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DEVELOPMENTAL DYNAMICS 247:492–508, 2018
DOI: 10.1002/DVDY.24589

Endothelial-to-Mesenchymal Transition in Cardiovascular Diseases: Developmental Signaling Pathways Gone Awry

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The process named endothelial-to-mesenchymal transition (EndMT) was observed for the first time during the development of the chicken embryo several decades ago. Of interest, accumulating evidence suggests that EndMT plays a critical role in the onset and progression of multiple postnatal cardiovascular diseases. EndMT is controlled by a set of developmental signaling pathways, very similar to the process of epithelial-to-mesenchymal transition, which determine the activity of several EndMT transcriptional effectors. Once activated, these EndMT effectors regulate the expression of endothelial- and mesenchymal-specific genes, in part by interacting with specific motifs in promoter regions, eventually leading to the down-regulation of endothelial-specific features and acquisition of a fibroblast-like phenotype. Important technical advances in lineage tracing methods combined with experimental mouse models demonstrated the pathophysiological importance of EndMT for human diseases. In this review, we discuss the major signal transduction pathways involved in the activation and regulation of the EndMT program. Furthermore, we will review the latest discoveries on EndMT, focusing on cardiovascular diseases, and in particular on its role in vascular calcification, pulmonary arterial hypertension, and organ fibrosis. *Developmental Dynamics 247:492–508, 2018.* © 2017 Wiley Periodicals, Inc.

Key words: EndMT; EMT; fibroblast; TGF-β; inflammation; flow; Wnt; FGF; calcification; PAH; fibrosis

Submitted 3 May 2017; First Decision 30 August 2017; Accepted 30 August 2017; Published online 11 September 2017

Introduction

The cardiovascular system supplies all tissues within the body with sufficient oxygen and nutrients and removes waste metabolites. Endothelial cells (ECs) are the main constituents of the blood vessels and form a monolayer to enclose the vessel lumen. Adjacent to the ECs are the mural cells (pericytes and vascular smooth muscle cells [vSMCs]), which provide stability (Pober and Sessa, 2007). During embryonic development, ECs arise from the mesoderm germ layer and generate a heterogenous population of cells (Garlanda and Dejana, 1997; Aird, 2012). Perhaps the most intriguing form of endothelial plasticity refers to their ability to de-differentiate into a multipotent mesenchymal progenitor. This process is known as *endothelial-to-mesenchymal transition* (EndMT) and somehow recapitulates a phenomenon largely studied in epithelial cells, named *epithelial-to-mesenchymal-transition* (EMT), that has been shown to take part in the onset and progression of diverse pathologies, including cancer (Saito, 2013).

Microscopy studies have shown that ECs undergoing EndMT progressively change their concrete, compact and well-structured (so called cobblestone-like) shape and acquire a less organized and elongated morphology. This change is partially due to cytoskeletal rearrangements in cellular actin filaments that, among other events, suggest the loss of their apical-basal orientation, destabilization of cell junctions, and the remodeling extracellular matrix (ECM). Early publications have merely relied on these morphological observations to describe the phenomenon. Currently, EndMT is characterized by the down-regulation of endothelial markers (e.g., CD31 or platelet EC adhesion molecule-1 (Pecam-1), vascular endothelial (VE)-cadherin, vascular endothelial growth factor receptor (VEGFR), and the angiopoietin receptor Tie-2) and the increase in the expression of mesenchymal proteins, including fibroblast specific protein-1 (FSP-1), α-smooth muscle actin (αSMA) and fibronectin (Fig. 1). EndMT-

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ABBREVIATIONS: AP-1, activator protein-1; ALK, activin receptor-like kinase; αSMA, alpha smooth muscle actin; BMP, bone morphogenetic protein; EC, endothelial cell; EMT, epithelial-to-mesenchymal transition; EndMT, endothelial-to-mesenchymal transition; FGF, fibroblast growth factor; FPAH, familial PAH; HIF, hypoxia inducible factor HIMEC human intestinal microvascular ECs; HLH, helix-loop-helix; HO, heterotopic ossification; HUVEC, human umbilical vein EC; IL, interleukin; IPAH, idiopathic PAH; LV, left ventricular; MAPK, mitogen activated protein kinase MEnT mesenchymal-to-endothelial transition; miR, micro-RNA; miRNA, micro-RNA; MMP, matrix metallo proteinase; NF-κB, nuclear factor kappa B; Pecam-1, platelet EC adhesion molecule-1; PAEC, pulmonary arterial EC; PAH, pulmonary arterial hypertension; PAVEC, porcine aortic valve EC; Smad, homolog of the *Drosophila* protein mothers against decapentaplegic (MAD) the *C. elegans* protein SMA; TAZ, transcriptional coactivator with a PDZ-binding domain; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; VE-cadherin, vascular endothelial cadherin; VEGFR, vascular endothelial growth factor receptor; vSMC, vascular smooth muscle cell vWF von Willebrand factor; YAP, Yes-associated protein

Article is online at: http://onlinelibrary.wiley.com/doi/10.1002/dvdy.24589/abstract

derived mesenchymal cells acquire a highly migratory potential and are susceptible, when receiving specific extracellular cues, to re-differentiate into several mesodermal cell lines (e.g., osteoblasts, chondrocytes, and adipocytes) (Sánchez-Duffhues et al., 2016; Medici, 2016).

EndMT During Cardiovascular Development

EndMT was first observed in developmental studies of the heart by Markwald et al., who described this phenomenon as an endothelial transformation (Markwald et al., 1975; Eisenberg and Markwald, 1995). These pioneering studies found that the embryonic endocardial ECs undergo EndMT between E8.5 and E12, subsequently invading the adjacent cardiac jelly, thereby generating the cardiac fibroblastic cushions. Such cushion tissue is the precursor of the atrioventricular (AV) valves and septum and the semilunar valves (reviewed in Kovacic et al., 2012).

Noteworthy, mice where EndMT has been genetically disrupted exhibit improper heart formation, mainly due to abnormal a scale of inflow and outflow tract formations (Timmerman et al., 2004; Wang et al., 2005; Mercado-Pimentel et al., 2007). Of interest, a recent study has expanded this original observation by demonstrating that ECs contribute to the accumulation of pericytes and vSMCs in the endocardium in mouse embryos (Chen et al., 2016). Furthermore, at the embryonic stage EndMT contributes to vascular development, at least in the case of the abdominal aorta (DeRuiter et al., 1997) and pulmonary vasculature (Arciniegas et al., 2005). Apart from this essential role during development, a partial EndMT process has been suggested to be necessary for physiological angiogenic sprouting (Welch-Reardon et al., 2015).

EndMT is a highly dynamic, gradual, and reversible process; the opposite mechanism is termed mesenchymal-to-endothelial transition (MEnT) (Ubil et al., 2014). This dynamism leads to a broad spectrum of undefined intermediate cellular phenotypes (thus enhancing EC heterogeneity) and hinders the detection of EndMT in fixed tissue biopsies. In this regard, the development of lineage tracing technologies in animal experimental systems has become crucial to demonstrate the in vivo occurrence of EndMT/MEnT.

Using this recombination technology with different reporter constructs (i.e., fluorescent or luminescent proteins), nowadays it is possible to permanently label individual cells which have expressed a particular endothelial-specific protein at one moment during their life time. This has enormously facilitated the identification of EndMT-derived cells, because mesenchymal cells usually down-regulate the expression of endothelial markers once transitioned.

As we will discuss below, EndMT appears as a developmental process modulated by a common set of transcriptional effectors, which are induced by the cooperation between different signaling pathways, including transforming growth factor (TGF)-β (Nomura-Kitabayashi et al., 2009), Wnt (Liebner et al., 2004), Notch (Niessen and Karsan, 2008), hypoxia (Wikenheiser et al., 2005), Yap/Taz (Singh et al., 2016), fibroblast growth factor (FGF) (Zhang et al., 2008), and hemodynamics (Ma et al., 2016). Furthermore, cytokines like tumor necrosis factor (TNF)-α and interleukin (IL)-1β, that induce the canonical inflammatory signaling pathway, have been shown to induce EndMT in embryonic ECs. This process is normally shut down in adulthood, and it seems to be awakened in an increasing number of postnatal pathologies. In this review, we will first introduce the transcription factors involved in EndMT and describe the main developmental signaling pathways that mediate and regulate this process. Furthermore, we will discuss recent publications concerning the role of EndMT in vascular calcification, pulmonary arterial hypertension (PAH) and organ fibrosis.

Developmental Signaling Pathways Regulating EndMT

In the following sections, we will describe the mechanisms by which a variety of extracellular stimuli trigger a cascade of intracellular molecular events that result in the activation of the EndMT program (Fig. 2). Irrespective of the stimulatory signal, the activated signal transduction pathway leads to the induction of a specific set of transcription factors that mediate the repression of endothelial genes. However, the relative contribution of these factors to EndMT is dependent on cellular context and the pathophysiological process considered. Importantly, micro-RNAs (miRNAs) have been unveiled as important regulators of EndMT (Ghosh et al., 2012).

