Endothelial to Mesenchymal Transition in Cardiovascular Disease: Key Mechanisms and Clinical Translation Opportunities

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THE PRESENT AND FUTURE

JACC STATE-OF-THE-ART REVIEW

Endothelial to Mesenchymal Transition in Cardiovascular Disease

JACC State-of-the-Art Review

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ABSTRACT

Endothelial to mesenchymal transition (EndMT) is a process whereby an endothelial cell undergoes a series of molecular events that lead to a change in phenotype toward a mesenchymal cell (e.g., myofibroblast, smooth muscle cell). EndMT plays a fundamental role during development, and mounting evidence indicates that EndMT is involved in adult cardiovascular diseases (CVDs), including atherosclerosis, pulmonary hypertension, valvular disease, and fibroelastosis. Therefore, the targeting of EndMT may hold therapeutic promise for treating CVD. However, the field faces a number of challenges, including the lack of a precise functional and molecular definition, a lack of understanding of the causative pathological role of EndMT in CVDs (versus being a "bystander-phenomenon"), and a lack of robust human data corroborating the extent and causality of EndMT in adult CVDs. Here, we review this emerging but exciting field, and propose a framework for its systematic advancement at the molecular and translational levels. (J Am Coll Cardiol 2019;73:190–209) © 2019 The Authors. Published by Elsevier on behalf of the American College of Cardiology Foundation. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

The endothelium is arguably one of the largest organ systems, and data continue to emerge regarding its heterogeneity and the many complex functions that it performs. Importantly, substantial evidence has implicated "endothelial dysfunction" as contributing to a range of cardiovascular diseases (CVDs). However, the broader programs whereby "endothelial dysfunction" leads to CVD pathogenesis have been challenging to define. Here, we review the rapidly expanding published data implicating the endothelial to mesenchymal transition (EndMT) as a common and potentially disease-causal biological program in CVD, highlighting the gaps in knowledge and therapeutic opportunities (**Central Illustration**).

To place EndMT in context, it is important to first consider epithelial to mesenchymal transition (EMT). Our understanding of EMT has its origins in seminal studies of embryonic development from the 1920s and the work of Johannes Holtfreter (1). However, it was not until the 1960s that chick embryo studies conducted by Elizabeth Hay led to the understanding that epithelial cells can undergo a "transformation" and give rise to embryonic mesoderm (2). It was later appreciated that EMT is reversible (mesenchymal to epithelial transition [MET]), and gradually the term

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"transition" has replaced "transformation." Given these initial studies, it is not surprising that a great deal is known about the indispensable roles of EMT/MET during embryonic development (which we previously reviewed from a cardiovascular perspective [3]). However, an equally impressive body of research also attests to the importance of EMT/MET during adult life. While many examples exist, such as the role played by EMT in organ fibrosis (4), perhaps the most relevant from a translational perspective is the role of EMT in cancer (5). While EMT is implicated in multiple aspects of cancer, and in particular epithelial tumor metastasis (5), it is notable that multiple targeted therapies aiming to inhibit EMT in cancer are already undergoing clinical evaluation (5). Furthermore, the inhibition of EMT is a partial effect of several U.S. Food and Drug Administration–approved chemotherapeutic agents that are already in use (6).

Although a vast amount has been learned about EMT/MET, our knowledge of EndMT is far more rudimentary. However, the endothelium is a specialized subtype of epithelium, and therefore, as highlighted throughout this review, it has been possible to extend some of the prior knowledge regarding EMT to EndMT.

A FUNCTIONAL AND MOLECULAR DEFINITION OF EndMT

Conceptually, EndMT involves a transition from an endothelial to a mesenchymal-like cellular state. However, at a molecular level, there are no agreed upon criteria for defining EndMT. This is rapidly becoming a hindrance, as there is no standardization and often little cross-comparability among data from different model systems and laboratories. Moreover, with respect to both development and CVD, the field must take account of endothelial cellular origins and their significant heterogeneity when considering formal EndMT definitions. Here, we review the current methods and systems used to study and define EndMT.

IN VITRO EndMT MODELS. EndMT is readily studied using in vitro cell culture systems. Typically, primary endothelial cells (ECs) or EC lines are induced to undergo EndMT by chemical or physical stimuli, with the most widely used being the application of transforming growth factor (TGF)-β for 5 to 8 days. Again, while a lack of standardization is problematic, an increasing tendency has been to use TGF-β with an additional stimulus, such as interleukin (IL)-1β (7) or hydrogen peroxide (H_2O_2) (8). These in vitro models have the advantage of providing a convenient and controllable environment to test novel factors and study molecular aspects of EndMT. They also provide a supply of cells that have undergone EndMT, which can be studied in downstream molecular and functional assays. However, a major limitation is that cell culture conditions (e.g., media, supplements) affect the extent and phenotype of EndMT.

IN VIVO EndMT MODELS. At present, 3 principal methods are used for studying EndMT in vivo. The simplest is to perform immunostaining for endothelial and mesenchymal proteins, which allows colocalization of these markers on individual cells that is suggestive of "transitioning" cells undergoing EndMT. However, this approach cannot identify cells that have substantially reduced or lost EC marker expression, and it is also dependent on the specificity and sensitivity of the antibodies used for immunostaining. Furthermore, under light microscopy, the superimposition of an EC and mesenchymal cell can be erroneously interpreted as a single cell undergoing EndMT.

While generally only applicable to mouse models, endothelial-specific Cre-lox lineage tracking systems are a more rigorous approach for studying EndMT

ABBREVIATIONS AND ACRONYMS

α-SMA = α-smooth muscle actin

BMP = bone morphogenetic protein

BMPRII = bone morphogenetic protein type II receptor

circRNA = circular ribonucleic acid

EC = endothelial cell

ECM = extracellular matrix

EFE = endocardial fibroelastosis

EMT = epithelial to mesenchymal transition

EndMT = endothelial to mesenchymal transition

FAO = fatty acid oxidation

FGF = fibroblast growth factor

GRB2 = growth factor receptor-bound 2

IL = interleukin

lncRNA = long noncoding ribonucleic acid

LOXL2 = Lysyl oxidase homolog 2

miRNA = microribonucleic acid

ncRNA = nonprotein coding ribonucleic acid

NOS3 = nitric oxide synthase 3 (also termed endothelial nitric oxide synthase)

PAH = pulmonary arterial hypertension

TGF-βR = transforming growth factor-β receptor

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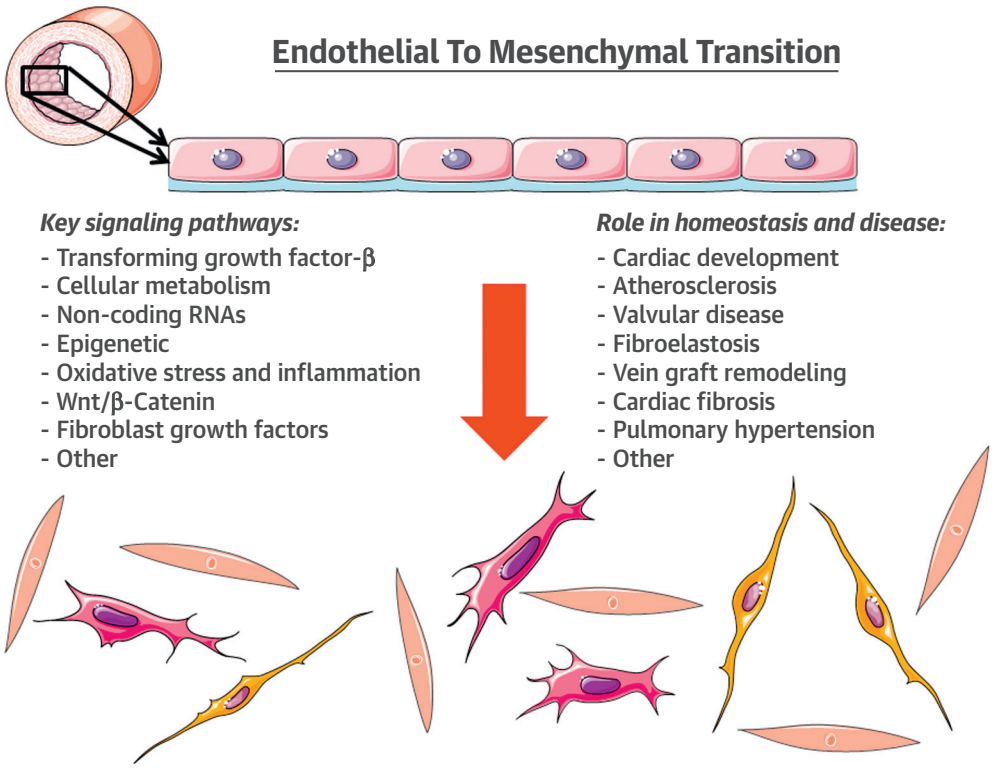
CENTRAL ILLUSTRATION **Endothelial to Mesenchymal Transition in Cardiovascular Disease: Key Mechanisms and Clinical Translation Opportunities**



Kovacic, J.C. et al. J Am Coll Cardiol. 2019;73(2):190–209.

