Endothelial-Mesenchymal Transition in Cardiovascular Disease

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Abstract

Endothelial-to-mesenchymal transition (EndMT) is a dynamic process in which endothelial cells suppress constituent endothelial properties and take on mesenchymal cell behaviors. To begin the process, endothelial cells loosen their cell-cell junctions, degrade the basement membrane and migrate out into the perivascular surroundings. These initial endothelial behaviors reflect a transient modulation of cellular phenotype, i.e., a phenotypic modulation, that is sometimes referred to as partial EndMT. Loosening of endothelial junctions and migration are also seen in inflammatory and angiogenic settings such that endothelial cells initiating EndMT have overlapping behaviors and gene expression with endothelial cells responding to inflammatory signals or sprouting to form new blood vessels. Reduced endothelial junctions increases permeability, which facilitates leukocyte trafficking, while endothelial migration precedes angiogenic sprouting and neovascularization; both endothelial barriers and quiescence are restored as inflammatory and angiogenic stimuli subside. Complete EndMT proceeds beyond phenotypic modulation such that mesenchymal characteristics become prominent and endothelial functions diminish. In pro-adaptive, regenerative settings the new mesenchymal cells produce extracellular matrix and contribute to tissue integrity whereas in mal-adaptive, pathologic settings the new mesenchymal cells become fibrotic, overproducing matrix to cause tissue stiffness, which eventually impacts function. Here we will review what is known about how transforming growth factor beta (TGFβ) influences this continuum from junctional loosening to cellular migration and its relevance to cardiovascular diseases.

Graphical Abstract:

TGFβ$_{1-3}$ and Wnt signaling initiate EndMT while laminar shear stress-induced ERK5 and KLF2/4 along with FGFR1 and BMPR2 signaling prevent EndMT. Acetyl CoA, by enhancing fatty acid oxidation, also minimizes EndMT. Endothelial cell morphology changes dramatically during EndMT as transcription factors Snail1 and Slug suppress endothelial genes and increase mesenchymal genes.

Introduction

EndMT was first described in the endocardial cushions of the embryonic heart by Markwald and colleagues in the mid-1970s[1, 2]. For decades this endothelial transition was referred to as epithelial-to-mesenchymal transition (EMT) but in recent years the terms EndMT

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and EndoMT have become widely used to refer specifically to endothelial versus epithelial transitions. Hallmarks of EndMT are down-regulated adherens junction molecules such as VE-cadherin, increased cellular migration aided by increased matrix metalloproteinases (MMPs), and up-regulated myofibroblastic markers such as α-smooth muscle actin (α-SMA). Correspondingly, EndMT results in diminished endothelial properties such as cytokine-induced leukocyte adhesion and tube formation and increased mesenchymal properties such as collagen deposition and contractility. The transcription factors Snai1, Snai2 (also known as Slug), Twist and nuclear factor of activated T-cells, cytoplasmic 1 (NFATc1) orchestrate the changes in gene expression needed to induce or attenuate EndMT. Endothelial cells from different parts of the vasculature show varying propensities to undergo EndMT[3], which may be in part due to embryonic origin. Heart valves are lined with a specialized endothelium derived from FLK1^{+} (a.k.a.VEGFR2^{+}) progenitor cells that originate from cardiac mesoderm[4]; these valvular endothelial cells (VEC) are among the most readied to undergo EndMT. Yet, heterogeneity exists as some clonally expanded VECs show robust transforming growth factor beta (TGFβ)-mediated EndMT while other VEC clones resist EndMT signals and maintain an endothelial phenotype[5-7]. EndMT propensity may also be influenced by the location along the leaflet or cusp, as VECs facing onward blood flow (ventricular side the aortic valve; atrial side of the mitral valve) experience different hemodynamic forces compared to the downstream side. Indeed, distinct molecular phenotypes have been described in VECs on either side of the aortic valve[8, 9]. At present, the molecular basis for the differential susceptibility to EndMT signals among valvular, arterial, venous, microvascular and lymphatic endothelial cells is not fully understood.

TGFβ driven EndMT

TGFβ is well known for its role in maintaining homeostasis among cells within a given environment[10] yet it can become chronically elevated in disease settings, where it activates fibroblasts to become myofibroblasts, increases angiogenesis and suppresses immune responses[11]. It is also a major driver of EndMT during development and in pathological settings in adult life, where inflammatory cytokines such as interleukin-1β (IL1β) and mechanical forces imparted by abnormal shear stress contribute as well[12]. Endothelial TGFβ1 signaling was recently shown to drive cardiac valve regeneration in adult zebrafish by increasing cell-cycle entry and differentiation into new valve cells[13]. Other TGFβ family members, including bone morphogenic proteins (BMP), modulate TGFβ signaling in intricate ways[14]. For example, elegant studies by Hiepen and colleagues have shown BMP9-BMPR2 signaling maintains vascular quiescence and blocks EndMT by preventing mixed heterodimeric receptor complexes and TGFβ autostimulation[15]. Here we will focus on TGFβ and its direct role in EndMT.

Three TGFβ family members, TGFβ1, TGFβ2 and TGFβ3, can each stimulate EndMT in cultured endothelial cells, with TGFβ2 playing a prominent role. Indeed, TGFβ1 and TGFβ3 were shown to increase endogenous TGFβ2 in endothelial cells, including VECs [16], indicating TGFβ1 and TGFβ3 work indirectly by elevating TGFβ2. *In vivo* TGFβ2 signaling was shown to initiate EndMT in chick endocardial cushion[16, 17] and this was further demonstrated in mice[18]. TGFβ2 stimulates expression of the transcription factor Slug, which then initiates EndMT[19]. While the direct role of TGFβ3 in EndMT induction

was questioned when TGFβ3^{−/−} mice showed no valve defects[20], a recent study noted abnormally formed valves among the several morphological defects in TGFβ3^{−/−} mice[21]. Recent work shows knocking out endothelial TGFβ receptors type I and II (TGFβRI and TGFβRII) reduces vascular permeability[22], implicating TGFβ signaling in the earliest steps in EndMT. Endothelial TGFβ signaling can reduce cell-cell contacts and promote angiogenesis[23,24]; this suggests commonality in TGFβ-stimulated endothelial behaviors in EndMT, permeability and angiogenesis[25].

