Perspectives on endothelial-to-mesenchymal transition: potential contribution to vascular remodeling in chronic pulmonary hypertension

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ALL FORMS OF CHRONIC PULMONARY HYPERTENSION are characterized by cellular and structural changes in the walls of pulmonary arteries. Virtually all of these changes are characterized, to a greater or lesser degree, by increased numbers of cells expressing α-smooth muscle (α-SM) actin (44, 76, 109). Albeit, neither the origin(s) of these cells nor the molecular mechanisms operating to cause their accumulation have been fully elucidated.

Traditionally, it has been thought that the SM-like cells that express α-SM actin and accumulate in vascular lesions were derived from the proliferative expansion of resident vascular smooth muscle cells (SMC) or adventitial fibroblasts through the processes of dedifferentiation of the former or differentiation of the latter. Over the years, however, this concept has been challenged by new experimental data suggesting many other possible sources of α-SM actin-expressing cells (SM-like cells and/or myofibroblasts) in various vascular diseases including pulmonary hypertension. Circulating progenitor cells have been shown to be recruited to sites of vascular injury and to assume SM-like phenotype (29, 57, 87). Resident vascular progenitor cells have also been demonstrated to express SM-like characteristics in several vascular injury states (41, 95). Finally, the possibility that endothelial cells have the capability of transitioning into a mesenchymal or SM-like phenotype has been raised. It is toward this last possibility (i.e., endothelial-mesenchymal transition) that this review is directed.

Many terms have been used to describe the process through which endothelial cells lose endothelial characteristics and gain expression of mesenchymal, myofibroblast-like characteristics. In this review, we will use the term endothelial-mesenchymal transition (EnMT). We will name it transition, rather than transformation or transdifferentiation, to keep this review in line with the current thinking of epithelial biologists, where the process of epithelial-mesenchymal transition (EMT) has been more thoroughly investigated (38, 48, 74, 81, 88, 97). The term transition seems more appropriate than transformation or transdifferentiation, because transformation classically describes oncogenic conversion, and transdifferentiation refers to a phenotypic switch of cells from one differentiated phenotype into another differentiated phenotype. Our intention is to first, briefly review EMT, the transition of epithelial cells toward a mesenchymal phenotype, since this process can lay the groundwork for understanding EnMT. We will then review the evidence for EnMT in vascular endothelial cells and address its potential mechanisms. The mechanisms identi-
fied so far in EnMT will be compared with those of EMT, noting that there are a great many similarities (Table 1).

**EMT.** EMT is a process in which epithelial cells lose their cell-to-cell contacts and polarity and undergo a dramatic remodeling of the cytoskeleton (37, 48, 52, 74, 81, 88, 97). During this process, which can be initiated by factors including transforming growth factor (TGF)-β, fibroblast growth factor (FGF)-2, epithelial growth factor (EGF), hepatocyte growth factor (HGF), RTK, integrins, MMPs, it should be noted that during development, mesenchymal cells can also participate in the formation of epithelial organs through a mesenchymal-epithelial transition (MET) (38, 97). This concept has potential relevance to treatment of diseases where EMT may be playing a role and is discussed below (74, 81).

In addition to its known role in development, recent data suggest EMT is important in cancer biology, especially as it relates to the metastatic capabilities of epithelial tumors. EMT is an important event in the progression of many carcinomas (37, 74, 81, 97), where epithelial cells transition toward a mesenchymal phenotype, leave the organized epithelial layer, and acquire an invasive phenotype, thus initiating metastasis. This transition has been shown to confer resistance to apoptosis in murine epithelial cell lines (82). It has even been speculated that EMT is a permanent feature in mixed carcinoma/sarcoma tumors (74).