Summary of the major concepts elucidated in this paper. Adapted with permission from Kovacic (144).

in vivo (9). Such mice are able to activate Cre-recombinase, which can be placed under the control of an endothelial-specific gene (e.g., *VE-Cadherin*). Cre activation is used to trigger defined genetic events, like the expression of a fluorescent marker protein that can be used to track ECs. With careful selection, Cre-lox systems can achieve permanent fluorescent marking of ECs, such that they continue to exhibit the fluorescent signal even if they undergo EndMT and suppress endothelial gene/protein expression. Alternatively, EC-specific Cre mouse strains can be crossed to "floxed" strains, where Cre activation leads to the deletion of a gene of interest. EC-specific Cre-lox gene deletion strategies can be used to selectively delete genes of interest that regulate EndMT, and thus, the effects of these genes and EndMT on differing biological processes can be determined (10,11).

As a further method for studying EndMT in vivo (and also in vitro), high-throughput RNA sequencing, of bulk or single cell preparations, is a powerful tool for studying the cellular transcriptome, whereby endothelial and mesenchymal gene expression patterns can be profiled to define the extent of EndMT. For example, bulk RNA deep sequencing of purified murine cells showed that following myocardial infarction or tissue hypoxia, ECs undergo clonal expansion and express mesenchymal genes such as *SM22α* in vivo (12). In addition, due to its potential to resolve EC signatures while concurrently showing mesenchymal gene up-regulation at the single-cell level, it is anticipated that single-cell RNA sequencing will be another useful tool for studying EndMT in vivo in human samples. Furthermore, RNA sequencing holds promise for providing insights on EC plasticity, which is the ability of an EC to switch its identity, including to additional phenotypes other than mesenchymal cells and also, having changed identity, to revert back to an EC state (see review [13]).

CELLULAR AND MOLECULAR ANALYSIS OF EndMT. A diverse selection of readouts has been used to

demonstrate EndMT, but obligatory characteristics are either: 1) reduced expression of endothelial genes/proteins; 2) increased expression of mesenchymal genes/proteins; or 3) ideally, both of these. Typically, most investigators present 2 to 3 each of endothelial and mesenchymal genes/proteins. Common examples include: *Endothelial:* CD31, VE-Cadherin, and endothelial nitric oxide synthase (NOS3); *Mesenchymal;* α-smooth muscle actin (α-SMA), calponin, SM22α, and versican. However, there is no agreement on which genes/proteins should be studied or how many, and the level of change required. Additional features that are sometimes also studied include increased expression of EndMT-associated transcription factors such as TWIST, SMAD3, ZEB2, SNAI1 and SNAI2.

Looking ahead, we propose that future studies should seek to provide more comprehensive transcriptomic and proteomic profiles of any proposed EndMT phenomenon. Furthermore, in any individual cell or cell population undergoing EndMT, gradations of EndMT exist (i.e., partial vs. more complete EndMT, reversible, transient, and so on), and there may be relative differences in the extent of endothelial gene/protein down-regulation versus mesenchymal up-regulation (12). This heightens the importance of a complete portrayal of EndMT using high-throughput techniques, whereby the balance of endothelial gene/protein down-regulation versus mesenchymal up-regulation is fully appreciated.

Functional and phenotypic cell changes during EndMT are also fundamental to this process and, thereby, to a definition of EndMT. Accordingly, studies of EndMT are increasingly demonstrating relevant changes in phenotypic traits (**Table 1**). However, yet again, no standardization exists. Indeed, reaching a consensus on these functional cellular aspects may be challenging, because certain EndMT-related phenotypic features may be important in specific contexts, but irrelevant or even opposing in others. For example, reduced tubule formation (i.e., angiogenesis) has been associated with EndMT (14,15), but as an apparent paradox, at least partial EndMT is necessary for angiogenesis (16). Highlighting this paradox at a molecular level, the transcription factor SNAI2 is expressed in angiogenic ECs and mediates angiogenesis (16), but SNAI2 is also a key mediator of EndMT (11). A full reconciliation of these functional aspects will likely remain challenging until more is understood about EndMT as a whole.

TABLE 1 Functional Studies to Support an Altered Cellular Phenotype With EndMT

Assay	Cell Characteristic	(Ref. #)
Reduction of Endothelial Characteristics		
EC tubule formation in culture	Cellular ability to form tubules in culture—a defining EC characteristic	(14,15)
Thrombin generation	Cellular ability to inhibit thrombin formation	(14,15)
Lectin binding	Lectin binding is a defining EC characteristic	(15)
LDL-uptake	Ability of cells to uptake LDL cholesterol—a defining characteristic of ECs	(15)
Enhancement of Mesenchymal Characteristics		
Invasion	Ability to invade through matrix and other substrates	(8)
Migration	Ability of cells to migrate across a transwell and/or through micropores	(8,14)
Contraction	Enhanced cell contractility with mesenchymal phenotype	(14,15,60)
Collagen production	Enhanced collagen production with mesenchymal phenotype	(108)

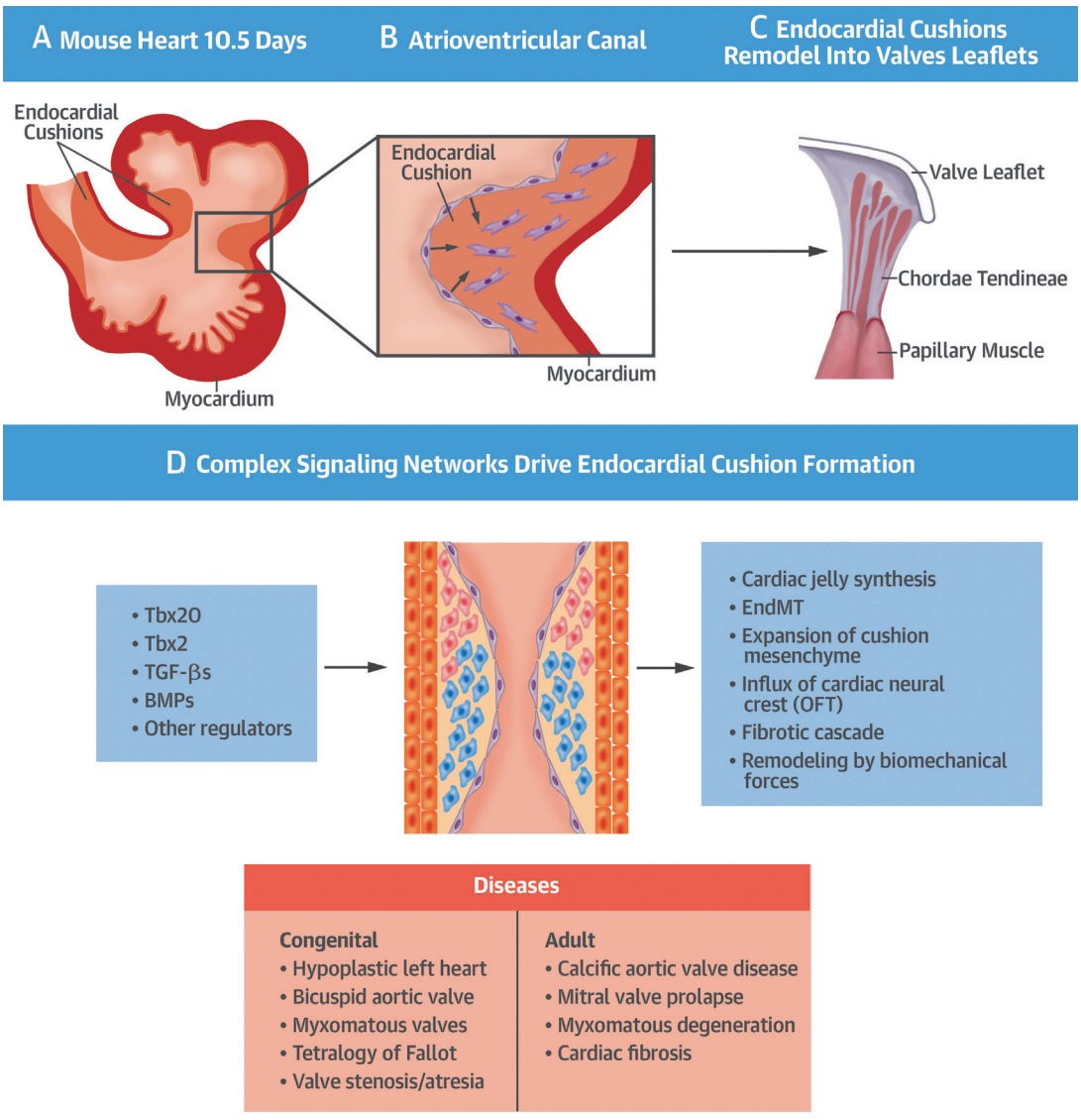
EC = endothelial cell; EndMT = endothelial to mesenchymal transition; LDL = low-density lipoprotein.

EndMT IN CARDIAC DEVELOPMENT

Heart progenitor cells arise within the embryo from newly formed mesoderm that originates from the primitive streak. After heart tube formation, the endocardium and endothelium of the great vessels are created by vasculogenesis (17-19), whereby vessels form de novo from endothelial progenitors. The endocardium likely has heterogeneous origins, arising from endocardial-myocardial heart field progenitors (17,18,20-22), and also cells that migrate in from the yolk sac mesoderm (an extraembryonic tissue) (19). Some endocardial cells express markers of hemogenic endothelium, perhaps reflecting their origins from yolk sac hemangioblasts (a common progenitor of blood and vessels) (19) and the activation of the hematopoietic program within embryonic endothelium (23).