At least five key transcription factors have been linked to EndMT, originally identified for their ability to repress the transcription of E-cadherin (*Cdh1*) and induce EMT in epithelial cells. In humans, these factors are SNAIL (encoded by *SNAI1*) (Cano et al., 2000), SLUG (*SNAI2*) (Bolós et al., 2003), ZEB1 (*ZEB1*) (Eger et al., 2005), ZEB2 or SIP1 (*ZEB2*) (Comijn et al., 2001), and TWIST (*TWIST1*) (Yang et al., 2004). The first four of these factors belong to the same SNAIL family of zinc finger transcription factors, sharing a highly conserved C-terminal region consisting of four to six E2-box DNA binding zinc fingers, a N-terminal protein-interaction domain (SNAG domain) required for the transcriptional repressor activity, and a central domain that can be posttranslationally modified to regulate protein stability or localization (Peinado et al., 2007). The fifth transcription factor, TWIST-1, is part of a large family of helix-loop-helix (HLH) transcription factors which, upon dimerization, are capable of binding to specific E-boxes in the DNA to regulate gene transcription (Teng and Li, 2013).

Although SNAIL has often been positioned upstream of the other four factors in cancer-related EMT (Peinado et al., 2007), studies in EndMT have indicated that all transcription factors can strongly influence one another's expression. Whether EndMT results due to the cooperative action of several of these factors, or if there is a leading factor, remains to be elucidated.

TGF-β Family

TGF-β is the prototype of a multifunctional family that also includes activins and bone morphogenetic proteins (BMPs). TGF-β is well-known for its role promoting EMT (Xu et al., 2009; Lamouille et al., 2014), and accumulating evidence has unveiled the importance of TGF-β in EndMT (van Meeteren and ten Dijke, 2012). Indeed, aberrant TGF-β signaling has been linked to multiple vascular diseases (Pardali et al., 2010; Goumans et al., 2017). Furthermore, various studies have shown that TGF-β can induce a mesenchymal-like phenotype in a variety of ECs (Arciniegas et al., 1992; Frid, 2002; Ishisaki et al., 2003; Kokudo et al., 2008; Díez et al., 2010; Mihira et al., 2012;), and this effect appears to be cell type-dependent (Hopper et al., 2016). The role

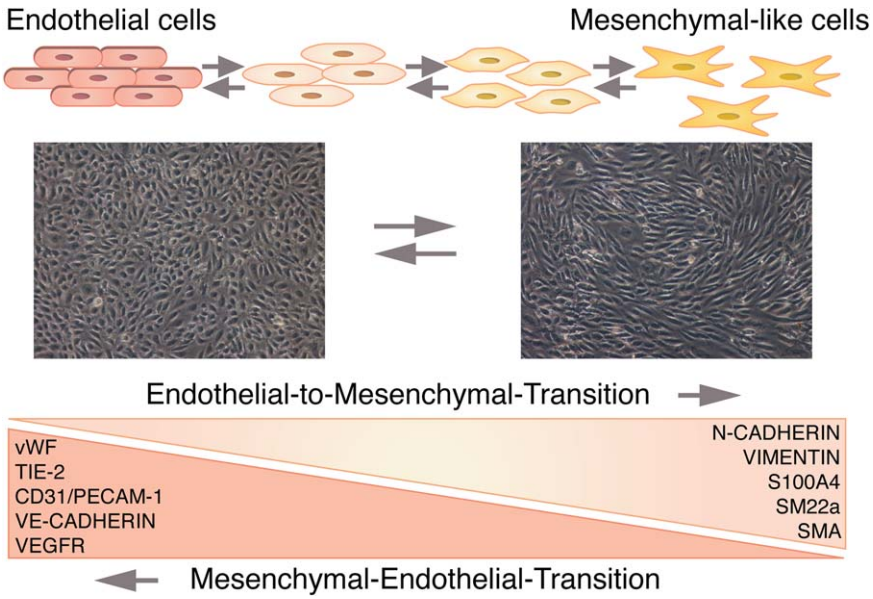

Fig. 1. EndMT consists in a progressive and dynamic loss of the endothelial phenotype and acquisition of a fibroblast-like appearance, as it occurs during vascular development and postnatal pathologies. MEnT, however, has been exclusively reported in adult tissues. Changes in cell morphology can be easily visualized by bright field microscopy, where in response to EndMT stimuli (such as TNF-α, as shown here) well-structured cobblestones become spindle-shape unorganized cells. This process can be monitored by the mRNA or protein expression of endothelial and mesenchymal specific genes, some of which are illustrated in the figure. This ultimately compromises endothelial cells junctions and most of their specific functions. In contrast, EndMT-derived cells exhibit multipotent abilities, including further differentiation into mesoderm cells (chondrocytes, osteoblasts, adipocytes).

of other TGF-β family members in EndoMT is an emerging area of research.

The TGF-β family members are multifunctional cytokines that elicit their effects by means of heterotetrameric complexes of specific type I and type II Ser/Thr kinase receptors and intracellular Smad transcription factors (Massagué, 1998). TGF-β signals through the TGF-β type I receptor, also termed activin receptor-like kinase 5 (ALK5) and TGF-β receptor II (TGF-βR-II). Activin transduces its effects via the activin type IB receptor (ActRIB, or also termed ALK4) and ActR-IIA and ActRIIB. BMPs elicit their responses by activating ALK1, ALK2 and BMP type IA and IB receptor (also termed ALK3 and ALK6) in complex with ActRIIA, IIB and BMP type II receptor (BMPRII) (Upton and Morrell, 2009). Upon TGF-β or activin-induced type I receptor activation, receptor regulated (R-) Smad2 and -3 become phosphorylated, whereas activated BMP type I receptors induce R-Smad1, -5 and -8 phosphorylation.

Phosphorylated R-Smads can form heteromeric complexes with Co-Smad (Smad4). These heteromeric Smad complexes can translocate to the nucleus where they, together with other transcription factors, bind (in)directly to gene promoters containing Smad binding elements. Typical target genes for TGF-β include *Serpin family E member 1* (*SERPINE-1*), *inhibitory (I)-SMAD7, Collagen type I α 1 chain* (*COL1A1*), and *connective tissue growth factor* (*CTGF*). Typical target genes of BMPs include *ID1, ID3*, and *inhibitory (I)-SMAD6*. Activation of BMP and TGF-β signaling is normally balanced and, in fact, BMP is frequently antagonized by TGF-β (Botney et al., 1994). Furthermore, TGF-β signaling cross-talks to other signaling pathways by means of Smad independent cascades, for example different limbs of the mitogen activated protein kinase (MAPK): extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK); phosphoinositide 3 (PI3)-kinase/AKT and protein kinase C (PKC) signaling pathways, as well as a variety of Rho-GTPases (Zhang, 2009).

TGF-β signaling and subsequently EndMT is modulated by the so-called TGF-β co-receptors (e.g., betaglycan, Endoglin). For example, TGF-β receptor III or betaglycan, was found up-regulated in cardiac ECs undergoing EndMT, and incubation with antibodies against the extracellular domain of betaglycan prevented EndMT in AV cushion explants. Furthermore, retroviral-mediated restoration of betaglycan expression in nontransformed ventricular ECs allowed these cells to undergo EndMT in response to TGF-β_2 (Brown et al., 1999). Endoglin, was shown to contribute to EndMT in the formation of endocardial cushions (Nomura-Kitabayashi et al., 2009). *Endoglin* knockout animals die during embryonic development due to endocardial cushion formation disruption.

Although *Endoglin null* endothelial embryoid bodies were still capable of forming new vessels and lead angiogenesis, they lost the ability to contribute to the mesenchymal population in AV cushions. Mechanistically, *Endoglin*-deficient hearts exhibited a decrease in *SNAI1* expression, whereas *SNAI2* and *Cdh5* mRNA levels were not affected. This suggests that the observed phenotype could be the result of a combination of different signaling pathways. Congruently, Endoglin can participate in multiple TGF-β family signaling complexes (i.e., TGF-$\beta_{1\text{-}3}$, activin A, BMP-2/-7/-9) (Barbara et al., 1999), which might affect EndMT in different manners.

Some manuscripts have illustrated the contribution of BMP signaling to endothelial functions, including EndMT (Ricard et al., 2012; Levet et al., 2013; Dyer et al., 2014; Morrell et al., 2016; Goumans et al., 2017). Of interest, *Gdf2* (encoding for BMP-9) knock-out mice displayed an improper closure of the ductus arteriosus after birth, a process that requires EndMT (Levet et al., 2015). Fibrodysplasia ossificans progressiva (FOP) is a rare

