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SPOTLIGHT REVIEW

Endothelial–mesenchymal transition in atherosclerosis

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Abstract Atherosclerosis is an inflammatory disease resulting in the hardening and thickening of the wall of arteries and the formation of plaques, which comprise immune cells, mesenchymal cells, lipids, and extracellular matrix. The source of mesenchymal cells in the atherosclerotic plaques has been under scrutiny for years. Current endothelial-lineage tracing studies indicate that the endothelium is a source for plaque-associated mesenchymal cells. Endothelial cells can acquire a mesenchymal phenotype through endothelial–mesenchymal transition (EndMT), wherein the expression of endothelial markers and functions is lost and the expression of mesenchymal cell marker and functions acquired. Furthermore, EndMT can result in delamination and migration of endothelial cell-derived mesenchymal cells into the underlying tissue. Here, we review the contribution of EndMT in vascular disease focusing on atherosclerosis and describe the major biochemical and biomechanical signalling pathways in EndMT during atherosclerosis progression. Furthermore, we address how the well-established systemic atherosclerosis risk factors might facilitate EndMT during atherosclerosis.

Keywords Atherosclerosis • Endothelial function • Endothelial–mesenchymal transition (EndMT) • Transforming growth factor beta (TGF-β) • Mechanotransduction • Regional blood flow • Remodelling • Shear stress

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1. Introduction

Atherosclerosis is a leading cause of death and morbidity. Atherosclerosis is an inflammatory disease resulting in the hardening and thickening of the wall of arteries and the formation of plaques. Atherosclerotic plaques are characterized by the accumulation of immune cells, smooth muscle cells (SMC) and (myo)fibroblasts, lipids, and extracellular matrix (ECM) in the artery wall. Unstable atherosclerotic plaques can rupture resulting in thrombosis and interruption of the blood flow. Despite the systemic nature of atherosclerotic risk factors—which comprise amongst others hypertension, diabetes mellitus (hyperglycaemia), obesity, dyslipidaemia, hyperhomocystinuria, and male gender—atherosclerotic lesions develop preferentially at vessel curvatures, branching points and bifurcations, where the blood flow is disturbed.[1,2] This suggests the importance of haemodynamic forces in the initiation of the disease.

Endothelial dysfunction plays a critical role in the development of atherosclerosis.[3,4] The endothelium is the cell layer lining the luminal surface of the blood vessels and, in healthy individuals, is a major regulator of vascular homeostasis.[5] The main function of the endothelium is to form a barrier controlling the passage of molecules and cells from the bloodstream to the vessel wall and vice versa. Additionally, the endothelium responds to a series of chemical and biomechanical cues by secreting factors regulating vascular tone, SMC proliferation and migration, immune cell adhesion, thromboresistance, and vessel inflammation.[6] Most atherosclerotic risk factors can activate the endothelium, resulting in the expression of chemokines and cytokines (e.g. IL1, IL6, IL8, MCP-1) and adhesion molecules (e.g. VCAM-1, ICAM-1, E-selectin) that attract and facilitate immune cell extravasation.[5] Critical to the activation of endothelial cells (ECs) is the switch from nitric oxide (NO) signalling to reactive oxygen species (ROS) signalling. NO promotes homeostasis and maintains the vascular wall in a quiescent state by the inhibition of proinflammatory cytokine secretion, immune cell extravasation, SMC proliferation, thrombosis and by preventing vascular leakage,[3] whereas ROS induces NFκB signalling, the main regulator of inflammation. This state of oxidative stress is a common underlying mechanism for EC dysfunction in response to (biochemical and biomechanical)

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pathophysiological stimuli.[7] Upon activation, the endothelium acquires a proinflammatory state and become more permeable, promoting the accumulation of leucocytes and lipids in the intima of the artery, culminating in foam cell formation and the formation of a fatty streak, hallmarks of atherosclerosis development.

EC can acquire myofibroblast-like properties through a specific form of epithelial-to-mesenchymal transition (EMT) known as endothelial-to-mesenchymal transition (EndMT). During EndMT, the expression of endothelial markers such as VE-cadherin and CD31 is reduced whereas the expression of mesenchymal markers such as alpha smooth muscle actin (αSMA), N-cadherin, and calponin is acquired.[8,9] EndMT is accompanied by the loss of cell–cell contact and cell polarity, which result in a spindle-shaped morphology and the acquisition of a migratory and invasive phenotype with enhanced ECM production.[10,11] In some instances, EndMT results in delamination and migration of EC-derived mesenchymal cells into the underlying tissue. EndMT was first described in embryonic development during angiogenesis and the formation of the mesenchymal heart cushion, the precursor of the cardiac valves.[12] In adulthood, EndMT is associated with disease including fibrosis in the heart,[13] kidney,[14] dermis,[15] vascular (re-)stenosis,[16] pulmonary arterial hypertension,[17] and cancer.[18] Recently, the contribution of EndMT to atherosclerosis development was established.[19–22]

In this review, we discuss the contribution of EndMT in vascular disease focusing on atherosclerosis and describe the major biochemical and biomechanical signalling pathways in EndMT during atherosclerosis progression. Furthermore, we address how the well-established atherosclerosis risk factors might facilitate EndMT during atherosclerosis.

2. Endothelial plasticity as origin of mesenchymal cells in atherosclerotic plaques

Plaque formation in atherosclerosis is associated with the accumulation of mesenchymal cells [i.e. vascular SMC, and (myo)fibroblasts] in the intima of the artery. These mesenchymal cells are critical in the progression of atherosclerosis as they secrete proinflammatory molecules and synthetize ECM proteins and metalloproteases which facilitate plaque build-up and regulate plaque stability.[23] The origins of the neointimal mesenchymal cells in the plaque have been under scrutiny for decades but have remained elusive. Under pathological conditions, SMC originating from the media[24] and fibroblasts from the adventitia[25] migrate, proliferate, and participate to the neointima thickening. Also, bone marrow-derived cells contribute to neointima formation under pathological conditions.[26] Recent observations in human, porcine, and mouse plaques suggest a substantial endothelial origin for neointimal mesenchymal cells, which express both endothelial (e.g. PECAM-1, Endocan, and VE-cadherin) and mesenchymal markers [e.g. αSMA, Notch3, Snail, SM22α, fibroblast-associated protein (FAP), FSP1 (S100A4), and Vimentin].[19–22] Such cells represent the transitioning states of EndMT, where endothelial markers continue to be expressed and new mesenchymal markers have been acquired. Thus, ongoing EndMT results in a panel of phenotypically distinct populations that may exhibit different functional characteristics.