As the heart develops, the endocardium retains remarkable cellular plasticity. For example, the endocardium associated with the forming ventricles undergoes a process that resembles angiogenic sprouting in developing vascular beds (24), leading to the formation of endocardial domes which, together with myocardium and extracellular matrix (ECM), define the morphological units of trabeculation. The endothelium of the coronary arteries and veins is also formed by sprouting, in this case from the sinus venosus endocardium into the myocardium (25). This process leads to formation of an endothelial plexus within the subepicardial ECM, which then extends deeper into the myocardial walls (26,27). Developmental patterning of the coronary vessels may then be supplemented by adaptive angiogenesis initiated by sprouting of endocardium from the intertrabecular crypts into the myocardial wall, driven by hypoxia (27-30). Trabecular endocardium also contributes to the coronary arterial tree postnatally, as the outer

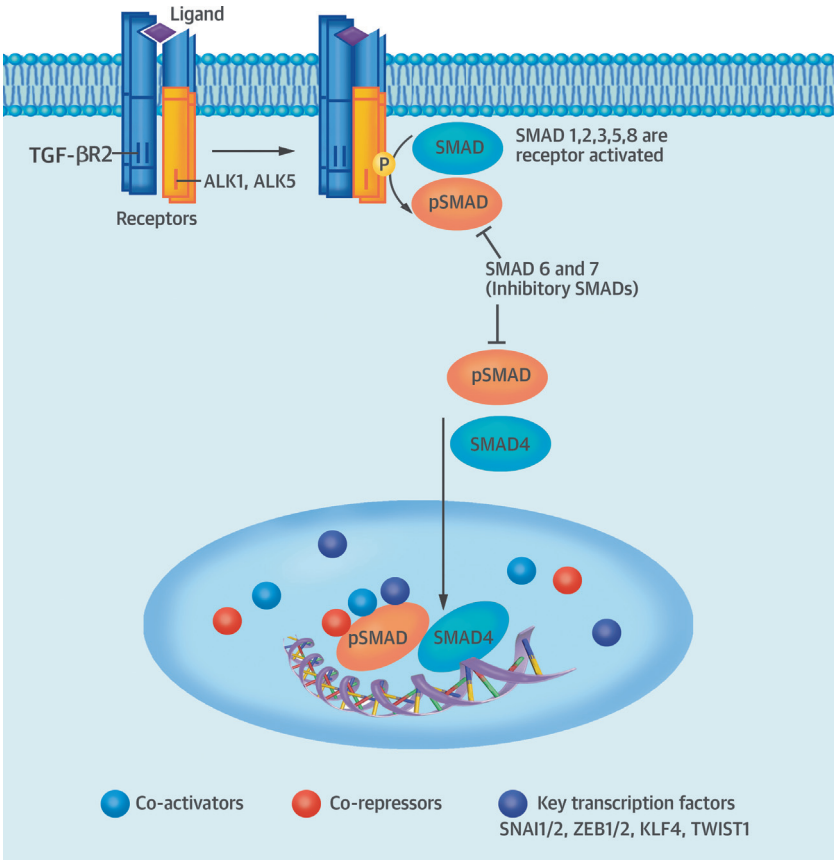
FIGURE 1 EndMT During Cardiac Development



(A) Schematic cross-section of the developing mouse heart at ~10.5 days showing major chambers, forming septa, and the outflow tract (OFT). **(B)** Schematic enlargement of one side of the AV canal **(boxed region in A)**. During EndMT, ECs of the AV canal and OFT become mesenchymal and occupy the prominent ECM swellings separating myocardium and endocardium (endocardial cushions) in those regions. **(C)** Cellularized endocardial cushions are later remodeled into stratified valve leaflets. Here, a mitral valve leaflet is shown, correctly tethered to the ventricle by papillary muscles and chordae tendineae. **(D)** Complex signaling networks drive endocardial cushion formation, EndMT, expansion of cushion mesenchyme and cardiac remodeling, which are further modulated by biomechanical forces associated with heart contraction and blood flow. Key molecular factors (see text and **Figure 2**) and relevant human congenital and adult valvular diseases are indicated. AV = atrioventricular; EC = endothelial cell; EndMT = endothelial to mesenchymal transition; TGF = transforming growth factor.

"compact" myocardial layer undergoes expansion (29). Blood islands form on the ventral surface of the heart through a budding process, and these contain an endothelium that is also derived from the endocardium (27). Blood island endothelium expresses hemogenic markers (31), suggesting that the blood cells found within these islands are derived from the endothelium (and hence endocardium).

FIGURE 2 **TGF-β Signaling and EndMT**



In ECs, TGF-β classically signals via TGF-βR2 (a type II receptor component) and AKL1 or ALK5 (type I receptor complexes). Receptor complexes combine on the cell surface and comprise 2 type I and 2 type II components. TGF-βR2 phosphorylates (activates) type I components, which then propagate the signal intracellularly via activation (phosphorylation; pSMAD) of SMAD 1, 2, 3, 5, or 8. Activated SMAD proteins form complexes that include the common mediator SMAD4, and which may be inhibited by SMAD6 or SMAD7. SMAD complexes shuttle to the nucleus, where they interact with coactivators, corepressors, and additional transcription factors, the latter including key EndMT gatekeepers SNAI1/2, ZEB1/2, KLF4, TCF3, and TWIST. These interactions culminate in chromatin rearrangements and transcription factor binding to endothelial, mesenchymal, and other gene promoter regions that ultimately bring about EndMT. Abbreviations as in **Figure 1**.

Endocardial lineage plasticity is further highlighted by its ability to transdifferentiate into adipocytes and mural cells in distinct settings (32,33).

The touchstone of EndMT occurs during the formation of the endocardial cushions, which are the precursor structures for the cardiac valves (**Figure 1**) (34,35). The endocardial cushions first appear as prominent swellings of ECM (called cardiac jelly) located between endocardium and myocardium in the valve-forming regions of the atrioventricular (AV) canal and outflow tract. Cushions are induced locally by TGF-β signaling from adjacent myocardium, which increases the synthesis of cardiac jelly ECM and pathways that induce EndMT (34,36). At around embryonic day 8.5 to 9.0 in the mouse, following heart looping, a subset of endocardial cells lining the cushions undergo EndMT and migrate into the cushion ECM (34). Genetic lineage tracking shows that the majority of mesenchymal cells infiltrating the cushions are derived from endocardium (37), although the lateral (parietal) AV valve leaflets are composed of epicardium-derived cells (38). Cushion infiltration is mediated by metalloproteinases and ECM receptor signaling, and is accompanied by both new synthesis and degradation of ECM. During further development, cellularized cushions are remodeled into valve leaflets with stratified mesenchymal and ECM layers (34), a process that relies on

genetic and hemodynamic cues (39). Cushion mesenchyme also contributes to the structural fibrotic tissue that knits together the interatrial and interventricular septal structures with the valvular complexes (36). The outflow tract cushions are also infiltrated by migratory cranial neural crest cells, which contribute to outflow tract septation forming the aortic and pulmonary trunks (34,35).

The involvement of EndMT in formation of the AV-septal complex, and its critical role in valvulogenesis, septation, alignment of the cardiac chambers and vessels, and hemodynamics, creates a vulnerability that underlies both congenital heart disease and adult valvular disease. At the severe end of the spectrum, aberrant cushion development may lead to complete AV canal defect, which is typically fatal. More subtle forms of endocardial and cushion mal-development may contribute to a variety of congenital heart diseases. For example, in the rare but serious hypoplastic left heart, aortic and/or mitral valve stenosis may be a contributing factor. Likewise, pulmonary valve stenosis is a part of the tetralogy of Fallot (**Figure 1**). We focus specifically on EndMT in valvular disease later in this paper.

SIGNALING PATHWAYS AND MECHANISMS CONTROLLING EndMT

EndMT SIGNALING DURING DEVELOPMENT. A large number of signaling pathways govern EndMT during cardiac development (34–36,40). Briefly, signaling through bone morphogenetic proteins (BMPs) and TGF-β ligands and receptors, which is modulated by the Hippo pathway (41), leads to endocardial expression of *Snai1*, *Snai2,* and *Twist*, which encode archetypal transcription factors regulating EndMT (36,40). The NOTCH pathway is also essential for EndMT, although it is not required for the initial formation of ECM swellings (42). NOTCH ligands Delta-like 4 and NOTCH receptors (NOTCH1-4) are expressed on AV canal and outflow tract endocardial cells before and throughout EndMT. When membrane-bound NOTCH1 receptor is engaged by ligand, sequential protease cleavages release the NOTCH1 intracellular domain (N1ICD), which migrates to the nucleus and acts as a transcription coregulator, activating and repressing genes that define cell identity. NOTCH1 signaling through an alternate ligand Jagged 1, expressed from myocardium, restrains BMP-mediated EndMT, highlighting the presence of negative feedback mechanisms (43). NOTCH1 intracellular domain binds directly to and positively regulates *Snai1* and *Snai2*, and the expressed Snai1 and Snai2 proteins repress *Ve-Cadherin* transcription to allow EndMT. Signaling pathways involving WNT/β-catenin, VEGFA/VEGFR, and neuregulin 1/ERBB2/ERBB3; as well as transcription factors NFATC1, GATA4, and SOX9; and ECM proteins hyaluronan and versican are also involved in EndMT and subsequent valve maturation (34–36,40). As a result of these signaling pathways, endocardial-derived cells within the cushions undergo EndMT and adopt a fibroblastic fate. Like fibroblasts in other connective tissues, valvular fibroblasts undergo a maturational process akin to bone, cartilage, and tendon formation, and the transcription factor SOX9, which is induced by BMPs, acts as a central regulator of ECM gene expression networks (44).