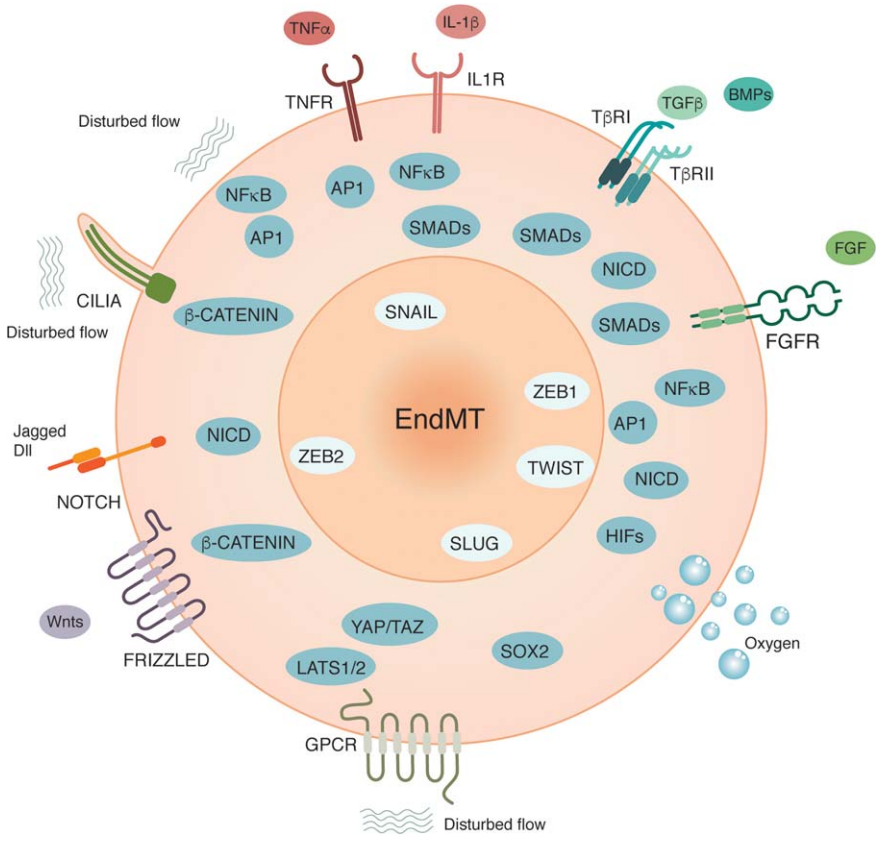

Fig. 2. Signal transduction cascades regulating EndMT. EndMT results as the integration of a variety of signal transduction pathways (including those induced by TGF-β family members, Notch and Wnt ligands, mechanical forces, and disturbed flow, and inflammation). Such signaling cascades modulate both embryonic and postnatal EndMT, and are misregulated under pathological conditions. The crosstalk between cascades always leads to the activation of EMT/EndMT transcription factors: SNAIL, SLUG, ZEB1/2, and TWIST-1. The relative importance of these factors varies according to the pathology and stimulus context.

heterotopic bone disease characterized by abnormally high levels of BMP signaling, due to a gain-of-function mutation in the BMP type I receptor ALK2 (Shore et al., 2006; Hatsell et al., 2015; Hino et al., 2015). Using patient-derived biopsies and a mouse model of the disease, combined with Tie-2-based endothelial lineage tracing techniques, an earlier publication demonstrated that up to 50% of the ectopic bone formed in FOP shares an endothelial origin. In vitro, the authors showed that EC lines stably expressing the mutant receptor undergo EndMT in response to TGF-β_2 or BMP-4, which eventually results in osteogenic or chondrogenic differentiation (Medici et al., 2010).

Furthermore, in addition to this form of inherited heterotopic ossification (HO), trauma-induced HO has also been linked to BMP-4 and TGF-β_2-induced EndMT, by means of down-regulation of miR-630 (Sun et al., 2016). This exemplifies the reciprocal regulation between TGF-β family signaling and miRNA expression. As such, TGF-β-induced miR-21 indirectly activates noncanonical TGF-β signaling and subsequent EndMT in cardiac fibrosis (Kumarswamy et al., 2012), while inhibition of miR-21 by Kallistatin inhibited TGF-β noncanonical signaling induced-*SNAI1* expression (Guo et al., 2015). Disturbed coronary arterial wall remodeling in Kawasaki disease was partially due to an up-regulation of *CTGF* by miR-483 inhibition (He et al., 2017). Altogether, miRNAs may be useful as therapeutic targets or biomarkers for TGF-β-related EndMT diseases.

Wnt Signaling

A large number of secreted Wnt ligands signal through a so-called canonical pathway with a key effector role for β-catenin. In the absence of interaction between an extracellular Wnt ligand and a membrane receptor complex (consisting of the seven-pass transmembrane receptor Frizzled and the co-receptor LRP5/6), β-catenin is phosphorylated and tagged for degradation by a proteasomal complex. This complex consists of adenomatous polyposis coli (APC), axin, glycogen synthase kinase 3 (GSK3) and casein kinase I (CK1) (Edeling et al., 2016). Upon Wnt activation, β-catenin is prevented from degradation and translocates into the nucleus, where it activates the expression of Wnt target genes through the formation of transcriptional complexes with hepatocyte nuclear factor 4-α (HNF4A) or T-cell factor (TCF) and lymphoid enhancer binding factor 1 (LEF1).

Furthermore, other less-characterized noncanonical signaling pathways can be activated in response to specific Wnt ligands, including JNK/activator protein 1 (AP-1), Dishevelled (Dsh)-nuclear factor of activated T cells (NFAT), and its related C3 botulinum toxin substrate 1 (Rac1) (Reis and Liebner 2013). Both canonical and noncanonical Wnt signaling pathways contributed to EndMT in lymphatic ECs in response to Wnt5B ligand secreted by oral squamous cell carcinoma cells. This pathway was able to promote tumor metastasis without affecting tumor growth (Wang et al., 2017). In this regard, a recent article by Chen et al. has

revealed that cardiac SMCs and pericytes derive from ECs that undergo EndMT in response to paracrine Wnt ligands (Chen et al., 2016). Taking advantage of both *Cdh5* and *Tie-2* reporter mice, the authors found a population of endocardial ECs that give rise to mesenchymal progenitors that further migrate into the heart valves or differentiate into vSMCs and pericytes that cover the larger arteries in the heart. Of interest, this article showed that conditional knockout of the Wnt receptor *Frizzled4* in ECs resulted in a reduced number of mesenchymal cells, suggesting that extracellular Frizzled 4 ligands are necessary for this process.

Both TGF-β family and Wnt pathways crosstalk to each other. With regard to EndMT, β-catenin-deficient mice exhibit a strong decrease in TGF-β-induced EndMT in the heart (Liebner et al., 2004). Moreover, our group showed that mouse embryonic ECs lacking primary cilia exhibit a higher tendency to undergo EndMT and further osteogenic differentiation in response to ectopic BMPs. The absence of primary cilia induced a nuclear accumulation of β-catenin. Accordingly, this process was abolished by BMP type I receptor kinase inhibition or siRNAs against *Ctnnb1* (encoding β-catenin) (Sánchez-Duffhues et al., 2015).

In recent years, EndMT has been implicated in the development of a brain vascular disease called cerebral cavernous malformations (CCM), which consists in abnormal and hemorrhagic blood vessels. This disease is caused by inactivating mutations in one of the three genes: *Ccm1, Ccm2*, or *Ccm3*. Elevated expression of BMP-6 and TGF-β mediated EndMT in *Ccm1*-deficient ECs (Maddaluno et al., 2013). In addition, endothelial-specific knockout of *Ccm3* led to increased β-catenin expression, which was subsequently responsible for enhanced BMP and TGF-β activity (Bravi et al., 2015). Conversely, a later study using a different mouse model for CCMs, identified Krüppel like factor (KLF)2/4 gain-of-function as a potential underlying mechanism for CCM; however, no evidence of EndMT or increased Smad signaling was found (Zhou et al., 2016).

Inflammation

During acute inflammation, ECs react by expressing cell surface adhesion molecules and secreting pro-inflammatory factors (e.g., arachidonic acid derivatives). This response is activated by two main cytokines: TNF-α and IL-1 (Pober and Sessa, 2007). Upon activation by their respective membrane receptor complexes, both TNF-α and IL-1 converge in the phosphorylation and consequent proteasomal degradation of the inhibitory κB (IκBα) protein, which sequesters NF-κB in the cytosol. Degradation of IκBα allows NF-κB to translocate to the nucleus, where it binds specific κB responding DNA elements to regulate gene expression. In addition to NF-κB, other signaling pathways are activated upon pro-inflammatory stimulation, including MAPKs (JNK, ERK, and p38) (Li et al., 2005b).

Inflammatory signaling has been associated with EndMT (Pérez et al., 2016). In the 1980s, it was shown that conditioned medium from activated peripheral blood lymphocytes (PBLs) could induce a fibroblast-like morphology in human umbilical vein ECs (HUVECs). Although the expression of endothelial or mesenchymal markers was not investigated, the formation of an extracellular proteoglycan matrix in response to TNF-α was reported (Montesano et al., 1984). A subsequent study focused on the effect of IL-1β on human dermal microvascular ECs. Incubation of these cells with IL-1β for 72 hours successfully induced a spindle-shaped morphology in these cells, which correlated with the loss of the surface expression of the endothelial proteins Pecam-1 and von Willebrand factor (vWF) (Romero et al., 1997).

Of interest, phorbol 12-myristate 13-acetate can directly induce the activation of downstream NF-κB and MAPKs signaling cascades and thereby induce EndMT. Another example was shown in human pulmonary arterial ECs (PAECs), in which the treatment with TNF-α induced changes in the cell morphology and cytoskeletal rearrangements, as well as decreased VE-cadherin expression on the membrane, leading to a reduction in transendothelial electrical resistance (Petrache et al., 2003).