TGFβ binds to a tetrameric complex on the plasma membrane composed of two TGFβRI and two TGFβRII, both dual specificity kinases. In endothelial cells, the prominent type I receptors are the activin receptor-like kinases 1 and 5 (ALK1 and ALK5). ALK1 is activated by bone morphogenetic protein-9/10 (BMP9/10) and usually promotes endothelial quiescence while TGFβ activates ALK5. ALK5 phosphorylates the transcription factors Sma genes and the Drosophila Mad, Mothers against decapentaplegic (SMAD)- 2 and −3 that, once phosphorylated, combine with SMAD4 and translocate to the nucleus to regulate expression of specific target genes. This is referred to as canonical TGFβ signaling pathway. The same TGFβ-bound receptor complex also activates the mitogen activated protein kinase (MAPK) pathway via recruitment and phosphorylation of the Src homology domain-containing protein ShcA, resulting in assembly of ShcA-Grb2-SOS complexes that in turn lead to phosphorylation and activation of ERK1/2. ERK1/2 then moves from the cytoplasm into the nucleus to activate transcription of non-canonical TGFβ target genes. Both canonical and non-canonical TGFβ pathways are functional in EndMT[26-29].

TGFβ is also known to induce EndMT via epigenetic reconfiguration. This occurs either through regulation of EndMT transcription factors such as Snail1[30-35] or via SMAD molecules that directly interact with genome modifiers such as tripartite motif containing 33 (TRIM33)[36], forkhead box H1 (FOXH1)[37], histone acetyltransferases (HATs) including CREB-binding protein (CBP/P300)[38] and P300/CBP-associated factor (P/CAF)[39], histone deacetylases (HDACs)[40], and SWItch/Sucrose Non-Fermentable (SW1/SNF)[41]. More recently, FOXM1 was found to drive TGFβ-induced EndMT by binding to the promoter of *SNAI1* in human umbilical vein endothelial cells (HUVECs), which increased SNAIL1 expression[42].

In addition, miRNA-mediated modulation of TGFβ-induced EndMT has been studied in the context of development and disease. Some examples of inhibitory roles of miRNA on TGFβ-induced EndMT include: miR-23, which was shown to restrict endocardial cushion formation[43]; miR-126, which was shown to inhibit EndMT in rat bone marrow-derived endothelial progenitor cells[44], and miR-148b, which blocked EndMT and angiogenesis in a skin wound healing model[45]. miRNAs were also found to promote EndMT: examples are miR-494[46], miR-21[47] and miR-374; the latter works by downregulating MAPK7 (see below) at sites of developing atherosclerosis[48]. In depth review of the role(s) of miRNAs in (TGFβ-induced) EndMT can be found in these recent publications[49, 50].

EndMT and endothelial permeability

The integrity of the endothelial barrier is strongly influenced by laminar shear forces and the molecular signals induced by shear stress. In particular, extracellular signal-related kinase (ERK) 5, also called MAPK7 and big-mitogen kinase-1 (BMK-1), has long been known to promote vascular integrity. ERK5 is increased in response to normal laminar shear stress (15 dynes/ cm^2) and this promotes endothelial barrier integrity by increasing expression of two transcription factors - the Kruppel-like factors KLF2 and KLF4 - both of which exert anti-inflammatory and atheroprotective effects on the endothelium. These effects are mediated by the ability of KLF2 and KLF4 to inhibit nuclear factor kappa B (NF-κB), a master transcriptional regulator that orchestrates many aspects of leukocyte trafficking across the endothelium. In addition, KLF2 and KLF4 inhibit TGFβ signaling directly by interfering with SMAD2 and SMAD3 respectively; this action maintains endothelial VE-cadherin and prevents mesenchymal gene transcription[51]. In contrast, disturbed shear stress favors TGFβ signaling, which promotes EndMT[51]. Abnormally low shear stress (4 dynes/cm2) was shown to induce EndMT by increasing the transcription factor Snai1, which inhibits expression of VE-cadherin. Diminished VE-cadherin results in loosened adherens junctions, which in turn increases vascular permeability[52]. As such, inflammatory cytokines promote EndMT by perturbing endothelial barrier function via disassembly of adherens and tight junctions. For example, interleukin-6 (IL-6) was found to increase the permeability of HUVECs by causing a redistribution of tight junction proteins and the actin-cytoskeleton[53]. Interestingly, IL-6 is strongly increased in BMPR2-deficient endothelial cells, which are predisposed to EndMT[15]. Tumor necrosis factor alpha (TNFα) combined with interferon gamma (IFNγ) also increased permeability in HUVECs by reducing junctional adhesion molecule[54]. Chen and colleagues provide further insights on the impact of cytokines on permeability and EndMT[55]. In summary, dismantling of endothelial cell-cell junctions is a first step in EndMT (see Figure 1) as well as inflammation and angiogenesis.