TGF-β AND THE TGF-β SUPERFAMILY. The TGF-β superfamily is an extensive signaling network that is considered a master regulator of EndMT and which comprises TGF-β isoforms 1 to 4, BMPs, activins, and related proteins (**Figure 2**). Among these, while TGF-β3 and -β4 are less studied, both TGF-β1 (11,45) and TGF-β2 (8) promote EndMT. TGF-β and other ligands from the superfamily signal via TGF-β receptor complexes (46). These receptor complexes combine and are comprised of 2 type I and 2 type II receptor components (4 components in total), which include activin receptor-like kinases (ALKs) and BMP receptor components. Also, among these are TGF-β receptor 1 and 2 (TGF-βR1 and TGF-βR2), with TGF-βR2 being a type II receptor component. Type I receptor components are comprised of the ALK family, which include TGF-βR1 (also known as ALK5). In the complex, type II receptors phosphorylate and active type I components, which then propagate the signal intracellularly. There are 7 type I and 5 type II receptor complexes in humans; however, the binding possibilities are restricted in ECs, where TGF-β binding to TGF-βR2 can activate either of 2 type I receptors; ALK1, which is largely restricted to ECs, or the broadly expressed ALK5 (47,48). Accessory TGF-β receptors may also become involved, like endoglin or betaglycan, which modulate signaling through type I and II receptors.

Upon type I receptor activation, TGF-β family members regulate gene expression via SMAD transcription factor activation (i.e., via phosphorylation) (46,49). Activated SMAD proteins form complexes and shuttle to the nucleus, where they interact with additional transcription factors that include key regulators of EndMT: SNAI1, SNAI2, ZEB1, ZEB2, KLF4, TCF3, and TWIST. These interactions culminate in chromatin rearrangements and transcription factor binding to endothelial, mesenchymal, and other relevant gene promoter regions which induce EndMT (46,49) (**Figure 2**).

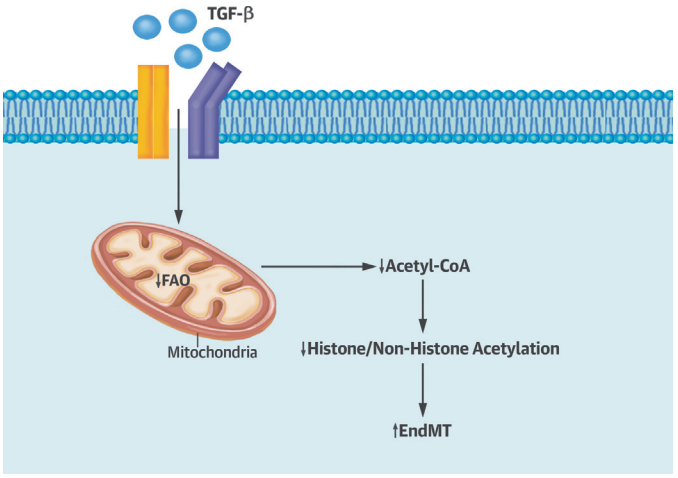
There are multiple checkpoints in this system including the ligand BMP7 which inhibits EndMT (45), and SMAD7, which exerts an inhibitory effect at the transcriptional level (7). In addition, although TGF-βs signal mainly via the SMADs ("canonical TGF-β signaling"), they may also activate other complimentary pathways ("noncanonical TGF-β signaling").

The TGF-β signaling system also acts as a final common mechanism for other pathways. Important factors that intersect with TGF-β signaling include mitogen-activated protein kinases (MAPKs), the phosphoinositide 3-kinase (PI3K) pathway, inhibitory microRNAs (miRNAs) such as the miR-200 family, and others. Therefore, as well as canonical and noncanonical TGF-β signaling, the TGF-β signaling system serves to integrate these other pathways and to fine-tune the ultimate regulatory changes governing EndMT (7).

METABOLIC REGULATION OF EndMT. There is a growing appreciation that cellular fate is mechanistically associated with intracellular metabolism. However, the mechanisms linking these processes are imprecisely understood. As a new development, a recent study suggests that EndMT may have metabolic underpinnings (7). Using TGF-β1 to induce EndMT in vitro, it was shown that TGF-β1 triggered a reduction in mitochondrial-dependent fatty acid oxidation (FAO) (7). In other cell types and paradigms, TGF-β signaling had been shown to modulate glucose metabolism (50), lipid metabolism (51), and mitochondrial function (52). For the case of ECs, the TGF-β-stimulated inhibition of FAO resulted in a decline in acetyl-CoA (7). Indeed, this fall in acetyl-CoA was an important metabolic stimulus for EndMT, as other genetic or pharmacological strategies that reduced cytosolic acetyl-CoA levels could recapitulate the effects of TGF-β signaling (7) (**Figure 3**). Notably, although FAO inhibition would be expected to primarily alter mitochondrial acetyl-CoA levels, the authors found that it was the cytoplasmic pool of acetyl-CoA that was modulating cellular fate. These pools are not in equilibrium, and there is growing evidence that acetyl-CoA modulates its effects under strict spatiotemporal control (53). These observations likely have in vivo relevance, because it was further shown that genetic disruption of endothelial FAO augmented the contribution of EndMT to mitral valve development in a mouse model (7), suggesting that targeting of endothelial metabolism might be a therapeutic strategy to modulate EndMT in other pathological settings.

Finally, there is an additional potential link between metabolism and EndMT. There is increasing evidence for a role of EndMT in fibrotic disease, including the fibrosis associated with chronic kidney disease (54). In that sense, other studies have suggested that fibrosis in chronic kidney disease is somehow mediated by a fall in FAO (55). It is tempting to speculate that the mechanistic link between a fall in FAO and the increase in fibrosis is somehow related to an altered threshold for EndMT, or through the related process of EMT.

FIGURE 3 Fatty Acid Oxidation Regulates EndMT

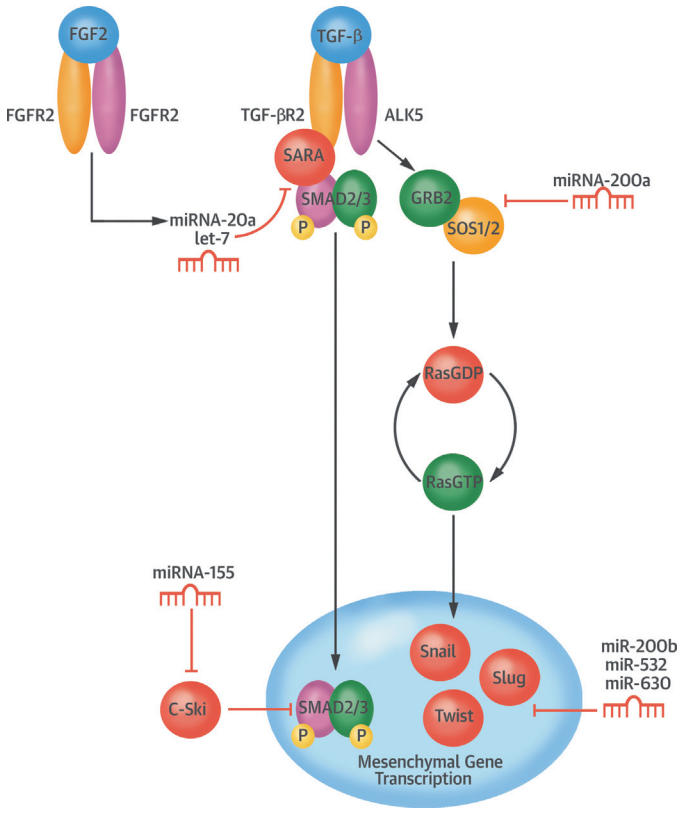


Key elements of the role of fatty acid oxidation (FAO) in regulating EndMT. EndMT = endothelial to mesenchymal transition.

NONCODING RNAs IN EndMT. Nonprotein coding ribonucleic acids (ncRNAs) play a major role in cell fate decisions, and recent advances have also underlined their critical role in regulating EndMT. ncRNAs include miRNAs, long noncoding ribonucleic acids (lncRNAs) and circular ribonucleic acids (circRNAs), which together could influence the entire EndMT regulatory program.

miRNAs are small, noncoding RNAs that inhibit the expression of their gene targets, predominantly by inducing messenger RNA degradation or inhibiting messenger RNA translation. In the context of EndMT, TGF-β induces a distinct shift in EC miRNA expression (56), suggesting their importance in the overarching regulation of EndMT. Notably, several miRNAs have been identified that antagonize the EndMT transcriptional program, which are transcriptionally suppressed by TGF-β signaling (57–62). For example, fibroblast growth factor (FGF) 2, an antagonist of TGF-β signaling in ECs (63), induces the expression of miR-20a which then silences TGF-βR1 and -βR2 expression, effectively blunting canonical TGF-β

FIGURE 4 The Role of Noncoding RNAs in Regulating EndMT



Key elements of what is known about how EndMT may be controlled by noncoding RNAs. FGFR = FGF receptor; SARA = SMAD anchor for receptor activation; SOS = Son of sevenless homolog; other abbreviations as in **Figure 1**.