In vivo lineage tracing experiments, employing endothelial-specific Cre-lox systems such as Cdh5-CreERT2 and SCL-CreERT2 in combination with an $ApoE^{-/-}$ or $LDLR^{-/-}$ mouse model of atherosclerosis have been instrumental in understanding the link between EndMT and atherosclerosis.[21,22] In these mice, EC and their derivatives are irreversibly labelled with GFP or YFP upon induction with tamoxifen while a high-fat diet induces the formation of atherosclerotic lesions. After 4 months of high-fat diet, approximately 30% of luminal aortic EC are undergoing EndMT as assessed by co-expression of the lineage tracer and the mesenchymal markers Notch3[21] or FAP[22] (*Figure 1*). Half of the endothelial-derived mesenchymal cells completely lost the expression of VE-cadherin, indicating that these cells have undergone a complete EndMT,[22] yet the proportion of cells that undergo EndMT and delaminate into the plaque still needs to be defined.

Interestingly, in healthy animals on chow diet, approximately 20% of endothelial-derived cells express the mesenchymal marker FAP.[22] These observations suggest that EndMT occurs in normal vascular homeostasis, albeit to a lesser extent than in atherosclerosis. Corroboratively, in healthy arteries, a proportion of EC express both endothelial markers and the EndMT-related transcription factors Snail, Twist1, and Gata4.[20,27] However, the role of EndMT in the context of physiological vascular turnover warrants further investigation.

Assessing the full extent of EndMT *in vivo* is challenged by (i) the lack of specific fibroblast markers (e.g. vimentin is a marker of activated EC and fibroblasts[28]), (ii) the potential loss of endothelial marker expression by EC at the end of the transition, and (iii) the heterogeneity of EndMT-derived cell populations.[22] Moreover, (iv) the classical endothelial markers (e.g. CD31, Tie-2) are also expressed by monocytes[29] which can also express markers of the mesenchyme.[30] Caution must thus be taken when double immunofluorescence is used to analyse EndMT as the lack of cell-specific markers can underlie overinterpretation of the data. Thus, the use of a large panel of markers in combination is crucial to assess the full extent of EndMT and to better characterize the contribution of the resultant cells to plaque composition.

In summary, several independent studies have revealed the prevalence of EndMT in atherosclerosis.[19–22] Although the contribution of EndMT to disease initiation and progression requires further scrutiny, the extent of EndMT observed in the human plaque strongly correlates with the severity of the disease, implying clinical relevance of the EndMT process.[21,22] EndMT may consist of multi-step fate changes, most likely regulated in differential and sequential manners, capable of giving rise to mesenchymal cells in the plaque. Understanding this multi-step process as well as a better characterization of the resultant cells are of great interest in order to control the EC fate and be able to eradicate the emergence of potential detrimental cell populations.

3. Biochemical signals in EndMT

The discovery that EndMT is an active contributor to pathologies such as atherosclerosis and fibrosis has intensified research into the molecular mechanisms that drive EndMT. In this section, we discuss the biochemical signals that induce EndMT, modulate its progression and the currently identified endogenous inhibitors of EndMT.

3.1 Developmental pathways in EndMT: the Gata4–Twist1–Snail pathway

Gata4, Twist1 and Snail1 (Snai1) and Slug (Snai2) are key transcription factors that govern EMT during embryonic development. Notably, Snail, Slug, and Twist1 regulate EndMT during the formation of the endocardial cushions in the atrioventricular canal of the heart.[31–33] Snail, Slug, and Twist repress endothelial marker gene expression, such as VE-cadherin, PECAM-1, and Claudin-5,[32,34] and facilitate the expression of mesenchymal genes.

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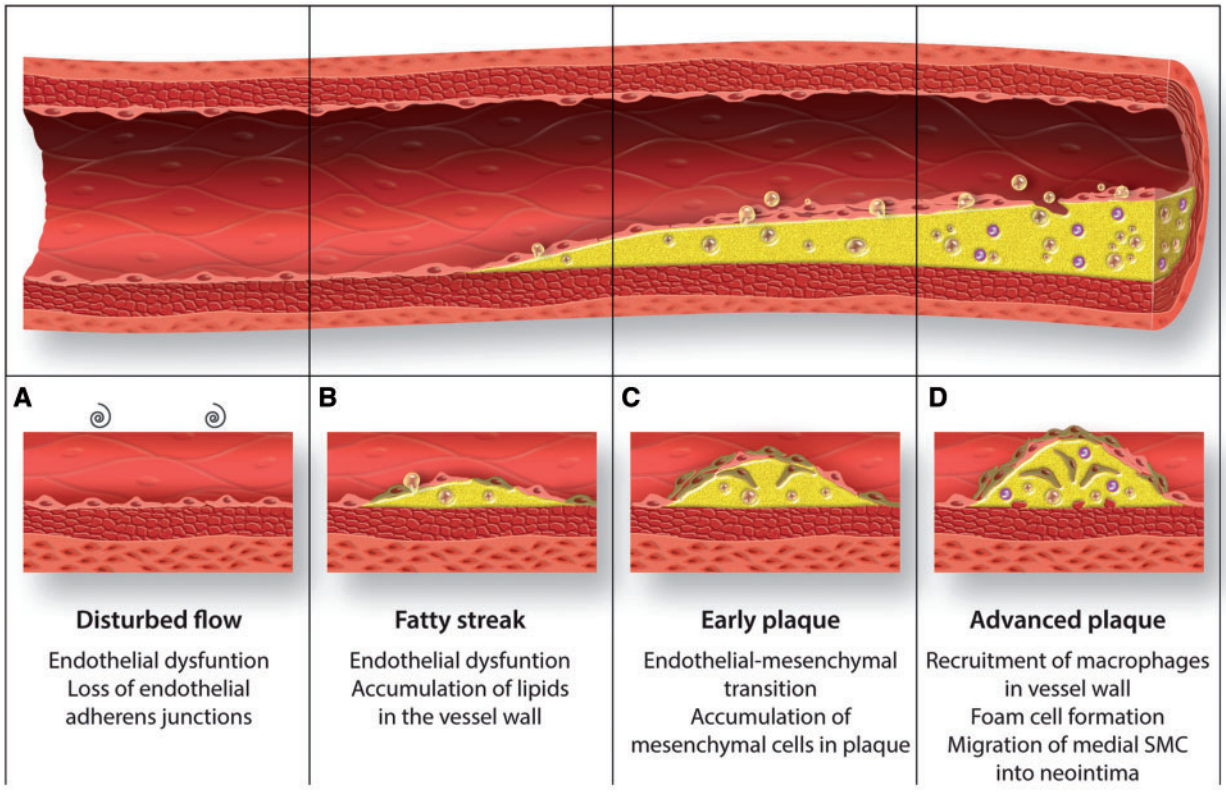

Figure 1 Potential contribution of EndMT to atherosclerosis progression. At areas of disturbed blood flow, ECs are activated (*A*), and adherens junctions dissociate resulting in lipid accumulation in the vessel wall (*B*). Persistent activation of ECs induces EndMT, wherein ECs start to form a multicellular layer and some ECs delaminate and start to migrate into the underlying tissue (*C*). Recruitment of inflammatory cells, foam cell formation, and adventitial smooth muscle cell proliferation contribute to plaque progression (*D*).