EndMT and endothelial migration

Endothelial cell migration is a hallmark of EndMT, angiogenesis and vascular repair. In EndMT, the cells migrate individually whereas collective migration is seen in sprouting angiogenesis where endothelial tip and stalk cells migrate in a coordinated manner[56]. In EndMT, loosened cell-cell junctions allow subsets of endothelial cells to migrate as single cells and invade the sub-endothelial mesenchyme. TGFβ increases MMPs and endothelial podosomes, actin-rich microdomains that protrude into the basement membrane; this likely facilitates breach of the basement membrane by focal degradation of matrix proteins and the cellular migration that ensues. The zinc-finger transcription factor SNAI2 (i.e., SLUG), known for its role in cell movement, is consistently increased in cells undergoing TGFβ-mediated EndMT and is required for EndMT in endocardial cushions[57]. Welch-Reardon and colleagues showed SLUG is also expressed in migrating angiogenic EC and that reducing SLUG decreased membrane type 1 MMP (MT1-MMP) and in turn MMP2 and MMP9, which reduced the ability of endothelial cells to sprout and migrate into various extracellular matrices[58]. This again underscores overlapping cellular behaviors between angiogenesis and EndMT, and as proposed by these authors, angiogenesis requires a transient adoption of mesenchymal features which they refer to as partial EndMT[25]. Despite the known

requirements for SLUG and MMPs in EndMT, at present we have only a nascent molecular understanding of how the increased migration in EndMT is achieved. One might speculate that EndMT-specific migration involves SLUG-mediated transcription of genes needed for podosome formation and that complex interactions between the podosomes and the extracellular matrix, as well as surrounding cells in that milieu, provide important guideposts for migration[59].

Restraints on EndMT

Endothelial phenotype and function are maintained by signaling pathways that counter TGFβ-driven EndMT. For example, fibroblast growth factor-2 (FGF-2, aka basic FGF), a growth factor known to play important roles in vascular integrity[60] and endothelial proliferation[61, 62], was shown to inhibit TGFβ signaling in endothelial cells in 1990[62]. Subsequent studies showed removal of basic FGF from culture media stimulated EndMT in aortic VECs[5] and in HUVECs[63]. When a variety of growth factors were analyzed for effects on mitral VECs, basic FGF proved to be the strongest negative regulator of TGFβ induced EndMT[64]. Recent mechanistic studies have shown FGF signaling through endothelial FGF-receptor 1 (FGFR1), as opposed to FGFR3 or FGFR4, inhibits TGFβ signaling and EndMT. It was shown that activated FGFR1 recruits FGFR substrate 2a (FRS2a), an adaptor protein, and this leads to increased levels of the microRNA let-7, which in turn reduces expression of TGFβRI and its signaling[65, 66]. Inflammatory cytokines IFNγ, TNFα and IL1β, however, diminish FGFR1 signaling[65], which could explain how inflammatory cytokines augment EndMT in some cultured endothelial cells. TNFα and IL1β potently activate NF-κB, which leads to rapid induction of leukocyte adhesion molecules (e.g., E-selectin, vascular cell adhesion molecule 1; VCAM1, intercellular adhesion molecule 1; ICAM1). These molecules attract leukocytes to the endothelium at sites of inflammation. In contrast, endothelial cells that have undergone EndMT show diminished cytokine-induced E-selectin, VCAM1 and ICAM1 and concomitantly decreased adherence of leukocytes[6, 67]. Thus, the loss of an endothelial function that occurs during EndMT would appear to blunt leukocyte trafficking across the endothelium to sites of inflammation. Indeed, TGFβ/Smad and NF-κB are known to antagonize one another in inflammatory responses[68].

Bone morphogenic protein-receptor 2 (BMPR2) also prevents EndMT by dampening endothelial TGFβ signaling. BMPR2 loss of function mutations in patients with pulmonary arterial hypertension (PAH) are associated with increased TGFβ signaling and expression of EndMT markers[69]. When BMP2R is absent, formation of a mixed heteromeric BMPR1/TGFβR type I/TGFβR type II complex is favored, and this leads to increased canonical TGFβ signaling and EndMT[15]. These authors describe endothelial BMPR2 as a gatekeeper that maintains homeostatic BMP/TGFβ signaling. Reduced BMPR2 was shown to result in increased chromatin factor high mobility group AT-hook 1 (HMGA1), which in turn increased SLUG and initiated EndMT in pulmonary arterial endothelial cells[70].

Endothelial phenotype is also maintained by acetyl-CoA levels generated by fatty acid oxidation. Xiong and colleagues showed that acetyl CoA increases SMAD7, an inhibitory SMAD that dampens TGFβ signaling; this inhibits EndMT[71]. Endothelial-specific knock out of carnitine palmitoyltransferase II (Cpt2), an enzyme required for the shuttle of fatty

acids into the mitochondria for oxidation, resulted in thickened mitral valve leaflets and aortic valve cusps. Lineage tracing showed endothelial contribution to the increased valve interstitial cells in the Cpt2-deficient mice, demonstrating that decreased endothelial fatty acid oxidation increased EndMT during valve development. *In vitro* studies in which human pulmonary microvascular endothelial cells were treated with 10 ng/ml TGFβ1 + 1 ng/ml IL1β for 7 days, conditions used in prior studies[72], showed that increases in mesenchymal cell markers could be reversed by supplying acetate into culture medium to replenish acetyl CoA. The acetate increased SMAD7 and decreased phospho-SMAD2 levels, consistent with reduced TGFβ signaling[71]. In summary, laminar shear stress, FGFR1 and BMPR2 signaling and fatty acid oxidation suppress EndMT –whereas disturbed shear stress, TGFβ and Wnt signaling (see below) stimulate EndMT.

Wnt signaling in EndMT

In contrast to inhibitory pathways, Wnt (wingless-type mammary tumor virus integration site family of proteins) signaling can augment TGFβ signaling. Wnt ligands bind to Frizzled receptors on the plasma membrane, which triggers changes in intracellular β-catenin, a primary effector of canonical Wnt signaling. In quiescent, non-Wnt-stimulated endothelial cells, β-catenin interacts with VE-cadherin at the cytoplasmic face of adherens junctions. Cytosolic levels of β-catenin are kept low by constitutive ubiquitination and proteasomal degradation such that in the absence of Wnt ligands and/or presence of Wnt inhibitors, the canonical Wnt pathway is in an 'off state'. Wnt ligand-receptor binding releases β-catenin from the adherens junction and reduces its turnover by proteasomal degradation, resulting in cytosolic accumulation that then favors β-catenin translocation into the nucleus where it increases transcription of the lymphocyte enhancer factor/T-cell transcription factor (Lef/TCF). In some settings, Wnt and TGFβ signaling converge in the nucleus where β-catenin interacts with SMAD transcriptional regulators as well as Lef/TCF to orchestrate the transcriptional regulation of shared target genes[68].