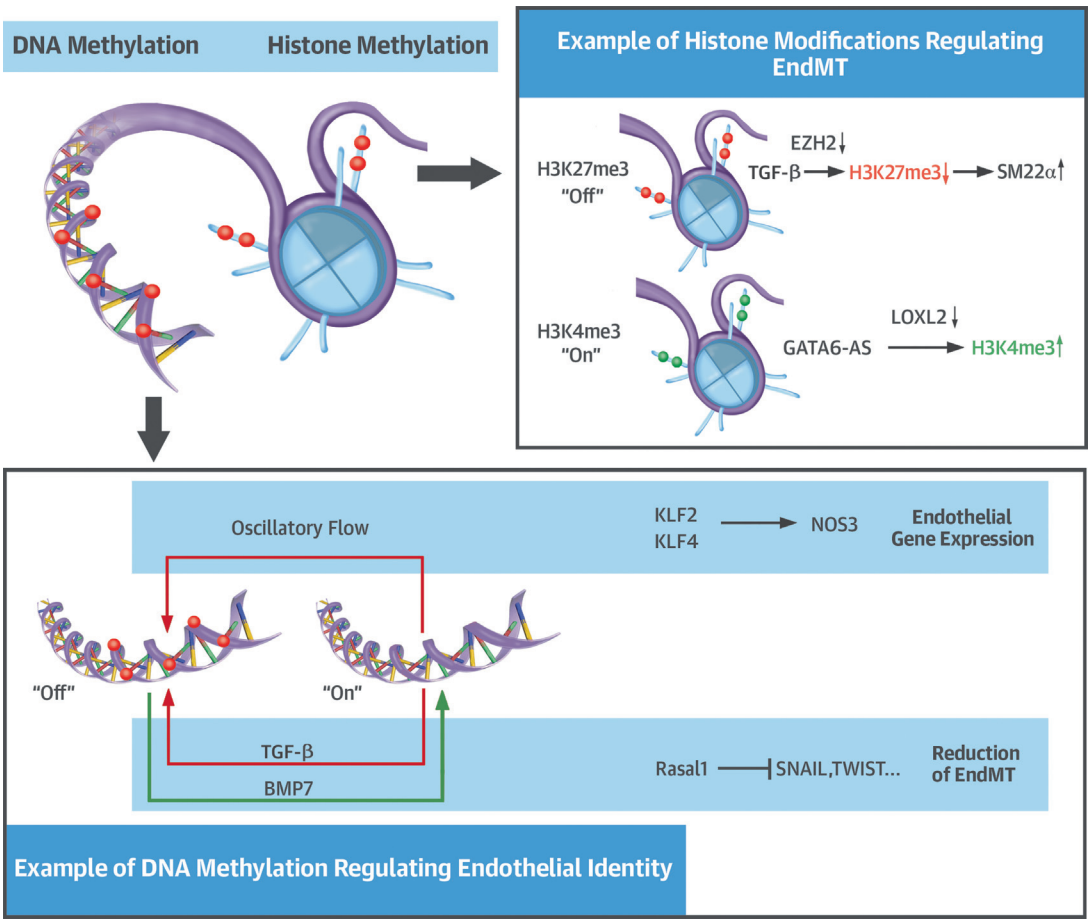
signaling (60). Chen et al. (59) also showed that miRNA let-7 negatively regulates TGF-βR1 expression. Similarly, miR-200a can reduce the expression of growth factor receptor-bound 2 (GRB2), a mediator of noncanonical TGF-β signaling (62). GRB2 plays a vital role in the development of cardiac fibrosis (64), a condition wherein EndMT may be present (45), and the ectopic expression of miR-200a in ECs treated with TGF-β blunted the EndMT response (62). Downstream of TGF-β receptors, miRNAs also affect the expression of SNAI1 (e.g., miR-200b and miR-532 [57,58]), and SNAI2 (e.g., miR-630 [61]).

As well as miRNAs that directly suppress EndMT, TGF-β induces the expression of miRNAs that affect endothelial gene expression or that suppress inhibitors of mesenchymal gene transcription (65-67). In ECs, mesenchymal gene transcription is kept inactive by transcriptional repressors, including the SKI proto-oncogene (c-Ski) and the ternary complex factor ELK1. C-SKI represses TGF-β signaling by stabilization of inactive SMAD complexes on SMAD-binding elements (68), which is inhibited by miR-155 upon TGF-β signaling (67). Similarly, ELK1 is repressed by miR-27b upon TGF-β signaling (66). ELK1 competes with the mesenchymal transcription factor MRTF in binding to serum response factor, thereby acting as a myogenic repressor (69). The loss of ELK1 from ECs leads to increased MRTF activity (70) and mesenchymal gene transcription (71). Besides miRNAs that affect mesenchymal gene expression, TGF-β also increases the expression of miRNAs that suppress endothelial protein expression. Sustained AKT activation facilitates EndMT (72) and culminates in elevated expression of matrix metalloproteinases (73,74) that can degrade VE-Cadherin (75). PTEN is an endogenous inhibitor of AKT activation (76) and a target of miR-21 (65), suggesting that miR-21 inhibition can inhibit EndMT. Similarly, the systemic delivery of miR-21 antagonists reduced the number of cells undergoing EndMT in the cardiac microvasculature, and altered cardiac fibrosis in mice (65). Notably, the regulation of EndMT by miRNAs is not limited to these examples (**Figure 4**), and the list of miRNAs implicated in EndMT appears certain to expand.

LncRNAs are a vast additional class of ncRNA that regulate gene transcription by a variety of mechanisms. Recently, GATA6-AS, a long noncoding antisense transcript of GATA6, was shown to facilitate EndMT by interacting with the histone deaminase Lysyl oxidase homolog 2 (LOXL2) to regulate endothelial gene expression via chromatin remodeling (77). Moreover, the lncRNA MALAT1 was shown to suppress the function of miR-145, which culminated in increased expression of TGF-βR2 and SMAD3, facilitating EndMT (78). However, little is currently known about how lncRNAs are regulated and functionally relevant in EndMT; an area that is important to pursue with the improving knowledge of lncRNA biology. Notably, lncRNAs are generally poorly conserved across species, adding difficulty to proving in vivo evidence of their function. This may be particularly relevant when considering translational animal studies targeting lncRNAs as a route to human therapeutics.

CircRNAs are a poorly understood subset of lncRNA that are characterized by their covalently closed loop structures (79), with current research suggesting a possible regulatory role for circRNAs in EMT (80). If a regulatory role for circRNAs in EndMT is also demonstrated, this will assuredly be a rich area for further basic research.

FIGURE 5 Epigenetic Mechanisms and Control of EndMT



Key elements of what is known of how epigenetic changes modulate EndMT. EndMT = endothelial to mesenchymal transition.

EPIGENETIC CONTROL OF EndMT. "Epigenetic" refers to heritable control of gene expression that does not involve changes to the underlying DNA sequence. Epigenetic control can occur at the level of DNA, where DNA methylation induced by DNA methyltransferases results in silencing of gene expression, a process that can be reversed by DNA demethylases (e.g., TETs). In addition, various histone modifications, including acetylation and methylation, control accessibility of transcription factors to target gene promoter regions. Whereas multiple studies have elucidated the epigenetic control of EMT, little is known regarding the epigenetic control of EndMT (**Figure 5**).

In EMT, expression of the *SNAI1/2* family, *TWIST* and *ZEB1/2*, is controlled by DNA methylation as well as histone acetylation and methylation (81). Also, the effects of transcription factors on their target genes (e.g., E-Cadherin) are regulated by corepressors, including the histone deacetylases, histone methyltransferase G9a or SUV39H1, and DNA methyltransferases (81). In ECs, epigenetic mechanisms at the level of DNA methylation or histone modifications play a crucial role in the expression of EC-specific genes and up-stream regulators. For example, DNA methylation represses the flow-induced transcription factors of the Krüppel-like family Klf2 and Klf4 (82-84), which are important for maintaining endothelial function and are involved in EndMT (13). Likewise, the promoter of *Nos3* is repressed in non-ECs by DNA methylation and is controlled by histone acetylation and methylation (**Figure 5**) (85,86). These mechanisms regulate endothelial-specific gene expression in response to differing stimuli; however, whether EndMT is associated with complete, direct, and long-lasting

silencing of endothelial genes via epigenetic mechanisms is unclear.

DNA methylation patterns are modulated under conditions of EndMT and can indirectly interfere with EndMT signaling. Altered DNA methylation in response to oscillatory flow was reported in aortic intima-media tissues from patients with aortic valve disease (87). This study showed that methylation patterns are distinct in dilated versus nondilated ascending aortas, and specifically that nondilated aortas from patients with bicuspid aortic valve disease show a methylation signature associated with cell transformation and differentiation. Conversely, the flow response in ascending aortas from patients with bicuspid aortic valves involved hypomethylation and increased expression of Wnt/β-catenin genes, whereas an angiogenic profile was observed in the aortas of patients with tricuspid aortic valves (87). Whether these changes in DNA methylation are solely due to ECs and how they causally contribute to aneurysm formation in patients with bicuspid aortic valves will be important to understand. Additional insights regarding the epigenetic control mechanisms of EndMT were gained in animal models of cardiac fibrosis. Here, TGF-β1 induced DNA methylation of the promoter of the Ras inhibitor *RASAL1,* thereby increasing the expression of SNAI1, SNAI2, and TWIST and promoting EndMT in vitro and in vivo. Interestingly, BMP7 reversed the TGF-β1-induced RASAL1 promoter methylation and subsequent silencing of gene expression via induction of the DNA demethylase TET3 (88). At the level of histones, enhancer of zeste homolog-2, a methyltransferase of the polycomb complex, was shown to regulate SM22α expression (89). TGF-β2 reduced enhancer of zeste homolog-2 levels in ECs, leading to a decrease in silencing H3K27me3 marks at the *SM22α* promoter (89). Furthermore, the histone deacetylase 3 isoform HD3α was shown to induce EndMT (90). However, this effect was likely not caused by epigenetic control mechanisms, but was mediated via HD3α interactions with Akt and regulation of TGF-β2 (90). Finally, as mentioned earlier, lncRNAs may control EndMT by interfering with histone modifications, where GATA6-AS was shown to regulate EndMT and modulate H3K4m3-dependent gene expression by binding to LOXL2 (77).