Taking advantage of a 3D culture system, it was shown that a fraction of the porcine aortic valve EC (PAVEC) population responded to TNF-α by down-regulating Pecam-1 and up-regulating αSMA expression, resulting in cells with an elevated migratory potential (Farrar and Butcher, 2013). Of interest, although *SNAI1* expression was transiently up-regulated upon TNF-α stimulation in all ECs, *SNAI1* remained elevated only in these cells that acquired migratory features. PAVECs were also used in a later study, in which ECs were shown to undergo EndMT in response to another pro-inflammatory cytokine, IL-6. In contrast to other publications, these authors used high concentrations of TNF-α or IL-6 (100 ng/ml), whose effects were inhibited by co-incubating with inhibitors of AKT and MAPK (Mahler et al., 2013). Remarkably, induction of EndMT by TNF-α in embryonic endocardial monolayers was blocked by co-incubation with the small molecule ALK4/5/7 kinase inhibitor SB-431542. However, TNF-α-induced EndMT in adult PAVECs was not affected by TGF-β receptor inhibition, suggesting that aging influences the mechanisms that underlie EndMT. Importantly, it should be noted that inflammation is a postnatal condition and, although embryonic ECs have been used to investigate the mechanisms driving EndMT, embryonic ECs are not exposed to an inflammatory environment in vivo.

Other publications demonstrated crosstalk between inflammatory cytokines and TGF-β family members in EndMT modulation. For example, Rieder et al. showed that human intestinal microvascular ECs (HIMECs) treated with combinations of TNF-α, IL-1β, and TGF-β displayed an enhanced EndMT response as measured by the expression levels of endothelial and mesenchymal specific proteins (Rieder et al., 2011). IL-1β was identified as the most potent EndMT inducer. Of interest, whereas TNF-α and IL-1β mainly induced the down-regulation of Pecam-1 and VE-cadherin, TGF-β-treated cells increased αSMA expression, suggesting a 2-steps process. Importantly, this result was verified in a later study using HUVECs. A combination of TGF-β_2 and IL-1β induced EndMT very efficiently and in a NF-κB dependent manner (Maleszewska et al., 2013).

Moreover, TGF-β_2-induction of *SNAI1* in human cutaneous microvascular ECs was blocked by U0126 (an ERK inhibitor), SB202190 (a p38 MAPK inhibitor), or LY294002 (a PI3K inhibitor). This inhibitory effect, was also achieved by ectopic expression of a dominant-negative Smad4 construct (Medici et al., 2011), which suggests that both canonical and noncanonical TGF-β signaling pathways are necessary for EndMT. Nevertheless, it has also been shown that inflammation and TGF-β signaling do not always cooperate, but rather that, inflammation inhibits TGF-β-induced EndMT. A study performed using an inducible endothelial specific (*Cdh5*) reporter mouse model of skeletal muscle tissue damage suggested that TNF-α secreted by muscle infiltrating macrophages blocked EndMT. Accordingly,

macrophages depletion resulted in increased phosphorylated Smad2 and *SNAI1* expression (Zordan et al., 2014). In general, it appears that inflammation provides the proper environment to facilitate EndMT in adult ECs, thereby recapitulating an embryonic phenomenon.

Notch

In contrast to the other signaling pathways described in this review, which exert their functions by means of the interactions of soluble ligands and membrane receptors, the Notch signaling pathway is dependent on cell–cell contacts. Four Notch receptors (1–4) on the membrane of the responsive cells intracellularly transduce signals initiated by the Notch ligands Jagged 1-2 (Jag1, Jag2) and Delta-like (Dll)-1, Dll-3, and Dll-4, which are expressed on the surface of juxta positioned cells (Ilagan et al., 2011). Upon ligand-receptor binding, the γ-secretase activity of the Presenilin complex mediates the cleavage of the Notch receptor, which releases the Notch intracellular domain (NICD).

Subsequently, the NICD translocates to the nucleus and regulates the expression of the Hairy and enhancer of Split (Hes) and Hes-related basic HLH transcription factor with the YRPW motif (Hey) families, among others (Ehebauer et al., 2006). To regulate gene transcription, the NICD binds the transcriptional repressor recombination signal binding protein for immunoglobulin kappa J region (RBPjκ). Because Notch signaling is strongly dependent on the expression of interacting proteins on the membrane of the cells, it can be assumed that EndMT-related cytoskeletal rearrangements would affect Notch signaling indirectly. In this regard, several publications have investigated the interplay between EndMT stimuli and Notch (Hurst et al., 2017; Gustafsson et al., 2005; Sieiro et al., 2016).

The association between Notch signaling and EndMT has been largely studied in heart development (Niessen and Karsan, 2008) and valve formation. Mutations in Notch1 have been linked to bicuspid aortic valves, one of the most common congenital cardiac defects. Notch regulates different stages of cardiac development, including the EndMT process that results in the formation of the AV canal. Most of the Notch signaling components, including the Notch1 intracellular domain (Yang et al., 2009), are expressed in the embryonic endocardium and their levels are severely down-regulated in *RBPjκ* mutants (Timmerman et al., 2004).

Of interest, mice lacking a functional *RBPjκ* exhibited reduced EndMT and lack of mesenhymal cushion cells, which correlated with a decrease in the expression of TGF-β signaling components, such as TGF-β_2 and its receptors. The authors demonstrated that *SNAI1* was the main EndMT effector in response to Notch signaling. Conversely, others showed that Notch-induced EndMT requires SLUG (*SNAI2*) (Niessen et al., 2008). Overexpression of Notch1,4 ICD in different EC lines induced the expression of *SNAI2*, but not *SNAI1*. Mechanistically, it was shown that the induction of *SNAI2* occurs at the transcriptional level by a process that requires the nuclear Notch1 partner RBPjκ. SLUG was found to directly bind the *Cdh5* promoter, thereby regulating EndMT. While SLUG overexpression failed to increase the expression of the mesenchymal marker αSMA, Notch1 ICD successfully increased αSMA in addition to down-regulating Pecam-1, Ve-Cadherin, and Tie-2. This result suggests that SLUG is only one branch of the EndMT program triggered by Notch signaling (Niessen et al., 2008).

Furthermore, EndMT mediators can also affect Notch signaling through a feedback loop. Mice with a conditional knockout of *SNAI1* in Tie-2 positive ECs displayed vascular defects and embryonic lethality, which seemed to be due to an abnormal up-regulation of Notch (Wu et al., 2014). Accordingly, the inhibition of Notch signaling in *SNAI1*-deficient mice by DAPT (a γ-secretase inhibitor) reversed the observed vascular defects. TWIST-1 has also been shown to act on Notch signaling, as overexpression of TWIST-1 induced endothelial differentiation of a head and neck cancer cell line by means of *Jag1-Klf4* (Chen et al., 2014a).

The interplay between TGF-β and Notch signaling pathways has been explored. A manuscript by Luna-Zurita et al., demonstrated that activation of Notch in BMP-2 treated-ECs in ventricular explants, was sufficient to induce EndMT in a *SNAI1*-dependent manner. In vivo, over-activation of Notch signaling in the myocardium led to increased BMP-2 expression, modulating endocardial cells that undergo EndMT (Luna-Zurita et al., 2010). Other TGF-β family member, activin A (*INHBA*) was found to be up-regulated by the Notch components *Jag1, Dll4*, or *NICD* in an RBPjκ-dependent manner (Chang et al., 2011). A recent publication using Tie-2-driven specific deletion of the Notch signaling components in the endocardium has shown that *Dll4*, and not *Jag1*, is necessary for EndMT during the formation of endocardial cushions (MacGrogan et al., 2016) through a mechanism that requires the function of BMPs to promote proliferation of the mesenchyme.

Hypoxia

Several physiological processes are modulated by oxygen (O_2) tension. Because the vascular system is progressively developed during the early stages of embryonic development, an O_2 gradient is established in tissues, which depends on the proximity to neighboring blood vessels. The relative O_2 tension modulates, for example, angiogenesis (e.g., vascularization of the placenta and the heart) and the development of the cardiac endothelium (Iyer et al., 1998; Ryan et al., 1998; Compernolle et al., 2003; Wikenheiser et al., 2005), and maladaptation often results in lethal cardiovascular defects (Dunwoodie, 2009). Additionally, hypoxia has been shown to participate in the onset or progression of several postnatal cardiovascular diseases, including PAH, cardiac fibrosis and coronary artery disease (CAD).

Two transcription factors are primarily responsible for coordinating cell responses to low O_2 level. Under normoxia, hypoxia-inducible factor (HIF)-1 and HIF-2 are targeted for proteasomal degradation by the E3 ubiquitin ligase von Hippel-Lindau protein (VHL). This activity requires the prior hydroxylation of HIF-1 and HIF-2 by prolyl hydroxylase (PHD)-1, PHD-2, and PHD-3. When the O_2 tension drops, PHDs are inhibited and HIFs are accumulated and translocated to the nucleus to induce gene transcription in cooperation with the aryl hydrocarbon receptor nuclear translocator (ARNT) factor. Hypoxia-inducible genes include genes that participate in metabolic pathways, cell proliferation and differentiation, among others (reviewed in Semenza, 2012).

Hypoxia has been shown to induce EMT. These studies are usually complemented with HIF overexpression experiments under normoxic conditions, and have revealed that HIF factors directly modulate the transcription of EMT-promoting genes by interacting with hypoxia response elements (HREs) in their promoters (Luo et al., 2011; Yang et al., 2008; Zhang et al., 2015). Similar

studies have been performed in ECs. For instance, human coronary artery ECs (HCAECs) exposed to hypoxic conditions for four days underwent EndMT, and genetic or pharmacologic inhibition of HIF1α inhibited EndMT in these cells (Xu et al., 2015a). Among all the EndMT factors up-regulated in response to hypoxic conditions, SNAIL was identified as the main regulator, probably due to the presence of HIF1α-binding elements in its promoter region.