Snail1 is present in luminal EC overlying human coronary artery plaques, and Gata4, Twist1, and Snail1 are all expressed in luminal EC in atheroprone areas of the mouse aorta.[20,27] At these sites, Gata4 regulates Twist1 expression, which in turn induces Snail1 in EC.[27] The upstream regulators as well as the downstream targets of this pathway are still unclear, but might include TGF-β,[32] Notch,[31] and Wnt[35] signalling (*Figure 2*). Endothelial-specific deletion of Gata4 or Twist1 reduces lesion size in atherosclerotic mice, suggesting that the Gata4–Twist1–Snail1 pathway contributes to atherosclerosis. As these TF are critical regulators of EndMT (*Figure 2*), understanding their regulations and downstream targets in the vascular endothelium is of great interest to understand the molecular basis controlling EndMT in atherosclerosis.

3.2 TGF-β and EndMT

Canonical TGF-β signalling is considered the driving force of EndMT.[36] TGF-β1 is the prototypic member of a large family of growth factors that includes the activins and bone morphogenetic proteins (BMPs). TGF-β family members mediate their effects by binding to heteromeric receptors consisting of a type I and type II receptors. Upon ligand binding, type I TGF-β receptors induce the phosphorylation of receptor-regulated (R-) SMADs. In the cytosol, activated R-SMADs form heteromeric complexes with SMAD4 and translocate to the nucleus where they regulate gene expression in cooperation with other transcription factors and transcription enhancers.[37]

All three TGF-β isoforms (i.e. TGF-β1, TGF-β2, and TGF-β3) can induce EndMT *in vitro*;[9,38,39] however, TGF-β2 is essential for EndMT induction during cardiac development. Mice deficient in TGF-β2 signalling do not undergo EndMT during cardiac development,[39,40] whereas a deficiency in TGF-β3 does not limit cardiac development.[41] Conflicting data exist on the necessity of TGF-β1 for embryonic EndMT. Single-knockout mutant mice lacking TGF-β1 are born without cardiac malformations,[42,43] yet a subset of TGF-$\beta1^{-/-}$ mice develop multiple cardiac malformations, resulting in embryonic lethality.[42] In atherosclerosis, all TGF-β isoforms are expressed, but with a marked spatial and cellular variability.[44,45] *In vitro*, TGF-β2 appears to have a higher efficacy for the induction of EndMT;[38] however, data exploring the distinct and overlapping functions of the TGF-β isoforms in EndMT induction and progression during adult pathology are lacking. Thus, during atherosclerosis development, EndMT might be induced by multiple TGF-β isoforms and further detailed molecular and quantitative studies are necessary to elucidate this conundrum.

In EC, activin-like kinases (ALK) 1 and ALK5 are the predominant type I TGF-β receptors.[46] The binding of TGF-β to ALK1 activates, i.e. phosphorylates, the R-SMADs SMAD1 and SMAD5, which promote vascular homeostasis, EC proliferation, and angiogenesis.[47,48] In contrast, binding of TGF-β to ALK5 induces the activation of the R-SMADs SMAD2 and SMAD3, which inhibit EC proliferation and facilitate EndMT.[48,49] Interestingly, activation of ALK1 by TGF-β antagonizes the ALK5 activity,[46,48] which might explain the pleiotropic responses of the EC to

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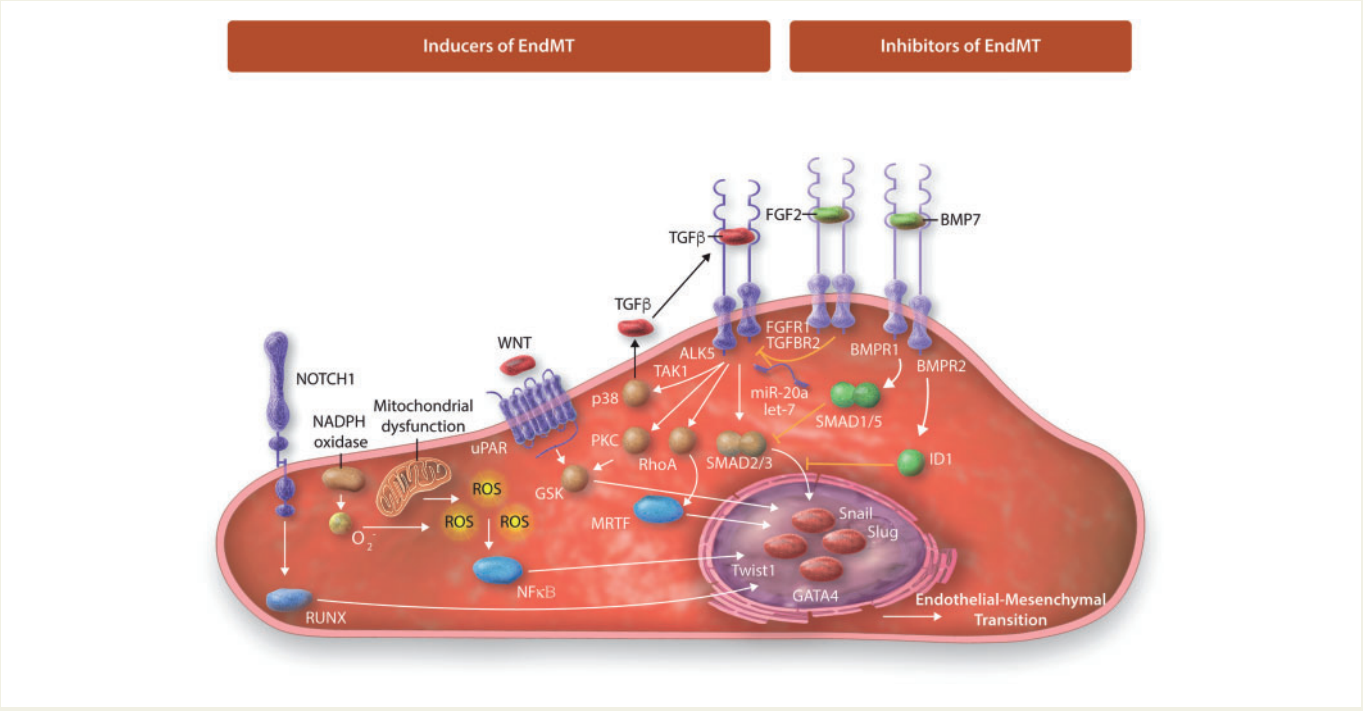