OTHER FACTORS AND PATHWAYS INFLUENCING EndMT. It is notable that TGF-β signaling only partly induces EndMT (8,11,45), suggesting that additional mechanisms are also involved. Although several stimuli, including glucose (91), endothelin-1 (92), angiotensin II (93), and advanced glycation end-products (94) induce EndMT by converging with TGF-β signaling, alternate pathways of EndMT induction also exist. Among these, Jagged/NOTCH signaling can directly induce the expression of SNAI2, TWIST, and the mesenchymal transcription factor RUNX3 (95–97). In addition, Wnt/β-Catenin signaling drives EndMT via increased SNAI2 expression (98). Interestingly, Wnt/β-Catenin–induced EndMT via SNAI2 induction does not change SNAI1 transcripts (99), indicating that not all transcription factors are required for EndMT induction.

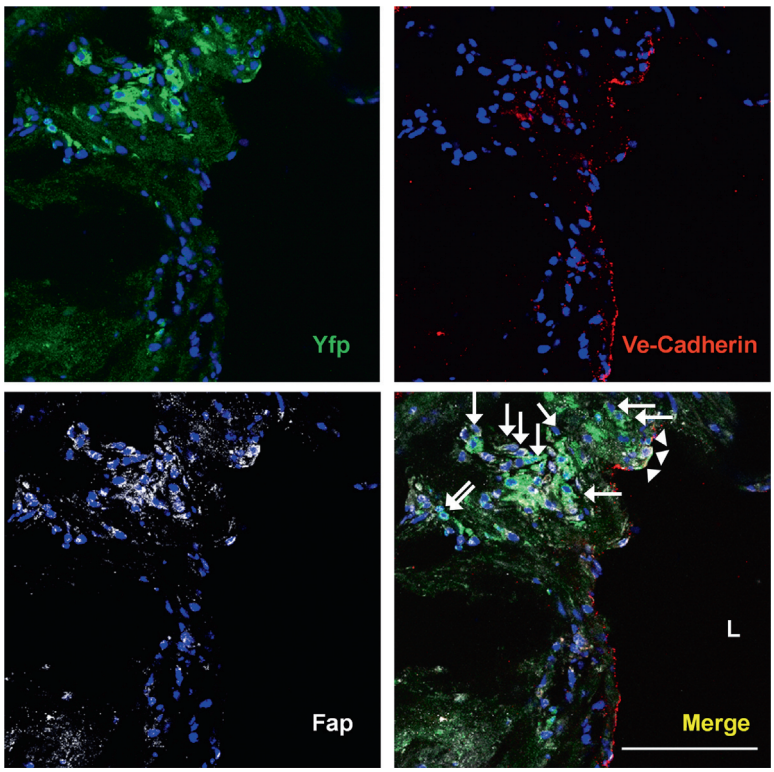
Oxidative stress is another factor that promotes EndMT. Specifically, hydrogen peroxide (H_2O_2), a classic inducer of oxidative stress, promotes EndMT (8). Furthermore, the effect of H_2O_2 is additive to TGF-β (8), and the inhibition of reactive oxygen species can decrease oxidative stress–induced EndMT in vitro (100). Consistent with this, EndMT is also promoted by the inhibition of nitric oxide synthase, which reduces the bioavailability of nitric oxide and enhances oxidative stress (101). The importance of oxidative stress in EndMT is being further explored, with recent studies suggesting that oxidative stress may promote EndMT in the setting of atherosclerosis and renal fibrosis (102,103).

As mentioned, endogenous inhibitors of EndMT also exist, although their mechanisms of action are incompletely understood. FGF signaling in ECs abrogates TGF-β signaling by suppressing the transcriptional activity of SMAD2 (104) and the induction of miRNAs that silence TGF-β receptor expression (59,60). BMP7 can antagonize TGF-β signaling by induction of ID proteins (105), which are dominant negative helix-loop-helix proteins that lack a DNA-binding domain. ID proteins can heterodimerize with SMAD2 and SMAD3, resulting in the formation of inactive transcription factor complexes (106). Of note, ID protein expression is reduced during EndMT (15) and the restoration of ID protein expression can inhibit EMT in certain tumors (107). Although these data suggest a role for ID proteins in EndMT, this is yet to be confirmed. Undoubtedly, many additional pathways controlling EndMT remain to be disclosed.

CVDs AND PATHOLOGIC PROCESSES WITH EndMT IMPLICATIONS

ATHEROSCLEROSIS AND PLAQUE EROSION. The accumulation of mesenchymal cells, including myofibroblasts, smooth muscle cells, and osteoblasts, is central to plaque formation and atherosclerosis. Mesenchymal cells play key roles in this disease including proinflammatory molecule secretion; matrix, collagen, and metalloproteinase production;

FIGURE 6 **EndMT in Atherosclerosis and Plaque Erosion**

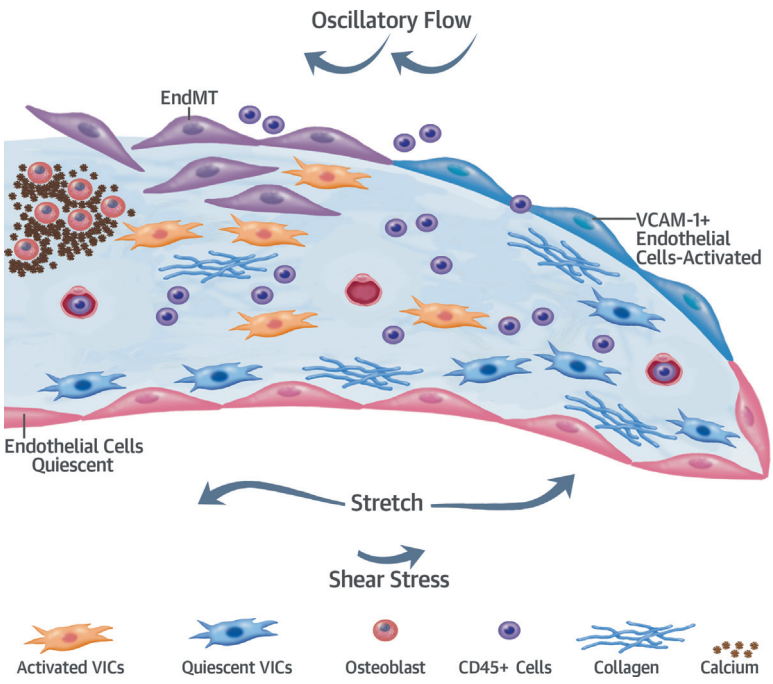


Confocal microscopy of thoracic aortic sections from tamoxifen-induced *end.SclCreER^{T};R26RstopYfp;ApoE$^{-/-}$* mice after Western diet feeding. In this model of advanced atherosclerosis, ECs are permanently marked using a Cre-lox system such that ECs, and all EC-derived cells, permanently express yellow fluorescence protein (Yfp). Staining for Ve-Cadherin is in **red**, with staining for fibroblast activation protein (Fap), a fibroblast marker, in **white**. DAPI nuclear staining is in **blue**. Yfp^{+}Fap^{+}Ve-Cadherin^{+} cells **(arrowheads)** represent endothelial-derived cells expressing endothelial and fibroblast proteins. Yfp^{+}Fap^{+}Ve-Cadherin^{-} cells **(arrows)** represent endothelial-derived cells that express Fap, but that have lost Ve-Cadherin expression. Scale bars, 100 μm. L = lumen. Reproduced with permission from Evrard et al. (8).

plaque calcification; and fibrous cap formation. As early evidence suggesting EndMT is involved in atherosclerosis, costaining of human atherosclerotic plaques and porcine vessels for endothelial and mesenchymal markers identified copositive cells in the intima and within neointimal tissues (108). In addition, while uniform laminar shear stress was found to inhibit EndMT, ECs exposed to disturbed flow (as is typical in atherosclerosis-prone regions) underwent EndMT and showed atherogenic differentiation. Gain- and loss-of-function studies established a role for ERK5 signaling in the inhibition of EndMT with uniform laminar shear stress (108). Supporting these findings, Mahmoud et al. (109) showed that low, oscillatory shear stress promotes EndMT, whereas high shear stress is protective. In this case, low-shear related EndMT was under the control of SNAI1 (109) and TWIST1 (110), whereas costaining was again suggestive of EndMT in human atherosclerotic plaques (109). Importantly, the link between EndMT and disturbed flow indirectly suggests that EndMT may be causal for atherosclerosis. As a sidebar, but also indirectly suggesting that EndMT may be causal for atherosclerosis, it was recently shown that atheroprotective high-density lipoproteins inhibit EndMT (111).