A role for EMT is also becoming increasingly clear during tissue injury leading to organ fibrosis (defined by deposition of collagens, elastin, tenasin, and other matrix proteins). For instance, EMT is associated with progressive fibrosis in kidney disease (48, 68, 69, 90, 112). In the normal kidney, fibroblasts are not particularly abundant. However, at the onset of fibrogenesis, there is a marked increase in the number of fibroblasts. It has recently been demonstrated in a model of renal fibrosis that at least one-third of the newly appearing fibroblasts are derived through a local EMT process (48, 112). Thus, it has been suggested that kidney fibrogenesis, to a great degree, is a local epithelial event (69). There is emerging evidence to suggest that EMT also contributes to the fibrotic responses observed in several lung pathologies, such as rejecting lung allografts, silica-induced lung carcinogenesis, and in idiopathic pulmonary fibrosis (14, 17, 103–105).

**EnMT.** Less is known regarding EnMT than EMT. However, there is increasing experimental evidence to suggest that EnMT plays an important role during cardiovascular development and in various vascular pathologies. In the developing vertebrate embryo, the heart is the first organ to be formed, and its endocardial cells, initially expressing an endothelial phenotype (VE-cadherin, Tie-1/2, VEGFR-1/2, and PECAM/CD31) in the atrio-ventricular canal, give rise to the mesenchymal heart cells through a process of EnMT and form the mesenchymal portion of cardiac septa and valves (10, 27, 54).

In addition to its role in heart development, several groups have provided evidence that EnMT is critical in early vascular development. Using ultrastructural, immunohistochemical, and cell culture approaches, Arciniegas et al. (4, 8) have shown that EnMT is an important event in aortic and pulmonary artery development. Using immunogold labeling techniques, DeRuiter et al. (24) showed that endothelial cells labeled at an early stage of development later (at the onset of SMC differentiation) appeared in the subendothelial space of the developing aorta and expressed α-SM actin. Development of the normal arterial intima, a process that begins relatively early in life, also appears to involve EnMT (4, 7). Morphological studies in human embryos suggest that endothelial-like cells may give rise to SMC during the maturation of both pulmonary arteries and veins (35, 36). Findings in experimental wound repair have suggested that EnMT may also take place in the adult, reporting that capillary endothelial cells could undergo...
conversion into interstitial connective tissue mesenchymal-like cells in granulation tissue (85). Others have observed that microvascular endothelial cells transitioned into mesenchymal cells in response to chronic inflammatory stimuli (56, 83). A possible role for EnMT in the neointimal thickening observed in transplant atherosclerosis and restenosis has also been suggested (11, 12, 62). In addition, it has recently been demonstrated that transdifferentiation of pulmonary arteriolar endothelial cells into SM-like cells occurs in hypoxia-induced pulmonary vascular remodeling and is regulated by myocardin (113).

A number of in vitro studies have demonstrated that endothelial cells from a variety of vascular beds retain the capability of transitioning into mesenchymal or even SM-like cells under a variety of culture conditions (4, 6, 30, 46). Endothelial cells derived from the adult bovine aorta convert to spindle-shaped \(\alpha\)-SM actin-expressing cells when treated with TGF-\(\beta\) (9). Certain murine endothelial-like cell lines irreversibly transform into mesenchymal cells upon overgrowth in culture (51).

One concern raised regarding the early reports describing EnMT was the possibility that the primary endothelial cell cultures were simply contaminated with small numbers of mesenchymal cells. The studies by Frid et al. (30) addressed this problem in more detail by using both fluorescein-activated cell sorting (FACS) and magnetic bead (Dynabeads) techniques to “purify” primary endothelial cell cultures based on elevated uptake of Dil-Ac-LDL or on PECAM-1/CD31 expression by endothelial cells to obtain endothelial cultures free of contamination with mesenchymal cells. These purified endothelial cell cultures, however, were still shown to give rise to colonies of mesenchymal and even SM-like cells when selection of endothelial cells was performed immediately (on day 1) after initiating primary cultures. In contrast, when endothelial cell selection was performed 5 or more days after establishing primary cultures, no mesenchymal cells were observed to appear. The possibility was raised that the endothelial-mesenchymal phenotypic switch had already occurred in these cultures before day 5, and the resulting endothelial-derived mesenchymal cells have therefore been eliminated by FACS or Dynabead sorting. Interestingly, these techniques are commonly used by investigators to eliminate mesenchymal contaminants in endothelial cultures derived from large arteries, and they are usually applied after expansion of cells in culture. In contrast, in studies of microvascular endothelial cells, sorting techniques are usually applied at the time of cell isolation, and therefore, the need for subsequent selective sorting has been noted as an essential requirement (39, 93, 101). Dermal microvascular endothelial cells, for example, are described as very susceptible to transitioning into mesenchymal cells (83). Thus these studies supported the idea that mesenchymal cells often observed in primary endothelial cultures can arise through the process of EnMT.

