaling and Wnt signaling, to modulate EMT process. Dysregulation of these miRNAs and lncRNAs may lead to tumor progression.

These lncRNAs function as ceRNAs (competing endogenous RNA) that antagonize miRNAs. lncRNAs may positively or negatively regulate EMT-associated proteins and miRNAs, as discussed below (Figure 2 and Table 2).

4.1. lncRNAs Regulate EMT through EMT-TFs. Most lncRNAs act as transcription inducers or form RNA-protein complexes that promote expression of EMT-associated genes at transcriptional or posttranslational levels [105]. The lncRNA, ZEB1-AS1, was found to be frequently upregulated in hepatocellular carcinoma (HCC) [103]. ZEB1-AS1, whose transcription locus is close to *ZEB1*, increases *ZEB1* promoter activity through an unknown mechanism. ZEB1, in turn, suppresses proteins that maintain the epithelial phenotype, such as E-cadherin, ZO-1, and occludin [103]. Thus, targeting ZEB1-AS1 may inhibit ZEB1-related EMT.

Expression of lncRNA-ATB, another lncRNA that upregulates ZEB1/2 expression and functions as a ceRNA, is enhanced by TGF-β. lncRNA-ATB promotes a metastatic cascade by competitively binding to members of the miR-200 family, thereby attenuating the inhibitory function of miR-200 on ZEB1/2. At the same time, lncRNA-ATB upregulates interleukin-11 (IL-11) and activates IL-11/STAT3 (signal transducer and activator of transcription 3) signaling, which enables cancer cell colonization [104].

lncRNA-HIT, a HOXA transcript induced by TGF-β, is a newly identified lncRNA upregulated by TGF-β that also mediates TGF-β-induced EMT [105]. It has been found that lncRNA-MEG3 associates with a PRC2 (polycomb repressive complex 2) complex through interactions with EZH2 (enhancer of zeste 2 polycomb repressive complex 2 subunit). lncRNA-MEG3 is recruited to a GA-rich sequence in target genes, forming a RNA-DNA triplex structure that regulates expression of the TGF-β receptor genes, *TGFBR1* and *TGFB2*, as well as *SMAD2* [106]. lncRNA-Hh, activated by Twist at the transcriptional level, directly targets GAS1 (growth arrest-specific 1) and activates hedgehog signaling [103]. The Twist/lncRNA-Hh signaling cascade enhances the stemness property of cells, suggesting a connection between EMT and stemness [107].

4.2. lncRNAs Regulate EMT through Their Interplay with miRNAs. EMT is a tightly controlled physiological and pathological process. Not only proteins but also lncRNAs and miRNAs are involved in fine-tuning EMT regulation. lncRNAs may serve as ceRNAs, which act as molecular "sponges" to regulate the harmony of miRNA pools and the biological signaling regulated by them. Interestingly, ceRNAs absorb target miRNAs without altering their total amount. Therefore, it is important to note that biological functions of miRNAs are simply determined not only by their measured abundance but also by their interactions with lncRNAs. lncRNAs are thus attractive therapeutic targets in miRNA-mediated diseases. Three lncRNAs—H19, MALAT1, and Hotair—will be discussed in this section.

4.2.1. H19. H19, which is highly expressed at the embryonic stage in mesodermal and endodermal tissues [155], and insulin-like growth factor II (IGF2) are reciprocally imprinted. After the early gestation period, H19 is solely expressed from the maternal-inherited allele whereas IGF2 is exclusively expressed from the paternal-inherited allele [156, 157]. Loss of IGF2 or H19 imprinting leads to IGF2 upregulation and subsequent H19 promoter hypermethylation, a phenomenon commonly found in cancers [158–160].

Studies have suggested that H19 possesses tumor-suppressor functions and have implicated chromatin insulator protein CCCTC-binding factor (CTCF) in methylating the promoter region of the *H19* gene [161, 162]. How H19 functions in cells has grown clearer with the introduction miRNAs [111, 163]. miR-675 is an intergenic miRNA embedded in the first exon of H19 and coexpressed with H19. H19/miR-675 negatively regulates insulin-like growth factor 1 receptor (IGF1R), nodal modulator 1 (NOMO1), and Twist proteins and suppresses TGF-β1/SMADs signaling [111–114, 164, 165]. Since TGF-β/SMAD signaling is a well-known EMT pathway, it is possible that H19/miR-675 plays a role in EMT regulation.

On the other hand, a cancer-promoting role of H19 has been suggested in colorectal and gastric cancers [115, 166]. In colorectal cancer, overexpressed H19 serves as a ceRNA that antagonizes miR-138 and miR-200a, leading to derepression of their endogenous targets, vimentin, ZEB1, and ZEB2 [115]. In addition, H19 was found to harbor several binding sites for Let-7 family miRNAs and act as a sponge that negatively regulates their activity [167]. Notably, expression of Let-7 miRNAs, which inhibit EMT by suppressing HMGA2, is

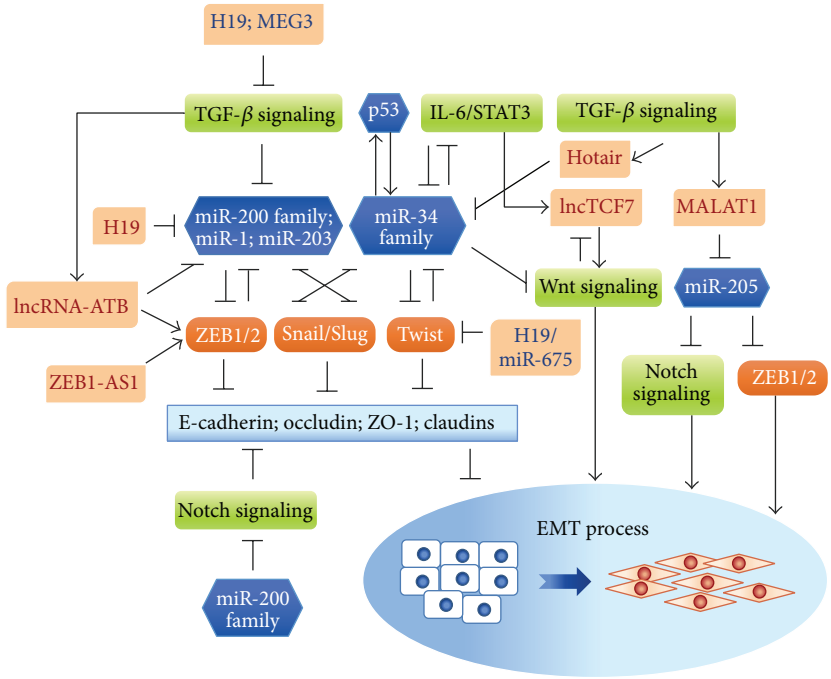

Figure 3: The molecular network composed of miRNAs/lncRNAs and EMT-TFs. miRNAs and lncRNAs form regulatory networks with EMT-TFs and EMT-associated signaling pathways that individually or cooperatively modulate EMT. EMT-suppressing miRNAs reciprocally suppress EMT-TFs and consequently downregulate the expression of epithelial markers. This negative feedback loop can be broken by TGF-β or IL-6/STAT3 signaling, p53, and lncRNAs (e.g., H19, lncRNA-ATB, and ZEB-AS1). Dysregulation of these miRNAs and lncRNAs may lead to tumor progression.

frequently downregulated in cancers with a mesenchymal phenotype. Thus, H19 may exert an EMT-promoting function through its role as a miR-200 and Let-7 family sponge [116, 117].

These results suggest that the dual roles of H19 in cancer EMT regulation are deciphered by cellular context, in which different sets of miRNAs are involved in disease pathogenesis.

4.2.2. MALAT1. MALAT1 (metastasis associated in lung adenocarcinoma transcript 1) has been reported to be a prognostic marker in several cancers, including lung, breast, pancreas, liver, colon, uterus, cervix, and prostate cancers [118]. In bladder cancer, TGF-β1 induces MALAT1 expression, whereas silencing of endogenous MALAT1 and its binding partner, SUZ12, suppresses TGF-β1-induced EMT [119]. In renal cancer, reciprocal crosstalk among MALAT1, miR-205, and EZH2 suppresses the expression of E-cadherin and enhances Wnt signaling activity, thereby promoting cancer metastasis [120]. EZH2 and SUZ12 are subunits of PRC2, which is responsible for the repressive histone 3 lysine 27 trimethylation (H3K27me3) chromatin modification. Previous studies have suggested that MALAT1 might be associated with the PRC2 complex and promote cancer EMT.

4.2.3. Hotair. Hotair (Hox transcript antisense intergenic RNA) epigenetically regulates its target sequences by recruiting PRC2, which in turn results in gene silencing [168, 169]. Hotair upregulation was found to be a prognostic indicator of poor outcome in various types of cancers [121, 122]. In addition, Hotair was shown to be required for TGF-β-mediated EMT in colon cancer [170]. Hotair epigenetically silences miR-34 transcription, resulting in augmentation of C-Met and Snail expression [123]. It was also found that miR-141, an EMT suppressor, decreases the expression of Hotair through complementary binding and thereby inhibits its oncogenic functions [124]. miR-141 negatively regulates Hotair target genes, including *SNAIL*, the nonreceptor tyrosine kinase *ABL2*, and *PCDH10* (protocadherin 10). In addition, the expression levels of miR-141 and Hotair were found to be inversely correlated in renal cancer cells [124]. These results suggest that crosstalk between Hotair and miRNAs play important roles in cancer EMT regulation.

5. Conclusion

EMT is recognized as the first step of cancer metastasis—the main cause of cancer mortality. Cancer EMT is a tightly controlled pathological process. Multilayered regulatory elements, including proteins, miRNAs, and lncRNAs, are involved in the complex EMT regulatory networks through RNA-protein, RNA-miRNAs, and RNA-DNA interactions at pretranscriptional, posttranscriptional, and posttranslational levels. ncRNAs modulate epithelial plasticity by targeting different signaling pathways, EMT-TFs, and/or EMT-associated proteins. Several important reciprocal feedback loops, composed of ncRNAs and EMT-TFs, are involved in establishing flexible control over EMT and MET. Therefore, peeling back the mysteries surrounding ncRNAs in EMT regulation will be important in furthering advances in cancer therapy strategies (Figure 3).

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Molecular Mechanisms Underlying Peritoneal EMT and Fibrosis

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Peritoneal dialysis is a form of renal replacement alternative to the hemodialysis. During this treatment, the peritoneal membrane acts as a permeable barrier for exchange of solutes and water. Continual exposure to dialysis solutions, as well as episodes of peritonitis and hemoperitoneum, can cause acute/chronic inflammation and injury to the peritoneal membrane, which undergoes progressive fibrosis, angiogenesis, and vasculopathy, eventually leading to discontinuation of the peritoneal dialysis. Among the different events controlling this pathological process, epithelial to mesenchymal transition of mesothelial cells plays a main role in the induction of fibrosis and in subsequent functional deterioration of the peritoneal membrane. Here, the main extracellular inducers and cellular players are described. Moreover, signaling pathways acting during this process are elucidated, with emphasis on signals delivered by TGF-β family members and by Toll-like/IL-1β receptors. The understanding of molecular mechanisms underlying fibrosis of the peritoneal membrane has both a basic and a translational relevance, since it may be useful for setup of therapies aimed at counteracting the deterioration as well as restoring the homeostasis of the peritoneal membrane.

1. Introduction

Peritoneum is a serosal membrane that forms the lining of the abdominal cavity. It is composed of a continuous monolayer of cells of mesodermal origin, the mesothelial cells (MCs). MCs have an epithelial-like cobblestone shape and cover a submesothelial region constituted of a thin layer of connective tissue composed mainly of bundles of collagen fibers with few fibroblasts, mast cells, macrophages, and vessels [1]. Peritoneum supports the abdominal organs and serves as a conduit for their blood vessels, lymph vessels, and nerves. Between parietal peritoneum, covering the abdominal wall, and visceral peritoneum, covering abdominal viscera, resides the peritoneal cavity, a virtual space filled of scarce interstitial fluid. This fluid facilitates peristaltic movements of abdominal viscera. Moreover, peritoneum is relevant for the control of local and intestinal immunity due to leukocyte recirculation [2].

Peritoneal membrane can be used as a dialysis membrane in therapeutic procedures for the treatment of end-stage renal disease, as an alternative to classical hemodialysis procedure [3]. Currently, peritoneal dialysis (PD) accounts for more than 10% of all forms of renal replacement therapy worldwide [3]. During PD, the peritoneal membrane (PM) acts as a permeable barrier across which ultrafiltration and diffusion take place [4]. Continual exposure to hyperosmotic, hyperglycemic, and acidic dialysis solutions, mechanical stress connected to dwelling practice, and episodes of catheter complications (including peritonitis and hemoperitoneum) may cause acute and chronic inflammation and injury of the PM. In these conditions, peritoneum undergoes progressive fibrosis, angiogenesis, and vasculopathy, eventually leading to discontinuation of PD.

A main role in the induction of peritoneal fibrosis during exposure to PD fluids is played by the epithelial to mesenchymal transition (EMT) of mesothelial cells (MCs),

named more properly mesothelial to mesenchymal transition (MMT) [5]. The EMT represents a complex phenomenon of cellular transdifferentiation that converts the epithelial phenotype into a mesenchymal one, with loss of cell polarization, disassembly of adherent and tight junctions, and, conversely, the acquisition of fibroblastic shape and ability to invade. The EMT process characterizes physiological (i.e., organogenesis, development, wound healing, and regeneration) as well as pathological (i.e., fibrosis, tumor progression, and metastasis) processes [6].

In this review, we highlight current knowledge about cellular players and molecular mechanisms triggering PM fibrosis. In particular, we summarize the evidence supporting the involvement of EMT in this phenomenon, with emphasis on the response to signals delivered by TGF-β family members and by Toll-like/IL-1β receptors, molecules playing a main role in EMT induction in the PM.

2. Induction of Fibrosis during PD

During practice of PD, modifications of the PM occur virtually in all patients. Signs of peritoneal fibrosis are detected in 50% to 80% of patients within one to two years on PD [7]. In many cases, the peritoneal alterations are limited and result in a simple peritoneal sclerosis (SPS). SPS is characterized by increased thickness of the submesothelial space, increased angiogenesis with hyalinizing vasculopathy, and presence of denuded areas with loss of MCs. In this form, the entity of fibrosis is generally limited; it correlates with the length of exposure to PD fluid and is reversible when PD is interrupted [8]. In some cases, the patients develop encapsulating peritoneal sclerosis (EPS), which is a potentially deadly form of peritoneal fibrosis characterized by severe peritoneal thickening, inflammation, calcifications, and fibrin deposits [9]. Fibrosis may progress even if the patient switches to another form of renal replacement and may evolve in visceral encapsulation with episodes of bowel obstruction. The pathogenesis of EPS is debated: it is uncertain whether EPS evolves as a progression of SPS or whether it is a primitive form of sclerosis [10].

3. Cellular Players of Peritoneal Fibrosis

When exposed to a wide range of exogenous or endogenous inflammatory/profibrotic stimuli, both cellular components of peritoneum (MCs, macrophages, mast cells dermal fibroblasts, endothelial cells, and resident macrophages) and other elements of innate and adaptive immunity actively participate in the induction of the inflammatory response.

In the case of acute peritonitis, a first wave of neutrophils recruited by chemoattractants of bacterial origin (LPS) is progressively replaced by a population of mononuclear cells, composed of monocytes/macrophages and lymphocyte subsets [11]. In this context, IL-6 plays a main role. IL-6 soluble receptor (s-IL-6R) shed by neutrophils favors, through a process called "transsignaling," the production of chemokines, including CXCL8 and CCL2, able to recruit mononuclear cells [12]. Besides directing leukocyte recruitment to inflamed peritoneum, these chemokines directly target MCs and other components of the peritoneum, and their inhibition may limit peritoneal fibrosis [13, 14].

Both MCs and peritoneal macrophages respond to the first neutrophil wave and the secondary mononuclear cell predominance producing a wide array of inflammatory cytokines such as IL-1β, TNF-α, IL-6, and other proinflammatory mediators (chemokines, endogenous Toll-like receptor (TLR) ligands) [15, 16]. At the same time, molecules with anti-inflammatory activities, such as IL-10 and TGF-β, are released in the peritoneal cavity. Mast cells have been demonstrated to play a role in kidney fibrosis through production of tryptase and chymase [17]. Moreover, their number is increased in peritoneum, and they produce fibrogenic factors in a model of peritoneal fibrosis in rats [18].

Besides components of the innate immunity, more recent studies performed using murine models demonstrated a main role of T helper 1 (Th1) cell response and of T lymphocytes expressing IL17A [19–21]. In these conditions, the presence of IL-6 is particularly relevant since it may shape the immune response in subacute-chronic conditions. IL-6, in combination with TGF-β, is the main cytokine involved in the T helper 17/regulatory T (Th17/Treg) balance [21]. The predominance of IL-6 favors the generation of Th-17 lymphocytes, which produce inflammatory cytokines. On the other hand, TGF-β in the absence of IL-6 promotes the Treg lineage, producing the anti-inflammatory cytokine IL-10. The modulation of the expression of these cytokines through biologic antibodies or recombinant cytokines is an attracting field for the design of new therapies aimed at counteracting peritoneal EMT and fibrosis [20, 22, 23]. The presence of proinflammatory and profibrotic cytokines determines the following: (i) the aberrant production of extracellular matrix (ECM) proteins, such as Fibronectin (FN) and type I collagen (Coll) in the submesothelial stroma, (ii) an unbalanced ratio between procoagulant and anticoagulant factors (plasminogen activator inhibitor- (PAI-) 1/plasmin), and (iii) an altered production of glycosaminoglycans and proteoglycans constituting the extracellular fluid, which are responsible for the lubrication of the two peritoneal sheets (parietal and visceral) [16]. In particular, during PD, expression of high molecular weight hyaluronan and decorin is reduced, whereas low molecular weight hyaluronan and versican are induced [24, 25]. Differential expression of glycosaminoglycans and proteoglycans has pathogenic significance, since decorin may modulate the bioactivity of TGF-β1, thus directly affecting the entity of peritoneal fibrosis, whereas hyaluronan fragments have been shown to induce multiple signaling cascades, cytokine secretion, and matrix metalloproteases (MMP) activity [26].

Moreover, neoangiogenesis detected in the peritoneal stroma is due mainly to the effect of VEGF production, whose levels correlate with alterations in transport rate [27].

4. EMT of MCs as a Main Cause of Peritoneal Fibrosis

Besides contributing to production of cytokines and other soluble factors relevant to sustaining and modulating the

inflammatory reaction, MCs through EMT play a central role in the alterations of the PM leading to fibrosis. A seminal study by Yáñez-Mó and collaborators first demonstrated that EMT of MCs plays a role in the onset of fibrosis in PD patients [5]. This study was followed by others, where with the help of animal models the main characteristics of MC EMT were elucidated [23, 28–30].

Not all the features of MC EMT parallel those of epithelial cells: due to their mesodermal origin, and differently from "true" epithelia, such as hepatocytes or keratinocytes, MCs coexpress in basal conditions epithelial and mesenchymal markers. This may explain their enhanced plasticity. With respect to epithelial markers, these cells express high amount of epithelial cytokeratins, such as cytokeratin 8-18, and proteins of tight and adherens junctions, such as junctional adhesion molecule 1 (JAM1) and zonula occludens-1 (ZO-1). E-cadherin has a peculiar distribution in MCs, since it is expressed both in membrane and in cytoplasm [28]. Similarly to mesenchymal cells, MCs express constitutively the intermediate filaments vimentin and desmin [2, 5].

The exposure to inflammatory/profibrotic stimuli leads to a rapid E-cadherin downregulation, which parallels an induction of N-cadherin (cadherin switch). While E-cadherin expression is rapidly downregulated, the expression of cytokeratins is only gradually lost; thus, transdifferentiated cells can maintain for long time trace of their origin.

E-cadherin downregulation parallels the induction of Snail, a master factor of EMT, directly inhibiting the E-cadherin transcription [5]. At the same time, the expression of the specific mesothelial differentiation factor Wilms tumor 1 (WT1) is reduced [31].

While epithelial features are lost, MCs rapidly gain expression of molecules related to EMT, such as α-SMA and FSP1. Moreover, MCs produce high levels of PAI-1, which plays a role in fibrin deposits and fibrosis. Also, ECM molecules such as FN and Coll are produced, as well as metalloproteases MMP2 and MMP9, which degrade the ECM favoring MCs invasive activity [32, 33].

The expression of α-SMA and FSP1 by MCs makes them a conceivable main source of myofibroblasts, the cells endowed with ability to contract the ECM and considered mostly responsible for the abnormal production of ECM in fibrosis of all organs [34, 35]. The myofibroblast in a fibrotic organ is thought to emerge by the activation and modification of different cellular components: lineage tracing studies demonstrated that epithelia may take a role in the generation of myofibroblasts in fibrotic kidney and lung, whereas endothelium is relevant in the production of myofibroblasts in heart through a process called Endothelial to Mesenchymal Transition (EndMT) [36–38].

Myofibroblasts are absent in normal peritoneum, whereas they are found in PM of patients undergoing PD or in mice exposed to PD fluids [28, 29]. In mice exposed to PD fluid, it has been demonstrated that myofibroblasts found in the PM have different origins, including resident dermal fibroblasts, endothelial cells, bone marrow derived cells, and MCs [23]. Cells coexpressing cytokeratin (as MCs marker) and FSP1 or α-SMA (as myofibroblast markers) invade the submesothelial stroma, where they take a role in regulating mesothelial thickness, angiogenesis, leukocyte chemotaxis, and perturbation of ultrafiltration function [23, 28, 39].

Interestingly, once the EMT-inducing stimuli have been removed, transdifferentiated MCs tend to maintain their "mesenchymal" state (Strippoli, unpublished). This observation, essentially based on *in vitro* studies, deserves further analyses since EMT reversal, the phenomenon named Mesenchymal to Epithelial Transition (MET), is a mechanism of peritoneal recovery that may take place *in vivo*. During mechanical or biochemical stresses including PD, areas of PM become devoid of cells. In these conditions, floating MCs (that have suffered a "bona fide" EMT) may reattach and restore cell-to-cell contacts, undergoing MET [40]. Interestingly, these mesenchymal-like MCs may be isolated from PD fluids and cultured *in vitro*. Upon exposure to soluble factors or inhibition of specific pathways, they may partially reacquire an epithelial-like state [32, 41, 42].

5. Extracellular Inducers of Fibrosis

5.1. Factors Related to Dialysis Fluid Bioincompatibility and Uremia. Nonphysiologic characteristics of conventional PD fluid, such as hypertonicity, the presence of high concentrations of glucose and lactate, and acidic pH, are associated with production of inflammatory cytokines and other molecules. High glucose (HG) itself may induce a proinflammatory and profibrotic reaction [43]. Many lines of evidence suggest that the local injury induced by classical glucose-based PD fluids is mediated, at least in part, by the presence of glucose degradation products (GDPs) and by the acidic pH. GDPs through the formation of advanced glycation-end products (AGEs) may stimulate the production of extracellular matrix components (ECM) as well as the synthesis of profibrotic and angiogenic factors [11, 44]. Several studies have demonstrated the appearance of AGEs in the peritoneal effluents of PD patients, which correlated with the time on PD treatment. Biopsy studies have confirmed the accumulation of AGEs in the peritoneal tissues of PD patients. AGEs accumulation is associated with fibrosis and ultrafiltration dysfunction [11]. AGEs accumulate also in condition of prolonged hyperglycemia not related to PD practice, such as in patients with diabetes mellitus and during kidney diseases [45]. Uremia *per se* is sufficient for inducing fibrosis in peritoneum, which is further increased when uremic patients undergo PD [46–48]. Various uremic solutes have been characterized. Among them, indoxyl sulfate, a derivative from tryptophan, plays a role in inducing fibrosis in kidney via ROS generation and TGF-β production [49].

The use of solutions with neutral pH and with low content of GDPs may represent a potential strategy to attenuate some of the PD-related adverse effects.

5.2. Release of Bacterial Molecules and TLR Ligands. Besides factors related to bioincompatibility of PD fluid, other inflammatory stimuli are linked to events connected to catheterization, such as hemoperitoneum and peritonitis. Both Gram-positive and Gram-negative bacteria may play a role in PM injury during PD. Administration of LPS in mice

peritoneum induces production of inflammatory cytokines and chemokines and PM damage [50, 51].