Canonical Wnt signaling is required for EndMT in endocardial cushions in the developing heart - cushion explants or endothelial cells deficient in β-catenin fail to undergo EndMT[73]. Wnt's role in EndMT has also been studied in cell culture models under mechanical stress. Balachandran and colleagues showed that β-catenin plays a role when mitral VECs undergo EndMT under high cyclic mechanical strain[74], and indeed EndMT occurs upon mechanical stretch of mitral valve leaflets in a large animal model[75]. Zhong and colleagues showed that Wnt-β-catenin can drive EndMT in aortic VECs and is influenced by matrix stiffness[7]. Endothelial Wnt signaling and EndMT have also been analyzed within days after myocardial infarction (MI), produced by ligating the left anterior descending coronary artery in mice[76]. In this elegant study, lineage tracing of endothelial cells expressing the TOPGAL reporter, a readout for canonical Wnt-β-catenin signaling, showed Wnt signaling in α-SMA-positive cells of endothelial origin, indicating a post-MI Wnt activation coincident with EndMT. Interestingly, activation of Wnt/β-catenin in cultured bovine aortic endothelial cells, without TGFβ or FGF in the culture medium, was sufficient to induce mesenchymal markers and reduce endothelial markers[76]. The authors proposed that Wnt signaling in endothelial cells produces a migratory CD31+/αSMA+ intermediate phenotype that reflects cells that have partially transitioned to a mesenchymal state. Cells that have undergone partial EndMT

can either undergo angiogenesis to produce new blood vessels, reverting to an endothelial phenotype, or proceed to a CD31-/α-SMA+ collagen-producing myofibroblast. The authors concluded that the endothelial cells contributed to cardiac repair after MI by both partial and complete EndMT pathways[76]. The mechanisms that control whether endothelial cells revert to an endothelial phenotype after partial EndMT or complete a full transition to mesenchymal phenotype will be important to elucidate as this information could be used to both increase angiogenesis and provide matrix-producing cells to enhance tissue repair.

EndMT in different types of endothelial cells

The extent to which the location of endothelial cells within the cardiovascular system influences EndMT and what type of stimulus or stimuli are needed to induce partial or complete EndMT can be gleaned from the literature. First, endothelial cells isolated from heart valves – mitral, aortic or pulmonic - show robust EndMT *in vitro* when treated with 1-2 ng/ml TGFβ for 4-5 days. In these studies, EndMT is defined as cells: 1) co-expressing endothelial and mesenchymal markers, 2) exhibiting increased cellular migration, 3) showing a loss of endothelial properties such as leukocyte adhesion and tubule formation, and 4) exhibiting increased mesenchymal/myofibroblastic properties such as collagen production and contractility[5-7, 16, 77]. *In vivo* studies of the mitral valve in large animal models show EndMT is associated with valve leaflet growth and thickening[16, 78-80]. The propensity of VECs to undergo EndMT may be enhanced by the inherent plasticity of endocardial endothelial cells[4, 81] and possibly expression of the Type III TGFβ-receptor, which was shown to necessary for EndMT in atrioventricular cushions and sufficient to drive EndMT in ventricular endocardium[82].

Some arterial ECs can be induced to undergo EndMT. Endothelial lineage tracing provides compelling evidence for arterial EndMT in mouse models of atherosclerotic plaque development[83] as well as partial EndMT behaviors in formation of collateral vessels in injured neonatal hearts[84]. In contrast, some sources of arterial endothelial cells - isolated from the coronary artery, carotid artery or pulmonary artery and analyzed *in vitro* - showed no increase in EndMT markers in response to TGFβ[3, 16, 26]. In contrast, primary aortic ECs were shown to undergo robust EndMT in response to TGFβ2[3]. Venular endothelial cells such as HUVECs, treated with TGFβ1, showed increased expression of transgelin (SM22α) and SLUG, and decreased expression of CD31 and VE-cadherin, compared with no treatment, indicating EndMT[29]. In another study, HUVECs treated with TGFβ2 plus IL1β for 24 hours showed EndMT characteristics that were attributed to p65-dependent NF-κB activation[85]. Microvascular intestinal endothelial cells were non-responsive to TGFβ unless TNFα, another NF-κB activating inflammatory cytokine, was included[72]. In another study, human dermal microvascular endothelial cells did not undergo EndMT in response to TGFβ2 alone, whereas in parallel, human pulmonary VECs showed robust EndMT[6]. In summary, the findings suggest inflammatory signals contribute to changes in endothelial phenotype, perhaps by priming the cells to respond to EndMT inducing stimuli such as TGFβ. Interestingly, lymphatic endothelial cells have been reported to undergo EndMT. For example, Kaposi's sarcoma herpesvirus (KSHV) infection of lymphatic endothelial cells stimulates EndMT through viral protein activation of the NOTCH pathway and MT1-MMP[86]. Further, FGF2 helps maintain a differentiated lymphatic endothelial cell identity by

opposing TGFβ-activated SMAD2 via Ras/ERK MAPK signaling[87]. In summary, we have much to learn about the requirements and conditions under which endothelium in different vasculature beds undergoes EndMT.