Figure 2 Biochemical signals that induce EndMT. EndMT is driven by the transcription factors Snail, Slug, and Twist1. The expression of the EndMT transcription factors can be induced by multiple upstream signalling cascades. Canonical TGF-β signalling through Smad2/3 directly induces Snail, Slug, and Twist1 expression. Non-canonical TGF-β signalling through RhoA activates the myocardin-related transcription factors (MRTF) that transcriptionally regulate Snail and Slug, whereas TGF-β signalling through TAK1 induces the expression of TGF-β, creating a positive feedback loop for EndMT. Notch signalling, Wnt signalling, and oxidative stress all facilitate in EndMT by either the induction of TGF-β expression or enhancing the nuclear accumulation of transcription factors essential for EndMT.

TGF-β stimulation and suggests a fine balance for TGF-β signalling in vascular homeostasis.

Signalling of TGF-β through ALK5 and the subsequent nuclear translocation and binding of activated SMAD2/3 to SMAD-binding elements (SBE) in the proximal promotors of mesenchymal genes induce their expression. ALK5 activity induces expression of Snail,[32,50] Slug,[49,51] and Twist1,[33] the transcriptional regulators of EndMT (*Figure* 2). Endothelial deficiency in Snail[50] or Slug[31] abrogates EndMT induction by TGF-β, yet overexpression of Snail[50] or Slug[31] alone is not sufficient to induce EndMT, which suggest cooperation between factors in EndMT induction.

Besides canonical TGF-β signalling through Smad2/3, ALK5 activity can activate several non-canonical kinases, such as (i) TGF-β-activated kinase 1 (TAK1),[52] whose activity results in the activation of p38 MAPK[53] that in turn elevates the endothelial TGF-β expression,[54] providing a positive-feedback loop for EndMT. Concurrently, pharmacological inhibition of p38 MAPK limits EndMT induced by TGF-β;[50] (ii) protein kinase C (PKC)-δ and c-Abl induce the phosphorylation of GSK3β, resulting in the increased stability of Snail and thus EndMT.[55] Besides, PKC-δ activity promotes the activity of protein phosphatase 2a (PP2A),[56] resulting in the dephosphorylation of occludins and their subsequent loss from the tight junctions.[57] Indeed, blocking PP2A signalling with neutralizing peptides reduces EndMT;[58] (iii) RhoA,[59] which results in the expression of genes encoding cytoskeletal proteins as well as the reorganization of the cytoskeletal structure.[59,60] RhoA activation triggers multiple downstream cellular events, including the nuclear accumulation of the myocardin-related transcription factors,[61] which facilitate the expression of cytoskeletal genes (e.g. transgelin, αSMA, and calponin) and the transcription factor Slug,[60] facilitating EndMT.

TGF-β signalling is more complex than solely receptor-ligand binding and subsequent biochemical signalling. First, all TGF-β isoforms are secreted as inactive precursors containing a C-terminal peptide that renders the TGF-βs inactive. This latency-associated peptide (LAP) needs to be enzymatically removed from TGF-β before signalling can commence.[62] Secondly, within the ECM, multiple matrix-associated proteins such as betaglycan (also known as TGF-β Receptor 3) bind the secreted TGF-β and act as reservoir for the pericellular storage of TGF-β.[63] Thirdly, even when the LAP is removed, within the ECM, the association of TGF-β with the latent TGF-β-binding proteins (LTBP) renders TGF-β inactive, until mechanical forces disrupt the TGF-β–LTBP complex and liberate TGF-β from this inactivation.[64] Finally, endoglin, a type III TGF-β receptor, directs the actions of TGF-β in ECs by favouring ALK1 signalling and blunting ALK5–SMAD2/3 activation,[65] which suggests that high endoglin expression might inhibit EndMT initiation or progression. Future in-depth molecular investigations are necessary to elucidate the enigma of the contribution of TGF-β-binding proteins on the regulation of EndMT during atherosclerosis development and progression.

Thus, TGF-β signalling via ALK5 induces a series of canonical and non-canonical signalling cascades that induce expression of the EndMT transcriptional programme. In coronary artery disease patients, the extend of EndMT (as revealed by Notch3 expression) and the extend of TGF-β activation (as revealed by the presence of activated SMAD2) are strongly associated in the luminal endothelium,[21] which suggest that endothelial TGF-β activity participates to plaque progression by stimulating EndMT.

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However, definitive proof for the contribution of endothelial TGF-β signalling in atherosclerosis development is missing.

3.3 Alternative pathways in the induction of EndMT: Notch and Wnt/β-catenin signalling

Although TGF-β signalling is considered the main driver of EndMT,[36] antagonism of TGF-β signalling in EC by, amongst others, TGF-β neutralizing antibodies,[49] BMPs,[13] knockdown of SMAD2/3[49] or microRNA-mediated silencing of TGF-β signalling[66,67] only partially inhibit EndMT. Likewise, SMAD3 haploinsufficiency[13,49] or EC-specific SMAD2 deletion[49] decreases, but does not abolish, EndMT during vein graft remodelling. Therefore, it is conceivable that other signalling mechanisms might induce EndMT.

During development, Notch signalling promotes endocardial EndMT by the activation of a mesenchymal gene expression programme.[68,69] Notch can directly activate Twist1 expression[70] and facilitates the recruitment of SMAD3 to SMAD-binding elements in the promoters of a variety of mesenchymal genes,[71] including Slug.[31] Additionally, Jagged-1-induced activation of Notch signalling results in the nuclear accumulation of the mesenchymal transcription factor RUNX3 that induces the expression of several mesenchymal genes.[72,73]

Wnt/β-catenin signalling is essential for EndMT during heart valve development.[74,75] The inhibition of GSK3β by Wnt/β-catenin results in the stabilization of Snail and thus increased EndMT;[50,76] however, Wnt/β-catenin signalling might also induce EndMT in a direct manner. Indeed, Wnt3a induces the expression of transgelin[77] and other mesenchymal proteins in EC,[35] which can be inhibited by the Wnt-inhibitor DKK1.[78] Contrastingly, signalling through Wnt7a inhibits EndMT.[79] These conflicting data indicate that further studies are necessary to conclusively establish the role of Wnt-signalling in EndMT. Currently, a role for Notch and Wnt signalling in EndMT during atherosclerosis has not been established.