Besides inducing a response mediated by Toll-like receptor (TLR) 4, LPS may take a role in the release of HMGB1, ubiquitous nonhistone nuclear protein capable of activating innate immune response through engagement of TLRs [50]. MCs may sense bacterial pathogens also through cytoplasmic Nod-like receptors, which may also induce production of inflammatory cytokines and chemokines [52]. Also, fragments of hyaluronic acid released during inflammation can induce EMT in MCs through engagement of TLRs [16, 53, 54].

5.3. TGF-β1 and Other Cytokines. Among different cytokines and inflammatory mediators elicited during peritoneal inflammation, TGF-β1 is considered the main mediator of peritoneal fibrosis. TGF-β1 belongs to a family of growth factors that includes TGF-βs, activins, and bone morphogenic proteins (BMPs) [55, 56]. Among all the members, TGF-β1 and BMP-7 are key determinant factors in peritoneal cell plasticity and, in particular, the predominance of one or the other may determine the epithelial or mesenchymal phenotype of MCs. TGF-β1 is present in fluids from patients undergoing PD and its levels correlate with deterioration of peritoneal membrane [57]. The role of TGF-β1 has been demonstrated in animal models, in which the intraperitoneal injection of adenovirus carrying TGF-β1 gene induced a peritoneal fibrosis similar to that induced upon exposure to PD fluids [29]. In a mouse model of peritoneal fibrosis, TGF-β1 blocking peptides preserved the peritoneal membrane by PD fluid induced damage [23].

The epithelial-like phenotype of MCs, together with their metastability and plasticity, is the result of a balance between constitutively secreted factors (including TGF-β1 and its "counteracting" BMP7, whose expression has been shown to interfere with fibrogenic activity of TGF-β1) and other extracellular stimuli [41, 43, 58]. In this regard, BMP7/TGF-β1 balance may be altered by other cytokines produced during the inflammatory response. For example, CTGF is produced in response to TGF-β1 and inhibits BMP7 effects [59]. Also, gremlin concentration in the peritoneal effluent correlated with measures of peritoneal membrane damage and may modulate BMP7-mediated effects [60]. HGF may stabilize the epithelial phenotype inhibiting EMT in MCs [43]. EGF which supports the epithelial state in some experimental systems, fostering EMT, and invasion in others has been recently demonstrated to promote peritoneal fibrosis through a cross talk with TGF-β mediated signals [61]. Besides these inflammatory mediators, many other cytokines that cooperate in peritoneal EMT/fibrosis induction (i.e., IL-1β, IL-6, and TNF-α, VEGF, and endothelin-1) are secreted by MCs and other cells in peritoneum [16, 62].

6. Molecular Mechanisms of EMT and Fibrosis

The complexity of proteome reprogramming occurring during EMT-MET dynamics, often involving dysregulation of specific differentiation processes, suggests the occurrence of cell-specific molecular mechanisms driving EMT and fibrosis [63]. Moreover, the molecular mechanisms driving EMT in different processes (i.e., embryogenesis or tumor) may be different even in the same cell type. In the case of the MCs, only a limited number of studies focused on the understanding of molecular mechanisms underlying EMT induction, compared to other experimental systems. Cell specificity is evident in the case of HGF, a cytokine which is generally considered a "pro-" EMT factor, whereas in MCs it has an anti-EMT activity [43, 64]. To complicate the picture, the same pathway may induce both pro-EMT and anti-EMT effects depending on the experimental conditions. This is the case of p38 MAPK, which is a main inducer of inflammatory cytokine production, thus potentially favoring EMT, but also promoting E-cadherin expression and the epithelial-like phenotype in MCs [65, 66]. The study of the role of a specific signaling pathway has been often performed using pharmacological inhibitors. Although the interpretation of the results obtained should be carefully evaluated considering the "caveat" of a possible lack of specificity, the "pharmacological approach" is especially relevant from a translational point of view, since it is possible to hypothesize the design of pharmacological treatments designed to specifically preserve or recuperate the PM homeostasis in PD patients.

6.1. TGF-β1 Induced Signaling Pathways. With TGF-β1 being the main factor controlling fibrosis in all organs, it is not a wonder whether the main signaling pathways responsible for EMT induction in MCs are induced by this cytokine. Signaling pathways induced by TGF-β1, as well as TGF-β family members, are generally divided into Smad-dependent and Smad-independent ones. TGF-β factors signal via heterodimeric serine/threonine kinase transmembrane receptor complexes. The binding of the ligand to its primary receptor (receptor type II) allows the recruitment, transphosphorylation, and activation of the signaling receptor (receptor type I). Receptor type I of TGF-β1, or activin receptor-like kinase 5 (ALK5), is then able to exert its serine-threonine kinase activity phosphorylating Smad2 and Smad3. Receptor type I of BMP-7 (ALK3) phosphorylates instead Smad1, Smad5, and Smad8. Upon phosphorylation, they form heterodimers with Smad4, a common mediator of all Smad pathways [15, 55, 56, 67]. The resulting Smad heterocomplexes translocate into the nucleus where they bind directly to DNA and activate specific target genes (Figure 1). A third group of Smads composed of Smad6 and Smad7, called also inhibitory Smads, limit BMP-7- and TGF-β1-triggered Smad signaling, respectively, by preventing the phosphorylation and/or nuclear translocation of Smad2/3 or Smad1/5/8 complexes and by inducing their degradation through the recruitment of ubiquitin ligases [55, 56, 67].

The role of Smad3 signaling in TGF-β1 induced EMT and fibrosis is demonstrated *in vivo* in Smad3 knockout mice, which are protected from peritoneal fibrosis, show reduced collagen accumulation, and display attenuated EMT [39]. On the other hand, Smad2 may play an antagonistic role in the EMT process *in vivo*. Data in peritoneum are lacking; however Smad2 deficiency increases EMT in keratinocytes and hepatocytes [68, 69]. Despite their relevance in EMT/MET induction, the transcriptional activity of Smads alone is low, compared to other transcription factors: they display their

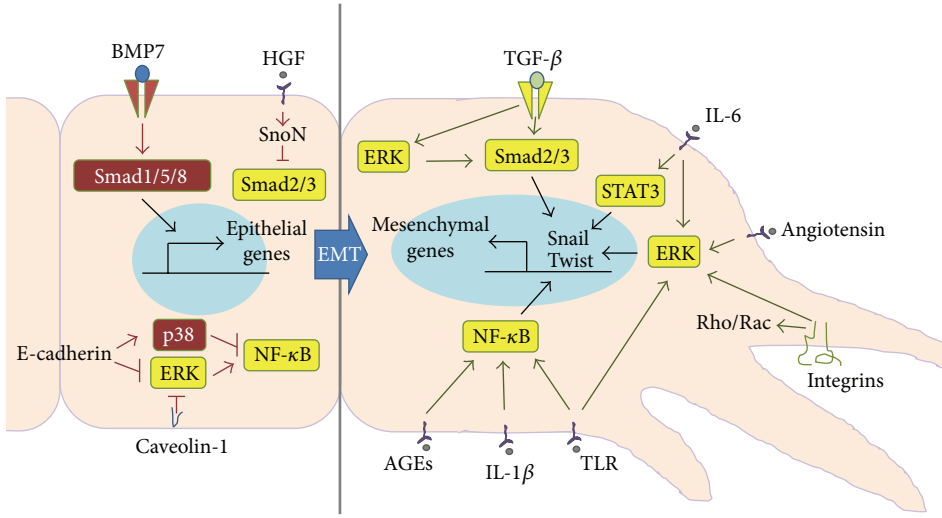

Figure 1: The epithelial/mesenchymal status of MCs is due to the balance of signals delivered by multiple receptors. Stimuli promoting EMT are delivered by TGF-β in cooperation with inflammatory cytokines and other mediators such as IL-1β, IL-6, TLR ligands, AGEs, and angiotensin. Smad2/3 pathway plays a main role in combination with ERK1/2 and NF-κB pathway and all converge on the expression of Snail, the master gene of EMT. Integrin activation promotes the induction of conformational changes and the invasivity of MCs. On the other hand, signals delivered by BMP7 and HGF favor the epithelial phenotype through the activation of Smad1/5/8 and the inhibition of the Smad2/3 signaling. Also, signals delivered by cell-to-cell confluency (E-cadherin omotypic junctions) may lead to predominance of p38 MAPK over ERK1/2 and to the inhibition of NF-κB activity. Caveolin-1 organizes signaling platforms favoring the stability of membrane receptors and inhibiting the Ras/MEK/ERK1/2 pathway.

activity when other transcription factors such as those from Snail, bHLH, or NF-κB families are present [70].

Targeting Smad signaling by inhibitory Smad7 blocks EMT and reduces peritoneal fibrotic lesions [71]. Moreover, HGF and BMP-7 display their effect of EMT inhibition limiting Smad2/3 activity in MCs (Figure 1) [43, 58].

Indeed, HGF may interfere with TGF-β1 mediated EMT inducing the expression of the transcriptional corepressor SnoN, which interacts with activated Smad2/4 complex and blocks the expression of Smad-dependent genes [72]. BMP-7 inhibitory effect on EMT is dependent on the activation of Smad1/5/8 proteins that counteract TGF-β activated Smad2/3 activity [58].

MCs constitutively express BMP-7 and display basal activation of Smad1/5/8, which contribute to the maintenance of the epithelial-like phenotype. EMT induction by TGF-β1 results in BMP-7 downregulation and inactivation of BMP-7-specific signaling [58].

TGF receptors may also activate signaling pathways independently of Smads (Figure 2) [55, 73]. Mitogen activated protein kinases (MAPKs), Rac and Rho GTPases, phosphatidyl inositol 3 kinase (PI3Kinase)/Akt pathways are relevant in different cellular function elicited by TGF-β1 in different EMT experimental systems. TGF-β induced MEK/ERK1/2 is particularly relevant in EMT and fibrosis [74]. TGF-β RI may induce ERK1/2 pathway through tyrosine phosphorylation of ShcA adaptor protein and subsequent recruitment of Grb2/Sos complex [75]. In MCs, inhibition of the MEK/ERK1/2 pathway limited EMT induced by TGF-β1 in combination with IL-1β, a cytokine mimicking an inflammatory stimulus, and induced MET in MCs from PD patients that had undergone EMT *in vivo* [32]. Moreover, pharmacological inhibition of MEK/ERK1/2 pathway rescued E-cadherin and ZO-1 altered expression, reduced fibrosis, and restored peritoneal function in mice exposed to PD fluids [28].

Interestingly, TGFβ induced MEK/ERK1/2 pathways may alternatively enhance or limit Smad activities.

ERK1/2 may phosphorylate R-Smads in their linker region, thus inhibiting nuclear translocation and transcriptional activity [76]. More recently, it has been observed that ERK1/2 phosphorylation of the linker region of nuclear localized Smads resulted in increased half-life of C-terminal Smad2 and Smad3 phosphorylation and increased duration of Smad target gene transcription [77]. MEK/ERK1/2 pharmacological inhibition in MCs reduced Smad3 activity in luciferase assays, which correlated with reduced C-terminus Smad3 phosphorylation. Interestingly, in the same conditions Smad1/5 luciferase activity was increased, with increased C-terminus phosphorylation [28]. The intensity of MEK/ERK1/2 response can be modulated by intracellular factors. Caveolin-1, the principal marker of caveolae, plasma membrane specialized structures, limits the intensity of the EMT response through an effect on TGF-RI internalization or a direct effect on Ras/MEK pathway [28] (Figure 1).

TGF-β1 may induce p38 and JNK MAPK activation pathway through activation of TAK1 (TGF-β activated protein) [78]. Besides being a main driver of inflammation, p38 MAPK plays a role in the control of cell differentiation and apoptosis [65].

p38 is stably activated in quiescent MCs and, differently from ERK1/2, its activation levels are increased in conditions

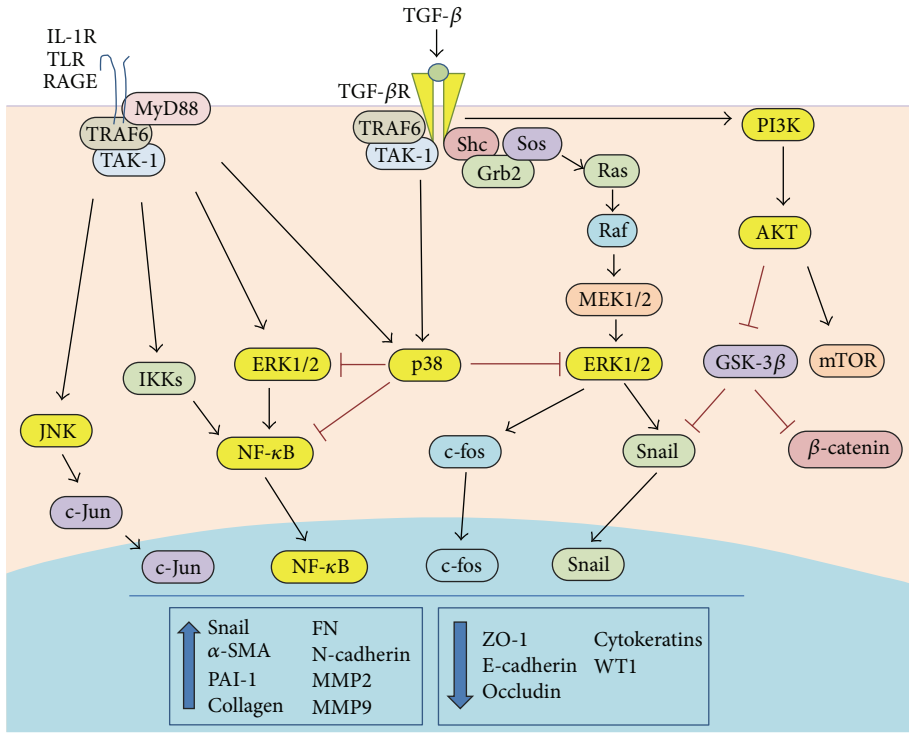

Figure 2: Cooperation between signals delivered by TGF-β and Toll-like/IL-1β receptors in the EMT of MCs. TGF-β delivers pro-EMT signals inducing the Smad2/3 (not described in this figure) and the non-Smad pathways, composed of MEK/ERK1/2 and PI3K pathways. IL-1β and TLR ligands activate redundant pathways leading to activation of NF-κB and ERK1/2. Also, pathways able to limit EMT induction, such as p38, are induced at the same time. Smad2/3 acted as transcription factor in combination with Snail, NF-κB, and AP-1 to induce the EMT program.

of cellular confluency in MCs [66]. p38 activity maintains E-cadherin expression in MCs and p38-mediated pathway modulates the mesenchymal conversion of MCs by a feedback mechanism based on the downregulation of ERK1/2, TAK-1/NF-κB activities (Figure 1) [66]. JNK inhibition leads to the maintenance of E-cadherin expression and block of EMT, similarly to ERK1/2 inhibition [66, 79].

Besides p38 and JNK, TAK-1 is an activator of NF-κB. NF-κB inhibition may limit EMT-related events in MCs [32]. Having a wide effect on TGF-β1 induced pathways, it is not surprising that TAK-1 inhibition may induce EMT reversal in MCs from PD patients [42].

Among the non-Smad mechanisms involved in EMT, also PI3K/Akt pathway has been extensively studied [80]. PI3K activates Akt through phosphorylation at serine 473. Once activated, Akt has multiple actions including the activation of mammalian TOR complex 1 (mTORC1) and mTORC2 [80]. Both complexes are involved in different aspects of EMT and invasion and are sensible to prolonged treatment with rapamycin [81]. On the other hand, mTORC2 phosphorylates and activates Akt. Treatment with rapamycin abrogated transition response, such as induction of α-SMA expression, in Smad3 deficient mice [39]. Moreover, it induced stabilization of β-catenin, another factor implicated in EMT induction [80]. Interestingly, rapamycin inhibited in the same experimental system hypoxia-induced VEGF expression and angiogenesis [82].

Both Smad and non-Smad pathways converge on activation of Snail, the master factor of EMT. Snail is a direct inhibitor of E-cadherin expression [83]. Moreover, Snail inhibits the expression of other proteins associated with cell junctions, such as claudins and occludin, with knock-on effects on the expression of other proteins such as metalloproteinases, integrins, and ECM proteins [84]. Besides Snail, also Slug (Snail2), ZEB1-2, and members of the basic helix-loop-helix (bHLH) family, such as Twist and E47, play a role in repressing E-cadherin expression and inducing EMT. They often have some tissue specificity but similar mechanism of action [85]. Snail is strongly induced in MCs upon treatment with TGF-β1. Immunofluorescence analyses show that Snail has a distribution mainly nuclear: this suggests that mechanisms favoring cytoplasmic accumulation are probably inactive in MCs [28]. Inhibition of Smad3, MEK/ERK1/2, and NF-κB results in reduced Snail expression in MCs [32, 39]. Compared to Snail, Slug is faintly induced in MCs treated with TGF-β1, whereas Twist is not induced by the same stimulus (Strippoli, unpublished). Interestingly, p38 inhibition parallels Snail inhibition in MCs, whereas induction of Twist is observed [66].

6.2. TLR Ligands Induced Signaling Pathways. During inflammatory EMT, signals elicited by mediators of inflammation cooperate with pathways elicited by activation of TGF-β (Figure 2). This is particularly relevant in the

case of peritoneum, which may undergo episodes of acute inflammation as in the case of bacterial peritonitis due to catheter implantation. In the case of PD dysfunction induced by high glucose or the presence of AGEs, peritoneum undergoes subacute inflammatory alterations, but the molecular mechanisms of damage are often similar.

Human MCs express ligands for both Gram-positive and Gram-negative TLR ligands, such as TLR1, TLR2, and TRL5, but not TLR4 [86]. TLRs have a cytoplasmic signaling domain homologous to that of IL-1 receptor (IL-1R), called the Toll/IL-1R domain [87].

Ligand binding to Toll-like receptor (TLR)/IL-1R family members results in the association of MyD88 with the cytoplasmic tail of receptors; this then initiates the signaling cascade that leads to the activation of NF-κB and MAPKs [87]. Besides IL-1 and TLR ligands, also signaling from AGEs to their receptors RAGEs converges on NF-κB and MAPK pathways (Figure 2) [88]. Moreover, signals delivered by IL-1/TLR ligands affect signaling from other pathways relevant in EMT induction, such as IL-6. IL-1β induces IL-6, and this cytokine may amplify IL-1 response in macrophages and synovial fibroblasts [89].

In MCs, IL-1β is a much stronger inducer of NF-κB response than TGF-β1, and their costimulation generates an additive response. Inhibition of NF-κB blocks EMT induction upon TGF-β1/IL-1β costimulation and partially reverses *in vivo* EMT in MCs from PD patients [32]. In the same cells, NF-κB nuclear translocation and transcriptional activity is enhanced by MEK-ERK1/2 pathway and is inhibited by p38 [66]. NF-κB controls Snail and Twist expression and cooperates with Snail in inducing FN transcription [32, 90, 91]. Moreover, NF-κB is a transcriptional inducer of cyclooxygenase-2 (COX-2), a main mediator of inflammation [88]. Inhibition of COX-2 with celecoxib resulted in reduced fibrosis and in partial recovery of ultrafiltration in mice exposed to PD fluids [92]. Interestingly, Twist is increased in MCs exposed to high glucose (probably due to NF-κB activation) *in vitro* and in the PM of mice exposed to high glucose PD fluids, and it is linked to MMP9 to MMP9 production and MCs invasion [33].

Overall, it is not surprising that inhibition of NF-κB and ERK1/2 pathways leads to block and reversal of EMT.

6.3. Noncoding RNA. The discovery of noncoding RNA unveiled a new layer of regulation of cellular function. Noncoding-miRNAs selectively bind mRNA, thus inhibiting their translation or promoting their degradation. Accumulating evidence shows that miRNAs regulate diverse biological processes, including cell proliferation, differentiation, and apoptosis [93, 94]. Recent studies have defined a large number of miRNAs associated with EMT and controlling the expression of EMT master transcription factors, suggesting a possible role also in peritoneal fibrosis.

In particular, miR29b and miR30A repress Snail1 expression [95, 96]. Moreover, members of the miR-200 family and miR-205 repress the translation of ZEB1 and ZEB2 miRNA. Notably, ZEB proteins repress the expression of miR-200 [97]. Interestingly, in hepatocyte cellular models Snail directly represses the miR-200c expression [98].

Studies on the role of noncoding RNA have been conducted mainly on experimental models of tumor EMT. Considering nontumoral experimental setting, studies have been performed mainly on experimental models of kidney and lung fibrosis. With regard to chronic progressive kidney disease, the roles of miR-21, miR-29, and miR-200 have been best established [99]. A mouse model of pulmonary fibrosis identified miR-31 as a direct modulator of integrin α5 and RhoA, proteins involved in migration and ECM deposition [100].

On the other hand, only a few data have accumulated so far on EMT/fibrosis of the peritoneum. PD-related peritoneal fibrosis is associated with a loss of miR-29b, and intraperitoneal delivering of plasmid expressing this miRNA in mice inhibited peritoneal fibrosis through an effect on TGF-β/Smad3 pathway [101]. Interestingly, expression of different miRNAs including miR-15a, miR-17, miR-21, miR-30, miR-192, and miR-377 from dialysis effluent correlated with peritoneal transport alterations in PD patients, suggesting a role of miRNA in PM damage [102]. In another study performed using MCs from effluent of patients undergoing PD, miRNA200c levels were found reduced in MC from PD patients [103]. A negative feedback mechanism involving TGF-β, miR-9-5p, NADPH oxidase 4 (NOX4), and playing a role in fibrosis of the mesothelial membrane has been recently described [104].

Besides miRNA, other noncoding RNAs (ncRNA) are abundantly transcribed in all cell types. Long noncoding RNAs can exert their effects on biological processes through a variety of mechanisms and can be involved in the pathophysiology of several diseases, including cancer and pulmonary fibrosis [105, 106]. Concerning peritoneal fibrosis, it has been recently reported that three lncRNAs target distinct mRNAs (Dok2, Ier3, HSP72, Junb, and Nedd9) involved in tissue inflammation and fibrosis [107]. Overall, the role of lncRNA in MCs EMT deserves future studies.

7. Conclusions

In the last years, the decrease in incidence rate of catheter complications coupled to the increased biocompatibility of dialysis solutions reduced the progressive damage to the PM during peritoneal dialysis. However, the incidence of peritoneal membrane problems remains high. To this purpose, current challenges are both the discovery of biomarkers (that could allow constantly monitoring the state of PM) and the understanding of molecular events underlying peritoneal damage in order to preserve or restore a peritoneal function. Thus, the study of molecular mechanisms involved in peritoneal fibrosis has both a basic and a translational relevance, appearing essential for the setting of more efficient therapies. Furthermore, it may conceivably be relevant in the possible treatment of other pathological conditions involving peritoneal fibrosis, such as postsurgical adhesions and peritoneal fibrosis induced by drugs, and peritoneal metastases [49, 108].

More efforts are needed to better elucidate the MCs molecular response to inflammatory/fibrogenic signals. Inhibition of main extracellular mediators as well as of specific players in the cascade of events triggered by TGF-β and by

TLR/IL-1β could represent possible drugs that can simultaneously affect multiple target genes. Moreover, the possible control of the levels of particular ncRNAs, for example, by simple antagomiRs approaches, could conceivably guarantee the specific regulation of gene expression for more targeted therapies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Endothelial Plasticity: Shifting Phenotypes through Force Feedback

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The endothelial lining of the vasculature is exposed to a large variety of biochemical and hemodynamic stimuli with different gradients throughout the vascular network. Adequate adaptation requires endothelial cells to be highly plastic, which is reflected by the remarkable heterogeneity of endothelial cells in tissues and organs. Hemodynamic forces such as fluid shear stress and cyclic strain are strong modulators of the endothelial phenotype and function. Although endothelial plasticity is essential during development and adult physiology, proatherogenic stimuli can induce adverse plasticity which contributes to disease. Endothelial-to-mesenchymal transition (EndMT), the hallmark of endothelial plasticity, was long thought to be restricted to embryonic development but has emerged as a pathologic process in a plethora of diseases. In this perspective we argue how shear stress and cyclic strain can modulate EndMT and discuss how this is reflected in atherosclerosis and pulmonary arterial hypertension.