A limitation of studying EndMT *in vitro* is that while many types of endothelial cells have been reported to undergo EndMT, the increase in mesenchymal markers is often not robust and functional assays are lacking. An overarching limitation for many *in vitro* EndMT assays is that the purity of the starting endothelial cultures in not readily apparent. This is a concern because a small number of contaminating cells in primary endothelial cell cultures, such as smooth muscle cells or fibroblasts, can lead to an appearance of EndMT on a western blot or in qPCR assays because TGFβ increases α-SMA, a commonly used marker for EndMT, in fibroblasts, for example. In addition to assays on whole cell lysates, techniques such as flow cytometry and/or immunofluorescence should be employed to 1) assess potential for other cell types in the endothelial cultures and to 2) verify co-localized endothelial and mesenchymal markers to document cells have initiated EndMT (Figure 2). In addition, functional assays that measure contractility (mesenchymal) and cytokine-induced leukocyte adhesion (endothelial) should be conducted to determine the extent to which cells have acquired mesenchymal functions and reduced endothelial functions. Cells that retain endothelial markers (e.g., VE-cadherin) and endothelial functions have undergone partial EndMT whereas cells that have lost endothelial markers and functions, and express mesenchymal markers and gained mesenchymal functions, have undergone complete EndMT.

EndMT in cardiovascular disease

The mechanisms that activate EndMT in settings of injury, inflammation or stress are under intense scrutiny in order to understand the extent to which EndMT is a driver or a consequence of cardiovascular pathology[55, 69, 88]. The dynamic nature of EndMT is also emerging, particularly from new single cell RNA-seq studies that have revealed a transient EndMT in the non-cardiomyocyte fraction after MI. Lineage tracing in the mice showed that within 3 days after MI, a subset of endothelial cells underwent a transient mesenchymal activation, termed endothelial to mesenchymal activation, that lasted approximately 10 days. HUVECs treated with TGFβ2 mimicked endothelial to mesenchymal activation, which was reversed upon withdrawal of TGFβ2[89]. The authors speculate that the transient mesenchymal state may facilitate angiogenesis, which would be beneficial for the ischemic myocardium, but also raise the possibility that matrix deposition from the temporary mesenchyme may have detrimental effects. These new observations, made possible by state-of-the-art transcriptomics, underscore the necessity for deeper understanding of the EndMT continuum. The role of BMP signaling and how dysregulation of this pathway contributes to cardiovascular diseases are discussed in detail elsewhere[90].

Ischemic mitral regurgitation (IMR)

EndMT is required for formation of the four cardiac valves during embryogenesis, but is strongly suppressed in adult valvular endothelium. Yet EndMT capability is not lost. Ovine, porcine and human VECs isolated from adult valves readily undergo EndMT in response

to TGFβ[5, 6, 75, 91], suggesting EndMT capability is retained, possibly throughout life, in order to replenish valve interstitial cells, major producers of the valve extracellular matrix. This speculation is not supported, however, by lineage tracing studies, which failed to find evidence of EndMT in mitral valves of mice at 4 months of age[92]. In contrast, recent studies in adult zebrafish indicate some endothelial cells undergo EndMT in uninjured valves[13]. Large animal studies devised to study IMR, which is the most common form of valvular heart disease in the U.S.[93] and is associated with excess mortality after MI[94, 95], showed that EndMT can be reactivated in adult valves. Mitral VECs undergoing EndMT were detected and quantified in mitral valve leaflets exposed *in vivo* to mechanical stretch designed to mimic the tethering of mitral valve leaflets that occurs when the left ventricle enlarges after MI. EndMT coincided with an increase in mitral valve leaflet area and thickness, leading to the concept that the mitral valve leaflets are not passive flaps but can adapt to changing hemodynamics, and that the valvular endothelium is central to this adaptive response[75]. When mechanical stretch was combined with MI, EndMT was further increased over that seen in mitral valves with mechanical stretch alone, but there was excessive sub-endothelial localized TGFβ on the atrial side, increased collagen deposition and leukocyte infiltration[78], all of which was blocked by losartan given over the 2 month post-MI time period[79]. The rationale for testing losartan therapy in this model was based on its negative effect on TGFβ signaling, in addition to its inhibitory effect on angiotensin II-receptor binding[96]. These studies suggest that preventing excessive TGFβ-driven EndMT may reduce fibrosis in the leaflets, which develops over several months post-MI, causing IMR.

Significantly increased EndMT was found at 6 months after inferior MI in sheep; this study led to the unexpected discovery of CD45, a protein tyrosine phosphatase, expressed along the MV leaflet endothelium and a corresponding increase in VE-cadherin-positive endothelial cells expressing CD45 and α-SMA, almost 15 times higher in IMI versus in sham animals. The increase was detected by flow cytometric analysis of mitral valve cells isolated immediately after mitral valves were removed from sham and post-MI sheep. *In vitro*, TGFβ induced expression of CD45, α-SMA, SLUG, collagen type I alpha 2 (COL1A2) and TGFβ2 in mitral VEC, all of which were blocked by inclusion of a CD45 phosphatase inhibitor[16]. We speculated that CD45 drives a maladaptive, pro-fibrotic form of EndMT in the mitral valve endothelium, possibly through post-translational mechanisms such as dephosphorylation of molecules involved in EndMT. EndMT has been recognized as a contributor to other types of valve diseases such as rheumatic heart disease[97] and calcific aortic valve disease (CAVD). In CAVD *in vitro* models, microRNA-483 was shown to protect against endothelial inflammation and EndMT, which can precede calcification, by downregulating ubiquitin E2 ligase C, resulting in decreased hypoxia inducible factor 1 subunit alpha (HIF1α)[98]. The authors went on to show a HIF1α inhibitor reduced calcification in a porcine CAVD model. CAVD and the role of EndMT in CAVD have been recently reviewed elsewhere[99, 100].