In summary, EndMT can be induced by a variety of signalling mechanisms, both canonical and non-canonical. How these mechanisms cross-communicate, or whether there is a hierarchical order between these mechanisms remains elusive. Yet, the high number of pathways resulting in EndMT suggests that EndMT plays a major role in normal physiology, which might become aberrant or aggravated during disease.

3.4 Modulation of EndMT by disease pathways: hypoxia, inflammation, and oxidative stress

Atherosclerotic plaques are characterized by an increase in inflammatory signalling, hypoxia, and oxidative stress.[22] Hypoxia aggravates EndMT by facilitating the nuclear accumulation of SMAD2/3.[80] Hypoxia-inducible factor (HIF)-1α is the archetype sensory protein for oxygen. HIF-1α is constitutively expressed in EC, but degraded under normoxic conditions. HIF-1α degradation is regulated by oxygen sensors that constitutively direct HIF-1α towards the proteasomal degradation route. During hypoxia, HIF-1α degradation is inhibited, resulting in its nuclear accumulation.[81] Interestingly, Snail, Twist1, and ALK5 are transcriptional targets of HIF-1α,[82–84] which might explain the increased sensitivity of EC to TGF-β signalling under hypoxic conditions. Indeed, pharmacological inhibition of HIF-1α reduces ALK5 expression and abolishes EndMT.[82] It must be noted that the contribution of hypoxia to EndMT of luminal ECs is contradictory as atherosclerosis development is not associated with a decrease in pO_2. However, the proportion of ECs that delaminate and migrate into the underlying neointima during EndMT might encounter tissue hypoxia and this hypoxia might aggravate the EndMT process.

Inflammatory signalling synergizes with TGF-β in the induction of EndMT[38,85] and inflammatory stress exacerbates atherosclerosis progression in mice.[86] Proinflammatory cytokines (e.g. IL-1β and TNFα) activate the transcription factor NFκB, resulting in elevated expression of TGF-β1 and TGF-β2,[38,54] the main inducer of EndMT. Besides the convergence of inflammation with TGF-β signalling, proinflammatory cytokines might also induce EndMT in a TGF-β-independent manner.[85,87] This inflammation-induced EndMT relies on the induction of Snail by NFκB (*Figure 2*),[88,89] a major transcriptional regulators of EndMT. The exact pathway of inflammation-induced EndMT has yet to be elucidated and how it converges with or diverges from canonical TGF-β-induced EndMT needs to be further investigated.

TGF-β induces oxidative stress in EC through the induction of mitochondrial dysfunction,[52] resulting in the activation of NFκB (*Figure 2*).[52] As stated previously, elevated levels of endothelial oxidative stress and NFκB activity increase the expression of TGF-β1 and TGF-β2 and consequently EndMT.[38,54] Moreover, oxidative stress can activate latent TGF-β in a number of ways; (i) oxidation of the LAP,[90,91] and (ii) matrix-metalloproteinase (MMP)-mediated degradation of LAP.[92] The reduction of oxidative stress by exogenous antioxidants reduces endothelial oxidative stress and consequently EndMT.[93] Interestingly, MMP-9, a TGF-β activating MMP, facilitates EndMT in kidney fibrosis, indicative of a role for oxidative stress responses in mesenchymal transition.[94] Together, these data imply that inflammation, hypoxia, and oxidative stress in the endothelium aggravate EndMT by the induction of canonical TGF-β signalling.

3.5 Endogenous inhibitors of EndMT: fibroblast growth factor signalling and ALK5 antagonism

In contrast to the factors that induce EndMT, endogenous factors that inhibit EndMT have received only limited attention. Fibroblast growth factor (FGF) signalling might be the best characterized endogenous inhibitor of EndMT. FGF signalling in EC induces the expression of several microRNAs that decrease the expression of ALK5 (*Figure 2*).[66,67] Moreover, FGF signalling directly antagonizes the ALK5-induced expression of mesenchymal genes in a Ras/MEK1-dependent manner.[95] Disruption of FGF signalling by EC-specific deletion of the FGF receptor 1 (FGFR1)[96] or FGF Receptor Substrate 2a[21] aggravates EndMT and the progression of atherosclerosis,[21,96] whereas restoration of FGF receptor signalling inhibits EndMT in diabetic nephropathy.[97]

Endothelial signalling through BMP-7 reduces EndMT (*Figure 2*), albeit through unidentified mechanisms.[13,98] Endogenous antagonists of ALK5 signalling, such as ALK1 agonists in EC, might block the induction of EndMT through a variety of mechanisms, namely (i) the induced expression of inhibitory Smads that prevent the phosphorylation of Smad2/3[99] and thereby EndMT,[100] and (ii) the ALK1-induced expression of inhibitor of DNA binding (ID) proteins (*Figure 2*).[46,48] ID proteins are dominant negative helix–loop–helix proteins that lack a DNA-binding domain. ID proteins heterodimerize with other transcription factors, resulting in the formation of non-functional complexes.[101] Interestingly, EndMT is associated with decreased expression of ID proteins,[11] and restoration of ID protein expression reverts EMT of certain epithelial tumours.[102] These data suggest a regulatory role for endogenous ALK1 ligands in the regulation of EndMT; however, data are currently unavailable.

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In summary, EndMT has emerged as a pathological process that can be induced by multiple signalling cascades (i.e. TGF-β, Notch, and Wnt/β-catenin). Moreover, the transcriptional regulation of EndMT, primarily regulated by Snail, Slug, and Twist, is facilitated by hypoxia, inflammation, and oxidative stress. Research into endogenous antagonists of the EndMT programme is in its infancy, yet FGF signalling is identified as a potent inhibitor of EndMT and the atherosclerotic process.[21,96]

4. Focal risk factors and EndMT in atherosclerosis

Biomechanical forces such as shear stress and (cyclic) wall strain play an important role in vascular development, homeostasis, and the pathology of several cardiovascular conditions including atherosclerosis.[103,104] In this section, we focus on how mechanical forces can influence EndMT and contribute to atherosclerosis.