1. Introduction

Mechanical forces, laminar shear stress (LSS), and cyclic strain (CS) are two major and well-established regulators of vascular development and adaptation (reviewed in [1–3]). Vasculogenesis, which marks the onset of embryonic vascularization, is driven by hypoxia and initiated by the differentiation of endothelial cells from mesodermal angioblasts [4]. Herein, hypoxia inducible factor 1 alpha (HIF1α) induces the expression of vascular endothelial growth factor (VEGFa) which results in endothelial differentiation and proliferation. The endothelial cells derived from the blood islands form a vascular network known as the primitive capillary plexus [5]. This plexus is highly uniform without a clear vascular hierarchy.

With the interconnection of the vascular plexus and major vessels a functional loop is formed and, as the heart begins to beat, the physical flow of fluid is introduced [6]. Arteriogenesis ensues the remodeling of the vessels to accommodate the physical forces associated with the increased pressures and flow. The pulsatile nature of the blood flow generated by the beating heart produces a complex interplay between two distinct mechanical forces, namely, fluid shear stress (FSS), the frictional force exerted by the flow of blood, and cyclic strain (CS), which results from the distensibility of the vessel wall caused by pulsations and increased hydrostatic pressures. Adaptation to these forces, known as angioadaptation, results in a hierarchical vascular tree composed of arteries, arterioles, capillaries, venules, and veins [7].

In the adult vasculature, hemodynamic forces play a major role in the maintenance of vessel homeostasis [8]. The most important intimation for a role of hemodynamic forces

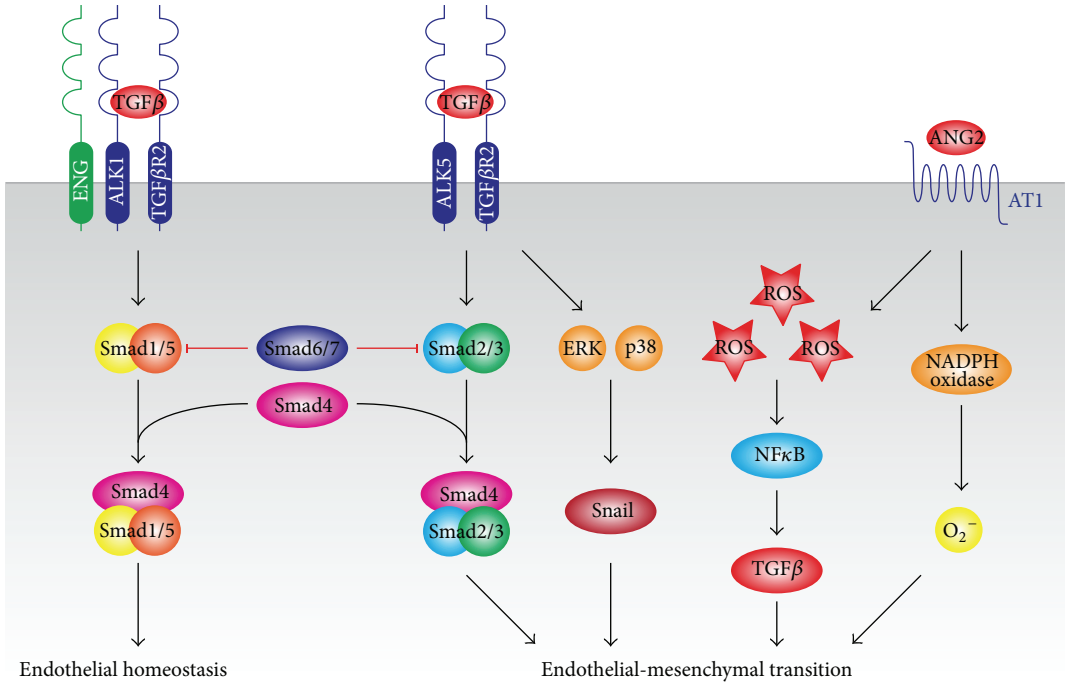

Figure 1: Mechanisms of endothelial-mesenchymal transition. EndMT is induced by TGFβ-dependent and TGFβ-independent mechanisms. Canonically, TGFβ-induced activation of SMAD2/3 induces the expression of mesenchymal genes and repression of endothelial genes. Noncanonically, TGFβ-induced activation of Erk1/2 and p38 MAPK activate the transcription factor Snail which induces mesenchymal differentiation and inhibits VE-Cadherin expression. Inflammatory signals and increased ROS production facilitate EndMT by increasing endogenous TGFβ expression, in an NFκB-dependent manner, creating a feed forward signaling mechanism. AT1 receptor can induce EndMT through activation of NADPH oxidase, resulting in increased ROS production and reduction of eNOS expression and activity.

in vascular homeostasis is found in the focal nature of most vascular diseases. For example, despite the fact that most risk factors for atherosclerosis development and progression are present at the systemic level, this inflammatory arterial disease preferentially develops in regions which are characterized by disturbed hemodynamic flow, typically encountered at the outer walls of vascular bifurcations and at the inner wall of vascular curvatures [9, 10]. Indeed, disturbances of fluid shear stress levels encountered at these locations are predictors of plaque location [11], associate with the increased mass transport of low-density lipoproteins (LDL) which build up the plaques [12], and are major determinants of plaque transition from stable plaques to high-risk unstable plaques and plaque rupture [13].

In recent years, it has become apparent that the endothelium plays a pivotal role in the development and progression of cardiovascular diseases [14–16]. The endothelial lining of the vasculature is exposed to a large variety of stimuli which require the endothelium to be highly plastic [17], as is reflected by the remarkable heterogeneity of endothelial cells in tissues and organs. Although this endothelial plasticity is essential during homeostasis, during disease, pathological stimuli might induce adverse plasticity which can contribute to disease. Herein, endothelial-mesenchymal transition (EndMT) and its contribution to neointima formation [18–21] is a perfect example.

In this perspective, we focus on how hemodynamic forces, that is, shear stress and cyclic strain, are sensed by the endothelium, how these forces modulate EndMT, and how this is reflected in vascular disease.

2. Endothelial-to-Mesenchymal Transition: The Hallmark of Endothelial Plasticity

Endothelial-to-mesenchymal transition (EndMT) was originally described as an embryonic phenomenon involved in cardiac valve formation [22]. EndMT has mainly been studied *in vitro*, where it is characterized by the loss of cell-cell adhesions and changes in cell polarity, resulting in a spindle-shaped morphology. Endothelial cell markers such as VE-Cadherin and PECAM-1 are repressed, whilst the expression of mesenchymal cell markers such as α-smooth muscle actin (α-SMA) and calponin is enhanced [23–26]. Functionally, endothelial cells acquire myofibroblast-like characteristics with contractile function, enhanced migratory phenotype, and increased extracellular matrix production [24]. In EndMT, endothelial functions, such as antithrombogenicity and angiogenic sprouting capacity, are lost [23, 24].

Through extensive studies on EndMT *in vitro*, we have gained in-depth knowledge of the signaling cascades that regulate EndMT (Figure 1). Many signaling pathways are involved in the induction and progression of EndMT, wherein the transforming growth factor β (TGFβ)/bone morphogenetic protein (BMP) superfamily plays a pivotal role.

In endothelial cells, canonical TGFβ signaling occurs through the type II TGFβ receptor (TGFβR2) which activates

the type I TGFβ receptor Activin-like kinase (ALK) 5, activating receptor-regulated Smad2/3, and leads to inhibition of cell proliferation and induction of EndMT [27–30]. In contrast, TGFβ and bone morphogenetic proteins (BMP) can also bind the BMP type I receptor, ALK1, which activates receptor-regulated Smad1/5/8 and induces proliferation and inhibits EndMT [27–29]. The common-mediator Smad4 interacts with the receptor-regulated Smads and is required for signaling. The inhibitory Smads 6 and 7 block activation of the receptor-regulated Smads and thus inhibit TGFβ signaling. The balance between ALK1 and ALK5 signaling pathways is partially regulated by an accessory type III TGFβ receptor called endoglin, which stimulates ALK1-induced Smad1/5/8 responses and thus indirectly inhibits ALK5 signaling [29].

Besides canonical TGFβ signaling, TGFβ can induce EndMT noncanonically through the activation of the ERK1/2 and p38 MAPK signaling pathways and the downstream induction of the mesenchymal transcription factor Snail [31, 32] (Figure 1). Furthermore, Notch is known to promote TGFβ-induced EndMT shifting the balance between different ALK1 and ALK5 signaling pathways in favor of ALK5 [33, 34].

Other signaling mechanisms can also induce EndMT, albeit often indirectly involving TGFβ signaling. Proinflammatory molecules, such as IL1β and TNFα, synergize with TGFβ in the induction of EndMT [35–37]. Besides, IL1β and TNFα induce the expression of Snail and Slug, two pivotal transcription factors in EndMT [38–40]. Reactive oxygen species (ROS) are potential stimulators of EndMT (Figure 1) by inducing endogenous TGFβ expression and activating latent TGFβ [41]. TGFβ also stimulates ROS production, resulting in a positive feedback loop [41]. Besides, ROS activates NFκB signaling which acts in synergy with TGFβ in induction of EndMT [36, 42].

The AT1 receptor (angiotensin II receptor type I), a member of the G-protein coupled receptor family, mediates EndMT of human aortic endothelial cells *in vitro* [43] as well as in cardiac endothelial cells *in vivo* [44, 45]. Angiotensin II-induced EndMT can be mediated both by release of cytokines such as TGFβ or through changes in the redox status of EC by the activation of NADPH oxidase system [45] which leads to higher superoxide production, NFκB activation [42], and lower NO bioavailability through uncoupling eNOS [46, 47] (Figure 1).

In summary, TGFβ signaling plays a dominant role as inducer of EndMT directly via both canonical and noncanonical signaling pathways. In recent years, it has become evident that EndMT is not restricted to embryogenesis but can also occur in adult life, where it contributes to organ and muscle fibrogenesis [48–52], cancer [53], and atherosclerosis [18–20]. In the following section, we argue how hemodynamic forces can modulate EndMT and how these mechanisms are reflected by vascular disease.

3. Hemodynamic Forces as Modulators of EndMT

3.1. Go with the Flow: Laminar Shear Stress Is a Hemodynamic Force Transduced by the Endothelium. Hemodynamic forces play a pivotal function in the maintenance of the vascular integrity. It is well established that high uniform laminar shear stress (LSS) has atheroprotective effects [54], which is evidenced by the occurrence of endothelial dysfunction [55], aneurysms [56], and atherosclerosis [57–59] at sites where LSS is reduced or worse, absent or disturbed.

A variety of signaling cascades are involved in endothelial mechanotransduction of LSS; however, identifying which pathways are primary or secondary to LSS sensing remains elusive as the initial mechanosensory complexes are not completely identified.

Endothelial cells sense LSS through a number of mechanisms including the endothelial glycocalyx [60–62], growth factor receptors [63, 64], cell-cell adhesion molecules [65, 66], integrins [67], and G-protein-coupled receptors [68–70] (Figure 2). The endothelium is covered by a slimy layer of plasma proteins and glycoproteins, known as the glycocalyx, which is an organized hydrated mesh of negatively charged membranous macromolecules, proteoglycans, and glycosaminoglycans [71]. The proteoglycan core proteins are the membrane-bound glypican and the transmembrane syndecans. Syndecans directly associate with the cytoskeleton [72] and may thus directly transmit mechanical stresses to the nucleus or remote mechanotransducers [73, 74] (Figure 2). Indeed, disruption of the endothelial glycocalyx either genetically or by pharmacological inhibitors of glycocalyx producing enzymes renders the endothelium shear insensitive [60–62]. Although insights into glycocalyx signaling remain elusive, syndecans and glypicans are known to associate with the β1 integrins and mediate the activity of focal adhesion kinase (FAK) through protein kinase C-alpha (PKCα) [61, 62, 67, 73], resulting in the activation of Akt, Rho, and the endothelial nitric oxide synthase (eNOS). Combined, glycocalyx signaling enhances endothelial survival and maintenance of the endothelial barrier (Figure 2).

The protein complex consisting of PECAM-1, VEGFR2, and VE-Cadherin is a junctional mechanosensory complex that transduces hemodynamic forces into biochemical responses [75]. In this mechanosensory complex, VE-Cadherin functions as an initial adaptor which initiates the formation of the signaling complex, whereas PECAM-1 can directly transduce mechanical forces to the cytoskeleton [65]. VEGFR2, in association with VEGFa, initiates bidirectional activation of PI3K and MEKK3 which cause the downstream activation of Akt and MEK5/Erk5 signaling (Figure 2). Collectively, signaling through this junctional complex results in the activation of a number of transcription factors [63, 64] described below and loss of any one of its components ameliorates the endothelial response to LSS [2].

Additionally, the membrane bound G-protein-coupled receptors of type $G\alpha_q/G\alpha_{11}$ transmit mechanical forces in part through the activation of PI3K and intracellular calcium signaling [76–78]. Activation of $G\alpha_q/G\alpha_{11}$ proteins results in the activation of Akt and increased activation of eNOS (Figure 2).

Thus, the endothelium is highly sensitive to laminar shear stress and endothelial mechanosensing and transduction is achieved through a variety of cell-matrix and cell-cell receptor complexes. Endothelial mechanotransduction by

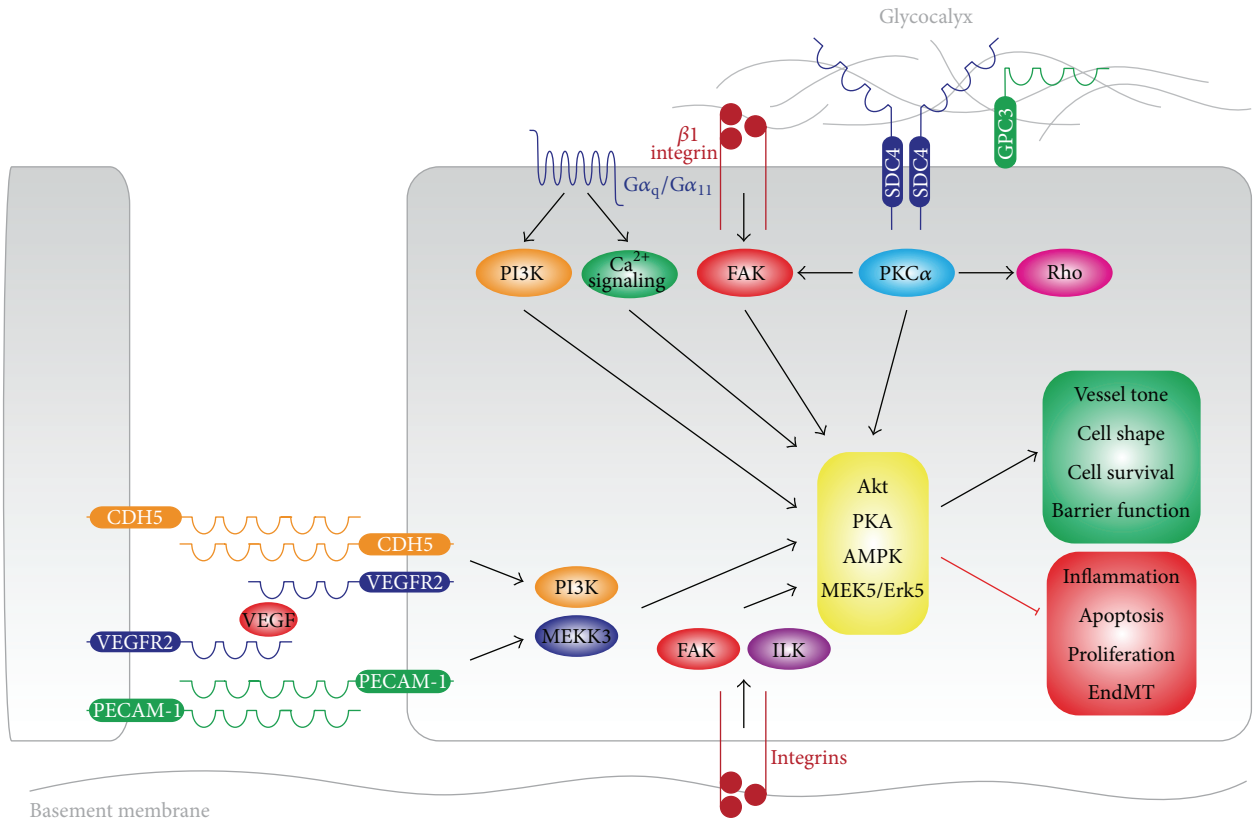

FIGURE 2: Endothelial shear stress sensing. Endothelial cells sense LSS through a number of mechanisms. Shear stress sensing through the endothelial glycocalyx is mediated by the syndecans and glypicans, which activate PKC signaling. Luminal β1-integrins also anchor to the endothelial glycocalyx and activate focal adhesion kinase (FAK). The G-protein-coupled receptors of $G\alpha_q/G\alpha_{11}$ sense shear stress and activate downstream PI3K and Ca^{2+} signaling. The junctional mechanosensory complex consisting of PECAM-1, VEGFR2, and VE-Cadherin mediates PI3K and MAPK (MEKK3) signaling. Signaling through these mechanosensors culminates in activation of Akt, PKA, AMPK, and MEK5/Erk5 which collectively maintain the endothelial phenotype.

these protein complexes culminates in the activation of the Akt, PKA, AMPK, and MEK5/Erk5 signaling cascades that ensure the maintenance of endothelial homeostasis and inhibit EndMT (Figure 2).

3.2. The Atheroprotective Effects of Laminar Shear Stress and the Inhibition of EndMT. It is well established that the extracellular signal-related kinase (Erk) 5, also known as MAPK7 and big-mitogen kinase-1 (BMK-1), has atheroprotective effects [54, 79, 80]. Erk5 is the only mitogen-activated protein kinase (MAPK) which is continuously activated by uniform LSS *in vitro* [18, 81] and several lines of evidence suggest that Erk5 transmits its atheroprotective effects through the activation of the transcription factors of the Krüppel-like factor (KLF) family [18, 82–84].

First, Krüppel-like factor 2 (KLF2) is an important shear stress-activated transcription factor, which exerts anti-inflammatory effects through inhibition of nuclear factor kappa B (NFκB) activation [85] and anticoagulant effects by induction of thrombomodulin and repression of tissue factor and plasminogen activator inhibitor (PAI-1) [86]. Besides, KLF2 regulates antioxidative signaling [87], induces quiescence [88], and inhibits VEGFa-induced angiogenesis, barrier disruption, and cell proliferation [89].

Second, KLF2 inhibits the phosphorylation and nuclear translocation of Smad2 through induction of inhibitory Smad7 [90]. Moreover, KLF2 also suppresses the activator protein 1 (AP-1), an important cofactor for TGFβ-dependent transcription [90]. Thus, KLF2 might directly inhibit EndMT by suppressing TGFβ signaling.

The expression of KLF4, a close family member of KLF2, is similarly induced upon exposure to LSS [91, 92]. Significant conservation exists between KLF2 and KLF4 [82, 93]. In fact, almost 60% of MEK5 regulated genes are coregulated by KLF2 and KLF4 [93]. Similar to KLF2, KLF4 exerts anti-inflammatory effects through suppression of NFκB activity [94]. KLF4-specific targets include forkhead box O1 (FOXO1) and vascular endothelial growth factor (VEGF) [93], both of which are known to inhibit TGFβ signaling [48, 95].

Third, KLF4 plays an important role in cell-cycle regulation and differentiation. KLF4 increases the expression of several inhibitors of proliferation, while genes that promote proliferation are repressed [88, 96, 97]. As such, KLF4 might partially be responsible for the quiescence observed in endothelial cells exposed to high levels of uniform LSS.

Fourth, KLF4 is known to suppress mesenchymal differentiation through several mechanisms. KLF4 inhibits myocardin expression, a potent coactivator of serum

response factor (SRF) [98, 99], and prevents SRF/myocardin from associating with mesenchymal gene promoters [100]. KLF4 can also directly bind the TGFβ control element (TCE) in the promoter of mesenchymal genes, preventing transcription [101]. Furthermore, KLF4 interacts with Smad3, inhibiting its binding to Smad binding elements (SBE) and preventing transcription of mesenchymal genes [102]. Interestingly, KLF4 is also known to transcriptionally regulate VE-Cadherin [103]. Thus KLF4 might inhibit EndMT via multiple mechanisms.

Last, KLF2 and KLF4 can inhibit EndMT through the inhibition of ROS formation by eNOS-induced stimulation of superoxide dismutase (SOD), scavenging of ROS through NO production [104, 105], and facilitation of mitochondrial biogenesis [106].

In addition to the atheroprotective effects mediated by the KLFs, LSS induces the expression of inhibitor of DNA binding/inhibitor of differentiation proteins 1–3 (Id1–3) [107]. These dominant-negative regulators of basic helix-loop-helix DNA-binding transcriptional regulators play a role in lineage commitment, cell cycle control, and cell differentiation [108]. Id2 and Id3 repress Smad2-mediated gene transcription [109] and ectopic expression of Id2 and Id3 inhibits EMT [110]. Besides, Id3 is downregulated during EndMT [24] and Id1 has been shown to promote survival of endothelial cells and preserve the integrity of lung microvascular endothelial cells [111].

In summary, LSS plays a major role in endothelial homeostasis, wherein mechanical stimuli are transduced into biochemical signals which culminate in the expression of the KLF transcription factors. KLF2 and KLF4 govern endothelial thrombogenicity, inflammatory phenotype, permeability, proliferation, and redox state through a variety of mechanisms. In addition, the KLF transcription factors inhibit mesenchymal gene expression. Hence, the LSS-induced expression of the KLF transcription factors is pivotal in the inhibition of EndMT.

3.3. Disturbed Shear Stress: Inducer or Permitter of EndMT? In contrast to the beneficial effects of LSS, oscillatory or nonuniform shear stress, further referred to as disturbed shear stress (DSS), has opposing effects on endothelial homeostasis; that is, DSS causes EC dysfunction. DSS is known to induce endothelial inflammatory activation [112–114]. DSS reduces the endothelial antithrombogenicity and increases the formation of reactive oxygen species (ROS), which further adds to the inflammatory activation and profibrotic signaling. During arteriogenesis, the screw-like curvatures of the collaterals convert LSS to DSS, which stimulates endothelial proliferation and MCP-1 activation, resulting in the recruitment of monocytes that are essential for remodeling of the vascular structure [115]. These mechanisms are reiterated during vascular pathology. Areas exposed to DSS are characterized by a high influx of leukocytes, which generates a microenvironment with excessive ROS production, high levels of TGFβ, and inflammatory mediators such as IL1β, which all favor EndMT [36].

DSS induces ROS formation through several mechanisms. DSS leads to increased nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity (Figure 4) which results in ROS production [116]. NADPH oxidase activity can induce xanthine oxidoreductase (XO), an important source of ROS [117]. Next, expression of NOX4, a subunit of vascular NADPH oxidase, is increased in response to DSS resulting in increased LDL oxidation [118]. Adding to this, intracellular glutathione, a powerful antioxidant, is greatly decreased by DSS [119].

DSS can also induce EndMT through direct activation of TGFβ signaling (Figure 4). BMP4 is a potent inducer of EndMT [48], expression of which is inhibited by LSS [120]. In contrast, exposure to DSS induces BMP4 [121] expression in endothelial cells and contributes to ROS production and NFκB activation [122]. Additionally, DSS induces TGFβ expression and activation in an NFκB-dependent manner [123].

Taken together, high LSS can inhibit EndMT via multiple mechanisms, either directly or indirectly interfering with TGFβ signaling. DSS suppresses these protective mechanisms making endothelial cells more prone to microenvironmental cues which favor EndMT. Besides, DSS can induce EndMT directly through induction of BMP4 or via increased ROS production. Indeed, exposing aortic endothelial cells (which normally are exposed to LSS) to DSS by aortic banding efficiently induces EndMT *in vivo* in the absence of other stimuli [18]. Therefore, DSS acts both as a permitter and inducer of EndMT by either suppressing protective signaling or by directly inducing the transition process.