Atherosclerosis –

Studies in murine models of atherosclerosis have revealed EndMT, with confirmatory studies in human atherosclerotic plaques[83, 101]. Evrard and colleagues employed inducible endothelial lineage tracing and single cell imaging to identify endothelial cells that had

undergone partial EndMT and those that had completely transitioned to mesenchymal cells. Three to nine percent of the intimal plaque fibroblast-like cells were endothelial-derived, and thus had undergone complete EndMT; and further, these cells were correlated with plaque instability[83]. Chen and colleagues discovered that endothelial FGFR1 prevented TGFβ-driven EndMT, neointimal formation, and the progressive development of atherosclerosis[101]. Moonen *et al.* showed that disturbed flow decreased the ERK5/MAPK7 signaling that normally protects endothelium from EndMT and led to neointimal hyperplasia and atherogenic differentiation of EC[102]. Boström *et al.* found aortic endothelial cells from ApoE^{−/−} mice fed a high fat/high cholesterol 'western diet' expressed mesenchymal markers and showed signs of calcification[103]. Helmke and colleagues reported partial EndMT in aortic arch sections from ApoE^{−/−} mice fed a western diet. The transitioning endothelial cells were identified by expression of the stem cell marker stem cell antigen 1 (Sca-1)[104]. Lastly, evidence in support of partial EndMT was reported in patients with critical limb ischemia, a hazardous manifestation of atherosclerosis[105]. In this study, endothelial cells of the patients' intramuscular arterioles were bulked up and re-oriented such that they narrowed or occluded the arterioles. The endothelial cells maintained CD31 expression and remained on the luminal side of the vessel, however, the mesenchymal markers S100A4 and N-cadherin expression were increased. Quantification showed 76% of arterioles in had at least one endothelial cell positive for nuclear localized SNAI1, and increased nuclear pSMAD2/3 was detected as well, indicating TGFβ signaling. Taken together these murine and human studies strongly implicate partial and complete EndMT in atherosclerotic lesions and point to EndMT as a potential target for limiting the development and progression of unstable plaques. An important question is at what point along the continuum from endothelial to mesenchymal do cells begin to exert deleterious effects on the vessel wall, and what are the molecular mechanisms at each juncture.

In a recent study, single cell RNA-seq analysis of carotid arteries exposed to disturbed blood flow (*d-flow*) versus stable flow (*s-flow*) revealed that endothelial cells respond differently at the genomic level to disturbed versus stable flow. Andueza and colleagues identified *s-flow-* and *d-flow*-dependent enrichment of specific transcription factor binding sites and cis-regulatory elements[106]. *D-flow* reprogrammed the carotid arterial ECs into an array of phenotypes from inflammatory, to mesenchymal (i.e., EndMT), stem/progenitor-like, hematopoietic, and immune cell-like phenotypes. Furthermore, the authors showed that *d-flow* rapidly induced, whereas s-flow prevented, robust atherosclerotic development within 2 weeks in hypercholesterolemic mice. This fascinating study underscores the complexity of the endothelium and suggests its plasticity may extend beyond EndMT.

Pulmonary arterial hypertension (PAH)

PAH is a fatal disease marked by the cancer-like proliferation of pulmonary vascular smooth muscle and endothelial cells that is strongly linked to germline loss-of-function mutations in *BMPR2*[107, 108]. Examination of pulmonary artery plexiform lesions from PAH patients revealed cells co-expressing both endothelial markers and α-SMA, indicating EndMT[109]. *BMPR2* loss of function was shown to increase the EndMT-related transcription factor Twist-1 and the mesenchymal intermediate filament protein vimentin, and repress VE-cadherin and p120-catenin, thus indicating a role for BMPR2 signaling in preventing

EndMT in the animal model of severe PAH[110]. Another study showed that when BMPR2 falls below 50% of its normal level, BMP9 switches from promoting endothelial quiescence to promoting endothelial overgrowth[111]. Recently, another group of researchers showed that BMP9 induced a partial EndMT in pulmonary microvascular endothelial cells isolated from PAH patients but not in pulmonary microvascular endothelial cells from non-PAH lungs. The authors traced the mechanism to BMP-9 induced expression of IL-6 in the PAH pulmonary microvascular endothelial cells. They showed that neutralizing anti-IL-6 blocked BMP9-induced increases in SNAI1 and SNAI2/SLUG, preserved the endothelial cobblestone morphology, sustained the expression and junctional organization of VE-cadherin and resulting barrier function, and blocked expression of SM22α[112]. This study identified autocrine IL-6 as an inflammatory mediator that can drive EndMT in the lung microvasculature and thereby enhances our understanding of how inflammatory molecules operate in EndMT.

PAH endothelial cells have also been shown to express stem cell markers. Suzuki and colleagues used endothelial-specific lineage tracing in a mouse model wherein Sugen 5416 and hypoxia were used to induce PAH[113]. The authors delineated partial EndMT as cells enriched for the stem cell marker CD133/Prom1 and endothelial marker CD34 whereas CD31$^-$CD45$^-$Sca-1$^+$ lung cells were designated complete EndMT. The cells identified as having fully transitioned to a mesenchymal phenotype – the CD31$^-$/CD45$^-$/Sca-1$^+$ cells - showed higher proliferative and migratory capacity compared to non-endothelial CD326$^+$ lung mesenchymal cells. Further, the complete EndMT cells could be induced to differentiate into smooth muscle-like cells when grown in appropriate media. This indicates a de-differentiation followed by redifferentiation pathway in this setting. Normal pulmonary vascular endothelial cells were shown to undergo EndMT, exhibit increased proliferation and express stem cell markers in a model of acute lung injury, yet the cells lacked the features of complete EndMT seen in PAH[114]. In summary, accumulating evidence suggests that partial and complete EndMT play important roles in initiation and progression of PAH.

Cerebral cavernous malformations (CCM)

EndMT has been suggested to play a driving role in cerebral cavernous malformation-1 (CCM1). CCM1 is a vascular malformation characterized by enlarged capillary-like channels, loss of function mutations in *KRIT1* and cerebral hemorrhage. In mouse models of CCM1, upregulation of Erk5, Klf4 and Bmp6 were shown to drive cerebral endothelial cells to undergo EndMT. The role of KLF4 in regulating EndMT appears to be dependent on the cellular context. Cuttano *et al.* showed that in the absence of *Krit1*, Klf4 was increased in ECs of different organs but the full upregulation of EndMT markers was detected only in brain ECs suggesting that Klf4 upregulation is critical to promote the EndMT, however, the strength of this effect is context-dependent and specific to the brain vasculature[115]. In contrast, another study demonstrated a causal role of Klf2/Klf4 in CCM formation but found no evidence of EndMT involvement[116]. The role of abnormal flow and low fluid shear stress in the malformed CCM vessels may have an impact on the development of abnormal endothelial responses and changes in phenotype, as proposed by Gamble and colleagues[117]. Taken together, the role of EndMT – either partial or complete - in vascular malformations

is not fully defined and requires further investigation. Many other EndMT-mediated human diseases are reviewed and discussed elsewhere[118].