4.1 EndMT and shear stress in atherosclerosis

EC are extremely sensitive to shear stress, the frictional force exerted by the blood flow oriented tangential to the EC. EC sense shear stress through a large variety of mechanosensory complexes which convert the mechanical stimuli into biochemical signals (reviewed in Ref. 105). Disturbed blood flow, characterized by low and oscillatory shear stress (LOSS) occurring at the branching point and curvatures, induces plaque initiation, whereas high uniform shear stress (HSS) is atheroprotective.[106–108]

Snail, Twist1, and Gata4 are expressed in the endothelium at sites of LOSS in healthy murine aorta[20,27] and mesenchymal markers have been detected in the luminal EC exposed to disturbed flow in the porcine abdominal aortic trifurcation.[19] Concurrently, a combination of signalling pathways favourable to EndMT is present in low shear stress area: FGFR1 expression is decreased in EC by LOSS at atheroprone regions, whereas TGF-β is activated,[21] suggesting that LOSS is an activating signal for EndMT. Indeed, the imposition of LOSS on carotid arteries in healthy animals triggers the expression of EndMT transcription factors in EC[20,27] and aortic constriction-induced LOSS causes co-expression of endothelial and mesenchymal markers.[19] These data imply that LOSS induces EndMT possibly by regulating the balance between FGF and TGF-β signalling and by activating Gata4–Twist1–Snail pathway.

LOSS induces ROS production and inflammatory signalling,[103,109] which both favour EndMT during atherosclerosis (*Figure 3*). LOSS enhances oxidative stress in EC by the production of ROS.[52,110] High levels of ROS in EC cause a change in morphology and the induction of EndMT markers with the concurrent down-regulation of endothelial gene expression,[22] suggestive of EndMT induction by LOSS-derived oxidative stress.

LOSS increases vascular inflammation and enhances EC proliferation, apoptosis, and senescence, thereby increasing vascular permeability and contributing to atherosclerosis development.[111–113] LOSS-induced EndMT may contribute to these alterations of EC function. Moreover, LOSS recruits monocytes to the endothelium and promotes the differentiation of M1-type foam cell which secrete CCL4, IL-1β, and TNFα. CCL4 can induce EndMT by increasing endogenous TGF-β expression in ECs,[114] suggesting that the interplay between ECs and foam cells is of importance to EndMT induction during atherosclerosis.

EndMT has been linked to an increased vascular inflammation as TAK1, Twist1, and Gata4 promote the endothelial expression of inflammatory molecules (i.e. VCAM-1 and ICAM-1) under LOSS conditions.[27,52] In turn, inflammatory signalling promotes EndMT in an NFκB-dependent pathway,[38,87] thus establishing a positive feedback loop. In addition, Twist1, Gata4, and Snail have all been shown to promote EC proliferation and permeability under LOSS conditions.[20,27]

In contrast to the areas of LOSS, EndMT does not occur in HSS regions of the vasculature, suggesting a protective effect of HSS.[19–21] HSS inhibits EndMT via activation of the MEK5/ERK5 pathway and KLF4 transcription factors,[19,115] which can inhibit Snail expression (*Figure 3*).[116] Interestingly, HSS-induced activity of KLF transcription factors induces the expression of the inhibitory Smad7,[117] thereby directly antagonizing the EndMT programme. Additionally, HSS stabilizes EC junction by controlling VE-cadherin localization at the membrane through Rac/Rho signalling (*Figure 3*).[118,119] Loss of adherent junctions is an initiating feature of EndMT.

In summary, it has become apparent that LOSS induces EndMT in atheroprone regions; however, the precise mechanisms linking wall shear stress and the activation of EndMT need further elucidation. On the contrary, HSS is a powerful inhibitor of EndMT via activation of protective ERK5/KLF pathway. Further work is now required to determine at which extend LOSS-induced EndMT contributes to EC dysfunction leading to focal atherosclerosis and to define the underlying mechanisms.

4.2 EndMT and cyclic strain

Cyclic strain (CS) is the blood pressure-derived force causing a repetitive deformation of the artery wall in the circumferential direction perpendicular to the EC as a result of the pulsatile blood flow. The physiological level of CS is around 10% in the human aorta, but this can vary highly during aging[120] (1.5–4%) or when the wall of the aorta becomes stiffer due to diseases such as hypertension (>10%).[121]

Mechanical stretch induces the expression of SMC markers in EC.[122,123] Pathological strain induces the expression of TGF-β and BMPs in ECs and drives cardiac valve calcification via EndMT.[124,125] Depending on the strain magnitude, different signalling pathways are activated that can induce EndMT. TGF-β is activated under a relatively low strain (10%), whereas β-catenin/Wnt signalling is increased at high pathological strains (15–20%) (*Figure 3*).[124] Corroboratively, endothelial TGF-β expression is elevated by hypertension,[126] a condition associated with pathological CS. These data suggest that CS might contribute to the activation of TGF-β signalling during atherosclerosis.

Physiological levels of CS increase the migration and proliferation of EC while inhibiting apoptosis via PI3-kinase-mediated activation of Akt,[127,128] whereas elevated levels of CS (>15%) induce the expression of metalloproteases (MMPs) in ECs, such as MMP-1, MMP-2, and MMP-9.[128,129] Interestingly, MMP-9 expression by ECs activates the EndMT transcriptional programme through Notch signalling,[94] suggesting that elevated levels of CS in atherosclerosis[130] might aggravate EndMT.

The small GTPases Rac and Rho regulate junctional stability of EC in a CS-dependent manner,[131,132] wherein junctional stability is dependent on the membranous localization of VE-cadherin.[133] Physiological CS (5%) favours Rac activation, whereas pathological CS (>15%) suppresses Rac activity and enhances Rho-mediated signalling,[131] thereby causing the internalization of VE-cadherin and dissociation of endothelial adherens junctions,[134] initiating EndMT.