3.4. Cyclic Strain. Cyclic strain (CS) is defined as the repetitive mechanical deformation of the vessel during the cardiac cycle. Vascular CS can vary from 2 to 20% *in vivo* as a result of arterial wall expansion and contraction in response to pulsatile pressure changes [124]. CS plays an important role in the modulation of cell proliferation, migration, apoptosis, morphological changes, and alignment through the production of vasoactive substances such as nitric oxide (NO) [125], endothelin (ET-1), [126] and antioxidants [127, 128].

Physiologically, the level of CS is around 6–10% but this strongly varies throughout the vasculature. CS can increase with hypertension and artificial pulmonary ventilation (10% to 20%) and decrease with ageing due to vascular stiffening or acutely due to sepsis (2%–6%) [129]. CS levels up to 10% do not induce endothelial injury [130] but inhibit endothelial apoptosis through activation of PI3-kinase and Akt. In contrast, CS levels of over 15% induce apoptosis [131].

Endothelial cells sense CS through a number of mechanisms involving stretch-activated ion channels, integrins, and G-protein-coupled receptors. Stretch-activated ion channels play a pivotal role in CS sensing. Stretching of endothelial cells opens the ion channels allowing extracellular Ca^{2+} to enter the cell, thereby activating downstream phospholipase C (PLC) signaling [132] (Figure 3). Additionally, the cell-matrix adhesion families of integrins mediate CS signaling in endothelial cells by the activation of focal adhesion kinase (FAK) and integrin-linked kinase (ILK) complexes that result

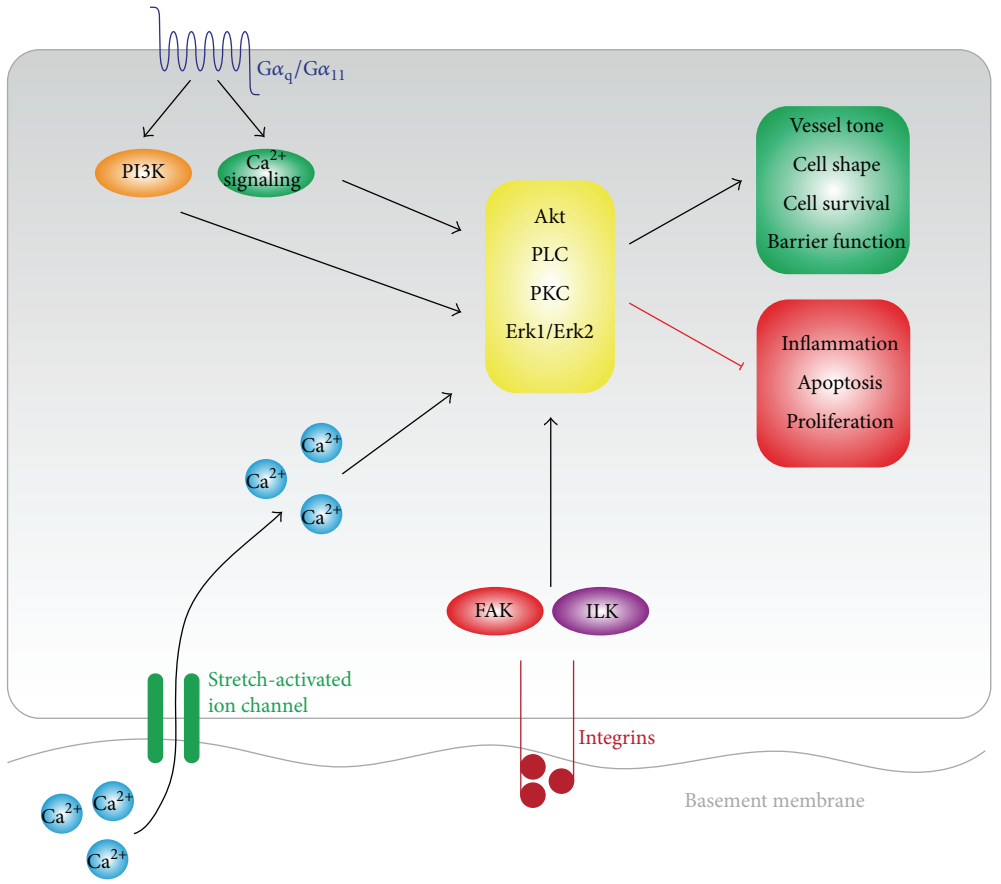

Figure 3: Endothelial cyclic strain sensing. Endothelial cells sense CS through a number of mechanisms. Stretch-sensitive ion channels undergo a conformational change during stretch which opens up the channel and induces Ca^{2+} flux from the extracellular space into the endothelial cell. Basal β1-integrins anchor to the endothelial basement membrane and activate focal adhesion kinase (FAK) and the integrin-linked kinases (ILK). The G-protein-coupled receptors of $G\alpha_q/G\alpha_{11}$ sense CS and activate downstream PI3K and Ca^{2+} signaling. Signaling through these stretch sensors culminates in activation of Akt, PKC, PLC, and Erk1/2 which collectively maintain the endothelial phenotype.

in the downstream activation of the small GTPase family Rho, PI3K, and Akt (Figure 3). Combined, these signals result in changes in endothelial morphology and the regulation of cell survival and cytokine production. However, to what extent CS induces these effects and the relation between the magnitude of CS and the endothelial integrin-mediated response remain elusive [133, 134]. The heterotrimeric $G\alpha_q/\alpha_{11}$ subunit of the G protein family is rapidly activated by CS and the intensity is related to the magnitude of the strain applied [135]. AT1 receptors bind and signal through all members of the G-protein family and are activated by mechanical forces *in vitro* and *in vivo*, independently of angiotensin II [136, 137].

Stimulation of the CS sensors causes downstream activation of small GTPases that are essential for the biochemical transduction of the mechanical stimulus. The most extensively characterized members are Rho, Rac, and Cdc42, which have distinct effects on actin cytoskeleton, cell adhesion, and cell motility [138]. Rho kinase induces assembly of stress fibers and focal adhesions and Rac plays an important role in junction formation and integrity of the endothelial barrier [139]. The activity of the Rho pathway determines the direction and extent of stretch-induced stress fiber orientation in endothelial cells. This demonstrates on one hand that physically stressing a cell determines Rho activity (in a linear fashion) and that Rho activation directly correlates with physical shape shifting of cells through cytoskeletal reorganization [140, 141]. Also, preconditioning of endothelial cells at 18% CS enhanced the effects of prothrombotic stimuli to induce permeability of the endothelial monolayer, while 5% CS prevents thrombin-induced disruptive response and accelerates barrier recovery [142]. Corroboratively, CS in the 10–20% range causes activation of Rho and a reduction of basal Rac activity (Figure 4). In contrast, 5% stretch maintains the balance between Rho and Rac activity [143, 144]. These studies suggest a major role for amplitude-dependent CS in regulation of the endothelial barrier.

3.5. Cyclic Strain Sets the Stage for EndMT. Enhanced CS (>10%) potentiates EndMT of valvular endothelial cells in a manner dependent on both strain magnitude and direction [145]. Modest CS (10%) induces EndMT via amplified TGFβ1 signaling, while high CS (20%) activates wnt/β-catenin signaling [145], a known inducer of EndMT in aortic endothelial cells [146, 147]. Both intensities decreased VEGFa signaling, a known inhibitor of EndMT [48]. Notably, mechanical stretch induces epithelial-to-mesenchymal transition (EMT)

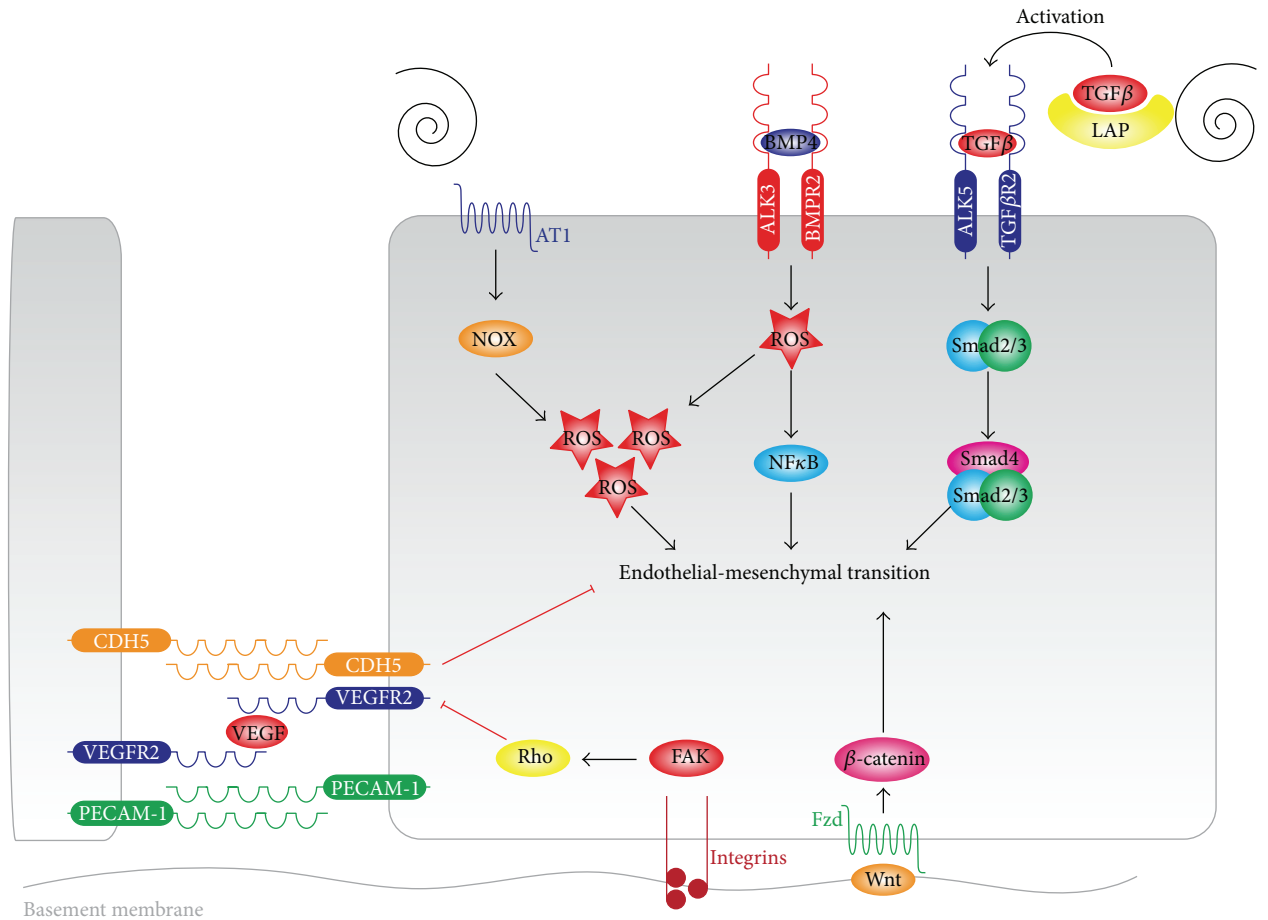

Figure 4: Disturbed shear stress and high cyclic strain signaling in endothelial-mesenchymal transition. Disturbed shear stress induces EndMT through several mechanisms. Disturbed shear stress activates latent TGFβ by liberating it from LAP, after which TGFβ can induce Smad2/3 signaling. Disturbed shear stress induces the expression of BMP4 which causes ROS formation and the activity of NFκB. Lastly, disturbed shear stress induces NOX and XO activity resulting in the generation of ROS. All these signaling intermediates culminate in EndMT. Increased cyclic strain (>10%) induces the FAK-dependent activation of Rho-kinases. Rho activity causes the translocation of VE-Cadherin from the cell membrane into cytoplasmic vesicles and causes a reduction in endothelial cell-cell contacts. Cyclic strain-dependent Wnt-β-catenin activity induces EndMT in part by the induction of Snail and Slug and the further activation of Rho activity.

of renal tubular epithelial cells by induction of TGFβ1 mRNA expression and activation of latent TGFβ [146].

VE-Cadherin is a central component of endothelial adherens junctions. VE-Cadherin is a transmembrane glycoprotein that complexes via its cytoplasmic tail to β-catenin, which links VE-Cadherin to the cortical actin cytoskeleton [148]. The cellular localization of VE-Cadherin is dependent on the small GTPases Rho and Rac that when appropriately balanced stabilize VE-Cadherin at the cell membrane [149].

EndMT results in a loss of barrier function, that is, increased permeability, which correlates with loss of VE-Cadherin at the cell surface (Figure 4). In response to the increase of Rho activity by high levels of CS [141], VE-Cadherin is translocated from the membrane into the cytoplasm. The concomitant loss of complexed β-catenin and its nuclear translocation might therefore be a secondary mediator of EndMT. Indeed, Rho activity plays a pivotal role in TGFβ1-induced EMT through induction of cytoskeleton remodeling and activation of the smooth muscle actin (SMA) promoter [150] and β-catenin/Rho signaling efficiently induces EndMT [32, 147, 151, 152]. This implies that supraphysiological CS is a direct inducer of EndMT (Figure 4).

Vascular remodeling is accompanied by changes in extracellular matrix (ECM) turnover, which result from alterations in the balance of matrix deposition and its proteolytic degradation. Matrix metalloproteinases (MMPs) represent the main group of proteases involved in remodeling of ECM [153]. An imbalance between the activity of MMPs and their tissue inhibitors (TIMPs) contributes to adverse remodeling. MMP2 induces matrix degradation during vascular remodeling [154] and TGFβ2-induced EndMT is characterized by a marked increase in MMP2 [151]. Interestingly, biaxial CS induces endothelial MMP2 expression and regulates its secretion and activity [130, 155]. Additionally, in a model for intimal hyperplasia, stretched human saphenous vein grafts increased expression and activity of MMP2 throughout the vascular wall [156]. Taken together, cyclic strain induces MMP2 expression in endothelial cells thereby facilitating

the migratory and proliferative phenotype acquired through EndMT.

Clearly, cyclic strain is not an ON/OFF switch of pathological vascular remodeling and EndMT, illustrated by the fact that endothelial cells react to CS in an amplitude-dependent manner. Stretching of endothelial cells may exert beneficial effects in physiological conditions (uniaxial, 5%–10%) but might induce advert effect during pathologies (strains <6% or >10%) such as hypertension. Reduced vascular distensibility is a common feature of vascular aging and is correlated with an increased risk of cardiovascular disease [157]. Loss of vessel wall compliance with age or increased CS during disease may blunt or aggravate the endothelial response to mechanical strain and induce EndMT. Whether and how this might contribute to pathophysiology remains to be determined.

3.6. Hemodynamic Forces Act in Concert. It should be emphasized that, for clarity reasons, LSS and CS were discussed as separate entities, yet they act in concert *in vivo*. For technical reasons, few studies have integrated both forces. However, fragmented evidence indicates that synchronicity between LSS and CS is important for vascular homeostasis, showing that asynchronicity between these hemodynamic forces induces a proatherogenic response through reduced expression of eNOS and cyclooxygenase-2 and increased expression of endothelin-1 and NFκB [158–160]. These findings illustrate the importance of studying both entities in combination.

4. Hemodynamic Forces, EndMT, and Vascular Disease

EndMT contributes to vascular pathologies such as cardiac fibrosis [44, 50, 161], atherosclerosis [18, 20, 79], vascular restenosis [162], and the remodeling observed with pulmonary arterial hypertension [26]. As stated previously, the focal nature of these diseases, despite their systemic or genetic risk factors, intimates a pivotal role for hemodynamic forces in modulating these pathologies.

In atherosclerosis, levels of activated Smad2 are elevated especially at areas exposed to DSS [163]. Also, endothelial BMP4 expression is elevated at the site of atheroma formation and in calcifed atherosclerotic plaques, characterized by DSS [164], where also increased levels of ROS production are found [165], which all favor EndMT induction and progression. Indeed, at these sites, EndMT contributes to intimal hyperplasia and atherosclerosis development [18, 20].

In patients with pulmonary arterial hypertension (PAH), elevations in pulmonary venous pressure or long-term increases in blood flow such as those produced by intracardiac shunts result in increased hemodynamic loads and cause structural changes of the pulmonary vasculature. The remodeling response is characterized by endothelial dysfunction, intimal hyperplasia, muscularization of small peripheral vessels, and wall thickening in proximal vessels [166]. The increased hemodynamic load results in increased CS and DSS originating from flow reversal in the pulmonary trunk due to the curved path of the blood flow and dilatation of the pulmonary artery [167]. Pulmonary artery endothelial cells from patients with PAH display a hyperproliferative, apoptosis-resistant phenotype [168] concomitant with EndMT [24]. Interestingly, expression of the Early growth response protein 1 (Egr-1) is elevated in experimental flow-associated PAH [169, 170] and in the vessels of PAH patients with media hypertrophy and neointimal lesions, including plexiform lesions [169]. Egr-1 induces the expression of Snail and Slug, two important mediators of EndMT [171–173], which argues for a definite role of EndMT in the pathogenesis of PAH, as was recently evidenced in human PAH and in experimental models for PAH [19, 174, 175].

Besides, certain patients with familial PAH have mutations of the bone morphogenetic protein (BMP) receptor type II (BMPR2) gene or Activin-like kinase 1 (ALK1) gene [176, 177]. Under normal conditions, these receptors can stimulate Smad1/5/8 signaling in endothelial cells which inhibits EndMT [27, 28, 178]. Hence, impaired BMPR2 or ALK1 signaling renders the pulmonary endothelial cells from these patients more prone to EndMT. BMPR2 deficient rats show spontaneous pulmonary vascular remodeling with enhanced expression of Twist-1, an inducer of EndMT [175]. Corroboratively, adenoviral BMPR2 gene delivery to the pulmonary vascular endothelium in experimental models of PAH reduces the vascular remodeling [179].

5. Conclusion

In conclusion, hemodynamic forces clearly modulate vascular homeostasis and endothelial plasticity. Exposure of endothelial cells to proper amplitudes of LSS and CS safeguard the endothelial integrity through a variety of signaling cascades. Deviations in these hemodynamic forces, both by ageing or pathology, results in adverse endothelial plasticity and culminates in EndMT. Current advances on endothelial mechanotransduction can provide us with many new insights into the regulation of endothelial plasticity.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Guido Krenning and Valerio G. Barauna contributed equally to this work.

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Review Article

Revisiting Epithelial-to-Mesenchymal Transition in Liver Fibrosis: Clues for a Better Understanding of the "Reactive" Biliary Epithelial Phenotype

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Whether liver epithelial cells contribute to the development of hepatic scarring by undergoing epithelial-to-mesenchymal transition (EMT) is a controversial issue. Herein, we revisit the concept of EMT in cholangiopathies, a group of severe hepatic disorders primarily targeting the bile duct epithelial cell (cholangiocyte), leading to progressive portal fibrosis, the main determinant of liver disease progression. Unfortunately, therapies able to halt this process are currently lacking. In cholangiopathies, fibrogenesis is part of ductular reaction, a reparative complex involving epithelial, mesenchymal, and inflammatory cells. Ductular reactive cells (DRC) are cholangiocytes derived from the activation of the hepatic progenitor cell compartment. These cells are arranged into irregular strings and express a "reactive" phenotype, which enables them to extensively crosstalk with the other components of ductular reaction. We will first discuss EMT in liver morphogenesis and then highlight how some of these developmental programs are partly reactivated in DRC. Evidence for "bona fide" EMT changes in cholangiocytes is lacking, but expression of some mesenchymal markers represents a fundamental repair mechanism in response to chronic biliary damage with potential harmful fibrogenetic effects. Understanding microenvironmental cues and signaling perturbations promoting these changes in DRC may help to identify potential targets for new antifibrotic therapies in cholangiopathies.

1. Introduction

Epithelial-to-mesenchymal transition (EMT) is a process of cellular reprogramming through which differentiated epithelial cells lose their native identity and acquire morphological and functional properties of mesenchymal cells, including a spindle-shaped ("fibroblast-like") appearance and the ability to detach from and to migrate outside the epithelial layer [1]. This process is relevant in physiological conditions, as seen during embryonic development, but it may occur also in pathological conditions, leading to organ fibrosis and malignant transformation in several organs [2].

The loss of epithelial cell-cell adhesion, caused by the relocalization and/or degradation of critical junction proteins, including E-cadherin, β-catenin, zonula occludens-1, occludin, and claudin, usually represents the first step of EMT. E-cadherin loss is often counterbalanced by the aberrant *de novo* expression of N-cadherin, an adhesion molecule enabling epithelial cells to establish dynamic interactions with surrounding mesenchymal cells. The disassembly of cell junctions, together with the ability to erode the basement membrane, results in a reduced intercellular cohesion within the epithelial layer [3, 4]. However, cells undergoing EMT also show a rearrangement of the cytoskeletal architecture, deriving from the downregulation of cytokeratins (K) along with the upregulation of cytoskeletal proteins belonging to the mesenchymal lineage, including vimentin, S100A4 (also called fibroblast specific protein-1), and, eventually, α-smooth muscle actin (α-SMA). These cytoskeletal and cell surface remodeling lead to the loss of the apical-basal polarity, typical of the epithelial phenotype, in favor of a front-rear polarity, prerequisite for the increased motility displayed by

mesenchymal cells. Additional EMT changes include the ability to produce extracellular matrix (ECM) components, such as fibrillar collagen, fibronectin, elastin, and tenascin, in conjunction with a range of matrix metalloproteinases (MMPs), particularly MMP2 and MMP9, and to increase the expression of integrin receptors mediating the interactions with ECM [1, 3, 5]. However, it must be underlined that the transition from an epithelial to a mesenchymal cell phenotype is not merely an "on/off" state but rather a highly dynamic process evolving gradually [6].

2. Molecular Players and Intracellular Pathways Regulating the "EMT Machinery"

Gene expression changes in EMT are orchestrated by a number of transcription factors actively engaged in embryogenesis, such as Snail (Snail1), Slug (Snail2), Twist1/2, and ZEB1/2. Their activation in response to growth factors, cytokines, and morphogens [13, 14] is an early event during EMT. Indeed, EMT can be induced by a number of extracellular signals, whose downstream transduction pathways extensively crosstalk with each other, share effector molecules, and converge on common endpoints [15].

Members of the transforming growth factor- (TGF-) β family (in particular TGF-β1) are the prototypical activators of EMT. TGF-β1 binding to the TGF-β type II receptor results in the activation of the Smad signaling pathway, which induces the expression of EMT transcription factors (especially Snail and ZEB family members), and cooperates with them in promoting gene reprogramming. However, TGF-β1 can also act through Smad-independent intracellular pathways, by activating Rho GTPases, mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K) [16, 17]. TGF-β1 may act upon local activation by integrin $\alpha v\beta 6$, which can be expressed at high levels by epithelial cells during tissue repair. Specifically, integrin $\alpha v\beta 6$ cleaves the latency-associated peptide from the latent precursor of TGF-β1, which is otherwise sequestered in the ECM as inactive form. This mechanism of action is potentially relevant to promoting EMT changes, since, once locally activated, TGF-β1 exerts its effects only within the limits of the epithelial cells displaying this specific receptor [18]. Alternatively, EMT initiation and progression can be regulated by tyrosine kinase receptors (RTKs), involved in signal transduction of epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin-like growth factor, hepatocyte growth factor (HGF), and platelet-derived growth factor (PDFG) [3]. RTKs stimulation has been widely linked to the activation of several regulatory molecules of EMT [19–23]. Notably, MAPK and PI3K pathways seem to play a major role in mediating RTKs-induced EMT [3]. Amongst morphogenetic signals, Wnt, Notch, and Hedgehog (Hh) signaling are well-established EMT inducers [24]. The ability of Wnt signals to trigger EMT relies on either the inhibition of the glycogen synthase kinase 3 (GSK3)-β, which prevents the destabilizing phosphorylation of Snail, or the nuclear translocation of β-catenin, whose gene targeting includes ZEB1 and Twist [25–27]. Notch and Hh signaling leading to the activation of EMT transcription factors (in particular the members of the Snail family) occurs through the activation of the Notch intracellular domain and the Gli family transcription factors, respectively [28–30].