Drugs that prevent EndMT

Several TGFβ blocking drugs have been proposed as EndMT inhibitors. Losartan, an angiotensin II type 1 receptor antagonist, impairs EndMT by blocking TGFβ signaling, as shown *in vitro* in TGFβ-treated mitral VEC[26]. Bartko and colleagues showed systemic Losartan therapy over 2 months allowed adaptive growth of mitral valve leaflets but prevented thickening, EndMT and fibrotic changes[79]. Moreover, the effect of Losartan in hypertensive cardiac fibrosis was studied using spontaneous hypertensive rat models where it was shown that Losartan downregulated TGFβ/Smad signaling, increased CD31 expression, and reduced collagen 1 in the left ventricle[119]. There is little evidence that Losartan inhibits angiogenesis per se and thus it may not affect partial EndMT in angiogenic sprouting. Indeed, Jain and colleagues have shown Losartan increases vascular perfusion in dense tumor stroma by reducing extracellular matrix[120, 121]; from this one might speculate that Losartan might increase vascular perfusion in fibrotic tissue. Other drugs include Kallistatin, which was shown to upregulate endothelial nitric oxide synthase (eNOS) via its active site domain and inhibit *miR21*, *SNAI*1 expression, and reactive oxygen species formation through its heparin-binding domain[122], Linagliptin, a dipeptidyl peptidase IV inhibitor (DPP-4) that impairs DPP-4 interaction with integrin β1, was shown to block TGFβ2-induced EndMT in cultured human microvascular endothelial cells via upregulation of *miR29*[123]. Macitentan, an endothelin-1 receptor inhibitor, was shown to impair EndMT induced by either endothelin-1 or TGFβ1[124]. Ideally, EndMT-inhibitors would be targeted to specific sites in a temporally controlled manner to avoid systemic inhibition of EndMT that may be needed for regeneration at other sites, as shown recently in regenerating atrioventricular valves in zebrafish[13]. Up-to-date lists of the drugs with EndMT-inhibiting properties are available[88, 125]. As the triggers and timing of mal-adaptive EndMT are elucidated, the possibility of targeted prevention of EndMT in cardiovascular disease – both temporal and site-specific - may become feasible using drugs therapies.

Summary

EndMT represents prototypical plasticity of endothelium. The onset and degree of EndMT capability is dictated by inherent features such as embryonic origin and genetics and external factors such as hemodynamics, cytokines and hypoxia[78, 126]. EndMT relies on loosened cellular junctions, sprouting, and migration to move into the extravascular milieu; these are activities that overlap with leukocyte diapedesis and angiogenesis. Partially transitioned cells have several fates – revert to endothelial phenotype, transition fully to a mesenchymal cell, or pause at an elusive 'partial EndMT' state. Molecular drivers of EndMT include TGFβ and Wnt signaling molecules while FGFR1, ERK5, and BMPR2 signaling and as recently shown, fatty acid oxidation, restrain EndMT. Expression levels of different receptors sensitize the cells to specific TGFβ isoforms to fine tune endothelial responses in different cellular contexts[127]. While lineage tracing strategies using cell-specific constitutive versus inducible Cre drivers have advanced our understanding of EndMT considerably – Zhou and colleagues provide an excellent summary of these tools[128] – there is still little

known about which molecules control the rate and extent to which partial versus complete EndMT occurs. There is also little to distinguish pro-adaptive versus maladaptive EndMT. The animal models described above for IMR, atherosclerosis and PAH provide essential tools to address these questions and develop strategies to harness the endothelial plasticity to achieve tissue repair and regeneration.

Sources of Funding

List of non-standard abbreviations and acronyms

ALK	activin receptor-like kinase
α-SMA	α-smooth muscle actin
BMP9/10	bone morphogenetic protein-9/10
BMPR2	bone morphogenic protein-receptor 2
CAF	CBP-associated factor
CAVD	calcific aortic valve disease
CBP	CREB-binding protein
CCM	cerebral cavernous malformations
COL	collagen
Cpt2	carnitine palmitoyltransferase II
DDP-4	dipeptidyl peptidase IV inhibitor
d-flow	disturbed blood flow
EMT	epithelial to mesenchymal transition
EndMT	endothelial to mesenchymal transition
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-related kinase
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FGRS	FGFR substrate
FOXH1	forkhead Box H1
HIF1a	hypoxia inducible factor 1 subunit alpha
HMGA	high Mobility Group AT-hook

HUVECs	human umbilical vein endothelial cells
IL1β	interleukin-1β
IMR	Ischemic mitral valve regurgitation
KLF	Kruppel-like factor
KSHV	Kaposi's sarcoma herpesvirus
Lef	lymphocyte enhancer factor
MAPK	mitogen activated protein kinase (MAPK)
MI	myocardial infarction
MMPs	matrix metalloproteinases
MT1	membrane type 1
NFAT1	nuclear factor of activated T-cells, cytoplasmic 1
NF-κB	nuclear factor kappa B
PAH	pulmonary arterial hypertension
PTH	parathyroid hormone
Sca1	stem cell antigen 1
Shc	Src homology domain-containing protein
s-flow	stable flow
SMAD	Sma genes and the Drosophila Mad, Mothers against decapentaplegic
TCF	T cell transcription factor
TGFβ	transforming growth factor beta
TGFβRI/II	TGFβ receptors type I/II
TNFα	tumor necrosis factor-α
TRIM33	tripartite motif containing 33
VEC	valve endothelial cells
Wnt	wingless-related integration site