Two studies have used Cre-lox mouse models to study EndMT in atherosclerosis, with both showing that EndMT plays an important role. Of these, Evrard et al. (8) showed that the predominant EndMT-derived cell population in atherosclerosis is fibroblast-like cells, with a lesser contribution to smooth muscle-like cells (**Figure 6**). Overall, EndMT-derived cells comprised almost one-half of the fibroblast population in advanced atherosclerotic lesions. In addition, they showed that EndMT is

FIGURE 7 EndMT in Heart Valve Disease



In normal valves, valvular interstitial cells (VICs) possess a quiescent phenotype. VICs become activated in disease. EndMT in heart valves could be initiated by mechanical stress and inflammation (CD45-positive cells). Activated VCAM1-positive endothelium undergoes endothelial to mesenchymal transition (EndMT), which generates more interstitial cells. Some of these cells may undergo osteogenic transformation and activation resulting in various diseases.

associated with increased plaque vulnerability. On the other hand, Chen et al. (10) studied a potential link between disrupted FGF signaling, EndMT, and atherosclerosis. In addition to lineage tracking, they also created atherosclerotic mice with endothelial-specific deletion of FGF receptor substrate 2α (Frs2α). These knockout mice exhibited extensive EndMT and developed atherosclerosis earlier than control mice, eventually demonstrating an 84% increase in total plaque burden. As a whole, their study suggested a link between loss of protective endothelial FGF signaling, development of EndMT, and progression of atherosclerosis.

It is provocative and exciting that these studies, involving mice, large animals, and humans, have consistently shown that EndMT is prominent in atherosclerosis. An important next step will be to define the exact functional role of EndMT in the development and progression of atherosclerotic disease (vs. being an epi- or bystander-phenomenon). Furthermore, we believe that another important step is to investigate the role of EndMT in "plaque erosion." In brief, plaque erosion may lead to arterial thrombosis and accounts for ~30% of acute coronary events (112). Mechanistically, plaque erosion occurs without fibrous cap disruption, where blood comes into contact with an intimal surface lacking ECs. Supporting the hypothesis that EndMT is involved, plaque erosion is more common in arterial bifurcations and areas of disturbed blood flow (112). We speculate that if a significant proportion of ECs undergo EndMT, this may lead to a disrupted endothelial layer that culminates in plaque erosion. Subjectively, images obtained during lineage tracking of EndMT in atherosclerosis (8) give the impression that the loss of ECs over the surface of plaques is related to their migration into the plaque's inner aspects (**Figure 6**).

VALVULAR DISEASE. While EndMT is critical to valve development, low levels of EndMT likely persist in postnatal and adult cardiac valves. As gauged by CD31/α-SMA coexpression, ~10% of ECs in human fetal valves undergo EndMT, decreasing to ~1% in human adult valves (113). Importantly, this raises the hypothesis that the adult valvular endothelium contains a subset of cells that can undergo EndMT to replenish the turnover of valvular

interstitial cells (114), thus maintaining valve tissue homeostasis.

Recent evidence implicates EndMT in valvulopathies (115), and many transcriptional regulatory mechanisms of heart valve development actively respond to valve injury, stress, and disease (**Figure 7**). Whereas interstitial valvular cells are quiescent fibroblasts in healthy adult valves, during disease progression they transform into activated myofibroblast-like cells that express α-SMA (116), and subsequently differentiate into osteoblast- and chondrocyte-like cells characteristic of calcific aortic valve disease (117). Since many of the previously mentioned fundamental pathways involved in valvulogenesis (e.g., NOTCH, Wnt, BMP, and TGF-β) also participate directly in valvular calcification, the question arises whether EndMT can generate osteogenic cells. The discovery that cadherin-11, which is important for cushion formation, is re-expressed in the endothelium and osteoblast-like interstitial cells in adult human aortic valves may support this notion (118). Moreover, in vivo and in vitro studies have demonstrated the osteogenic potential of a subpopulation of mitral valve ECs (119). Furthermore, Hjortnaes et al. (120) showed that EndMT precedes osteogenesis and that valvular interstitial cells suppress calcification of valvular ECs undergoing EndMT. Recent studies also implicated inflammation and mechanical stress in potentiating valvular EndMT (121–123). To recapitulate the microenvironment of mechanical strain, 2-dimensional microcontact printing was used to mimic regions of healthy and diseased leaflets, and to measure EndMT in sheep valve ECs responding to low (10%, healthy) and high (20%, disease) strain. The results suggest that dual strain-dependent pathways regulate EndMT: increased TGF-β yields low-strain EndMT and increased Wnt/β-catenin signaling yields high-strain EndMT. Furthermore, a surgical model of ischemic mitral regurgitation in adult sheep revealed elevated levels of α-SMA within the endothelium and interstitium, indicative of EndMT (122). After myocardial infarction, higher levels of collagen-producing α-SMA-positive cells in malfunctioning mitral valve subendothelium indicated a dramatically exaggerated EndMT process (123), which could be modulated by losartan without reducing adaptive growth (124).

Using clinically-relevant large animal models, collectively these reports suggest that: 1) EndMT participates in the initial adaptive response to an altered environment and may result in pathological processes such as fibrosis, leading to suboptimal valve function; and 2) proinflammatory conditions and mechanical stress/strain might regulate EndMT in adult valves. Furthermore, these studies demonstrate that EndMT plays an important role in maintaining the phenotype of valvular cells in adults, and that certain environmental conditions may predispose valvular endothelium to enhanced EndMT.

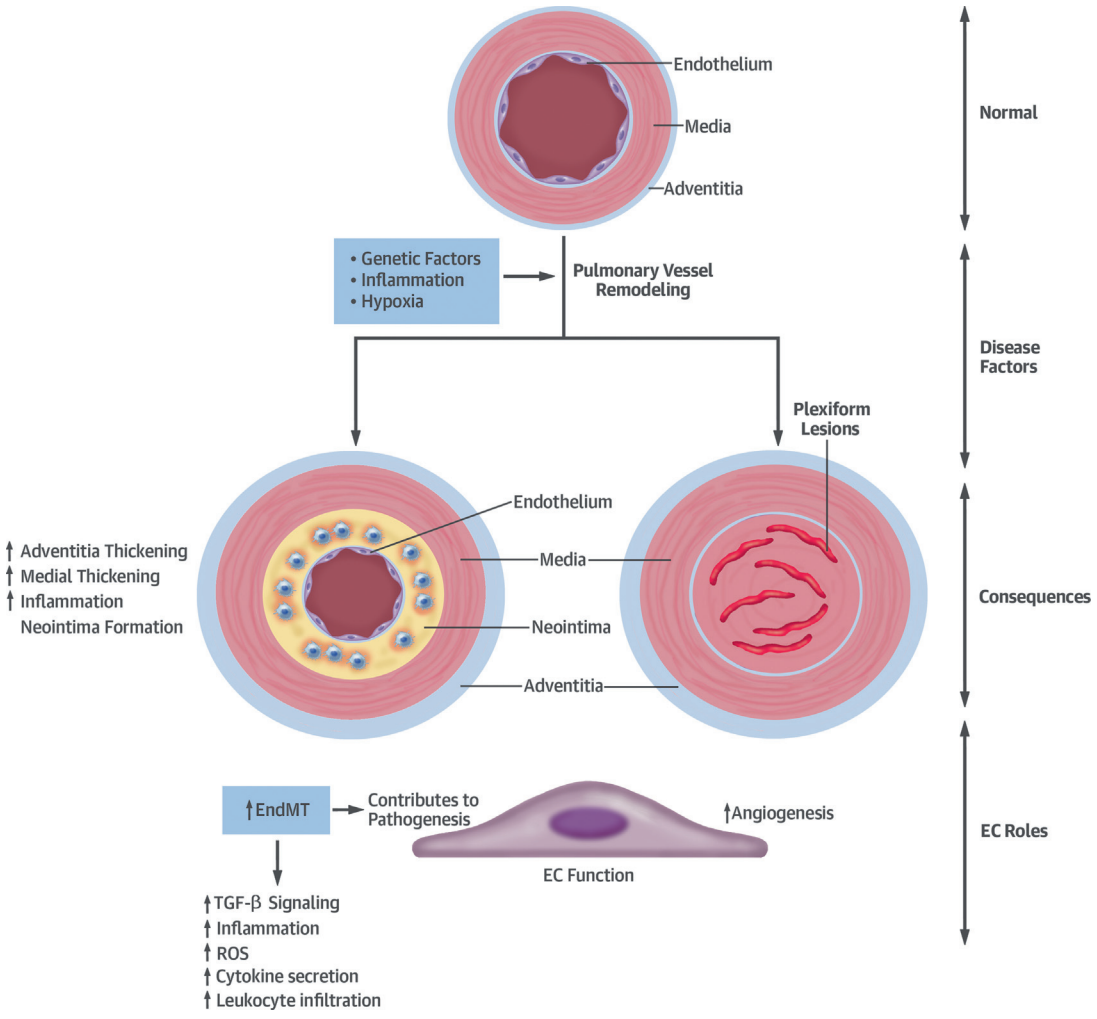
FIBROELASTOSIS. Endocardial fibroelastosis (EFE) is a rare disorder characterized by a unique fibrosis involving the ventricular endocardium, which restricts ventricular growth in infants and children. EFE is typically associated with prenatal cardiac abnormalities, most notably in lesions with left heart obstruction including Barth and hypoplastic left heart syndrome (125). Often, the only therapeutic option is surgical univentricular palliation, which is associated with high mortality rates (126). Hence, EFE is of major clinical importance, yet the mechanisms underlying this disease are poorly understood.

Novel mouse models that mimic human EFE now permit studies of the origin of EFE tissues and their mechanisms of formation (127,128). As discussed, during development, the endocardium undergoes EndMT to form the cardiac valves and septa (**Figure 1**). This indirectly suggests that, if aberrantly activated, the endocardium might also form the fibroelastic tissue found in EFE. Supporting this hypothesis, endothelial lineage tracking studies in EFE mice have shown that a proportion of EFE cells are derived via EndMT. Moreover, using immunofluorescence staining for endothelial and mesenchymal markers, EndMT was identified in human EFE tissues (129).