The EMT program is finely regulated at a posttranscriptional level, by specific microRNAs (miRNA), including miR-1, miR-29b, miR-34, miR-200, and miR-203. These are small RNAs with about twenty nucleotides, regulating stability and translational activity of mRNAs. In particular, miRNAs act in double-negative feedback loops with several EMT transcription factors, wherein they repress the expression of each other, thus providing epithelial cells with an additional, finely tuned mechanism aimed at maintaining EMT. Furthermore, a direct effect of miRNAs has been shown on the expression of critical biomarkers, such as E-cadherin, vimentin, and fibronectin (e.g., miR-9, miR-138, and miR-17), as well as on several EMT-promoting ligands and their related signaling pathways (e.g., miR-200a for β-catenin, miR-204 for TGF-βR2, miR-15, miR-16 for FGF, miR-198 for HGF [31], and miR-181a for TGF-β [32]). Recent data indicate that miR-181a acts as a downstream effector of the TGF-β signaling in hepatocytes where it modulates the expression of a number of EMT-related genes, among which are E-cadherin and vimentin [32]. In cholangiocytes, miRNA-15a downregulates Cdc25a, a cell-cycle regulator with potent proliferative effects, a mechanism possibly involved in hepatic cystogenesis [33].

It is important to underline that phenotypic changes resulting in EMT can be triggered by disease mechanisms, such as inflammation, hypoxia, ECM remodeling, and autophagy. In fact, proinflammatory cytokines, such as tumor necrosis factor-α (TNFα) and interleukin-1β, and hypoxia can activate EMT master genes, acting through nuclear factor- (NF-) κB and hypoxia inducible factor 1α, respectively [34].

The pathological remodeling of ECM also represents an additional mechanism of EMT progression [3]. In this regard, epithelial cells exposed to MMP-3 upregulate an alternative splice isoform of Rac1, which then enhances the expression of Snail by stimulating the production of reactive oxygen species [35]. Snail-induced EMT can be also triggered by type I collagen, which binds to $\alpha 2\beta 1$ integrin causing an integrin linked kinase-mediated increase in nuclear NF-κB activity [36].

Recent evidence suggest that also autophagy may behave as a critical regulator of EMT. Autophagy suppression by downmodulation of the autophagy-related gene 5 leads to the intracellular accumulation of the selective autophagy substrate p62, which then inhibits Twist1 protein degradation in both autophagosomes and proteasome, thereby decreasing E-cadherin expression and promoting cell motility [37, 38].

3. EMT in Liver Development

Acquisition of a mesenchymal phenotype endowed with migratory functions is a prerequisite of many morphogenetic processes. This concept is well established in renal biology, given the mesodermal origin of the tubular epithelium, while it is less defined in the liver, where, instead, the epithelial cells (hepatocytes and cholangiocytes) derive from the foregut

endoderm, and the mesodermal contribution is restricted to the generation of the stromal cells, including hepatic stellate cells (HSC). Only scant evidence suggest an EMT role in liver development. Studies performed in the late 1990s showed that cultured hepatocytes from neonatal rat livers underwent EMT changes, represented by the loss of specific differentiation markers, gain of a migrating morphology, and replacement of typical hepatocyte cytokeratins by vimentin, a property further stimulated by EGF [39]. EMT features were then reported in both hepatocytes and progenitor cells isolated from rodent and human fetal livers [40, 41]. Studies in mice from Lemaigre's group addressed the hypothesis that EMT is critically involved in the early process by which endodermal cells that line the hepatic diverticulum migrate through the basement membrane to invade the septum transversum, where they give rise to the hepatoblasts in the liver bud [42]. This process is controlled by the hematopoietically expressed homeobox factor (Hex), which acts in concert with the transcription factor GATA-6; their downmodulation is essential for hepatoblast clustering after liver budding. Expression of the prospero-related homeobox 1 (Prox-1), likely stimulated by the T-box transcription factor 3 (Tbx3), is an additional mechanism critically involved in hepatoblast migration, which interplays with Hex (Hex-Prox-1 axis) [42]. Starting from this hypothesis, coexpression of K18 and α-SMA was found in most nonhematopoietic cells of human fetal livers at early gestational ages; furthermore, multipotent stem cells expressing EMT features along with the stem cell markers Oct4 and Nanog were isolated in the human liver bud [43]. Unlike the early ontogenetic steps, data supporting an involvement of EMT in the morphogenesis of intrahepatic bile duct epithelium are even less evident. Expression of the SRY-related HMG box transcription factor 9 (SOX9) is critical for differentiation to a biliary epithelial phenotype. SOX9 is early expressed in endodermal cells of the hepatic diverticulum, but it is then downregulated as these cells are invading the septum transversum. SOX9 is reexpressed in the hepatoblasts switching to the ductal plate cell phenotype, and it is then maintained by cholangiocytes during the progressive maturation of bile ducts. When SOX9 is defective, epithelial cells become hyperresponsive to TGF-β [44], thereby being susceptible to mesenchymal changes. Thus, it seems that cholangiocytes express an active program to suppress EMT.

4. In Chronic Cholangiopathies, Mesenchymal Markers Are Expressed by Ductular Reaction

Broadly speaking, whereas activation of an EMT program may play a physiological role in embryonic development [2], its actual impact in disease conditions evolving to scarring is quite controversial, particularly in the liver [45]. Cholangiopathies may provide important clues to clarify whether and how EMT may really contribute to liver fibrogenesis. Cholangiopathies are a heterogeneous group of genetic and acquired liver disorders primarily targeting the epithelial cell lining the bile ducts (cholangiocyte). Most cholangiopathies typically follow a chronic, progressive course, characterized by an excessive matrix deposition confined to the portal tract (portal fibrosis), starting from the closest peribiliary area, and ultimately leading to portal hypertension, often before the development of full-blown cirrhosis. In contrast with other chronic liver diseases, treatment of cholangiopathies is mainly symptomatic, reflecting the limited knowledge on their pathogenesis. Nowadays, liver transplantation remains the only curative opportunity, especially in children and young adults [46, 47].

Fibrogenesis is the main determinant of disease progression, as well as of the most severe clinical manifestations related to portal hypertension, in both chronic hepatocellular damage and cholangiopathies. Fibrogenesis is a consequence of the excessive and sustained activation of tissue repair mechanisms driven by the ductular reaction [1]. Ductular reaction is a dynamic, multicellular reparative system that includes mesenchymal and inflammatory cells accompanying the expansion of the epithelial cells lining the smallest ramifications of the biliary tree, in continuity with the canals of Hering, which is the niche where the hepatic progenitor cells (HPC) is thought to reside. Expansion of the HPC compartment is a compensatory mechanism of liver repair activated when proliferative ability of mature liver cells is compromised because of a severe liver damage [48]. HPC are small cells marked with the bipotential capability to differentiate towards both biliary and hepatocyte lineages [49]. In ductular reaction, HPC-derived epithelial cells are arranged into irregular, highly branched ductules devoid of lumen, generally extending into the liver parenchyma and along the margins of the portal tract. During this process, ductular cells express a "reactive" phenotype (ductular reactive cells, DRC) and acquire the ability to produce cytokines, chemokines, growth factors, and angiogenic factors and to express a rich repertoire of receptors typically displayed by ductal plate cells in the early stages of liver development [50]. Thanks to these phenotypic changes, DRC may establish intense paracrine communications with multiple stromal cell types, including myofibroblasts (MFs), inflammatory cells, and endothelial cells, which dictate the functional consequences of ductular reaction [1]. To set in motion this multicellular reparative complex, DRC acquire a high degree of cell plasticity. Therefore, DRC need first to reduce the strength of cell-cell and cell-matrix contacts and then to acquire motile functions, enabling them to move from the HPC niche towards the site of damage whereby, by interacting with other inflammatory and mesenchymal cell elements, they build up the ductular reaction. A mainstay of the migratory properties of DRC is their increased production of polysialic acid (PolySia) in the course of biliary damage. PolySia is a highly polar ECM structural component with a strong binding affinity to the neural cell adhesion molecule (NCAM), commonly expressed by DRC [51]. PolySia turns NCAM adhesive properties into antiadhesive due to the size of the multiple PolySia chains and their high hydrophilic content [52, 53]. This process is an essential step to promote plasticity and migration of $NCAM^+$ cells in the generation of ductular reaction.

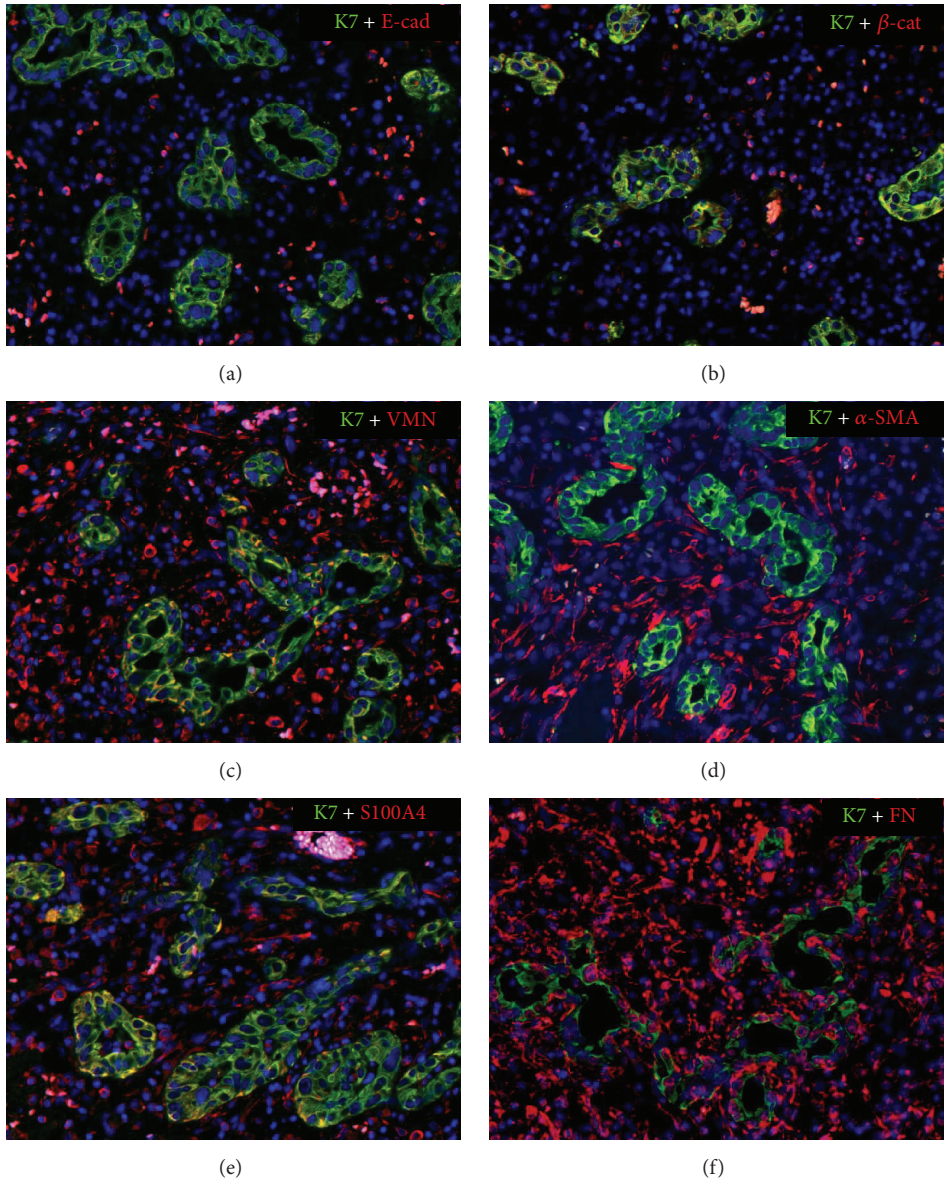

Figure 1: Partial expression of some mesenchymal features by ductular reactive cells. By dual immunofluorescence of a liver tissue section from a patient with ischemic cholangiopathy, with the cholangiocyte marker K7 (green), some mesenchymal features (red) are expressed by ductular reactive cells (coincident staining in yellow). They include downregulation of E-cadherin at the cell junctions of the epithelial layer (a) and upregulation in the cytoplasm of vimentin (c) and S100A4 (e) and in the basal side of fibronectin (f). In contrast, ductular reactive cells do not express typical markers of EMT, such as nuclear expression of β-catenin (b) and α-SMA (d) (M = 200x).

Although heavily involved in fibrogenesis, DRC lack the ability to actively secrete ECM components, such as type I or type IV collagen, and must cooperate with other effector cells by stimulating their profibrotic activities. Among them, DRC interactions with portal MFs are a crucial step in fibrogenesis [54]. Portal MFs are fibrogenic cells localized within the portal space, characterized by spindle-shape morphology, α-SMA expression, prominent motility, and contractility functions and strong capability to secrete ECM proteins, mostly type I collagen [55, 56]. They may originate from multiple cell sources, including HSCs and, to a lesser extent, portal fibroblasts and bone marrow-derived mesenchymal stem cells. These are recruited by paracrine signals (TGF-β, PDGF-B, vascular endothelial growth factor, angiopoietin-1, and sphingosine 1-phosphate) released in the site of damage by the DRC as well as by the other components of the ductular reaction, such as macrophages and inflammatory cells [1, 57, 58]. Whether the DRC themselves may be a further source of portal MFs via EMT as in the kidney [59, 60] and in the lung [61] has been hypothesized but never proven. However, several studies show that DRC express mesenchymal markers [62–64], as illustrated in Figure 1.

Initial studies showed that, in several cholangiopathies, cholangiocytes lining the small interlobular bile ducts and

the reactive ductules lose some epithelial markers and acquire, in turn, several mesenchymal traits. Neoexpression of S100A4, vimentin, Snail, and MMP-2, associated with downregulation of E-cadherin and K19 in the bile ducts, were observed in histological samples of patients with primary biliary cirrhosis (PBC), primary sclerosing cholangitis [65], and biliary atresia (BA) [62, 66]. Reduced expression of E-cadherin and increased expression of vimentin and S100A4 were also reported in bile ducts of patients with intrahepatic lithiasis, where these phenotypic changes strongly correlated with the extension of biliary fibrosis [67]. Epithelial expression of mesenchymal markers has been reported also in animal models of biliary fibrosis induced by bile duct ligation (BDL). In the BDL rat, cholangiocytes upregulate the expression of S100A4 and downregulate the expression of the specific epithelial markers and the membrane channel aquaporin-1, together with the cytokeratins K7 and K19 [7]. Similarly, in the BDL mice, small clusters of cholangiocytes showed immunoreactivity for both α-SMA and heat-shock protein 47, a surrogate marker of type I collagen production, as well as migratory aspects into the periductal region [10]. However, it must be underlined that reliability of S100A4 as marker of EMT is limited by its concurrent expression by many inflammatory cells, including macrophages, often infiltrating the bile duct profile, thus posing a risk of misinterpreting histological sections.

Paracrine Hh signaling has been proposed by Omenetti and colleagues as a major driver of the EMT changes associated with biliary fibrosis in both rodents and humans [7]. In PBC, where EMT was originally proposed also as a mechanism contributing to ductopenia [68], Gli2, a transcription factor activated by Hh ligands, decorated the nuclei of ductular cells coexpressing both mesenchymal (S100A4, vimentin) and epithelial markers (K7) [7]. In BDL rats, relief of ductal obstruction reduced Hh pathway activity, an effect accompanied by reduction in EMT phenotype and biliary fibrosis [7]. Interestingly, mouse cholangiocytes cocultured with MFs, a rich source of soluble Hh ligands, acquired increased expression of several mesenchymal markers, including a migratory phenotype, while concomitantly repressing epithelial markers, and these effects were abolished by Hh antagonism [7]. Furthermore, EMT changes induced by BDL were exacerbated in transgenic mice harboring an overactivation of the Hh pathway caused by a defect in the Hh inhibitor Ptc [7]. The interplay between Hh activation and EMT was also reported in BA, a cholangiopathy featuring a pronounced ductular reaction associated with a rapid development of biliary fibrosis. In BA, a marked activation of the Hh signaling was associated with an excessive accumulation of ductular cells displaying an immature, mesenchymal-like phenotype responsive to Hh [69]. Hh ligands could be also secreted by hepatocytes both in human liver diseases and in mice models of liver damage. Hh ligands, Sonic and Indian Hh, both were overexpressed by hepatocytes in nonalcoholic steatohepatitis [70] and in chronic cholangiopathies such as primary biliary cirrhosis, respectively [71], in keeping with findings supporting a proapoptotic effect of Hh signaling [72]. Similarly, chronically liver injured mice by thioacetamide treatment showed an increased hepatocyte expression of Sonic and Indian Hh [73].

Noteworthy, Hh signaling may potentiate the pro-EMT effects of TGF-β1, by interacting with its downstream effectors at several levels (Smad3, Snail, and Twist), and, in turn, liver cell expression of Hh ligands can be stimulated by TGF-β1 [74]. The crosstalk between the two signaling pathways is particularly relevant in biliary fibrosis, since TGF-β1 is strongly upregulated in several cell types populating the ductular reaction, such as HSCs, endothelial cells, and Kupffer cells [1]. Given the effects on HPC and cholangiocytes, TGF-β1 seems to play a pivotal role in the generation of ductular reaction. Within the HPC niche, some cells display mesenchymal-like features, which are modulated by TGF-β1 [75]. At high doses, TGF-β1 is toxic to most epithelial cells, but, at low doses (1–10 ng/mL), it stimulates cultured cholangiocytes to acquire some mesenchymal markers, such as S100A4, vimentin, and α-SMA, to lose expression of K7, K19, and E-cadherin and to gain invading abilities through the basement membrane [10, 65, 66].

There is evidence suggesting that TGF-β may drive expression of mesenchymal markers in cholangiocytes also *in vivo* in specific disease settings. For example, cholangiocytes display some mesenchymal features in both rat [76] and mouse (personal data) models of congenital hepatic fibrosis (CHF), a genetic cholangiopathy caused by mutations of the ciliary protein fibrocystin. CHF is characterized by progressive peribiliary fibrosis, accompanied by biliary dysgenesia. In the CHF mouse model, fibrocystin-defective cholangiocytes possess increased migratory functions [9], upregulate integrin $\alpha v\beta 6$, and respond to TGF-β1, by producing collagen type I. This feature is not observed in cultured normal cholangiocytes and may contribute to matrix deposition in the adjacent peribiliary area, where fibrogenesis starts (personal data). These phenotypic changes appear to be dependent upon an activation of the β-catenin signaling caused by a noncanonical phosphorylation at Ser^{675}. Ser^{675} phosphorylation prevents β-catenin from degradation, thereby allowing its nuclear translocation and, subsequently, its transcriptional activity. Activation of β-catenin as observed in fibrocystin-defective cholangiocytes is paradigmatic of the intracellular signaling perturbations induced by the loss of the tubular architecture and, consequently, of the cell polarity, which results in an increased secretion of cytokines, chemokines, and growth factors (personal data). This condition is likely reproduced by DRC, which, unlike normal cholangiocytes, are equipped with a number of ligand/receptor systems shared with the other cell elements involved in liver repair, mediating an extensive crosstalk ultimately leading to portal fibrogenesis [1].

Even in the model of fibrocystin deficiency, mesenchymal-like changes are induced in cholangiocytes by TGF-β1 released in the peribiliary space by progressively infiltrating macrophages, whose recruitment is regulated by a range of chemokines (CXCL1, CXC10, and CXCL12) secreted by cholangiocytes in a β-catenin-dependent fashion. Importantly, this signaling perturbation is specific to fibrocystin deficiency, as it is not observed in polycystic liver diseases

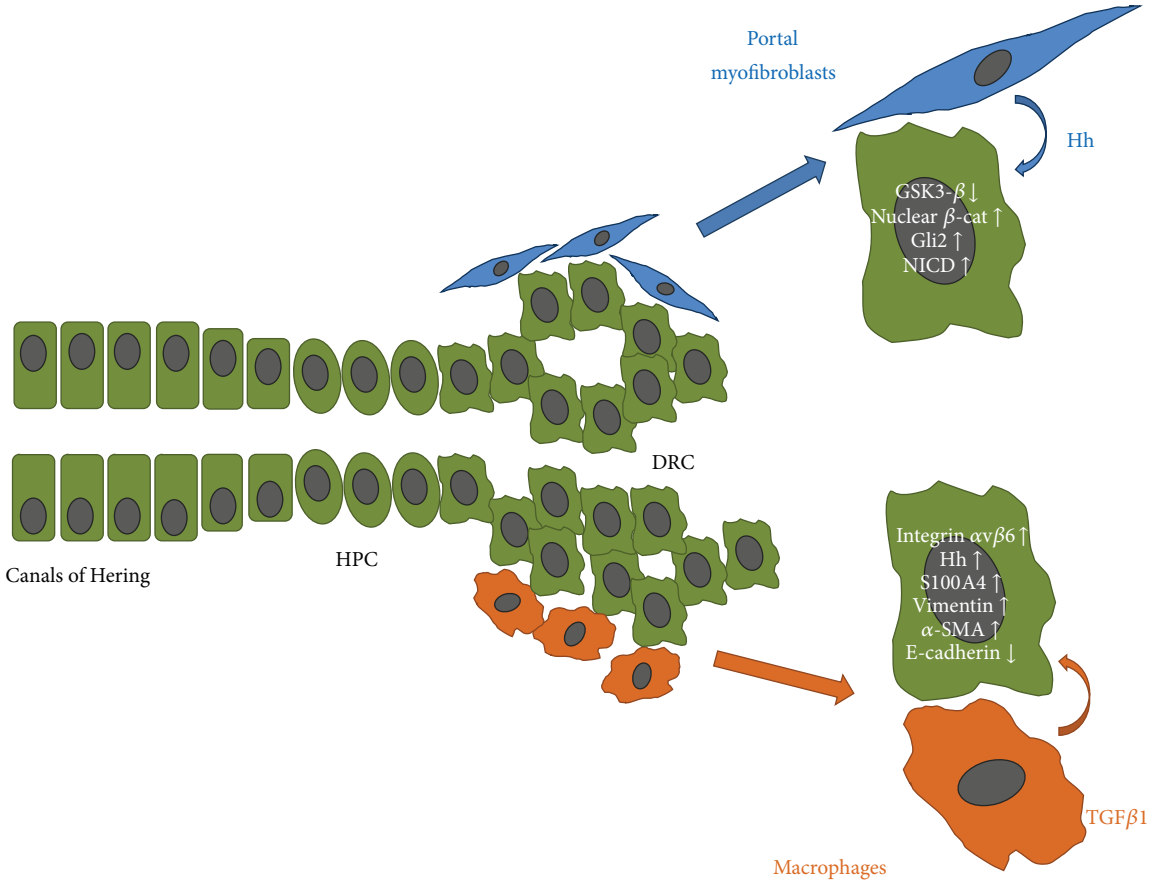

Figure 2: Epithelial-mesenchymal cell interactions promote ductular reaction. Crosstalk mechanisms with portal myofibroblasts and macrophages mediated by Hedgehog (Hh) and TGF-β1, respectively, are critical in generating ductular reactive cells (DRC) from activation of the hepatic progenitor cell (HPC) compartment, residing in the niche nearby the canals of Hering. Hh and TGF-β1 stimulate DRC to gain a range of mesenchymal changes typical of a "reactive" phenotype.

related to different ciliary protein defects, affecting polycystins [9]. However, even in this case, a full transdifferentiation of cholangiocytes into an activated mesenchymal phenotype (α-SMA neoexpression) was not found [76]. Crosstalk mechanisms driven by Hh ligands and TGF-β1 released in the inflammatory microenvironment, promoting mesenchymal changes in DRC, are outlined in Figure 2. Collectively, the pro-EMT body of evidence in cholangiopathies is summarized in Table 1.

5. Evidence against EMT in Biliary Fibrosis

Most studies supporting the occurrence of EMT in cholangiocytes are based essentially on a morphological approach, even when taking advantage of elegant *in vitro* methodologies and well-characterized animal models. These findings were not confirmed *in vivo* by lineage-tracing experiments. In these studies, mice harboring a Cre recombinase under a cholangiocyte- or oval cell-specific promoter were crossed with a reporter strain carrying the yellow fluorescence protein (YFP) reporter gene preceded a floxed Stop cassette, and the progeny was then subjected to a cholestatic, fibrogenetic liver injury, caused by BDL.