Pathological CS induces mitochondrial ROS release and the subsequent activation of NFκB signalling.[135] The resulting state of oxidative

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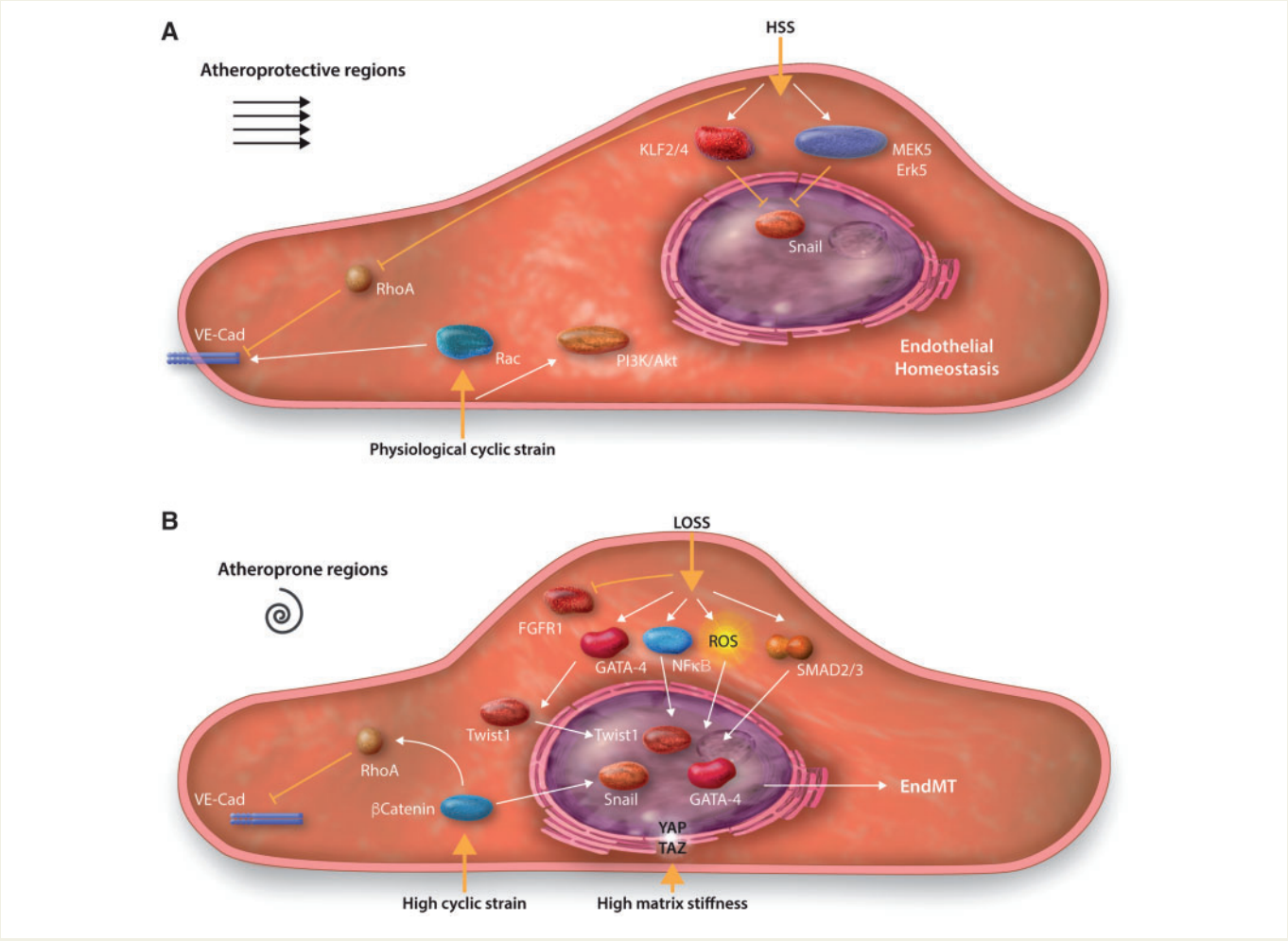

Figure 3 Mechanical forces and EndMT in atherosclerosis. ECs sense mechanical stimuli thanks to a multitude of mechanosensors (thick black arrows; reviewed by Hahn and Schwartz[109]). (*A*) HSS and physiological CS inhibit EndMT. HSS induces MEK5/Erk5 pathway and KLF transcription factors, which inhibit Snail expression. Both HSS and CS control VE-cadherin localization at the cell membrane by regulating the balance between the small GTPases Rac and Rho. Although Erk5 limits Rho activation, physiological strain stimulates Rac, stabilizing VE-cadherin at the cell junction. (*B*) LOSS, high CS, and matrix stiffness induce EndMT. LOSS inhibits FGFR1 expression which blocks FGF signalling, a potent inhibitor of EndMT. LOSS activates TGF-β and Gata4 and Twist1. LOSS is associated with increased inflammation and oxidative stress. All these pathways culminate in the expression of Snail1 and/or Slug, which trigger EndMT. High CS increases Wnt/β-catenin activity, which can induce and stabilize Snail1. Increased matrix stiffness promotes nuclear localization of YAP/Taz, which retains activated SMAD2/3 in the nucleus. High CS induces activation of Rho, which causes the translocation of VE-cadherin to the cytoplasm weakening cell–cell junctions, thus facilitation EndMT.

stress and inflammation facilitates EndMT by enhancing the expression of TGF-β and the activation of its signalling through ALK5.[54]

In summary, EC homeostasis is highly dependent on CS, wherein at physiological levels homeostasis is maintained and at pathological levels multiple EndMT-inducing signalling cascades are activated. Therefore, it is highly likely the interplay between EC and CS contributes to the focal development of atherosclerosis.

4.3 Matrix stiffness and EndMT

Vascular stiffening accompanies several cardiovascular conditions, such as hypertension[136] and atherosclerosis.[137] Arterial stiffening results from ECM remodelling within the vessel wall, characterized by degradation of elastin in the ECM and deposition and cross-linking of collagen molecules,[138] and occurs as a result of aging, diabetes, renal disorders, and atherosclerosis.

EC sense matrix stiffness through focal adhesions.[139] Focal adhesions are adhesion plaques, primarily formed by integrins complexes that act as an interface between the actin cytoskeleton and ECM, transmitting mechanical forces across the cell membrane. Matrix stiffness influences a variety of cell functions including proliferation, differentiation, migration, and apoptosis.[140–143] Vessel stiffness promotes the adaptation of an atherogenic phenotype in EC,[144,145] yet little is known about how arterial stiffness affects ECs within blood vessels where arteriosclerosis initiates. High matrix stiffening promotes the activation of RhoA,[146] a regulator of Slug-induced EndMT[60] and nuclear translocation of Twist1.[147] Matrix stiffness also regulates Yes-associated protein (YAP) and the transcriptional coactivator with PDZ-binding motif (Taz) signalling pathway potentially through activation of integrins.[148]

YAP/Taz signalling collaborates with Snail and Slug to induce the mesenchymal transcription factor Runx2.[149] Moreover, increasing matrix