Interestingly, hypermethylation of *BMP7* (an endogenous EndMT inhibitor) was found in human EFE tissues, and exogenous recombinant BMP7 was able to inhibit EndMT and EFE development in the mouse model (129), suggesting that drugs targeting epigenetic mechanisms (DNA methyltransferase inhibitors or DNA demethylase activators; see Epigenetics section) might be efficacious for preventing EFE.

VEIN GRAFT REMODELING. Veins are commonly used conduits in arterial bypass graft surgery; however, 20% to 30% of vein grafts may fail within 12 to 18 months (130). Vein graft failure is largely due to adverse vascular remodeling, and the modulation of "early" activators of this process could be targeted to block the entire downstream complications that lead to graft failure (130). Cooley et al. (11) have shown that EndMT is important in vein graft remodeling and neointimal formation, which is the maladaptive smooth muscle cell hyperplasia that arises after a vein is exposed to arterial pressure. Specifically, with the adaptation to arterial pressure, they observed that ~50% of neointimal cells were EndMT-derived (11).

FIGURE 8 EndMT in PAH



Key features of PAH and the role of EndMT. FPAH = pulmonary arterial hypertension; other abbreviations as in Figure 1.

EndMT-derived cells were found to be typical synthetic SMCs, expressing α-SMA and SM22α. EndMT in this setting was dependent on TGF-β signaling, with early activation of Smad2/3-Snai2. Correspondingly, antagonism of TGF-β signaling resulted in decreased EndMT and less neointimal formation. Cooley et al. (11) further identified that both Smad2 and Smad3 regulate *Snai2*, with Smad3 shown to directly bind the *Snai2* promoter. Histological examination of post-mortem human vein grafts corroborated these findings, suggesting that EndMT is operative during human vein graft remodeling (11).

CARDIAC FIBROSIS. If there is a controversial aspect of EndMT, it is its contribution to cardiac fibrosis. In 2007, the first major publication emerged about EndMT in adult animals, suggesting that cardiac fibrosis was associated with EndMT (45). Using a *Tie1* Cre-lox endothelial lineage tracking system in a model of cardiac overload and fibrosis, cells that once expressed *Tie1* (an endothelial marker) contributed to 27% to 33% of cardiac fibroblasts. The use of *Smad3*-deficient mice or administration of BMP7 inhibited EndMT and cardiac fibrosis in vivo (45). This study catalyzed significant interest in the field and subsequently, using cellular costaining (65,88,92,131) and Cre-lox systems (132), other investigators recapitulated the finding that EndMT contributes to cardiac fibrosis. For example, Murdoch et al. (131) used costaining and changes in protein expression to conclude that EndMT is involved in cardiac fibrosis and

diastolic dysfunction, which was mediated by endothelial nicotinamide adenine dinucleotide phosphate oxidase-2 activation (131). However, other studies have refuted these claims, suggesting that de novo EndMT plays little role in cardiac fibrosis in the adult (133,134). Adding complexity, a challenge faced by these studies is the need to distinguish between cardiac fibroblasts that are developmentally derived via EndMT from the endocardial cushions (for which there is consensus agreement [133,134]) versus de novo cardiac EndMT from adult ECs (where the controversy resides).

A potential explanation may lie in the fact that during EndMT in the adult, cells with a fully mature mesenchymal phenotype may be rarely achieved (8). In other words, as already mentioned, EndMT in the adult is likely associated with a partial transition to a mesenchymal-like phenotype, but not fully mature mesenchymal cells. This is consistent with recent studies in the kidney, where de novo EMT gave rise to partially transitioned fibroblast-like cells (4). Nevertheless, the fact the EndMT in the adult may be an incomplete process likely cannot account for all of the discrepancies in these studies, and further research is required to fully define the contribution (or not) of de novo EndMT to cardiac fibrosis in the adult.

TABLE 2 Additional Disease States Where EndMT Has Been Implicated

Disease	Potential Role of EndMT	(Ref. #)
Fibrodysplasia ossificans progressiva	Murine lineage tracking and human cell characterization experiments showed an endothelial origin of osteoblasts and chondrocytes via EndMT	(145)
Kidney fibrosis and kidney transplant failure	EndMT may participate in renal fibrosis	(146)
Cardiac transplant vasculopathy	Somewhat similar to atherosclerosis, EndMT may participate in cardiac transplant vasculopathy	(63)

EndMT = endothelial to mesenchymal transition.

PULMONARY HYPERTENSION. Primary pulmonary arterial hypertension (PAH) is a rare condition mediated by distal pulmonary vasculature vasoconstriction, aberrant vascular remodeling, vascular occlusions, and the formation of characteristic plexiform lesions (**Figure 8**) (135). In addition, endothelial dysfunction is a hallmark of PAH (135). Many cases of PAH are caused by BMP type II receptor gene (*BMPR2*) mutations, resulting in increased TGF-β signaling, including both noncanonical and canonical Smad-mediated signaling (135), although other rare variants are also implicated (136).

EndMT was first identified in PAH based on in situ analyses of endothelial and mesenchymal markers, as

TABLE 3 Major Near-Term Obstacles and Challenges That Remain to Be Overcome in the Investigation and Clinical Translation of EndMT

Challenge/Obstacle	Comments	Solution
Lack of a robust functional and molecular definition of EndMT	The lack of a functional and molecular definition of EndMT is fostering scientific confusion, hampering research and interpretation, limiting comparability of data, and facilitating the publication of studies with suboptimal endpoints.	Research teams should work together to achieve a functional and molecular definition in the near term. This will require sharing of data, pooling and combined analyses of high-throughput datasets (i.e., RNA sequencing, proteomics), and consensus agreement on definitions. This will be an ongoing process that will need refinement as further data and knowledge emerge. Embedded within this task is the understanding of additional molecular issues such as the reversibility of EndMT, or whether it is a clonal phenomenon.
Lack of understanding of the contribution of EndMT to disease causation (vs. being a disease association or epiphenomenon)	This problem is challenging to address in the human context, but genetic mouse models are well-suited to this task. However, these studies require significant resources and meticulous scientific approaches that must be relevant to EndMT in human pathology.	We propose extensive, well-designed, and meticulously conducted genetic mouse studies, with validation and reproducibility achieved among collaborating laboratories.
Lack of robust human data on EndMT	Although mouse studies are the most effective model system for rigorous proof of concept and for demonstrating causality, findings must be validated (ideally) in larger animals and (absolutely) in humans. Furthermore, some critical questions that drive our interest in EndMT, such as the role of EndMT in plaque erosion, can only be tackled in humans as there are no true animal models.	We propose detailed human studies using explanted and surplus surgical tissues from relevant disease states, with the application of cutting-edge techniques such as single-cell RNA sequencing to explore the contribution and extent of EndMT. Cross-validation among collaborating laboratories of key findings will be essential.
Lack of translational proof of concept	Few studies have attempted to manipulate EndMT in larger animals (122–124). Although large animal translational proof-of-concept studies are clearly necessary, they require extensive resources and clear scientific objectives. A critical consideration is the existence of a suitable model, and whether regulatory authorities would require large animal studies before clinical trials could commence. Moreover, this may be disease-specific and context-specific, because EndMT appears relevant across a range of CVDs.	Consortia should be formed with a view to prioritizing EndMT targets in disease states amenable to large animal models, with a view to systematically studying the utility of manipulating EndMT for therapeutic gain. It will be essential to engage with regulatory authorities to assess need and appropriate nature of such models in the functional disease context.

CVD = cardiovascular disease; EndMT = endothelial to mesenchymal transition.

well as an intervention study using rapamycin that reversed protein markers that are characteristic of EndMT (137). Further evidence demonstrated both the presence of EndMT in PAH and also the induction of EndMT by IL-1β, TGF-β, and TNFα. Notably, EndMT-derived cells secreted high levels of cytokines and supported a greater extent of immune cell transmigration (138). An association of pathological mechanisms came with the finding that EndMT in PAH was related to high motility group AT-hook 1 (HMGA1), demonstrated through association of HMGA1 protein expression with cells undergoing EndMT. Interestingly, and as a link to EndMT, HMGA1 expression was associated with reduced BMPR2 levels (139). Further mechanistic understanding has come through an association of EndMT in PAH with Twist expression and function (140). Moreover, a study of HIF-2α in human and experimental models has demonstrated a link with hypoxia, and mechanistically through induction of Snai1/2. Notably, endothelial loss of the prolyl hydroxylase domain protein 2 gene (this protein promotes HIF-2α degradation) led to severe PAH even in normoxia conditions (141). A separate study showed that loss of HIF-1α inhibited EndMT induction and normalized endothelial CD31 levels (142). Finally, a detailed characterization of pulmonary ECs undergoing EndMT has highlighted the contribution of the cells themselves, and also the paracrine signaling that such cells induce in the lung vasculature (143).

A growing list of other CVDs are also associated with EndMT. Although in some cases the evidence is perhaps not as robust as the studies and diseases mentioned previously, these are summarized in **Table 2**.

CONCLUSIONS AND FUTURE DIRECTIONS

EndMT is involved in numerous CVDs, which collectively are a major cause of global morbidity and mortality. Hence, the manipulation of EndMT for therapeutic gain is a tantalizing prospect. Nevertheless, a number of obstacles remain to be overcome before the full therapeutic potential of manipulating EndMT can be realized, as described in **Table 3**. Undoubtedly, beyond these issues there are still further unknown challenges to be met and unforeseen obstacles to be resolved. However, with a collaborative and focused effort, we believe that over the next decade enormous advances can be made with respect to our understanding and future manipulation of EndMT as a potential clinical therapy.

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