In the first study [11], using K19-CreERT × Rosa26-YFP mice, immunostaining revealed that after experimental liver injury, mesenchymal markers such as α-SMA, desmin (HSC biomarker), and S100A4 failed to colocalize in cholangiocytes tagged for K19 expression (i.e., YFP^+ cells), although, within the portal tract, $S100A4^+$ and $K19^+$ cells localized in close proximity to each other. These data indicate that, *in vivo*, cholangiocytes do not activate an EMT program. These results were confirmed in MFs isolated from BDL livers, where no YFP^+ cells could be detected. Similarly, in S100A4-green fluorescence protein (GFP) mice undergoing biliary damage, Pan-K^+ cholangiocytes never overlapped with S100A4-GFP^+ cells, thus confirming that cholangiocytes do not express S100A4. Even in this case, $S100A4^+$ cells purified from cholestatic livers did not express panK, thus suggesting that a mesenchymal conversion of cholangiocytes, also transient, does not occur during liver injury. The discrepancy with Omenetti et al.'s paper [7] may be related to the different biliary marker (K7 versus K19) used for coexpression studies with S100A4 [11]. In fact, the cholangiocyte

Table 1: Summary of evidence in favor of or against the existence of EMT in biliary diseases.

Model	Readouts	References
Pro-EMT		
Coculture of MFs and cholangiocytes	Cholangiocytes: ↑ S100A4, ↑ Fibronectin, ↑ N-cadherin, and increased motility	[7]
Cultured cholangiocytes from α-fetoprotein (Alfp)-Cre × Rosa26-YFP mice treated with TGFβ, or TNFα	↑ α-SMA, loss of cell-cell contacts, cellular reshaping, and E-cadherin delocalization	[8]
Cultured cholangiocytes from *Pkhd1*$^{del4/del4}$ mouse	↑ motility due to β-catenin activation	[9]
BDL rat	Coexpression of S100A4 and vimentin with K7	[7]
BDL rat	DRC (immunohistochemistry): ↑ S100A4, ↑ heat-shock protein 47, ↑ α-SMA, ↓ K7, ↓ K19, and ↓ Aquaporin-1	[10]
Against-EMT		
K19-CreERT × Rosa26-YFP mice, BDL	No coexpression of K19 YFP with α-SMA, Desmin, or S100A4	[11]
S100A4-CreERT × Rosa26-YFP mice, BDL	No coexpression of S100A4-GFP with Pan-K cells	[11]
α-fetoprotein (Alfp)-Cre × Rosa26-YFP mice, BDL	No coexpression of YFP with S100A4, vimentin, α-SMA, procollagen 1α2, or desmin	[8]
α-fetoprotein (Alfp)-Cre × Rosa26-YFP mice, DDC	No coexpression of YFP with S100A4, vimentin, α-SMA, procollagen 1α2, or desmin	[8]
Human EGI-1-EGFP xenograft in SCID mice	No K19/α-SMA coexpression; no expression of Y human chromosome on α-SMA$^+$ cells	[12]

population is highly heterogeneous and a distinct subset of K19$^+$/K7$^-$ cholangiocytes in the terminal bile ductules, activated in specific disease conditions, has been identified [77]. On the contrary, a different subpopulation of K7$^+$/K19$^-$ cholangiocytes close to the HPC niche and mainly triggered by biliary damage has been also reported [78].

Therefore, to elude the technical trick related to the K19$^+$ cell fate mapping, in a second study, Chu and colleagues used the alpha-fetoprotein (Alfp)-Cre × Rosa26-YFP mouse [8]. Taking this approach, the authors could track the cell fate not only of K19$^+$ cholangiocytes, but also of HPC. Again, no evidence of YFP colocalization with the mesenchymal markers S100A4, vimentin, α-SMA, procollagen 1α2, or desmin was observed in liver tissue following BDL. Furthermore, no coexpression of the same markers by YFP$^+$ cells could be observed after 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet, a model of biliary damage known to generate a robust HPC activation. Overall, these data support the finding that neither cholangiocytes nor their cell progeny may convert into fibrogenic MFs during experimental cholestatic liver injury. However, in contrast with the *in vivo* data, in the same study, cultured cholangiocytes isolated from the reporter mice and challenged with TGF-β1, alone or in combination with TNF-α, showed loss of cell-cell contacts along with a fibroblastoid cell reshaping, intracellular delocalization of E-cadherin, and increased expression of α-SMA. These apparent conflicting data clearly indicate that, under certain circumstances, cultured cholangiocytes may be committed to a complete EMT, unlike what happens in the *in vivo* condition, where the mesenchymal phenotype does not fully develop. This observation is in line with a recent study performed by our group to see if EMT contributes to the generation of the cancer-associated fibroblasts (CAF), usually accompanying the invasive growth of cholangiocarcinoma (CCA), a devastating malignancy originating from the biliary epithelium. A highly invasive human male CCA cell line (EGI-1) expressing an EMT phenotype, was xenografted by intraportal injection into a SCID male mouse, after transduction with lentiviral vectors encoding enhanced green fluorescence protein (EGFP). Liver tumors were analyzed by dual immunofluorescence for EGFP (serving as a CCA cell lineage marker) and α-SMA (CAF marker). Indeed, engrafted tumors were closely surrounded by abundant CAF, thus reproducing the native CCA characteristics. In this model, cancer cells that underwent a complete EMT would be expected to coexpress both markers. However, coincident labeling between EGFP and α-SMA was never observed in tissue samples from our xenograft models. Accordingly, FISH analysis further showed that the α-SMA$^+$ cells expressed the murine rather than the human Y chromosome, which, instead, was normally expressed by infiltrating CCA cells [12].

Taken together, results of these fate-mapping studies are in accordance with a previous report showing the lack of EMT even in hepatocytes in a model of chronic hepatocellular damage [79] (Table 1). However, it must be underlined that experimental models of DDC and BDL are not fully consistent with the clinical phenotype of the human disease, most likely because of the rapid establishment of biliary fibrosis, which in chronic cholangiopathies takes instead several years to become clinically overt [45, 80, 81]. From this point of view, the CHF mouse model better reproduces the slow evolving tissue scarring seen in most human cholangiopathies.

6. Conclusions

The controversy on the role of EMT in biliary fibrosis is substantially a matter of definitions [1]. The term EMT should be abandoned in cholangiocyte biology. Nevertheless, cholangiocytes may acquire, to a variable degree, some mesenchymal properties as part of a "reactive" phenotype,

which develops without the concurrent loss of the native epithelial identity. As pointed out by Kriz and colleagues, the main caveat of several studies favoring the EMT hypothesis is the *a priori* assumption of EMT as an established fact, leading to misinterpretation of data that are compatible with but not evidence for EMT [82]. Therefore, the risk is to overlook the high complexity of a process whose relevance is recognized only in renal biology.

In the setting of a reactive phenotype (ductular reaction and biliary dysgenesis), ECM components are abnormally represented in close vicinity of bile ducts. This feature is typical of several chronic cholangiopathies and represents the mechanism leading to portal hypertension and its severe complications. Therefore, it is expected that hampering fibrosis progression would lead to a significant improvement of patient's survival. However, the availability of effective antifibrotic therapies is still remote [83], especially for primary cholangiopathies [47]. The identification of factors released in the inflammatory microenvironment and able to activate DRC as well as of signaling perturbations modulating mesenchymal changes may provide a wide range of putative novel targets (soluble factors, morphogens, transcription factors, and miRNA) whose therapeutic interference might halt the progression of biliary fibrosis, an issue worth being investigated by future studies.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Vitamin D and the Epithelial to Mesenchymal Transition

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Several studies support reciprocal regulation between the active vitamin D derivative 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and the epithelial to mesenchymal transition (EMT). Thus, $1,25(OH)_2D_3$ inhibits EMT *via* the induction of a variety of target genes that encode cell adhesion and polarity proteins responsible for the epithelial phenotype and through the repression of key EMT inducers. Both direct and indirect regulatory mechanisms mediate these effects. Conversely, certain master EMT inducers inhibit $1,25(OH)_2D_3$ action by repressing the transcription of *VDR* gene encoding the high affinity vitamin D receptor that mediates $1,25(OH)_2D_3$ effects. Consequently, the balance between the strength of $1,25(OH)_2D_3$ signaling and the induction of EMT defines the cellular phenotype in each context. Here we review the current understanding of the genes and mechanisms involved in the interplay between $1,25(OH)_2D_3$ and EMT.

1. The Vitamin D System

The mammalian form of vitamin D is the prohormone vitamin D_3 (cholecalciferol), which is obtained from the diet or mainly synthesized in the skin from 7-dehydrocholesterol through ultraviolet B radiation. Vitamin D_3 is hydroxylated, first in the liver and then in the kidney and other tissues, to generate 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, calcitriol), the most active vitamin D_3 metabolite [1–5]. $1,25(OH)_2D_3$ is a major regulator of gene expression and exerts its effects by binding to a transcription factor of the nuclear receptor superfamily: the vitamin D receptor (VDR). VDR heterodimerizes with another member of the same family, the retinoid X receptor, and regulates gene expression in a ligand-dependent manner. The prevailing model holds that in the absence of $1,25(OH)_2D_3$ the heterodimer is bound to specific sequences on its target genes (vitamin D response elements) and to transcriptional corepressors that recruit complexes with histone deacetylase activity, thus maintaining the chromatin in a transcriptionally repressed state. $1,25(OH)_2D_3$ induces conformational changes in VDR that cause the release of corepressors and the binding of coactivators and chromatin remodelers. Together they mediate chromatin opening and permit the entry of the basal RNA polymerase II transcription machinery and transcription initiation [1, 6–9].

$1,25(OH)_2D_3$ is a pleiotropic hormone with many regulatory effects. It was classically known for its action on calcium and phosphorus homeostasis and bone mineralization [2, 10]. The seminal discoveries in 1981 that $1,25(OH)_2D_3$ induced myeloid leukemia cell differentiation and inhibited melanoma cell proliferation prompted the interest in $1,25(OH)_2D_3$ as an anticancer agent [11, 12]. Subsequent observations have shown that $1,25(OH)_2D_3$ induces differentiation and apoptosis and inhibits proliferation, migration, invasion, and angiogenesis in cancer cells of different origin and in several animal models of cancer [1, 5, 13–16]. However, the administration of $1,25(OH)_2D_3$ to cancer patients is restricted by its hypercalcemic effects at the therapeutic doses, enforcing the development of several analogs that maintain the antitumoral properties but have less calcemic actions. Currently, numerous clinical trials

are ongoing using $1,25(OH)_2D_3$ or its analogs, alone or in combination with other anticancer agents, against several neoplasms (https://www.clinicaltrials.gov/) [1, 4, 5, 13].

2. Epithelial to Mesenchymal Transition

Epithelial to mesenchymal transition (EMT) is the process by which epithelial cells are converted into mesenchymal cells. It takes place physiologically in several developmental situations such as mesoderm formation and neural crest migration. In the adult, it is reactivated in certain pathological conditions such as wound healing, fibrosis, and cancer progression [17, 18]. During EMT, epithelial cells lose cell-cell and cell-extracellular matrix junctions, change from an apical-basal to a front-rear polarity, reorganize their cytoskeleton, and undergo a gene expression reprogramming characterized by the downregulation of the epithelial gene signature and the activation of mesenchymal genes. This process generates motile individual cells that can degrade the extracellular matrix and thus develop a migratory and invasive phenotype [18–21]. EMT is a highly regulated, plastic, and reversible process. Thus, the mesenchymal to epithelial transition (MET) occurs under certain conditions and enables that mesenchymal cells acquire an epithelial state [22–25].

Typical EMT gene reprogramming is mainly orchestrated by key transcription factors including the zinc finger proteins SNAIL1 and SNAIL2, the double zinc finger and homeodomain factors ZEB1 and ZEB2, and the members of the basic-helix-loop-helix family TWIST1 and E47, all known as EMT transcription factors (EMT-TFs). They are repressors of E-cadherin (encoded by *CDH1*), that is the main component of adherens junctions and essential for the maintenance of the epithelial state. Thus, E-cadherin downregulation is considered a hallmark of EMT. In addition to the established EMT inducers, other transcription factors such as FOXC2, Goosecoid, KLF8, TCF4 (also known as E2-2), SIX1, HMGA2, Brachyury, and PRRX1 have been recently shown to induce or regulate EMT [18–21, 24]. Expression and/or activity of the transcription factors that drive EMT is induced and controlled by several signaling pathways that respond to extracellular cues, with a prominent role for transforming growth factor- (TGF-) β signaling. The contribution of each transcription factor to the EMT depends on the cell or tissue type involved and the signaling pathway that initiates the EMT. Moreover, EMT-TFs often exhibit reciprocal control of their expressions and functional cooperation [18–20, 22, 23].

3. $1,25(OH)_2D_3$ Inhibits EMT

3.1. $1,25(OH)_2D_3$ Induces the Expression of Epithelial Markers. $1,25(OH)_2D_3$ induces epithelial differentiation in several normal and cancer cells. Accordingly, it increases the expression of components of almost all types of cell adhesion structures that are essential for the acquisition and maintenance of the epithelial phenotype (Table 1). Remarkably, we found that the strong prodifferentiation effect of $1,25(OH)_2D_3$ in human colon cancer cells is associated with an increase in the expression of the key adhesion molecule E-cadherin. This is accompanied by the redistribution of β-catenin from the cell nucleus to the adherens junctions at the plasma membrane where it interacts with E-cadherin, thus inhibiting the Wnt/β-catenin signaling pathway that is aberrantly activated in most colon tumors and required for colon carcinogenesis [26]. In human colon cancer cells, $1,25(OH)_2D_3$ also induces the expression of the tight junction components occludin, claudin-1, claudin-2, claudin-7, claudin-12, *zonula occludens*- (ZO-) 1 and ZO-2, the desmosomal protein plectin, the focal adhesion members integrin α_3 and paxillin, the constituent of intermediate filaments keratin-13, and proteins associated with the actin cytoskeleton such as vinculin, filamin A, and ezrin [26, 27, 29–31, 49, 50]. Interestingly, $1,25(OH)_2D_3$ downregulates cadherin-17 [72], which induces cell proliferation and has protumoral and prometastatic effects in colon cancer cells [73].

Treatment of the $Apc^{min/+}$ colon cancer mouse model with $1,25(OH)_2D_3$ or analogs reduces polyp number and load, while it increases E-cadherin levels and reduces β-catenin nuclear localization and the expression of the β-catenin target genes *Tcf1*, *Myc*, and *Cd44* in the small intestine and colon [32]. Conversely, *Vdr* deficiency in $Apc^{min/+}$ mice enhances tumor size and the activity of the Wnt/β-catenin pathway in the lesions [65, 74]. Similar results were observed in other mouse and rat models of colon dysplasia, colon cancer, and colitis-associated neoplasia when treated with vitamin D_3 or $1,25(OH)_2D_3$ analogs [33, 63, 64]. Moreover, $1,25(OH)_2D_3$ increases and restores the normal level of Zo-1, occludin, and claudin-1 proteins in the colonic epithelium of the dextran sulfate sodium- (DSS-) induced colitis mouse model, protecting mice from intestinal mucosa injury and epithelial barrier disruption [27]. Conversely, *Vdr* deficiency potentiates DSS effects in this model, as DSS-treated $Vdr^{-/-}$ mice have severely disrupted and opened tight junctions and desmosomes in the colonic epithelium and develop more severe colitis than wild-type animals [29]. These data indicate that $1,25(OH)_2D_3$ contributes to the homeostasis and healing capacity of the colonic epithelium by preserving the stability and structural integrity of tight junctions [75]. Remarkably, a randomized, double-blind, placebo-controlled clinical trial showed that daily treatment of colorectal adenoma patients with 800 IU of vitamin D_3 for 6 months increases E-cadherin expression in normal-appearing rectal mucosa [34, 76].

In addition to colon cancer, E-cadherin is induced by $1,25(OH)_2D_3$ or analogs in normal mammary and bronchial epithelial cells and in tumor cell lines derived from breast, prostate, non-small cell lung, and squamous cell carcinomas, usually associated with an increase in epithelial differentiation, a reduction in cell migration and invasion, and the inhibition of Wnt/β-catenin signaling [35–41, 61, 77, 78]. We have described that the mechanism of E-cadherin induction by $1,25(OH)_2D_3$ in human colon cancer cells is transcriptional indirect and requires the transient activation of the RhoA-ROCK-p38MAPK-MSK1 signaling pathway [26, 31]. Phosphatidylinositol 5-phosphate 4-kinase type II β is also needed for E-cadherin induction by $1,25(OH)_2D_3$ in colon cancer cells [79]. In agreement with the transcriptional regulation, Lopes et al. showed that $1,25(OH)_2D_3$ treatment causes partial demethylation of CpG sites of *CDH1* promoter in MDA-MB-231 triple-negative breast cancer cells [78].

Table 1: List of $1,25(OH)_2D_3$-regulated proteins involved in EMT.

Protein	$1,25(OH)_2D_3$ effect	Reference
Tight junction components		
Occludin	Upregulation	[26–28]
Claudin-1	Upregulation	[27, 29]
Claudin-2	Upregulation	[29, 30]
Claudin-7	Upregulation	[31]
Claudin-12	Upregulation	[30]
ZO-1	Upregulation	[26, 27, 29]
ZO-2	Upregulation	[26]
Adherens junction proteins		
E-cadherin	Upregulation	[26, 29, 31–46]
N-cadherin	Downregulation	[37, 41, 42, 47, 48]
P-cadherin	Downregulation	[37]
Vinculin	Upregulation	[26, 31]
Focal adhesion members		
Integrin α_3	Upregulation	[31]
Integrin α_V	Upregulation	[37]
Integrin β_5	Upregulation	[37]
Integrin α_6	Downregulation	[37]
Integrin β_4	Downregulation	[37]
Paxillin	Upregulation	[31, 37]
FAK	Upregulation	[37]
Cytoskeleton-related proteins		
Filamin A	Upregulation	[49]
Ezrin	Upregulation	[50]
α-SMA	Downregulation	[37, 43–46, 51–54]
Keratin-13	Upregulation	[49]
Vimentin	Downregulation	[40–42]
Plectin	Upregulation	[49]
Extracellular matrix proteins		
Fibronectin	Downregulation	[44, 45, 51, 54]
Collagen type I	Downregulation	[44, 45, 51, 53–57]
Collagen type II	Downregulation	[56]
Collagen type III	Downregulation	[44, 51, 54, 58]
MMPs and inhibitors		
MMP2	Downregulation	[39, 41, 42]
MMP9	Downregulation	[39, 41, 42, 59, 60]
MMP13	Downregulation	[48, 61]
TIMP1	Upregulation	[59, 60]
TIMP2	Upregulation	[59]
EMT-TFs		
SNAIL1	Downregulation	[40–42, 44, 61, 62]
SNAIL2	Downregulation	[42, 61, 62]
ZEB1	Downregulation	[40]
TWIST1	Downregulation	[61]
Wnt/β-catenin target genes		
MYC	Downregulation	[26, 32, 63, 64]
TCF1	Downregulation	[26, 32]
CD44	Downregulation	[26, 32]
Cyclin D1	Downregulation	[31, 33, 55, 64]
AXIN2	Downregulation	[65]
LEF1	Downregulation	[65]
Other EMT-related proteins		
JMJD3	Upregulation	[66]
Cystatin D	Upregulation	[67]
Cathepsin L	Downregulation	[68]
Sprouty-2	Downregulation	[69]
PIT1	Downregulation	[70]
IL-1β	Downregulation	[71]
TGF-β	Downregulation	[44, 54, 56, 58]
TGF-β receptor type I	Downregulation	[44]
Cadherin-17	Downregulation	[72]

Moreover, protein kinase C inhibitors block E-cadherin, P-cadherin, α-catenin, and vinculin translocation to cell-cell contacts and the assembly of adherens junctions promoted by $1,25(OH)_2D_3$ in cultured human keratinocytes [80].

We reported that $1,25(OH)_2D_3$ induces cell adhesion, inhibits cell migration and invasion, and profoundly affects the phenotype of human breast cancer cells [37]. It promotes the formation of focal adhesions by increasing the expression of integrin α_V, integrin β_5, paxillin, and focal adhesion kinase (FAK) proteins and also by inducing FAK phosphorylation. Additionally, $1,25(OH)_2D_3$ reduces the expression of the mesenchymal marker N-cadherin and the myoepithelial proteins P-cadherin, integrin α_6, integrin β_4, and α-smooth muscle actin (α-SMA). Thus, $1,25(OH)_2D_3$ reverts the myoepithelial features that are associated with more aggressive and lethal forms of human breast cancer [37]. Likewise, N-cadherin expression is strongly suppressed by $1,25(OH)_2D_3$ in mouse osteoblast-like cells [47]. In line with these data, $1,25(OH)_2D_3$ treatment blocks the EMT-associated cadherin switch (from E-cadherin to N-cadherin) in pancreatic cancer cells [42]. Notably, $1,25(OH)_2D_3$ enhances corneal epithelial barrier function as corneal epithelial cells treated with $1,25(OH)_2D_3$ show increased occludin levels, reduced permeability, and elevated transepithelial resistance, a measure of the functional integrity of tight junctions [28].

3.2. $1,25(OH)_2D_3$ Inhibits the Expression of EMT-TFs. We and others have reported that $1,25(OH)_2D_3$ regulates the expression of certain transcription factors that induce EMT and of several modulators of the epithelial phenotype that can influence the expression of the EMT inducers (Table 1). $1,25(OH)_2D_3$ increases by a transcriptional indirect mechanism the expression of Jumonji Domain Containing 3 (JMJD3), a histone H3 lysine 27 demethylase with putative tumor suppressor activity. JMJD3 mediates the induction of a highly adhesive epithelial phenotype, the antiproliferative effect, the gene regulatory action, and the antagonism of the Wnt/β-catenin pathway promoted by $1,25(OH)_2D_3$ in human colon cancer cells [66]. Moreover, *JMJD3* depletion upregulates SNAIL1, ZEB1, and ZEB2, increases the expression of the mesenchymal markers fibronectin and LEF1, and downregulates

the epithelial proteins E-cadherin, claudin-1, and claudin-7. Accordingly, *JMJD3* and *SNAIL1* RNA expression correlate inversely in samples from human colon cancer patients [66]. The induction of ZEB1 by *JMJD3* depletion is associated with the downregulation of *miR-200b* and *miR-200c*, two microRNAs that target *ZEB1* RNA and inhibit ZEB1 protein expression [81].

$1,25(OH)_2D_3$ directly induces the expression of cystatin D, an inhibitor of cysteine proteases of the cathepsin family encoded by *CST5* gene. The binding of VDR to the *CST5* promoter induced by $1,25(OH)_2D_3$ is accompanied by the release of the NCOR2 corepressor and an increase in histone H4 acetylation [67]. We found that cystatin D mediates the antiproliferative and prodifferentiation action of $1,25(OH)_2D_3$ in human colon cancer cells. In addition, ectopic cystatin D expression inhibits proliferation, migration, anchorage-independent growth, and the Wnt/β-catenin pathway in cultured colon cancer cells and reduces tumor development in xenografted mice [67]. Cystatin D represses SNAIL1, SNAIL2, ZEB1, and ZEB2, whereas it induces the expression of E-cadherin and other adhesion proteins such as occludin and p120-catenin. Accordingly, cystatin D and E-cadherin protein expression directly correlate in human colorectal cancer, and loss of cystatin D is associated with poor tumor differentiation [67]. Notably, transcriptomic and proteomic studies comparing cystatin D-overexpressing and mock-transfected human colon cancer cells indicated that "cell adhesion, cell junction, and cytoskeleton" is one of the gene categories that englobes more cystatin D-regulated genes and proteins [82]. Remarkably, Swami et al. showed that the expression of cathepsin L, whose activity is inhibited by cystatin D, is downregulated by $1,25(OH)_2D_3$ in breast cancer cells [68], while Zhang et al. described that silencing of cathepsin L suppresses the cell invasion and migration, the actin cytoskeleton remodeling, and the increase in SNAIL1 expression associated with TGF-β-promoted EMT in breast and lung cancer cells [83].