Hypoxia modulates EndMT responses to TGF-β. For example, even though both TGF-β_1 and TGF-β_2 induced EndMT in bovine aortic ECs (BAECs) cultured under hypoxic conditions, only TGF-β_1-induced phosphorylated-Smad2 nuclear accumulation was enhanced under hypoxia. Whether the nuclear accumulation of phosphorylated Smad2 correlates with an increase in the TGF-β_1-induced transcriptional response was not investigated (Doerr et al., 2016). Such crosstalk between hypoxia and TGF-β signaling mechanisms can also be regulated by the expression of different miRNAs. For example, in hypoxia-induced persistent pulmonary hypertension of the newborn (PPHN), a cardiac syndrome that can potentially affect a considerable number of neonates, elevated expression of miR-126a-5p, which inhibits TGF-β signaling, was detected in a small set of serum samples of PPHN patients (Xu et al., 2017).

Hippo

Initially discovered in *Drosophila melanogaster*, the Hippo pathway has been shown to integrate different aspects of the cell cycle, including proliferation and differentiation (Hansen et al., 2015). Upon stimulation, the Ser/Thr kinases LATS1/2 become active and associate with 14-3-3 adaptor proteins to prevent the DNA binding of the Hippo effectors Yes-associated protein (YAP)/transcriptional coactivator with a PDZ-binding domain (TAZ), through a process that requires the association with the TEA domain family members (TEADs). Notably, the activation of LATS in response to distinct stimuli (i.e., shear stress, cellular oxidative or osmotic stress, DNA damage) seems to be highly influenced by the extracellular environment and cell type. Remarkably, an increasing amount of evidence suggests that this pathway coordinates the signals triggered by different transduction cascades, such as TGF-β (Varelas et al., 2008; Hiemer et al., 2014), Wnt (Varelas et al., 2010) or Notch (Rayon et al., 2014) or by biomechanical stimulation (Dupont et al., 2011) and inflammation (Ramjee et al., 2017).

In studies performed in cancer, members of the Hippo pathway have been shown to promote EMT, and several drugs have been developed to inhibit their oncogenic effect (Zhou and Lei, 2016). In the cardiovascular system, it has been demonstrated that genetic inhibition of YAP and TAZ caused severe defects in the formation of coronary vasculature due to abnormal cell proliferation and disturbed EMT in epicardial cells (Singh et al., 2016).

Hippo-mediated EndMT was demonstrated during cardiac development. For instance, endothelial-specific genetic inhibition of YAP1 caused down-regulation of *SNAI1, TWIST-1*, and *SNAI2* expression and subsequent EndMT failure, partially due to the inhibition of TGF-β signaling (Zhang et al., 2014). Consistent with Singh et al., the loss of YAP1 compromised the proliferation of endocardial cells, resulting in the aberrant formation of endocardial cushions. Furthermore, transgenic mice lacking either *SNAI1* or *SNAI2* or both displayed reduced levels of phosphorylated YAP/TAZ and total protein without affecting upstream LATS activation. Moreover, endogenous YAP/TAZ and SNAIL/SLUG comprise transcriptional complexes regulating TEAD target genes expression (Tang et al., 2016).

FGF

The FGF receptor (FGFR) signaling pathway is triggered upon the interaction of the receptor with extracellular FGFs, which leads to the activation of specific receptor tyrosine kinases (RTKs). At least four FGFRs (1–4) have been identified. The downstream receptor signaling involves, among others, the activation of MAPKs, PI3K/Akt pathways, signal transducer and activator of transcription (STAT) proteins (reviewed elsewhere Chae et al., 2016). FGF regulates multiple processes in ECs, including proliferation, migration and angiogenesis (Presta et al., 2005). Studies performed to decipher the effect of FGFR signaling on EndMT have highlighted its crosstalk with the TGF-β signal transduction pathway (Xiao and Dudley, 2016). FGFR-induced stimulation of miRNA *let7* was found to down-regulate TGF-β signaling by targeting ALK5.

One of the mechanisms by which inflammation promoted EndMT was attributed to inhibition of FGFR, thereby releasing ALK5 from *let7*-mediated down-regulation (Chen et al., 2012). Moreover, EndMT associated with atherosclerosis or vein graft adaptation models was found to be enhanced by endothelial-specific knockout of *FGFR1* (Chen et al., 2014b). A different group demonstrated that FGFR stimulation activated MAPK signaling in lymphatic ECs, eventually inducing the phosphorylation of Smad2/3 in the linker region. This process competes with the canonical C-terminal ALK5-induced R-Smad phosphorylation (Ichise et al., 2014), thereby preventing EndMT. In PAH, FGF signaling was identified as a node of a complex miRNA regulatory network controlled by the miR *130/301* family. This family was found to be up-regulated in cultured patient-derived PAECs, and serum samples, as well as in animal models of the disease. Using transfection of *miR-130* antagomirs and mimics in PAECs, the authors showed that *miR-130/301* indirectly targeted *mir-424/503*, thereby inducing FGF2 expression and consequent EC proliferation (Bertero et al., 2014).

Mechanotransduction

ECs in blood vessels are constantly exposed to hemodynamic forces triggered by the periodic contractions of the heart to pump blood all over the body. In larger arteries, ECs sense changes in blood pressure and release factors to modulate vSMCs to maintain the elasticity of the vessels (Hahn and Schwartz, 2009).

Several membrane-associated proteins participate in transducing shear stress signals into the cytosol. These include integrins, RTKs, primary cilia, the glycocalyx, heterotrimeric G proteins, and adhesion molecules (e.g., Pecam-1, VE-cadherin) (reviewed in Hahn and Schwartz, 2009). Consequently, shear stress has been linked to the activation of MAPK and NF-κB signaling, whose integrated outcome controls a multitude of cell responses, including apoptosis and proliferation, membrane permeability and migration (Li et al., 2005a).

Our group has addressed the role of primary cilia in EndMT. Primary cilia are nonmotile sensory organelles present in ECs (among other cell types) that translate environmental signals into intracellular signaling cascades (i.e., Wnt, MAPK) (Van der Heiden et al., 2011). Using cilia defective (*Tg737/ift88*) embryonic ECs, we found that nonciliated ECs undergo EndMT in a TGF-β-

dependent manner. This process was characterized by the up-regulation of KLF-4 (Egorova et al., 2011a) and KLF-2 by means of ERK5 (Egorova et al., 2011b). Notably, MEKK3-MEK5-ERK5-induced up-regulation of KLF-4 has been recently implicated in CCMs (Cuttano et al., 2016), and Moonen et al. have highlighted the role of ERK5 in disturbed flow-induced EndMT and subsequent osteogenic differentiation (Moonen et al., 2015).

The association between YAP/TAZ and signal transduction induced by mechanical forces has been reviewed elsewhere (Halder et al., 2012). For example, YAP/TAZ activity is modulated by changes in cell shape, ECM elasticity and cytoskeletal forces, in a LATS independent manner (Dupont et al., 2011). Inflammatory signaling is fine-tuned by blood flow (reviewed in Bryan et al., 2014). For example, ECs can sense blood flow very precisely and even induce different branches of the inflammatory cascade depending on the angle of the flow–cell axis (Wang et al., 2013). In general, it is well accepted that disturbed blood flow has a pro-inflammatory effect (Go et al., 2014) with the ability to induce EndMT (Cunningham and Gotlieb, 2004).

In summary, EndMT progresses through the integration and coordination between multiple signaling pathways. The exact mechanisms by which these pathways crosstalk, or are triggered, modulated and sustained is likely influenced by a myriad of factors, including aging, disease-specific gene mutations and tissue specific micro-environments. In the next section, we will describe and discuss the role of EndMT in several postnatal cardiovascular disorders, e.g., vascular calcification, PAH, and organ fibrosis (Fig. 3).

ENDMT in Cardiovascular Disorders

Vascular Calcification and Atherosclerosis

Vascular calcification is considered a common feature in many disorders, such as chronic kidney disease, diabetes, and hypertension. Calcification of the coronary arteries and atherosclerotic disease in particular, are currently considered a major cause of death and disability worldwide, because their associated vascular remodeling events usually lead to myocardial infarction, stroke, aneurysm, or pulmonary embolism (Alexopoulos and Raggi, 2009).

Mineralization of the blood vessels occurs through a coordinated crosstalk between different cell types and cytokines, but it ultimately relies on cells with osteogenic potential, which generate a calcified core in a process that somewhat resembles endochondral bone formation (Doherty et al., 2004; Johnson et al., 2006). Our group and others have pointed to ECs undergoing EndMT as a source of osteogenic progenitors in vascular calcification and atherosclerosis. Atherosclerotic plaques are irregularly distributed throughout the vasculature. One of the determinants of this irregularity could be the biomechanical forces affecting the arteries (Brown et al., 2016). Indeed, atherosclerotic plaques are observed more frequently at branching points within the vasculature, coinciding with oscillatory blood flow, rather than the straight sections where the luminal ECs undergo shear stress (Nigro et al., 2011).

ECs can sense changes in shear stress by means of primary cilia. Of interest, these primary cilia are also unequally distributed in the arteries. ECs located in high shear stress areas are devoid of cilia, whereas those at disturbed blood flow areas, i.e., the intersections of the vasculature tree or at the boundaries of the plaques (Chaldakov, 1985; Van der Heiden et al., 2008), expose their primary cilia to the lumen of the vessels. We have demonstrated that the shortening of the primary cilia by shear stress or genetic mutation can promote aortic ECs to undergo EndMT in a TGF-β dependent manner (Egorova et al., 2011a).