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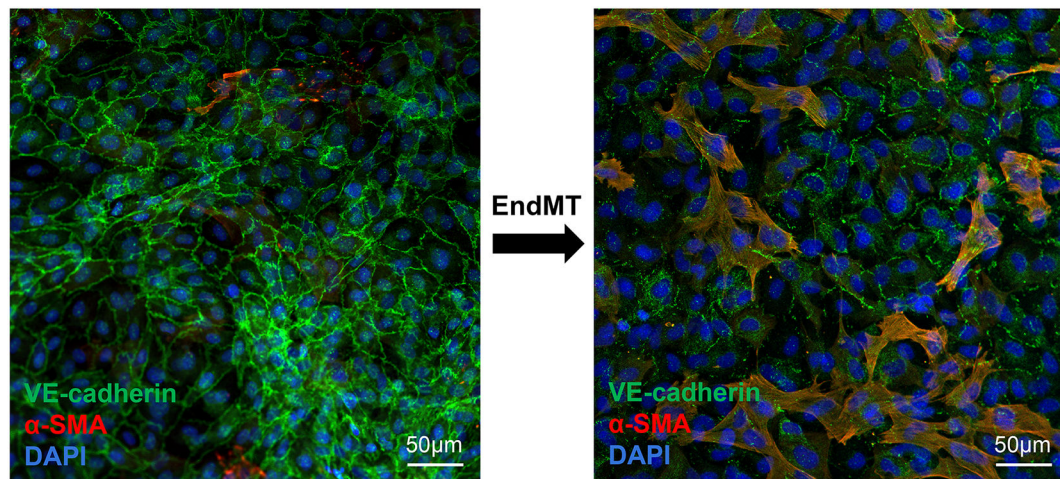
Highlights

- TGFβ, Wnt signaling, and disturbed shear stress promote EndMT.

- Normal laminar shear stress, FGFR1 and BMPR2 signaling and fatty acid oxidation suppress EndMT.

- Initial steps in EndMT – loosening of endothelial junctions and migration – are similar to initial steps of angiogenesis and leukocyte diapedesis.

- EndMT is associated with mitral valve leaflets thickening and mitral regurgitation after myocardial infarction.

- EndMT in pulmonary arterial hypertension is driven by loss of BMPR2 and dysregulated BMP-9 signaling.
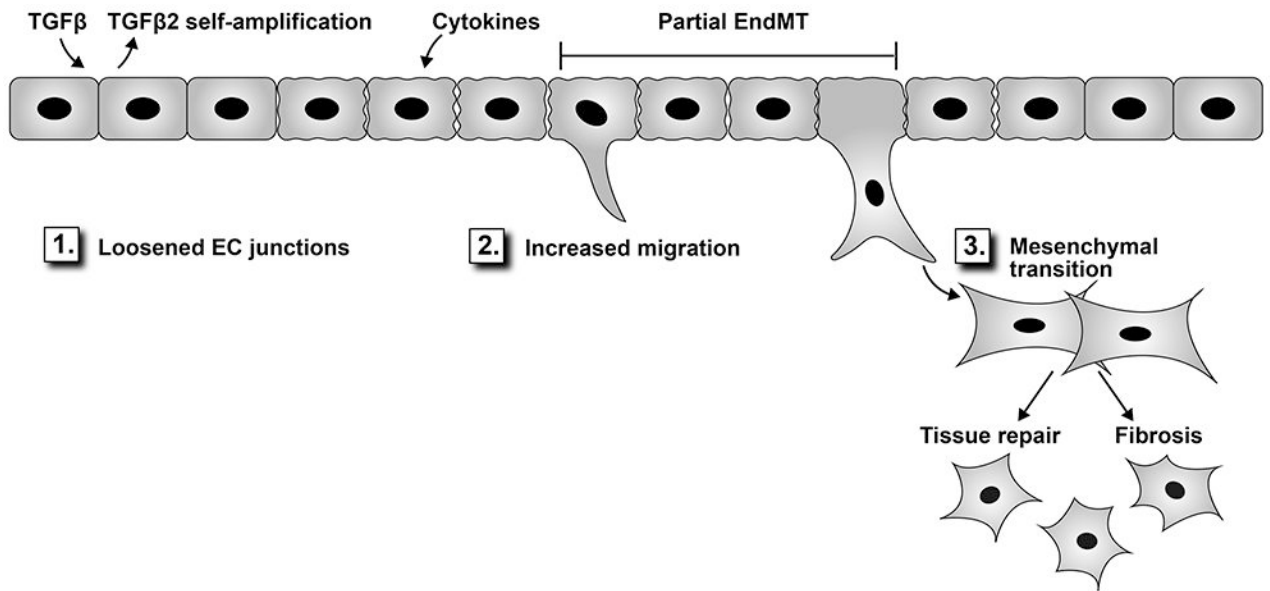
Figure 1.
Endothelial cells exposed to TGFβ and cytokines begin EndMT by loosening junctions between endothelial cells (1), which facilitates migration into the perivascular compartment (2). The mesenchymal transition (3) can be partial, wherein cells exhibit both endothelial and mesenchymal characteristics. This state can be transient – recently referred to as endothelial to mesenchymal activation[89]- as the cells revert to a functional endothelial phenotype. Fully transitioned cells with diminished endothelial and increased mesenchymal functions can contribute to tissue repair but in disease settings often exacerbate fibrosis.

Figure 2.
Mitral VECs in culture undergoing EndMT. Cells were co-stained with anti-VE-cadherin (green), anti-α-SMA (red) and DAPI (blue) to highlight nuclei. A 72-hour stimulus converted many VE-cadherin-positive VECs within the cobblestone monolayer to α-SMA-positive/VE-cadherin-positive cells (yellow/orange) with mesenchymal morphology, indicating partial EndMT. Scale bar, 50μm.