$1,25(OH)_2D_3$ reduces the expression of Sprouty-2, an intracellular modulator of growth factor tyrosine kinase receptor signaling involved in the regulation of cell growth, migration, and angiogenesis [69]. Sprouty-2 strongly inhibits the induction of intercellular adhesion and E-cadherin protein expression promoted by $1,25(OH)_2D_3$, and gain- and loss-of-function experiments indicate that Sprouty-2 and E-cadherin repress each other in colon cancer cells. Accordingly, the protein expression levels of Sprouty-2 and E-cadherin correlate inversely in cultured and xenografted colon cancer cells and in biopsies from human colon cancer patients. In line with this, we found that Sprouty-2 induces ZEB1 expression without affecting ZEB2, SNAIL1, or SNAIL2 levels [69]. ZEB1 upregulation by Sprouty-2 results from the induction of the transcription factor ETS1 and the repression of several microRNAs (*miR-200* family and *miR-150*) that target *ZEB1* RNA. Through ZEB1 upregulation, Sprouty-2 represses E-cadherin, claudin-7, occludin, the tight junction modulator matriptase, the cell adhesion molecule EPCAM, and the epithelial splicing regulatory protein ESRP1 that inhibits EMT [84]. Taken together, these data point to Sprouty-2 as a potent inhibitor of the epithelial phenotype that is downregulated by $1,25(OH)_2D_3$ in colon carcinoma cells.

Recently, effects of $1,25(OH)_2D_3$ on the expression of several EMT-TFs have been described. $1,25(OH)_2D_3$ inhibits SNAIL1 and ZEB1 expression in non-small cell lung carcinoma cells, accompanied by an increase in E-cadherin expression, vimentin downregulation, maintenance of the epithelial morphology, and inhibition of cell migration [40]. The low calcemic $1,25(OH)_2D_3$ analog MART-10 inhibits EMT and cell migration and invasion in breast and pancreatic cancer cells through the downregulation of SNAIL1 and SNAIL2. In addition, MART-10 inhibits TWIST1 expression in breast cancer cells [42, 61]. Accordingly, Findlay et al. reported the inhibition of SNAIL1 and SNAIL2 by $1,25(OH)_2D_3$ in human colon cancer cells [62]. Kaler et al. found that colon cancer cells stimulate tumor-associated macrophages to secrete interleukin- (IL-) 1β, which in turn promotes Wnt/β-catenin signaling, stabilizes SNAIL1 protein, and confers resistance to TRAIL-induced apoptosis in colon cancer cells [71]. They also found that $1,25(OH)_2D_3$, by inhibiting the release of IL-1β by macrophages, downregulates SNAIL1 protein expression in colon cancer cells [71]. Similarly, Zhang et al. showed that tumor-associated macrophages induce EMT in breast cancer cells and that high VDR expression in cancer cells abrogates the macrophage-promoted E-cadherin loss, α-SMA upregulation, and increase in cell migration and invasion [43]. Furthermore, $1,25(OH)_2D_3$ attenuates the enhancing effect of TGF-β1 on cell motility and on SNAIL1, N-cadherin, and vimentin expression in human bronchial epithelial cells [41] and inhibits the TGF-β1-stimulated EMT in rat lung epithelial cells [51].

Matrix metalloproteases (MMPs) are a family of zinc-dependent proteases that degrade components of the extracellular matrix and basement membrane. MMPs are regulated by the action of specific inhibitors: the tissue inhibitors of metalloproteases (TIMPs). Increased MMP activity is often associated with the EMT and confers invasive properties to cancer cells. Consistently with its inhibitory effect on EMT, $1,25(OH)_2D_3$ downregulates the secretion of MMP2, MMP9, and MMP13 in prostate, breast, pancreatic, and squamous cell carcinoma cells and increases TIMP1 and TIMP2 activity in prostate and breast cancer cells [39, 42, 48, 59–61]. In addition, $1,25(OH)_2D_3$ reduces the increase in MMP2 and MMP9 induced by TGF-β1 in human bronchial epithelial cells [41]. Through these mechanisms, $1,25(OH)_2D_3$ inhibits the capacity of cancer cells to degrade the extracellular matrix and invade the surrounding tissue and may thus reduce tumor cell metastatic potential. Remarkably, several studies from Pérez-Fernández's group have demonstrated that $1,25(OH)_2D_3$ represses the expression of the gene encoding the pituitary transcription factor 1 (PIT1) in breast cancer cells and that *PIT1* silencing downregulates SNAIL1, MMP1, and MMP13 proteins [70, 85, 86]. In agreement with this, high PIT1 protein expression correlates with elevated MMP1 and MMP13 levels, SNAIL1 protein expression, and presence of distant metastasis in invasive ductal breast carcinoma [85, 86].

Recent studies have established a link between the induction of EMT and the acquisition by epithelial cells of molecular and functional traits of stem cells. As stem cells can both self-renew and differentiate, these stemness-related

properties confer tumor-initiating capacities to carcinoma cells that could be crucial for cancer cell survival during dissemination and for the establishment by the disseminated cancer cells of metastatic foci at anatomically distant sites [19–25]. Interestingly, Pervin et al. found that manipulation of VDR levels modulates the expression of key EMT-related proteins and dictates the stem cell characteristics of breast cancer cells. Thus, *VDR* overexpression in these cells upregulates E-cadherin, downregulates SNAIL1, TWIST1, and MMP9, and reduces cell ability to form mammospheres, an attribute of breast normal and cancer stem cells. Conversely, *VDR* silencing has the opposite effect [87].

3.3. 1,25(OH)$_2$D$_3$ Inhibits Fibrosis. In addition to cancer progression, EMT is reactivated in adult life during other pathological processes such as organ fibrosis. This process occurs in certain epithelial tissues after trauma or inflammatory injury and is characterized by excessive deposition of extracellular matrix and increased fibrous connective tissue. In this context, the EMT is part of the repair program and originates fibroblasts and other related cells for tissue regeneration. However, the disease usually progresses and the organ is finally composed mainly of activated fibroblasts and extracellular matrix, which may eventually lead to organ failure. The EMT inducer TGF-β is also involved in the fibrotic process [17, 23, 88]. Several studies from Liu's group showed that vitamin D compounds attenuate renal interstitial fibrosis by inhibiting EMT in tubular epithelial cells. These compounds decrease collagen and fibronectin deposition, downregulate the expression of SNAIL1, α-Sma, β-catenin, TGF-β1, and its type I receptor, reduce β-catenin nuclear localization, and preserve Vdr and E-cadherin levels in kidneys from an obstructive nephropathy mouse model that develops interstitial fibrosis. Moreover, treatment with vitamin D compounds or *VDR* overexpression in human renal proximal tubular epithelial cells abolishes the EMT promoted by TGF-β1, while *VDR* silencing has a sensitizing effect [44, 45, 89]. Interestingly, combination of the 1,25(OH)$_2$D$_3$ analog paricalcitol with trandolapril, an inhibitor of the angiotensin-converting enzyme used as standard treatment for chronic kidney disease, leads to additive reduction of renal fibrosis in the obstructive nephropathy mouse model [90]. Additionally, Nolan et al. indicated that paricalcitol inhibits TGF-β1-induced tubular EMT also under the hypoxic conditions commonly associated with chronic kidney disease [46], and Kim et al. reported that it attenuates the tubular EMT exogenously induced by 4-hydroxy-2-hexenal, an aldehyde product of lipid peroxidation [52].

Beneficial effects of 1,25(OH)$_2$D$_3$ have also been reported in liver fibrosis. Hepatic stellate cells play a central role in liver fibrosis as upon injury-induced activation they proliferate and secrete many extracellular matrix components. Vitamin D compounds reduce extracellular matrix deposition and ameliorate liver fibrosis in rat and mouse models [55, 56]. In addition, 1,25(OH)$_2$D$_3$ suppresses cell proliferation and downregulates cyclin D1 and α_1 type I collagen expression in cultured hepatic stellate cells [55]. Potter et al. reported that the downregulation of α_1 type I collagen by 1,25(OH)$_2$D$_3$ is mediated by VDR binding to a proximal Sp1 site and a distal vitamin D response element in the human *COL1A1* gene promoter [57]. Notably, a study from Ding et al. revealed that ligand-activated VDR antagonizes TGF-β1-dependent transcription of profibrotic genes in hepatic stellate cells [56]. TGF-β1 changes ligand activated-VDR binding sites in the genome promoting VDR binding to SMAD3 sites in the regulatory regions of profibrotic genes, which decreases SMAD3 occupancy at these sites causing transcriptional silencing of the genes and inhibiting fibrosis [56]. Thus, VDR ligands limit fibrosis by modulating the tissue response to TGF-β1. Ito et al. described that a similar mechanism takes place in renal fibrosis and showed that the C-terminal α-helix 12 of the ligand-binding domain of VDR is necessary for the interaction with SMAD3 and the suppression of TGF-β pathway [53]. Furthermore, they designed VDR ligands that selectively inhibit TGF-β signaling without activating VDR-mediated transcription and significantly attenuate renal fibrosis in mice without hypercalcemic effects [53]. Another mechanism involved in the inhibition of TGF-β pathway by vitamin D analogs in renal fibrosis has been described: maxacalcitol blocks the autoinduction of TGF-β1 expression through the recruitment of a complex between VDR and the SMAD3 phosphatase PPM1A to the *TGFB1* promoter, causing SMAD3 dephosphorylation and release from the promoter and, consequently, attenuating *TGFB1* gene expression [54].

Peritoneal dialysis induces changes in mesothelial cells that are reminiscent of those occurring during EMT, and that may finally lead to the development of fibrosis. Vitamin D compounds prevent the progression of peritoneal fibrosis in mouse and rat models and inhibit the TGF-β1-induced EMT-like process in human peritoneal mesothelial cells [58, 91]. Thus, a large body of evidence indicates that vitamin D compounds protect against organ fibrosis in different tissues by inhibiting EMT and/or TGF-β profibrotic action.

4. The Transcription Factors SNAIL1 and SNAIL2 Repress *VDR* Gene Expression and Inhibit 1,25(OH)$_2$D$_3$ Action

Cell responsiveness to 1,25(OH)$_2$D$_3$ mainly relays on VDR expression levels. VDR protein is expressed in almost all normal human cell types and tissues, and also in cancer cell lines and tumors of several origins [8, 92]. Remarkably, elevated VDR expression is associated with high tumor differentiation, absence of node involvement, and good prognosis in colon cancer [93–95], with lower tumor grade, late development of lymph node metastases, and longer disease-free survival in breast cancer [43, 96–98], and with improved overall survival in prostate and non-small cell lung cancer and melanoma [99–101]. However, certain cancer cell lines do not express VDR and are unresponsive to 1,25(OH)$_2$D$_3$. Accordingly, VDR downregulation has been observed in a proportion of melanomas and colon, breast, lung, and ovarian tumors [43, 94, 99, 102–104], which may jeopardize the response to therapy with vitamin D, 1,25(OH)$_2$D$_3$, or its analogs.

These lines of evidence prompted us to study the mechanisms responsible for VDR downregulation in cancer. We found that SNAIL1 represses the expression of VDR by binding to three E-boxes in the human *VDR* gene promoter.

Moreover, SNAIL1 reduces *VDR* RNA half-life [105]. As a result, SNAIL1 overexpression in human colon cancer cells blocks the induction of E-cadherin expression and the acquisition of an epithelial phenotype promoted by $1,25(OH)_2D_3$. Consequently, β-catenin is not relocated from the nucleus to the plasma membrane adherens junctions and the Wnt/β-catenin signaling remains active. SNAIL1 also abrogates the inhibitory effect of $1,25(OH)_2D_3$ on cell proliferation and migration in cultured cells and the antitumoral action of the $1,25(OH)_2D_3$ analog EB1089 in xenografted mice [105, 106]. Consistently, Knackstedt et al. have shown that the downregulation of *Vdr* observed in the colon of DSS-induced colitis mouse model is associated with an increase in the expression of SNAIL1 and its upstream regulator tumor necrosis factor- (Tnf-) α [107].

In addition to SNAIL1, we reported that its family member SNAIL2 represses *VDR* gene expression through the same E-boxes in the human *VDR* gene promoter and blocks the induction of an epithelial phenotype by $1,25(OH)_2D_3$ in human colon cancer cells. Moreover, SNAIL1 and SNAIL2 show an additive repressive effect on *VDR* gene promoter [108]. Remarkably, *SNAIL1* and/or *SNAIL2* RNA upregulation was detected in 76% of colon tumors and significantly correlated with diminished *VDR* RNA expression. Indeed, the lowest *VDR* RNA levels were observed in those colon tumors that overexpress both EMT-TFs [95, 105, 108]. We also showed that *SNAIL1* RNA overexpression in colon tumors diminishes *VDR* RNA expression in the histologically normal tissue adjacent to the tumor, suggesting that SNAIL1-expressing colon cancer cells secrete signals that modulate VDR expression in neighboring cells [109].

The repression of *VDR* gene by SNAIL factors is not exclusive to colon cancer. It has been shown that SNAIL1 and SNAIL2 downregulate *VDR* gene expression and abrogate the antitumoral action of $1,25(OH)_2D_3$ in human osteosarcoma and breast cancer cells [110, 111]. The two proximal E-boxes of the human *VDR* gene promoter are conserved in rat and mouse, while the most distal box is only partially conserved with one base substitution. Bai et al. found SNAIL1 binding only to the most proximal E-box of the rat *Vdr* promoter accompanied by deacetylation of histone H3 in samples from rat intestine and kidney. Accordingly, an inverse correlation between SNAIL1 and *Vdr* levels was observed in those tissues [112]. de Frutos et al. showed that the sustained activation of SNAIL1 in transgenic mice represses *Vdr* gene expression in osteoblasts. This downregulation blocks the Vdr-mediated induction of the osteoclast differentiation factor Rankl and inhibition of osteoprotegerin, a decoy Rankl receptor that inhibits osteoclastogenesis. Thus, *Vdr* gene downregulation by SNAIL1 in osteoblasts reduces the osteoclast population due to an impaired osteoclastogenesis. In addition, chromatin immunoprecipitation assays indicated that *Vdr* gene repression in mouse osteoblasts is mediated by SNAIL1 binding to the two proximal E-boxes of murine *Vdr* promoter [113].

VDR downregulation takes place also and contributes to E-cadherin loss during the EMT promoted by the proinflammatory cytokine TNFSF12 in renal tubular epithelial cells [114]. Similarly, VDR repression by TNF-α sensitizes breast cancer cells to TGF-β1-induced EMT. Of note, $1,25(OH)_2D_3$ treatment protects against TNF-α-induced VDR loss, suppresses TGF-β1-promoted increase in the migration capacity of cultured breast cancer cells, and inhibits lung metastasis in an orthotopic breast cancer mouse model [43]. Conversely, the MET induced by the enforced reexpression of the putative tumor suppressor KLF4 in hepatocellular carcinoma cells was accompanied by VDR upregulation and an increase in the inhibitory effect of $1,25(OH)_2D_3$ on cell proliferation. As a result, KLF4 and VDR protein expression correlate directly in human hepatocellular carcinoma [115].

Other EMT-TFs such as ZEB1, ZEB2, TWIST1, or E47 have no effect on the expression of human *VDR* gene promoter in SW480-ADH human colon cancer cells [108]. However, Lazarova et al. reported that ZEB1 binds to two distal E-boxes in the murine *Vdr* promoter and activates its expression in COS-7 monkey kidney fibroblasts and SW620 human colon cancer cells, but not in human LNCaP prostate or HCT116 colon cancer cells [116]. Other studies showed absence of correlation or a significant direct correlation between *ZEB1* and *VDR* RNA expression in colon cancer [95, 109, 117]. Furthermore, Peña et al. observed that such direct correlation was stronger in colon tumors with high level of the transcriptional coactivator p300 [117]. Globally, these data suggest a cell- and context-dependent positive regulation of VDR by ZEB1.

5. Conclusions and Perspectives

Cell fate and phenotype are strictly regulated by extracellular signals. $1,25(OH)_2D_3$ and EMT-TFs have opposite effects on epithelial cell phenotype and they antagonize each other (Figure 1). $1,25(OH)_2D_3$ induces epithelial differentiation while it inhibits the expression of several EMT inducers. Conversely, expression of key EMT-TFs in epithelial cells promotes the acquisition of a mesenchymal phenotype, which in the case of SNAIL1 and SNAIL2 is associated with *VDR* gene repression and the blockade of $1,25(OH)_2D_3$ action on epithelial differentiation. Thus, a double negative feedback loop operates between $1,25(OH)_2D_3$ and EMT inducers that may contribute to the complete acquisition of the phenotype dictated by the extracellular cues. The loop may first amplify the signal and later stabilize cell fate once the process is completed. Hence, the balance between $1,25(OH)_2D_3$/VDR and SNAIL family of transcription factors determines cell fate, and its imbalance may explain the reversibility of the EMT process. Of note, the transition between epithelial and mesenchymal phenotypes is also governed by similar double negative feedback loops among EMT-TFs and certain microRNAs, such as the ZEB/*miR-200* and the SNAIL1/*miR-34* regulatory circuits [22, 118–120].

The implication of EMT in cancer progression and organ fibrosis and the inhibitory effect of $1,25(OH)_2D_3$ on EMT have opened the possibility of a therapeutic use of VDR agonists against these diseases. However, the downregulation of VDR expression found in several types of cancer, frequently associated with advanced stages of the disease, limits the applicability of vitamin D compounds to prevention in high-risk populations and treatment in patients at early stages of tumor progression. In addition, EMT is a transient event during tumorigenesis, and it has been proposed that the reverse

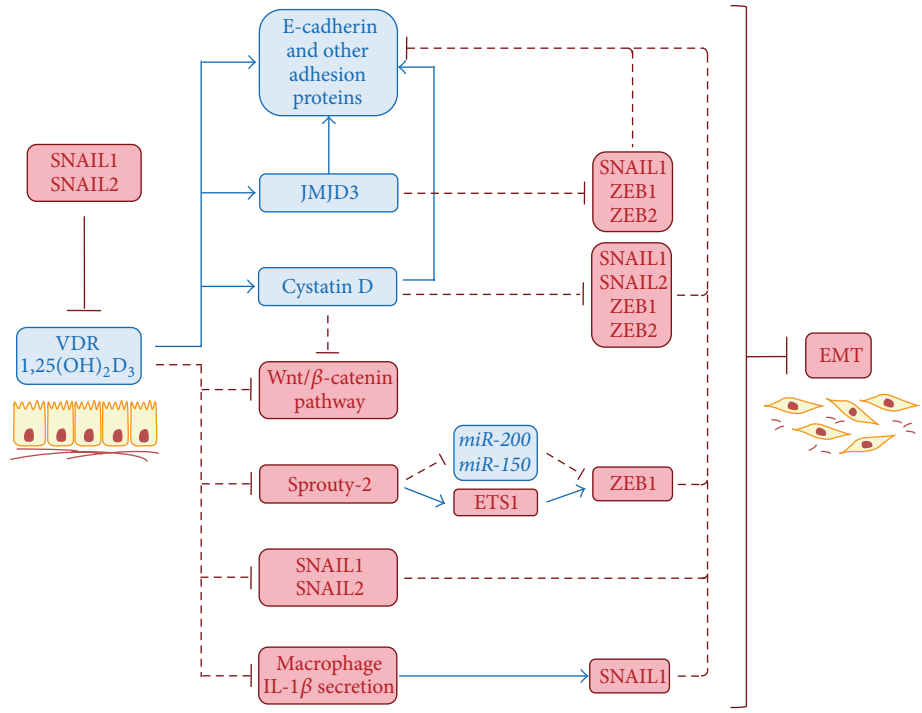

FIGURE 1: Scheme showing the mechanisms involved in the reciprocal regulation between $1,25(OH)_2D_3$ and EMT in human colon cancer cells. Proteins and pathways displayed in blue are associated with an epithelial phenotype, while those shown in red are related with a mesenchymal phenotype. Blue and red lines are used to indicate induction or repression, respectively.

process (MET) is required for the establishment of metastasis at distant sites [121, 122]. These lines of evidence have led to controversy about anticancer therapeutic strategies designed to inhibit EMT, as they may favor the formation of metastases, and suggest that these therapies may be limited to patients diagnosed at early stages of the disease to prevent invasion and dissemination [23]. Nevertheless, vitamin D compounds as inhibitors of EMT may be interesting therapeutic agents for fibrosis-associated pathologies, in which the EMT process is not reverted and the mesenchymal phenotype is maintained during disease progression.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Endothelial Transdifferentiation of Tumor Cells Triggered by the Twist1-Jagged1-KLF4 Axis: Relationship between Cancer Stemness and Angiogenesis

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Tumor hypoxia is associated with malignant biological phenotype including enhanced angiogenesis and metastasis. Hypoxia increases the expression of vascular endothelial cell growth factor (VEGF), which directly participates in angiogenesis by recruiting endothelial cells into hypoxic area and stimulating their proliferation, for increasing vascular density. Recent research in tumor biology has focused on the model in which tumor-derived endothelial cells arise from tumor stem-like cells, but the detailed mechanism is not clear. Twist1, an important regulator of epithelial-mesenchymal transition (EMT), has been shown to mediate tumor metastasis and induce tumor angiogenesis. Notch signaling has been demonstrated to be an important player in vascular development and tumor angiogenesis. KLF4 (Krüppel-like factor 4) is a factor commonly used for the generation of induced pluripotent stem (iPS) cells. KLF4 also plays an important role in the differentiation of endothelial cells. Although Twist1 is known as a master regulator of mesoderm development, it is unknown whether Twist1 could be involved in endothelial transdifferentiation of tumor-derived cells. This review focuses on the role of Twist1-Jagged1/Notch-KLF4 axis on tumor-derived endothelial transdifferentiation, tumorigenesis, metastasis, and cancer stemness.

1. Introduction

Metastasis and angiogenesis are among the hallmarks of malignant behavior of cancer cells. Cancer metastasis has been shown to be responsible for the majority of cancer-related deaths. It is established that survival rate of cancer patient is low during metastatic stage [1]. Metastasis proceeds through the progressive acquisition of traits that allow malignant cells originating in one organ to disseminate and colonize a secondary site. Metastasis is a multistep process that divides into several steps: loss of cellular adhesion, increased motility and invasiveness, entry and survival in the circulation, exit into new tissue, and eventual colonization in a distant site [2]. A developmental program termed epithelial–mesenchymal transition (EMT) has been shown to play a critical role in promoting metastasis by enhancing cancer cell motility and dissemination. Activation of EMT is considered essential to allow cancer cells to lose cell-cell junctions and dissociate from each other for single-cell migration and invasion [3]. Moreover, gene expression patterns in human cancers indicated that cancer cells combine EMT properties with a stem-cell-like phenotype [4]. A direct molecular link between EMT and stemness has demonstrated that the EMT activator, Twist1, can coinduce EMT and stemness properties [5]. Furthermore, induction of EMT in more-differentiated cancer cells can generate CSC-like cells, providing an association between EMT, CSCs, and drug resistance [6, 7]. Increasing evidence suggests that EMT plays an important role in therapeutic resistance. For example, in *EGFR* mutated non-small cell lung cancers (NSCLC), EMT has been associated with acquired resistance to EGFR inhibitors [8]. EMT also contributes to drug resistance to 5-FU in pancreatic cancer and colon cancer [9, 10]. Due to the clinical importance of the EMT-induced processes, inhibition of EMT is an attractive therapeutic approach that could have a significant effect on disease outcome.