Furthermore, we found that cells with defective cilia exhibit up-regulated β-catenin expression, leading to elevated *SNAI2* mRNA levels. Under these circumstances, nonciliated aortic ECs easily became mineralized in a BMP receptor kinase activity-dependent manner (Sánchez-Duffhues et al., 2015). We confirmed these in vitro studies using ex vivo staining on the aortas of high-fat fed LDLR-deficient animals and in human biopsies, whereby we showed that the ECs in advanced atherosclerotic lesions expressed higher levels of phosphorylated Smad1 and SLUG. In addition to the ability of the primary cilia to induce cell responses to changes in blood flow, ECs also respond to the oscillatory shear stress by down-regulating FGFR signaling. The down-regulation of FGFR signaling by inactivating FGF receptor substrate 2α (Frs2a) leads to enhanced nuclear localization of the TGF-β Smads (Chen et al., 2015).

Furthermore, increased phosphorylated Smad-2/3 signaling was shown to be accompanied by elevated Notch3 expression in the ECs transitioning to MSCs. These findings were obtained by comparing apolipoprotein E (*ApoE*) knockout mice with green fluorescent protein (GFP)-labeled ECs (*Cdh5-gfp*) to double knockout $ApoE^{-/-}Frs2a^{ECKO}$ *Cdh5-gfp* mice and were validated in biopsies of human coronary arteries. The Hippo pathway effectors YAP/TAZ have also been recently implicated in the onset of atherosclerosis in the induction of EndMT in response to disturbed blood flow. YAP is less potently activated and retained in the cytosol in the outer tract of the aorta, where ECs are under unidirectional shear stress (USS), in contrast to the inner arc of the aorta, which is characterized by disturbed blood flow and increased nuclear localization of YAP (Wang et al., 2016).

Of interest, it has been shown that USS induces YAP phosphorylation by means of the activation of integrinβ_3. After dissecting the underlying signaling pathway leading to the integrin-mediated YAP phosphorylation, which includes G protein subunit 13 ($G_{\alpha 13}$) and RhoA, the authors demonstrated that the EC-specific knockout of *Yap* reduced the formation of atherosclerotic plaques in $ApoE^{-/-}$ mice. In summary, both gain-of-function and loss-of-function experiments in vivo have shown the importance of YAP/TAZ activation in EndMT related atherogenesis.

In a recent study, based on ex vivo immunolabeling of aortic tissues, cells co-expressing endothelial and mesenchymal markers were identified in the *ApoE*-deficient atherosclerotic plaques (Boström et al., 2016). Moreover, the aortic ECs isolated from high-fat fed mice displayed a strong induction of mesenchymal and osteogenic markers, which correlated with higher expression of several BMP receptors and ligands. By contrast, the BMP antagonists Chordin and Noggin were also up-regulated. None of the TGF-β signaling members or TGF-β/BMP target gene expression levels were studied.

Of interest, they showed that the inhibition of *Sox-2* by systemic injection of shRNAs led to smaller calcified lesions. Sox-2 was also found to be involved in atherosclerosis progression in Matrix Gla protein (MGP)-deficient mice. These mice are prone to develop vascular calcifications, partly due to enhanced BMP signaling (Luo et al., 1997). When studying the aorta of $Mgp^{-/-}$ mice, the authors discovered cells co-expressing endothelial (CD31 and vWF) and osteogenic (Cbfa1 and Osterix) markers. Remarkably,

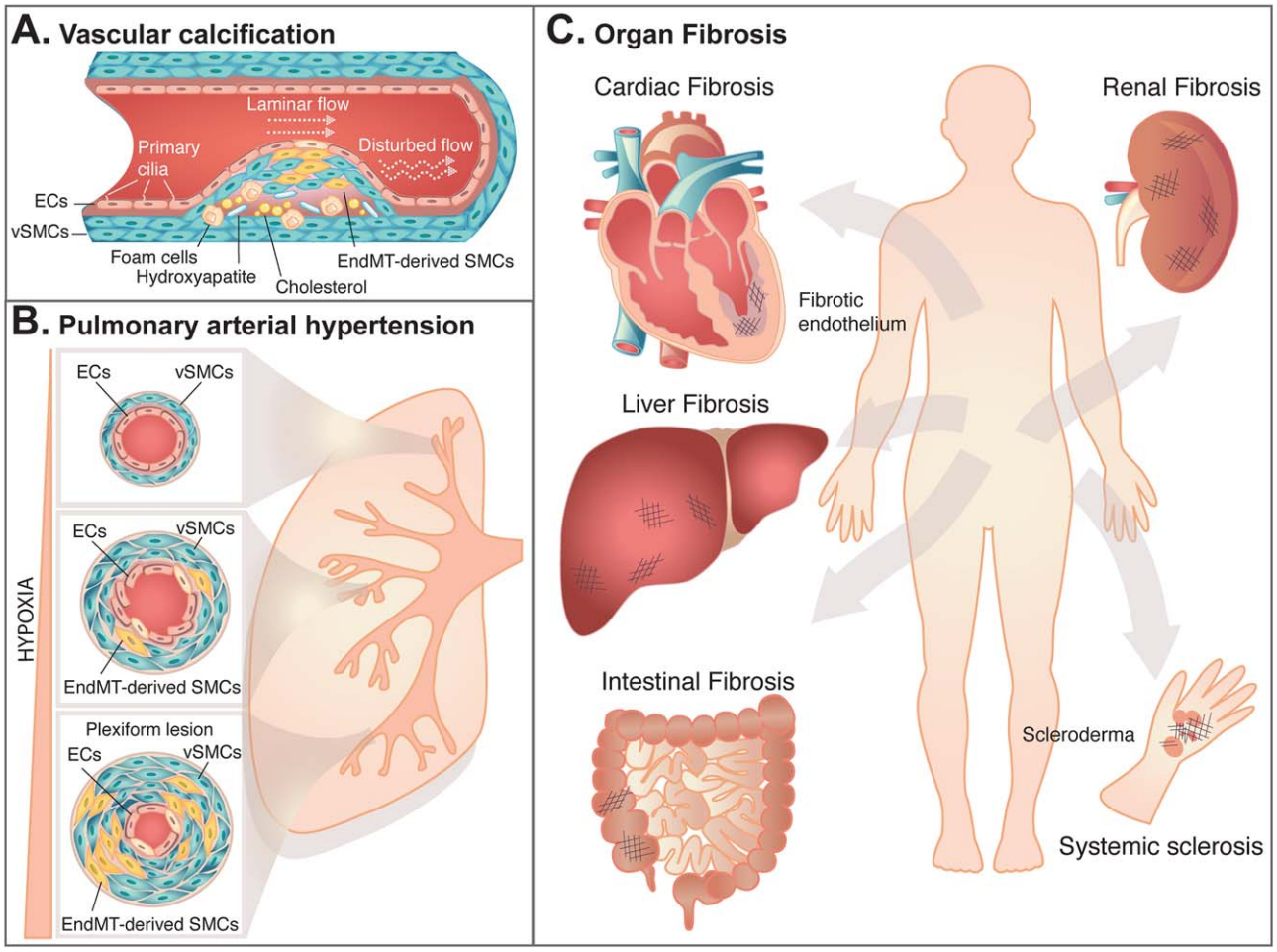

Fig. 3. EndMT contributes to the onset and progression of cardiovascular diseases. Initially described as an essential process for the formation of the heart during embryonic development, increasing evidence has highlighted the role of EndMT in several human postnatal disorders. **A**: Vascular calcification. EndMT derived cells have been shown to contribute to a population of SMC-like cells that can be further differentiated into osteogenic cells. In response to BMP ligands, such osteogenic cells are responsible for plaque formation, in a process that resembles that of endochondral bone formation. **B**: PAH. At the cellular level, PAH is characterized by the progressive accumulation of ECs and SMC-like cells in the pulmonary arteries, eventually leading to an increase in the blood pressure and ventricular hypertrophy. A fraction of the appearing SMC-like cells has been shown to be originated by means of EndMT. Genetic studies have demonstrated that inactivating mutations or decreased expression of BMPR2 favor the development of PAH, which is also affected by local inflammation and increasing hypoxia, due to the progressive closure of the vessels. **C**: Organ fibrosis has been suggested as a source of fibroblast-like cells in (i) cardiac fibrosis in mouse models of systemic and local inflammation; (ii) in renal fibrosis, as shown in different models of kidney failure; (iii) in IPH or liver fibrosis; in (iv) inflammatory-associated intestinal fibrosis; and finally, in (v) systemic sclerosis, which is an autoimmune disease affecting the vasculature in multiple organs all over the body.

the ECs in *Tie-2-gfp Mgp*$^{-/-}$ mice exhibited an increased expression of Sox-2, in addition to Nanog and Oct3/4 (Yao et al., 2013). This elevated expression was further enhanced by stimulation with recombinant BMP-4.

Of interest, in a follow-up study, the authors demonstrated that one of the mechanisms by which these mice developed arterial calcification is the induction of specific serine proteases that can regulate the formation of ECM in cells undergoing EndMT. Sox-2 was found to be necessary for the induction of these proteases and the endothelial specific inhibition of *Sox-2* prevented high-fat-diet-induced vascular calcification in the *Mgp*$^{-/-}$ *mice* (Yao et al., 2015).