**EnMT and EMT require loss of cell-cell contacts.** Recent studies have provided evidence that loss of endothelial cell-cell contacts is a necessary step in progression of EnMT. We have reported that loss of cell-cell contacts (including loss of VE-cadherin expression) consistently preceded changes in endothelial cell morphology and the subsequent expression of \(\alpha\)-SM actin (30). Alterations in cell morphology and upregulation of \(\alpha\)-SM actin have also been observed in sparsely plated endothelial cell cultures maintained in the presence of TGF-\(\beta\)-1 (9).

During chick embryo development, loss of cell-cell contacts, cell migration, and upregulation of \(\alpha\)-SM actin expression (involving TGF-\(\beta\)-2 and -3 and BMP-2) have also been reported in the conversion of a subset of endocardial endothelial cells into mesenchymal cells (15, 54, 63). Based on these observations and other reports, it was proposed that the TGF-\(\beta\)-signal transduction pathway is critically involved in the loss of cell-cell contacts and therefore in the EnMT process (4, 7, 9).

Similarly, studies in human epithelial cell EMT provided evidence that TGF-\(\beta\) induced a marked change in epithelial cell morphology that was associated with a loss of cell-cell contacts, including decreased E-cadherin expression, inhibition of ZO-1 expression, and loss of \(\beta\)-catenin association with both E-cadherin and \(\alpha\)-catenin (98). Comparing EnMT and EMT, we must keep in mind, though, that the adherens junctions in endothelial cells are composed of VE-cadherin and ADAM 15, whereas a central component of epithelial cell-cell adherens junctions is E-cadherin, and a hallmark of EMT in development, cancer, and fibrosis is the loss of E-cadherin expression (Fig. 1) (53, 74, 81). Tian and Phillips (98) have proposed that the cellular events observed during the EMT are the result of the interaction between two different intercellular signaling pathways, one initiated by TGF-\(\beta\) and the other by Wnt. Recent studies demonstrated a participation of members of the Wnt-signaling pathway (\(\beta\)-catenin and TCF/Lef) in addition to TGF-\(\beta\) pathways in the heart cushion development in the mouse, again suggesting an interaction between TGF-\(\beta\) and Wnt pathways in the EnMT process (54) (Fig. 1).

In addition to TGF-\(\beta\) and Wnt intercellular signaling pathways, the Notch pathway has also been proposed to be involved in EnMT. Notch signaling has been shown to operate in both vascular development and intimal lesion formation processes where EnMT is proposed to be involved (47). Studies on Notch signaling pathways in the vascular system have revealed that these pathways comprise the receptor families Notch1 and Notch4 and that Jagged1, Jagged2, and Delta4 are among their ligands (47). Of particular interest, Lindner et al. (55) demonstrated the expression of Jagged/Notch genes during the vascular response to injury and reported that the soluble Jagged1 protein modulated cell phenotype via alterations in the intercellular junctions as well as in focal adhesions. However, few studies have focused specifically on the role of the Notch signaling pathway in the EnMT process, particularly with regard to endothelial cell-cell interactions. Recent work by Noseda et al. (66, 67) suggests that expression of activated Notch1 or Notch4 in endothelial cells can cause transition to a mesenchymal phenotype. An important role for Notch-mediated EnMT during endocardial cushion formation has also been suggested (10, 99). Furthermore, Liebner et al. (54) have found that the complex Jagged/Notch promotes EnMT via transcriptional induction of Snail during heart cushion development. Snail is a DNA-binding factor that recognizes E box motifs in target promoters, including E-cadherin, and has been shown to be a critical mediator of EMT (53, 65). Other repressors of E-cadherin and important inducers of epithelial EMT are the zinc finger protein SIP1 and Twist (42, 81, 107). However, little work has been done examining specifically the role of these or analogous repressors in EnMT.