The generation of new capillaries from preexisting blood vessels is called angiogenesis. The angiogenesis process takes

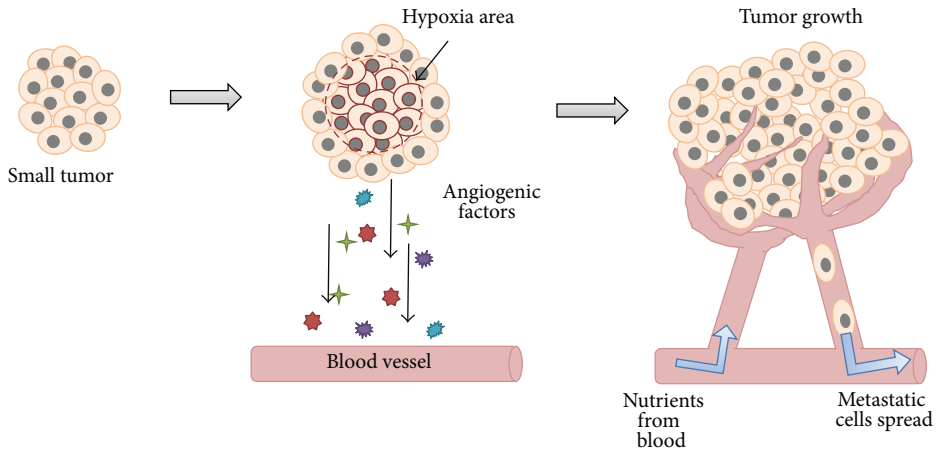

Figure 1: Angiogenesis is the process through which new blood vessels form and grow. Tumor cells activated by a lack of oxygen (or a gene mutation) release, among other things, angiogenic factors that attract inflammatory and endothelial cells and promote their proliferation. The endothelial cells that form existing blood vessels respond to angiogenic signals in their vicinity by proliferating and secreting proteases, which break open the blood vessel wall to enable them to migrate toward the tumor site. Proliferating endothelial cells then organize themselves into new capillary tubes by altering the arrangement of their adherence-membrane proteins. Finally, the capillaries provide a continuous blood flow that sustains tumor cell metabolism and sets up escaping avenues for metastatic tumor cells.

place during embryogenesis and in the adult, for example, in the female reproductive system and wound healing. Additional angiogenesis occurs in pathological conditions such as cancer, macular degeneration, psoriasis, and rheumatoid arthritis [11, 12]. Angiogenesis and tumor progression are very closely linked to each other. Tumor cells are dependent on angiogenesis because their growth and expansion require oxygen and nutrients, which are made available through the angiogenic vasculature (Figure 1). In 1971, Folkman proposed that an alteration in the blood supply can noticeably affect the tumor growth and its metastasis, which led to the idea that blocking tumor angiogenesis could be one of the strategies to prevent tumor cells spreading [13–15]. Tumor stem-like cells belong to a subpopulation of tumor cells that have acquired the stemness properties associated with normal stem cells. Cancer stemness property has been used to explain cancer initiation, progression, recurrence, and resistance to chemotherapy or radiation therapy. Recent research in tumor biology has focused on the model in which tumor-derived endothelial cells can arise from tumor stem-like cells [16–18], but the detailed mechanism is not clear. Furthermore, the evidence showed that about 70% of endothelial cells from the inner portion of the tumor were tumor-derived endothelial cells which were stained by human-specific antibody, whereas nearly all the endothelial cells in the tumor capsule were recruited from preexisting vessels which were stained by mouse-specific antibody inside glioblastoma xenografts [16].

Twist1, a basic helix-loop-helix (bHLH) transcription factor, is characterized by a basic DNA binding domain that targets the consensus E-box sequence 5′-CANNTG-3′ [19]. Consistently, bHLH members are transcription factors acting in various differentiation processes, as either positive or negative regulators, and play key roles in different developmental events like neurogenesis and myogenesis [20]. Twist initiates *Drosophila* mesoderm development and results in the formation of heart, somatic muscle, and other cell types [19]. Recent evidence implicates that Twist1 gene is overexpressed in a large of human tumors including a variety of carcinomas as well as sarcomas, melanomas, glioma, and neuroblastoma [21]. Functional studies have indicated that Twist1 may play a major role in tumor promotion and progression, by inhibiting differentiation, interfering with the p53 tumor suppressor pathway and favoring cell survival, and inducing epithelial-mesenchymal transition (EMT) [22].

Here, we discuss the relationship of the EMT regulator, Twist1, cancer stemness, and tumor angiogenesis. We also review the new role of Twist1 in angiogenesis and new downstream targets of Twist1.

2. Cancer Stemness and Angiogenesis

Cancer arises from cells accruing multiple mutations which initiate uncontrolled proliferation or resistance to apoptosis by both genetic and epigenetic aberration within unique microenvironments. Moreover, these cells, so-called cancer stem-like cells (CSCs), obtain self-renewing ability as stem-cell-like properties [23]. Some of the pathways activated in CSCs just like in normal stem cells are Notch, Hedgehog, and Wnt/β-catenin [24]. They also share similar gene and epigenetic profiles and express related surface and functional markers in different tumors, such as CD44, CD133, ALDH1, Sca1, and ABCG2. Some of these genes or markers also have been proposed for metastasis, angiogenesis, drug resistance, and tissue differentiation [25].

Cancer stem cells are well known for their greater potential of tumor initiation and formation than non-stem tumor cells. Recently, more and more reports support that CSCs, as well their self-renewal and proliferative capabilities, may promote tumor angiogenesis. First, in stem-cell-like glioma cells (SCLGC), Bao et al.'s group observed that the VEGF expression in CD133+ SCLGC was 10–20-fold upregulated, combined with a dramatically increased vascular

density identified by CD31 staining [26]. Then, Folkins et al.'s group also revealed that tumor with larger CSC population recruited a higher amount of endothelial progenitor cells (EPC), suggesting that CSCs promote tumor angiogenesis and EPC recruitment via stimulating VEGF and SDF-1 [27]. Recently, the evidences further showed that the presence of cancer-derived endothelial-like cells and suggested that the differentiation of cancer stem-like cells into endothelial cells might be mediated by vascular endothelial growth factor (VEGF) and Notch. These new findings provide new insight into the mechanisms of tumor neoangiogenesis [16, 18]. However, in order to discover the entire network of signals within CSCs and angiogenesis, more research is still needed.

3. Hypoxia-Induced EMT

Hypoxia is an important physiological factor that correlates with tumor progression including an increasing probability of recurrence, locoregional spread, and distant metastasis [28]. Furthermore, recent studies suggest that tumor hypoxia is associated with malignant biological phenotype such as angiogenesis, migration, invasion, and metastasis [29]. The key factor involved in adaptive responses to cellular hypoxia is HIF-1 and its activity is tightly regulated by the cellular oxygen tension [30]. HIF-1 is a heterodimeric protein that is composed of an O2-regulated HIF-1alpha subunit and a constitutively expressed HIF-1beta subunit. Both of them belong to the basic helix-loop-helix-per-arnt-sim (bHLH-PAS) family [31]. Hypoxia mediates EMT and metastasis. Twist1 is a direct gene target of HIF-1alpha and Twist1 mediates the invasion, migration, and metastatic activity of different cancer cell types, including head and neck (HNSCC), breast, and lung carcinoma [32].

4. Hypoxia-Induced Tumor Angiogenesis

Typically, tumor-associated angiogenesis goes through two phases: an avascular and a vascular phase that are separated by the "angiogenic switch." In the avascular phase, tumors are small and survive on diffusion of nutrients from the host microvasculature. In order for tumors to grow beyond 1-2 μm^3 [33], they need a continual supply of blood to supply nutrients and oxygen to overcome hypoxia and starvation. Hypoxia of tumor cells will occur if the tumor grows beyond the maximum distance of diffusion from local vessels around 200 μm [34]. When a condition such as hypoxia is present in the tumor tissue, the tumor cells receive the signal and promote the angiogenic switch and induce angiogenesis. In the case of hypoxia, the signal is mediated by hypoxia inducible factor-1 (HIF-1). HIF-1 binds to hypoxia-response elements (HREs) and activates a number of hypoxia-response genes such as VEGF. Thus hypoxia upregulates the expression of angiogenic factors, like VEGF, stromal derived factor 1 (SDF1), angiopoietin 2 (ANGPT2), placental growth factor (PGF), platelet-derived growth factor B (PDGFB), and stem cell factor (SCF) [35–40]. Receptor-ligand interaction activates these cells and promotes the recruiting endothelial cells into hypoxic area and stimulates their proliferation, for increasing vascular density [41].

5. Role of Twist1 in EMT and Angiogenesis

The mechanisms leading to the aberrant activation of Twist1 appear to be various and complex. They result from the deregulation of signaling pathways (e.g., transforming growth factor-beta (TGF-β), Wnt, and nuclear factor κB (NF-κB) signaling pathways) that normally mediate the expression of the genes during embryonic development [42]. Interestingly, stress conditions seem to control both the physiological and aberrant expression of Twist1. Hypoxic conditions are similarly defined as potent inducers of Twist1 expression in cancer cells, thereby promoting cell dissemination to other friendlier environment, presumably through its role in promoting the EMT and metastasis [32]. Besides EMT and metastasis, the recent finding provides a crucial link between less differentiated stem cells and the mesenchymal-appearing cells generated by EMTs [43]. Our results demonstrated that Twist-induced EMT and tumor-initiating capability in cancer cells occur through direct regulation of the polycomb group protein BMI1, which is involved in the self-renewal of neuronal, haematopoietic, and intestinal cells [5]. In addition, it was found that upregulation of Twist1 may play an important role in the angiogenesis of breast and hepatocellular carcinoma [44, 45]. But so far, the molecular mechanism of Twist1 gene on angiogenesis in human cancers remains unknown. The identification of downstream activators of Twist1 could provide valuable information about tumor angiogenesis and metastasis.

6. The Role of Notch Signaling Pathway in EMT and Angiogenesis

The Notch-signaling pathway is a cell-cell communication pathway that is evolutionarily conserved from *Drosophila* to human and modulates cell fate and differentiation [46–48]. To date, four different notch receptors (Notch1, Notch2, Notch3, and Notch4) and five different ligands (Jagged1 and Jagged2 and Delta-like-1, Delta-like-3, and Delta-like-4) have been identified in mammalian cells. Notch signaling is initiated when the extracellular domain of the Notch receptor binds their ligand on neighboring cells that are in close proximity to one another. This leads to a cascade of enzymatic cleavages and the Notch intracellular domain (NICD) is released and then translocated to the nucleus where it interacts with CSL (CBF1, Su(H), and Lag-2) transcriptional repressors and converts them to transcriptional activators.

Recently, it is believed that Notch signal pathway is a key regulator to induce EMT and endothelial-to-mesenchymal transition (EndMT) processes [49, 50]. Notch activation in endothelial cells results in morphological, phenotypic, and functional changes consistent with mesenchymal transformation. These changes not only include downregulation of endothelial markers (VE-cadherin, Tie1, Tie2, platelet-endothelial cell adhesion molecule-1, and endothelial NO synthase), but also upregulation of mesenchymal markers (α-SMA, fibronectin, and platelet-derived growth factor receptors) [51]. Moreover, Jagged1 stimulation in endothelial cells

also induced a similar mesenchymal transformation, suggesting that Jagged1 mediated activation of Notch signaling is important during the induction of EMT [51]. In EndMT and EMT processes, Notch cross-talks with several transcription and growth factors relevant to EMT, including Snail, Slug, TGF-β, FGF, and PDGF [52].

It is clear that the Notch family is critically important for the proper construction of the vascular system. Global as well as endothelium-specific knockouts of Notch receptors or ligands induce embryonic death with vascular defects [53–55]. These results suggest that Notch pathway components have also been shown to be required for postnatal angiogenesis. However, information about Notch signaling in tumor angiogenesis is limited. Notch signaling components are expressed in tumor endothelial cells, but the most notable component in this class is DLL4. It is known that DLL4 is upregulated in the vasculature of human xenografted tumors in mice and in human breast and kidney cancers [56]. Reduction of basal DLL4 level in ECs by siRNA led to the inhibition of multiple endothelial functions *in vitro* including proliferation, migration, and network formation, implying the potential role of this pathway in cancer [57]. In fact, blockade of DLL4-Notch signaling is an emerging therapeutic approach to inhibiting tumor angiogenesis [58–60]. Besides, recent findings suggest that the role of Jagged1 expression in head and neck squamous cell carcinoma and breast cancer can be diverse, influencing tumor cell growth, tumor angiogenesis, and/or the inflammatory response [61, 62].

7. The Role of KLF4 in EMT and Angiogenesis

KLF4 is member of the Sp1/KLF family, which are evolutionarily conserved zinc finger-containing transcription factors and function as regulators in diverse cell processes of cell growth, proliferation, and differentiation [63–65]. Earlier studies indicated that KLF4 is highly expressed in epithelial tissues including the gut and skin [66, 67]. Because KLF4 functions as an antiproliferative factor in differentiated epithelia, it seems that KLF4 might act as a tumor suppressor. In general, KLF4 seems to inhibit both EMT and invasion [68]. While loss of KLF4 function induces EMT-like morphological changes, forced expression of KLF4 in the highly metastatic MDA-MB-231 breast tumor cell line was sufficient to restore E-cadherin expression and suppress migration and invasion [69]. Furthermore, NFI-C, a member of the nuclear factor I (NFI) family of transcription factors, increased the expression of KLF4 and E-cadherin and led to a more pronounced epithelial cell phenotype. In contrast, NFI-C knockdown induced migration and invasion [70]. Notably, the research revealed that a number of mesenchymal genes, such as N-cadherin (Cdh2), vimentin (Vim), and β-catenin (Ctnnb1), are direct targets of KLF4 transcriptional repression by using a combinatorial approach of gene expression profiling and chromatin immunoprecipitation/deep sequencing (ChIP-Seq) analysis [71]. KLF4 significantly decreases lung and liver metastases in a murine model of mammary cancer [69, 72]. Indeed, loss of KLF4 occurs at early stages in the progression of gastric cancer [73, 74]. However, recent evidence suggests that KLF4 might also act as an oncogene in breast cancer, head and neck cancer (HNSCC), and pancreatic cancer [75–78]. It indicated that KLF4 expression and activity are altered in human cancers and KLF4 can be tumor suppressors or oncogenes depending on tissue, tumor type, or cancer stage.

It was found that overexpression of KLF4 along with Myc, Sox2, and Oct4 could transform mouse fibroblasts into the state resembling embryonic stem cells (ES cells). These cells have been termed "inducible pluripotent stem cells" (iPS cells) [79]. There are also some studies implying that KLF4 played an important role in the differentiation and function of endothelial and vascular smooth muscle cells [59, 80–82]. Furthermore, it is demonstrated that KLF4 can regulate sprouting angiogenesis and may be a therapeutic target in regulation of tumor angiogenesis [83].

8. Twist1 Induced Tumor-Derived Endothelial Differentiation

There are some evidences that glioblastoma stem-like cells differentiate into endothelial cells [16, 17], but the detailed molecular mechanisms are still unclear. We demonstrated that Twist1 overexpression in the HNSCC cell lines not only mediates the expression of the endothelial-specific markers including CD31 [84], CD144 [85], von Willebrand factor (vWF) [86], Tie2 [87], endoglin (CD105) [88], and intercellular adhesion molecule 1 (ICAM1) [89], but also exhibited obvious ability of capillary-like network formation and the ability of DiI-AcLDL (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanide perchlorate-labeled acetylated low density lipoproteins) uptake [90, 91]. It is a new vision that Twist1 can induce transdifferentiation of tumor cells into endothelial cells and promotion of tumor-derived vascular formation [18]. This observation of tumor-derived endothelial transdifferentiation is different from the traditional angiogenesis process contributed by sprouting and proliferation of formerly quiescent endothelial cells on nearby blood vessels and lymphatics that are triggered by soluble growth factors, cytokines, and proangiogenic factors secreted from tumor cell (Figure 1) [92]. Induction of tumor-derived endothelial differentiation by Twist1 was also different from the vasculogenic mimicry mechanism [93], because vasculogenic mimicry is the process by which aggressive tumor cells generate nonendothelial cell-lined channels delimited by extracellular matrix. Knockdown of Twist1 expression decreased not only cell mobility but also the tube-forming ability. Tumor-derived endothelial differentiation is important for Twist1-induced tumor metastasis, and inhibition of the angiogenesis process may be equally important to treat metastasis [18]. Finally, how classical angiogenesis versus endothelial transdifferentiation contributes to tumor angiogenesis and whether these two different mechanisms occur sequentially or have any tumor type preference remain to be determined through examination of different types of human tumors.

9. Regulation of the Jagged1-KLF4 Axis by Twist1

Recent study showed that the Twist1 functions upstream of Jagged1 in the process of development [94], but the regulatory mechanism was not provided. Our results indicate that Twist1 can activate Jagged1 expression and downstream Notch signaling pathway. In addition, the reporter assay and chromatin immunoprecipitation (ChIP) assay were performed and confirmed that Twist1 activated the expression of Jagged1 by directly binding to the E-box element in the Jagged1 promoter. Knockdown of Jagged1 not only decreased the levels of endothelial markers including CD31, CD144, vWF, CD105, and ICAM1 induced by Twist1 overexpression, but also abolished the activity of tube formation and DiI-AcLDL uptake activity induced by Twist1. Then, downregulation of Jagged1 caused the reverse shift in expression of mesenchymal markers (vimentin and N-cadherin) to epithelial markers (E-cadherin and plakoglobin) and abolished Twist1-mediated migration/invasion activity. Taken together, these results demonstrated that Jagged1 plays an essential role in Twist1-induced endothelial differentiation, EMT, and metastasis. Furthermore, the relationship among Notch, STAT3, and Twist1 pathways in the control of tumor progression was studied, and the results suggested that Notch1/STAT3/Twist signaling axis is involved in progression of human gastric cancer [95]. It provides an idea that there might be a positive feedback loop between Twist pathway and Notch signaling to promote tumor progression.

As Twist1 overexpression was shown to generate cells with stem-like properties [5], there are more and more evidences showing that Notch signaling pathway is involved in adult stem cell self-renewal and differentiation [96–98]. Moreover, recent researches indicated that tumor stem-like cell differentiation to endothelial-cell progenitors occurs trough Notch-mediated signaling [99]. Some pluripotency factors had an essential function in this network by actively directing differentiation for endoderm specification [100]. To further identify the transcription factors as downstream targets of the Twist1-Jagged1/Notch signaling to regulate the expression of various endothelial and vascular markers, we screened the expression of different stemness-related transcriptional factors including OCT4, SOX2, NANOG, KLF4, GFI1, WNT1, and BMI1. The results showed that Jagged1/Notch pathway can regulate the expression of KLF4 by directly binding to the KLF4 promoter using the qChIP assay. Although KLF4 is very likely an important regulator of ES cell self-renewal and pluripotency, our results demonstrate a role of KLF4 in endothelial differentiation and vasculogenesis. The direct regulation of KLF4 also showed the connection between the Notch pathway and KLF4. The potential downstream targets of KLF4 (e.g., Wnt5A, CCND2) may give us a new thought in the mechanism of KLF4-induced stem-like property that contributes to the tumor-initiating ability. Finally, KLF4 mediates Twist1-induced metastatic activity through an EMT-independent mechanism, suggesting that regulation of different targets (e.g., motility genes) other than the typical EMT marker genes also contributes to the metastatic activity induced by Twist1. All these results indicate the role of KLF4 in Twist1-induced endothelial differentiation, stem-like property, and metastasis.

10. Clinical Impaction of Twist1-Jagged1/KLF4 Axis

Furthermore, we also examined the correlation between the expression of Twist1, Jagged1, and KLF4 in head and neck cancer patient samples. Immunohistochemistry staining of Twist1, Jagged1, and KLF4 in 242 head and neck cancer patient samples showed there was significant correlation between Twist1, Jagged1, and KLF4. Meanwhile, the expression of Twist1-Jagged1-KLF4 axis was also confirmed in primary culture samples derived from head and neck samples. Overall, these results indicated that Twist1-Jagged1-KLF4 axis existed in real patient samples.

Cetuximab was recently approved in combination treatment with cisplatin for the treatment of patients with squamous cell carcinoma of the head and neck, but the survival benefit of adding cetuximab to standard chemotherapy was almost only three months [101, 102]. This means that there is still room for further improvement of treatment approach to treating head and neck cancer. It is well established that the angiogenic switch is a critical step in carcinogenesis [103]. With the clinical application of multiple inhibitors of vascular endothelial growth factor (VEGF) signaling, angiogenesis is a validated therapeutic target [13, 92]. However, the overall clinical benefit of agents targeting VEGF has been less than what was hoped. This lack of benefit appears to be substantially due to primary or acquired resistance to these drugs [104]. The tumor-derived endothelial differentiation might be responsible for this resistance. Because the Twist1-Jagged1-KLF4 axis seems to play an important role in angiogenesis, blocking Notch signaling activation by γ-secretase inhibitors might be a potential treatment.

Over the past decades, γ-secretase inhibitors have been investigated for their clinical potential to block the generation of Aβ peptide that is associated with Alzheimer's disease [105]. Because γ-secretase inhibitors are also able to prevent Notch receptor activation, several forms of γ-secretase inhibitors have been tested for cancer therapy. Treatment with one of γ-secretase inhibitors, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), either reduced medulloblastoma growth in a SmoA1 mouse model or induced G0-G1 cell cycle arrest and apoptosis in a T-ALL mouse model [106, 107]. Furthermore, a Notch inhibitor, MK0752, has been used for T-ALL patients and advanced breast cancers for a phase I clinical trial [108, 109]. To investigate whether the existence of Twist1-Jagged1-KLF4 axis might provide a potential new strategy treatment for the patients with Twist1-overexpressing tumors, we tested the drug response on Twist1-overexpressing OECM-1 cells. Xenotransplantation experiments showed that combined treatment of cetuximab and DAPT additively inhibited the tumor growth induced by Twist1 [18]. These results indicate the benefit of the γ-secretase inhibitor (DAPT) in combination treatment for Twist1-overexpressing tumors. However, further development of a specific type of γ-secretase inhibitor

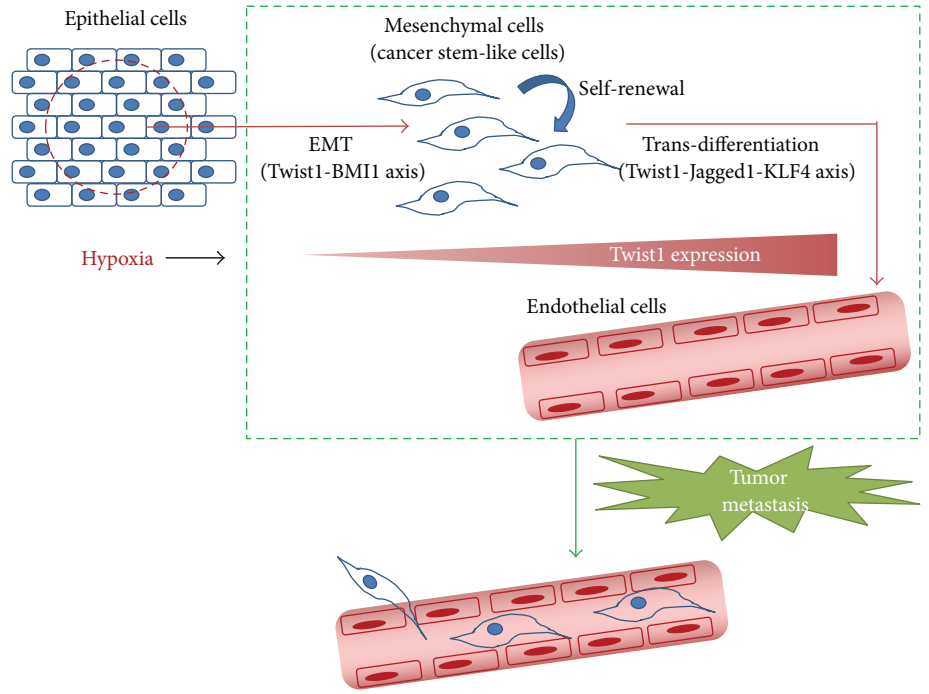

Figure 2: A model explains the crucial role of hypoxia-induced Twist1 to mediate different important processes of tumor progression including EMT, metastasis, cancer stemness, and endothelial differentiation through regulation of BMI1 or Jagged1/Notch-KLF4 axis.

that can specifically inhibit certain human tumors needs to be initiated in order to guarantee the success of target therapy of human cancers.

11. Conclusion

Tumor hypoxia is associated with malignant biological phenotype including enhanced invasiveness, angiogenesis, migration, and metastasis. HIF-1alpha, a key transcription factor that is induced by hypoxia and is implicated in tumor progression/metastasis, induces EMT through direct activation of Twist1 [32]. Twist1 plays a crucial role in epithelial-mesenchymal transition (EMT), metastasis, and cancer stemness through direct regulation of BMI1 [5]. Cancer stem cells have been described to be critical in tumor initiation tumor growth and metastasis. More evidences have shown that CSCs interact closely with angiogenesis and have the potential to develop the blood vessels [99]. Furthermore, our results indicate that the Twist1-Jagged1-KLF4 axis plays an important and essential role in inducing tumor-derived endothelial differentiation inside the tumors in addition to traditional angiogenesis and in creating better opportunities for tumor metastasis (Figure 2). These results also provide significant therapeutic implications to combine γ-secretase inhibitors with established chemotherapeutic agents for cancer treatment.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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