Furthermore, the authors showed a reciprocal regulation between Sox-2 and the EndMT factor TWIST-1 in the *Mgp*$^{-/-}$ cells, because the knockdown of *Sox-2* prevented BMP-4/glucose-induced *TWIST-1* expression and the knockdown of *TWIST-1* partially blocked the expression of *Sox-2*. The association between Sox-2 and the serine protease axis was studied in a mouse model of vascular calcification arising from diabetes, the Insulin deficient Ins2$^{Akita/+}$ mice (Yao et al., 2013). Guihard et al. showed that the CD31^{+}CD45^{-} sorted cells from the aorta of those mice exhibited increased expression of multipotent factors, including *Sox-2*, and that the endothelial specific deletion of *Sox-2* prevented aortic calcification. Also in this model, the pharmacological inhibition of serine proteases using diisopropyl fluorophosphate (DFP) blocked aortic calcification (Guihard et al., 2016).

Even though endothelial-based reporter lines are currently being routinely used for EndMT studies, ECs are a heteogenous population of cells, so that all the reporter strategies tend to overestimate the number of ECs undergoing EndMT (typically due to nonendothelial expression of the chosen Cre-driver). This issue has been elegantly addressed in a study by Evrard et al. (2016). The authors created a tamoxifen-inducible endothelial-specific

lineage tracing system end.SclCreER^T;R26RstopYfp in a pro-atherosclerotic *ApoE*$^{-/-}$ background. With this system, it was possible to avoid the nonendothelial Tie-2-Cre-mediated expression of the reporter constructs, mainly affecting the immune cells (Wosczyna et al., 2012).

The induction of the Cre recombinase activity was also performed sufficiently late to avoid YFP expression in bone marrow-derived cells and circulating leukocytes. This novel approach was crucial to determine the contribution of EndMT-derived cells in atherosclerosis, as the infiltrating immune cells might have otherwise been incorrectly identified as ECs. The authors were able to locate and characterize the double-positive FSP-1/vWF or FAP/CD31 cells in vulnerable atherosclerotic lesions. In addition, they analyzed the expression of genes encoding several collagen varieties as well as matrix metalloproteinases in the fibroblasts adjacent to the HUVECs undergoing EndMT in response to TGF-β and H_2O_2. Remarkably, the EndMT-derived fibroblast-like cells displayed higher MMP activity in their supernatants, which was likely due to the increased protein expression of several MMPs (e.g., MMP-1, MMP-9, MMP-10, TIMP-2, and TIMP-4).

PAH

PAH is a devastating disease characterized by abnormal remodeling of pulmonary vessels, progressive increase in the pulmonary artery pressure, and right heart failure, eventually. Although PAH is rare (a prevalence of 26 cases per million adults; Peacock et al., 2007), many other chronic conditions are associated with an elevated pressure in the pulmonary circulation, including severe left ventricular (LV) systolic dysfunction, isolated LV diastolic dysfunction, chronic obstructive pulmonary disease, and chronic thrombo-embolic pulmonary hypertension. All forms of PAH are characterized by vasoconstriction and vascular remodeling, although the underlying mechanisms are incompletely understood. Two types of PAH have been described: sporadic or idiopathic PAH (IPAH) and hereditary or familial PAH (FPAH). Heterozygous germline mutations in the *BMPRII* gene are found in more than 70% of the patients with FPAH and 20% of the patients with IPAH (International PPH Consortium et al., 2000; Deng et al., 2000). Notably, down-regulated BMP signaling is a common characteristic of PAH irrespective of a *BMPRII* mutation.

It is well-reported that the normal functions of ECs and vSMCs are disrupted in the lungs of PAH patients, and this effect may be a consequence of down-regulated signaling (Morrell, 2006; Goumans et al., 2017). Mice expressing a mutant BMPR2 lacking the C-terminal tail in the pulmonary SMCs develop pulmonary vascular lesions similar to PAH, characterized by exacerbated proliferation of the αSMA-positive cells. Although the resident vSMCs were traditionally thought to be the only source of the newly appearing αSMA-expressing cells, rapidly emerging experimental evidence suggests alternative sources contributing to the enlarged SMC layer.

Among them, ECs have been proposed to give rise to mesenchymal cells expressing αSMA in PAH (Arciniegas et al., 2007; Good et al., 2015). This hypothesis is supported by the demonstration that trans-differentiation of the pulmonary arteriolar ECs into SMC-like cells occurs during hypoxia-induced pulmonary vascular remodeling and is regulated by myocardin (Zhu et al., 2006).

Of interest, by using lineage tracing, Qiao et al. recently showed that some of the αSMA-positive cells constituting the neointima in occlusive PAH vessels have an endothelial origin (Qiao et al., 2014). This finding was later followed up by another group that identified double-positive lung CD31$^+$ αSMA$^+$ ECs from PAH patients who exhibited increased TWIST-1 expression (Ranchoux et al., 2015). In addition to TWIST-1, up-regulated SLUG was found in the PAECs from IPAH patients (Hopper et al., 2016). Of interest, knockdown of either *SNAI2* or the High Mobility Group AT-hook 1 (*HMGA1*) in BMPR2-deficient cells successfully prevented EndMT.

The association of the PAH pathology with disturbed BMP signaling stimulated the search for approaches to restore BMPR2 signaling as a therapy for PAH. To that end, the administration of recombinant BMP-9 increased BMP signaling. *BMPRII* is a BMP target gene and its mRNA and protein levels were increased in patient-derived ECs exposed to BMP-9 (Long et al., 2015). Signaling pathways that negatively target phosphorylated Smad1 (for example, phosphorylation of GSK3β or MAPK-mediated Smad-linker domain) may contribute to PAH. In this regard, the inflammatory cytokine TNF-α was found to inhibit the expression of *BMPRII*. Of interest, the mechanism causing the down-regulation of BMPR2 is cell-dependent e.g., in PAECs, *BMPRII* gene expression is down-regulated by TNF-α, whereas in vSMCs, this effect is enhanced by ADAM-mediated posttranslational cleavage of BMPR2 (Hurst et al., 2017).

Organ Fibrosis

Fibrosis is a nonphysiological wound healing process that occurs in multiple organs. Fibrosis is characterized by an excessive synthesis and accumulation of ECM proteins leading to destruction of the normal architecture of the affected organ, loss of tissue homeostasis and ultimately functional failure (Piera-Velazquez et al., 2016). Fibrosis can be observed as an end-stage pathological symptom in a large number of systemic disorders, such as systemic sclerosis (SSc), nephrogenic systemic fibrosis (NSF), sclerodermatous graft *versus* host disease (GVDH), IgG_4-associated sclerosing disease or chemotherapy- and radiation-induced fibrotic diseases, as well as the organ-specific disorders affecting the heart, lungs, liver, or kidneys (Piera-Velazquez et al., 2016).

Several studies have shown that myofibroblasts are the essential cell type in the pathogenesis of fibrotic disorders (Piera-Velazquez et al., 2016; Gilbane et al., 2013). However, their cellular origin is debatable. In addition to the expansion and activation of resident fibroblasts, myofibroblasts in fibrotic tissues have been shown to have a heterogeneous origin. Recent studies have highlighted several tissue-resident cells, including epithelial, SMCs and ECs as potential contributors to the myofibroblast population that emerge in fibrotic tissues (van Meeteren and ten Dijke, 2012). In this regard, EndMT may be a key contributor in pathological fibrosis.

Systemic Sclerosis

Systemic sclerosis (SSc) is an autoimmune disease characterized by progressive vascular damage and fibrosis of the skin and several internal organs. In lung samples from patients with SSc-associated pulmonary fibrosis, immunohistochemical analysis revealed cells co-expressing EC and mesenchymal markers (vWF and αSMA) in small- and medium-sized arterioles. Furthermore,

gene expression from ECs isolated from lungs of patients with SSc-associated pulmonary fibrosis showed higher expression of mesenchymal cell-specific genes and genes encoding EndMT-associated transcription factors than the controls (Mendoza et al., 2016).

In a recent study, the involvement of EndMT in the development of dermal fibrosis in SSc was investigated in two mouse models (bleomycin-induced dermal fibrosis and uPAR-deficient mice). Analysis of the number of cells co-stained with CD31 or VE-Cadherin and αSMA to detect the cells undergoing EndMT showed that these animals displayed a higher frequency of these cells than the controls (saline-treated or wild-type littermates, respectively) (Manetti et al., 2017). In addition, the treatment of healthy dermal microvascular ECs with SSc sera or TGF-β_1 induced an EndMT phenotype in these cells.

Cardiac fibrosis.

Cardiac fibrosis is an important health issue and a common feature linked to the end-stage of almost all kinds of cardiovascular diseases. It is characterized by an excessive deposition of ECM. Fibroblasts are the main cell type producing ECM proteins during physiological and pathological conditions such as cardiac fibrosis. However, the origin of these cells recruited to the fibrotic heart is still unclear. Several studies have elicited EndMT as the mechanism responsible for the observed increase in the fibroblast population.

In one of the pioneering studies, Zeisberg and collaborators investigated the origin of the fibroblasts in experimentally induced cardiac fibrosis by taking advantage of lineage tracing techniques. Two strains of transgenic mice were used, one in which the cells of endothelial origin (Tie-1) were marked by lacZ expression, and the other strain in which GFP was expressed under the control of the *Fsp-1* promoter. Cardiac fibrosis was induced by either aortic banding or allograft rejection. Quantitative analysis of the fibrotic lesions revealed that up to 35% of the fibroblasts had an EC origin (Zeisberg et al., 2007a).