**Cell adhesion, migration and EnMT.** Acquisition of a migratory phenotype, preceded by changes in cell shape and in interactions with the extracellular matrix (ECM), has been
proposed as critical during the EMT process (48, 88, 97). Whether the same is true for EnMT is unclear but very likely. We will therefore briefly review some general aspects of cell adhesion, spreading, interactions with ECM, and migration as they might relate to EnMT.

It is now well established that most ECM molecules participate in the regulation of cytoskeletal organization, cell adhesion, spreading, migration, cell differentiation, growth, and apoptosis in response to various soluble factors and mechanical stimuli (42). Both in vivo and cell culture studies have suggested that these cellular events are determined through the action of tractional or tensile forces that are generated within the cell cytoskeleton and are physically resisted by ECM attachment points, focal adhesions. The balance between tensile forces and ECM resistance has been termed tenserigrity, and alterations in this balance might actively contribute to control of cell shape and might be a critical determinant of cell migration (45). Focal adhesions, like adherens junctions, are thought to be important not only as structural links between the ECM and the cytoskeleton but also as sites for signal transduction through integrins present at these sites (86). Integrins, in addition to mediating cell binding to ECM components, also govern focal adhesion formation, cell spreading, migration, and differentiation (23, 96). These cellular events include the formation of ruffles, lamellipodia, filopodia, and microspikes at the leading edge of migrating cells and have been associated with activation and regulation of Rho family GTPases, including Rac and RhoA (23, 70, 72, 96).

Of particular note, a recent study in human microvascular endothelial cells provides evidence suggesting that Rac, but not Rho or Cdc42, is activated during endothelial cell adhesion to laminin-8 and that it is crucial for integrin-mediated cell spreading and migration (31). An important role for RhoA in the formation or disassembly of focal adhesions and endothelial migration during angiogenesis in vivo and in vitro has also been recently proposed (40, 49). Of further interest, recent work demonstrates that shear stress significantly enhances endothelial cell migration by modulating the tractional forces through the small GTPase pathway (100). Most recently, studies show that Rho-associated kinases have a critical role in the mesenchymal cell invasion/migration that occurs late in the EnMT process (84).

Given that changes in cell shape, interactions with the ECM, and acquisition of migratory capabilities have been proposed as critical during the EMT process, we suggest that the experimental findings reviewed above represent an important frame of reference for a better understanding of the EnMT process. Studies are necessary to gain insights into mechanisms that regulate the endothelial cell migration and transition in EnMT.

Proteases, endothelial cell migration, and EnMT. As discussed above, the balance between tensile forces generated within the cytoskeleton and the resistance to these forces offered by the ECM is a determinant not only of cell shape and growth but also of cell migration (21, 45). Cell migration is dependent on degradation of ECM (pericellular proteolysis), and cells produce proteases exhibiting different activities and substrate specificities. Most of the proteases involved in releasing cells from their localized state within the ECM belong to the metalloproteinase and serine protease families (80).

Numerous studies have suggested an important role for matrix metalloproteinases (MMPs) in morphogenesis, migration, and tissue remodeling during embryonic development and in various pathological conditions (106). However, few studies have addressed the role of MMPs in the EnMT process, and controversy exists regarding participation of MMPs in this process. For instance, MMP-2 has been proposed to play an essential role in producing epithelial-mesenchymal transformations in the avian embryo, and whereas certain studies proposed that MMP-2 participates in the loss of cell-cell contacts by removal of cadherins, other reports suggested that MMP-2...
is only expressed as the cells begin to migrate and acquire mesenchymal characteristics and not specifically during the loss of cell-cell contacts (26, 32, 91). Several studies have reported that MMP-2 directly affects cell migration by degradation of specific ECM components (1, 32, 91, 92). Other studies showed the localization of MMPs at the focal adhesion sites and lamellipodia during cell detachment on the onset of migration (16) and therefore proposed the role of MMPs in EMT. These reports are certainly compatible with observations showing increased MMP expression in cells exhibiting a mesenchymal compared with epithelial phenotype and suggest the role of MMPs in the EnMT process.