In vitro experiments also demonstrated TGF-β_1-induced EndMT in adult human coronary ECs. In both mouse models, the ectopic systemic administration of recombinant human BMP-7 prevented the progression of cardiac fibrosis by inhibiting EndMT. A subsequent study also showed that EC-derived endothelin-1 (ET-1) induced cardiac fibrosis in a type 1 diabetes model by stimulating fibroblast accumulation by means of EndMT, and that this process was mediated by the activation of TGF-β signaling in ECs (Widyantoro et al., 2010). In high-fat-fed *ApoE*$^{-/-}$ mice, EndMT was shown to play a crucial role in cardiac fibrosis and could be aggravated by chronic inflammation (Ma et al., 2013).

In another study, analyses of the fibroblast population present in the cardiac fibrotic lesions from patients with chronic kidney disease revealed that up to 17% of the fibroblasts/myoblasts were originated by means of EndMT and were double-positive for CD31/FSP1 (Charytan et al., 2014). Kanisicak et al. (2016) made use of a tamoxifen-inducible Cre-line under control of the *periostin* promoter to label interstitial fibroblasts. While hardly any cells were labeled in the uninjured heart, after myocardial infarction, periostin expression was restricted to the myofibroblasts. Cardiac fibrosis due to pressure overload also resulted in periostin labeled myofibroblasts.

To elucidate the origin of these cells, endothelial lineage tracing using the *Cdh5*-Cre line was combined with periostin expression, showing that less than 1% of the myofibroblasts share an endothelial origin (Kanisicak et al., 2016). Xu et al. showed that aberrant DNA promoter methylation and silencing of *RASAL1* (an inhibitor of Ras-GTP activity) led to the enhancement of EndMT and cardiac fibrosis. In vitro, they showed that human coronary ECs could undergo EndMT after prolonged exposure to TGF-β, and that the EndMT process persisted when TGF-β was removed from the culture due to a sustained activation of Ras-GTP. Upon TGF-β treatment, *RASAL1* promoter was found to be hypermethylated. Down-regulation of *RASAL1* by siRNA following TGF-β stimulation led to an enhanced EndMT, characterized by decreased Pecam-1 expression and increased *SNAI1, SNAI2*, and *TWIST1*. Moreover, immunohistochemical analysis of fibrotic heart samples from end-stage heart failure patients corroborated the reduction in the expression of RASAL1 correlated with the enhanced expression of *SNAI1, SNAI2*, and *TWIST1* (Xu et al., 2015b).

Renal fibrosis.

Several studies have provided evidence to indicate that EndMT is a prominent pathway involved in the development of fibrosis in different models of kidney fibrosis (Kizu et al., 2009; Li and Bertram, 2010). A study by Zeisberg et al. showed that up to 50% of fibroblasts co-expressed the endothelial marker CD31 with mesenchymal cell markers in three different mouse models of end-stage kidney disease. Using EC lineage tracing in *Tie-2-Cre;R26R-stop-EYFP* transgenic mice, they demonstrated the occurrence of EndMT and further showed that the fibroblasts present in the fibrotic tissue had an EC origin (Zeisberg et al., 2008). Using a transgenic mouse strain (*Tie-2-Cre/LoxP-EGFP*) to track the cells of endothelial origin, Li and colleagues identified a substantial population of myofibroblasts expressing αSMA that originated from ECs in fibrotic kidneys in mice with streptozotocin-induced diabetic nephropathy.

Quantitative analysis using confocal microscopy revealed that 10–23% of the myofibroblasts co-expressed the endothelial-specific marker (Li et al., 2009). More recently, LeBleu and colleagues have corroborated the occurrence of EndMT during the development of kidney fibrosis. The results of their study, using several engineered mouse strains and endothelial lineage tracing, showed that 10–15% of the activated myofibroblasts have originated from ECs (LeBleu et al., 2013). In addition to animal models, the analysis of kidney biopsy samples obtained from patients with diabetic kidney disease revealed the presence of glomerular cells co-expressing EC-specific (CD31) and mesenchymal cell-specific (αSMA) markers corroborating previous data on the occurrence of EndMT in vivo during the development of kidney fibrosis associated with diabetic kidney disease (Li et al., 2015). Nevertheless, as reported in several international conferences, several independent groups have communicated controversial results regarding the contribution and relevance of EndMT in renal fibrosis. Therefore, whether EndMT participates in the onset and/or development of fibrosis in the kidney is still under debate.

Liver fibrosis.

The involvement of EndMT has also been investigated in idiopathic portal hypertension (IPH) (Kitao et al., 2009). Patients with

IPH can develop other clinical manifestations, such as systemic sclerosis, in which EndMT has been suggested as a possible mechanism leading to excessive collagen deposition. Histological analysis using liver tissue sections of IPH donors showed the co-expression of CD34 (nonmature EC marker) and S100A4 (myofibroblast marker). In these cells, an increased nuclear expression of phosphorylated Smad2 was observed, indicating the possibility that EndMT of the portal vein endothelium might occur by means of TGF-β/Smad activation, and this might ultimately be the mechanism responsible for portal vein stenosis and obliteration in IPH.

Intestinal fibrosis.

Fibrosis is a common complication in several gastrointestinal diseases, including inflammatory bowel disease (IBD). EndMT can give rise to fibrogenic cells in the fibrotic intestine. Rieder et al. showed that HIMECs undergo EndMT in vitro when exposed to inflammatory cytokines (TGF-β_1, IL-1β, and TNF-α) or supernatants of mononuclear cells from the intestinal lamina propria. In the same study, the authors demonstrated the occurrence of EndMT in the intestinal mucosa in mouse models of experimental colitis and human intestinal samples from IBD patients (Rieder et al., 2011).

Concluding Remarks

As we have described above, accumulating evidence points at EndMT as an important player in the progression of cardiovascular diseases. In fact, the number of publications including the term *endothelial mesenchymal transition* in PubMed was doubled between 2012 and 2016.

Apart from the pathologies aforementioned, to date EndMT has been described in cancer. In this process, EndMT may contribute to tumor formation by modeling the surrounding vasculature and thereby facilitating tumor growth or metastasis (Xiao et al., 2015; Gasperini et al., 2012; Gasparics et al., 2016) or favoring the generation of cancer-associated fibroblasts (CAFs) (Zeisberg et al., 2007b), although these studies need further validation. Other conditions in which EndoMT has been implicated are sporadic (Sun et al., 2016) and hereditary heterotopic ossification (Medici et al., 2010), Kawasaki disease (He et al., 2017) and vein graft rejections (Cooley et al., 2014). In addition to a pathological role, the therapeutic potential of EndoMT has been explored as part of tissue engineering in regenerative medicine (Susienka and Medici, 2014; Medici, 2016).

Although the cellular mechanism of EndMT seems to occur in adult tissues in the same way as in embryonic development, it remains to be elucidated whether adult ECs undergoing EndMT were somehow sensitized at an embryonic stage. In vitro studies have suggested this in the case of diseases associated to genetic mutations, such as FOP, CCMs, where (over)expression of a mutant gene primes ECs to undergo EndMT. In these cases, the EndMT ability of ECs may be determined by the expression level of the mutant gene. In nongenetic diseases, however, other factors, such as local concentration of surrounding cytokines and growth factors, a particular extracellular matrix architecture or local shear stress conditions, may determine the induction of EndMT in a subset of ECs, but not in all of them.

The link between EndMT and other pathophysiological processes is likely to further increase due to better tools to investigate transdifferentiation, including lineage tracing methods in animal models. However, the animal models recapitulate at best only most of the characteristics of a human disease and serve as surrogate models. On the other hand, the endothelial labeling strategy used in lineage tracing experiments should be carefully chosen. First, as aforementioned, ECs comprise an extremely heterogeneous population expressing different subsets of markers, which in occasions up-regulate or completely shut down their expression in a time and tissue dependent manner. This is of particular interest in inducible animal reporter systems. Moreover, most of the endothelial markers used (e.g., Tie-2) are not exclusively expressed by ECs (Tang et al., 2010), therefore, the contribution of ECs to the mesenchymal population may be overestimated.

Finally, tissues under inflammatory or pathological conditions may tend to over-express endothelial proteins (Willam et al., 2000; Porat, 2004), leading to false positives. Therefore, validation with more than one reporter gene should be intended. Alternatively, emerging organ-on-a-chip platforms based on patient-derived cells will complement animal models to determine the significance of EndoMT in pathological human processes. Together, they will lead to the discovery of EndoMT biomarkers, development of new EndMT antagonists and agonists with therapeutic potential.

Acknowledgments

Research in the laboratory of PTD on TGF-β family is granted by the Netherlands Cardiovascular Research Initiative: the Dutch Heart Foundation, Dutch Federation of University Medical Centres, the Netherlands Organization for Health Research and Development, and the Royal Netherlands Academy of Sciences (PHAEDRA consortium). G.S.D. is supported by the RECONNECT consortium (belonging to the Netherlands Cardiovascular Research Initiative, as aforementioned) and a Postdoctoral Fellowship from AFM-Telethon. A.G.V. acknowledges the Dutch Arthritis Association (Reumafonds) for support. We thank Prof. Marie-José Goumans and Prof. Marco C. de Ruiter for critical reading of the manuscript.

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