One of the most studied members of the serine protease family is the urokinase type plasminogen receptor (uPAR)-urokinase type plasminogen activator (uPA) system and its inhibitor plasminogen activator inhibitor (PAI), which can convert plasminogen to plasmin. uPAR-uPA can degrade ECM inhibitor plasminogen activator inhibitor (PAI), which may urokinase type plasminogen activator (uPA) system and its family is the urokinase type plasminogen receptor (uPAR)-enchymal compared with epithelial phenotype and suggest the role of MMPs in the EnMT process.

Reversibility of EMT and EnMT. During embryogenesis, a process that is essentially the reverse of EMT, termed MET, occurs during organ morphogenesis (20, 38, 68, 69, 74). Control or upregulation of cadherin expression through developmental and spatially regulated expression of molecules such as BMP-7 and HGF appear critical for this process (68, 69). These observations suggested that the EMT process in mature organs may be reversible. Indeed, exciting new studies in the kidney suggest that a regenerative program can be initiated in the injured kidney that bears resemblance to renal development (53, 68, 69). It has been demonstrated that remission, as well as substantial regression, can be achieved by exogenous recombinant human BMP-7 (rhBMP-7) in the fibrotic kidney (68, 69, 111). rhBMP-7 reverses EMT, inhibits the release of proinflammatory/fibrogenic cytokines from tubular epithelial cells, and prevents apoptosis to repair and restore normal renal architecture. rhBMP-7 binds to Alk3 and Alk6, which function via phosphorylation of Smad 1, 5, and 8 to increase in E-cadherin expression, countering the TGF-β-induced Alk5- and Smad 2- and Smad 3-mediated decrease in E-cadherin expression. Thus it seems strategies aimed at reversing EMT may have a significant, positive effect on fibrotic organ injuries.

Less is known regarding the reversibility of EnMT. One study, in which EnMT was shown to be induced by withdrawal of FGF, showed that restoration of the endothelial phenotype could be restored by restoring FGF to the cells. More studies are obviously necessary to determine the reversibility of the EnMT process in vitro and in vivo.
Conclusions. The intent of this review was to present the current state of knowledge regarding the factors and mechanisms operating during the EnMT process, which occurs not only during embryonic development but also in the pathogenesis of various vascular diseases. We intended to reveal the complexity of the EnMT process, which seems to progress through a series of important steps (Fig. 1). Unfortunately, these have generally been analyzed separately and in different cell systems by different investigators. Additional studies are required for a better understanding of the molecular mechanisms of EnMT regulation operating in vivo and in vitro to control the maintenance of intercellular junctions and cell-cell and cell-matrix interactions. Proteome and genome level studies of the upstream regulators that participate in the control of transcription factors that repress VE-cadherin expression and thus initiate the EnMT are needed. Information is needed regarding the factors that regulate serine protease and MMP expression in endothelial cells, which ultimately affect the balance between the synthesis and degradation of ECM components and thus regulate cell migration and cell phenotype. Targeted pharmacological inhibition of serine-proteases and MMPs or knockedown with antisense or small interfering RNA technologies may provide a cornerstone for these investigations. Phospho-activated NF-κB is essential for EMT and tumor metastasis (43) and has been implicated in the progression of atherosclerosis and cancer, diseases in which EnMT may have pathological relevance. Therefore, studies targeting NF-κB may provide critical mechanistic insights into the EnMT process. Reporter-gene transgenic mice engineered to carry endothelium-specific reporters (such as flk-1/LacZ, /lacZ, tie-2/LacZ, and vWF/LacZ) provide a useful system for exploring EnMT. Conditional endothelial cell-specific transgenic knockout and/or knockin models for EnMT regulatory genes, such as β-catenin, may also provide fundamental insights. Finally, additional studies will be required to determine whether EnMT is a reversible process as has been proposed during EMT in tumorigenesis, embryogenesis, and fibrosis, and if so, whether treatments directed toward this process have significant effects on vascular disease (79).

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REFERENCES
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