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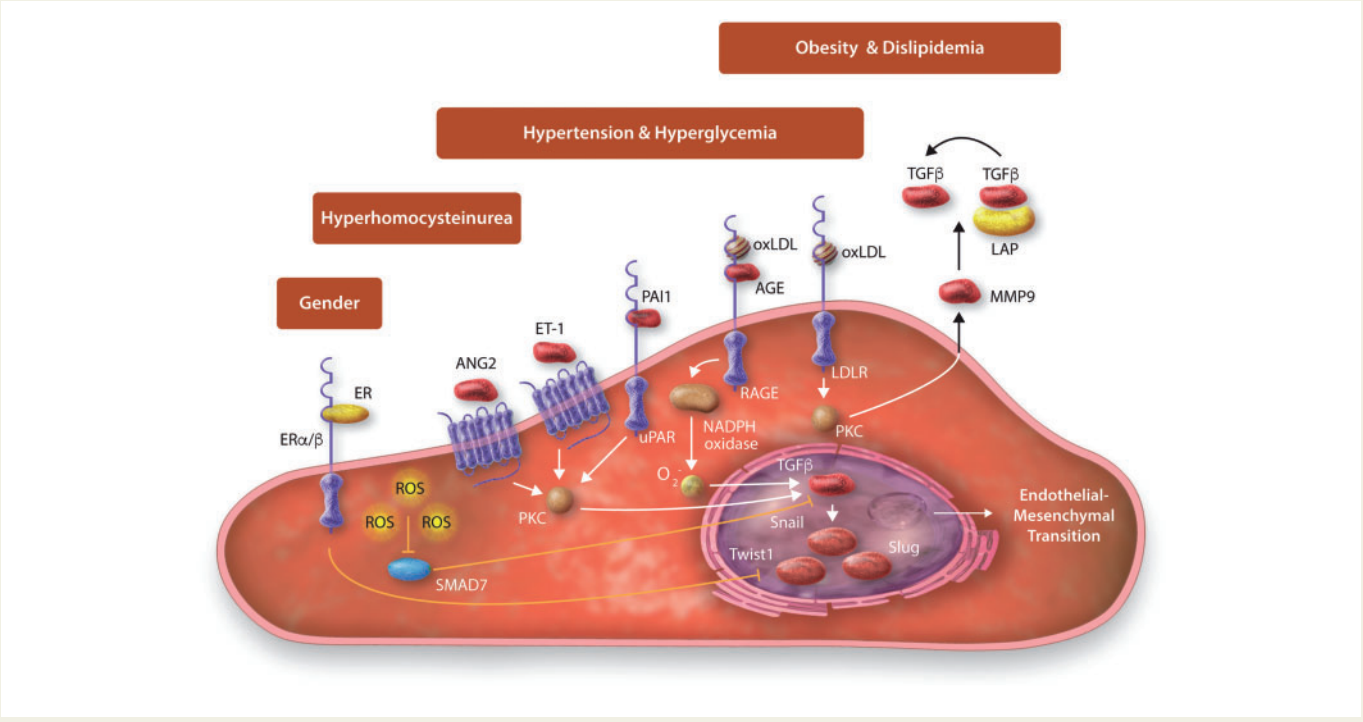

Figure 4 Systemic atherosclerosis risk factors and EndMT. Systemic risk factors for atherosclerosis might facilitate EndMT in a variety of ways. Hypertension and hyperglycaemia increase signalling through angiotensin, ET1 and PAI-1, all resulting in the activation of PKC. Likewise, obesity and dyslipidaemia are associated with increased signalling through PKC. PKC signalling culminates in the expression of TGF-β. TGF-β signalling, either canonical or non-canonical, induces the expression of the EndMT transcription factors Snail, Slug, and Twist1. Additionally, increased activity of RAGE signalling in diabetes, obesity, and dyslipidaemia activates NOX, resulting in oxidative stress-induced TGF-β expression. Hyperhomocystinuria is associated with increased endothelial oxidative stress and the concurrent reduction in SMAD7 expression, an important endogenous inhibitor of TGF-β signalling and thus EndMT. Oestrogen signalling reduces Slug expression in epithelial cells and reduces EndMT, albeit through unidentified mechanisms.

stiffness causes the nuclear translocation of YAP/Taz and promotes TGF-β signalling by the nuclear retention of SMAD2/3[150,151] in epithelial cells and fibroblasts. Corroboratively, endothelial YAP/Taz is increased in atheroprone areas,[152] which coincides with the nuclear accumulation of SMAD2 and EndMT.[21] Together, these data suggest an important role for YAP/Taz signalling in EndMT and atherosclerosis development, resulting in a vicious circle of increasing vessel stiffness and atherosclerosis progression.[153]

5. Systemic risk factors and EndMT in atherosclerosis

In the sections above, we have reviewed the biochemical and biomechanical factors that mediate EndMT at atheroprone sites. In the following section, we explore the mechanisms by which systemic atherosclerosis risk factors might induce or facilitate EndMT.

5.1 Hypertension and hyperglycaemia

Hypertension and hyperglycaemia are associated with increased endothelial signalling through endothelin (ET)-1, plasminogen activator inhibitor (PAI)-1, angiotensin (Ang)-II, and NOX, which all enhance TGF-β signalling in ECs (*Figure 4*). ET-1, PAI-1, and Ang-II signalling directly induce the expression of TGF-β in ECs through PKC,[154–156] whereas NOX induces endothelial TGF-β expression through the induction of endothelial oxidative stress.[157,158] In hypertension, endothelial Smad2 activity is increased,[159] indicative of active TGF-β signalling through ALK5. This increase in TGF-β activity associates with an increase in EC expressing mesenchymal marker proteins (e.g. FSP1, αSMA, and Collagen type I), indicative of EndMT.[160,161] Blocking of any of the signalling molecules upstream of TGF-β, i.e. ET-1,[162,163] PAI-1,[164] or Ang-II,[165] drastically reduces fibrogenesis, EndMT, and atherosclerosis progression.

In diabetes, the non-enzymatic reaction between glucose and serum proteins result in increased levels of advanced glycation end products (AGEs) that are biologically active and accumulate in the artery wall.[166] Binding of AGEs to their receptor (RAGE) initiates signalling event that induce or aggravate EndMT in diabetes,[167,168] primarily through the NOX-induced activation of PKC[169] and subsequent increase in TGF-β expression.[170]

5.2 Obesity, dyslipidaemia, and EndMT

Obesity and atherosclerosis are characterized by dyslipidaemia, including the increase in serum oxidized low-density lipoprotein (oxLDL) levels. Increased oxLDL signalling might induce EndMT through a variety of cascades all including oxidative stress.[171,172] OxLDL binding to its receptor LDLR induces signalling via PKC which increased endothelial MMP production in an oxidative stress-dependent manner.[173] Elevated levels of PKC signalling and MMP activity might increase endogenous TGF-β production[174] and activation,[92] and thus facilitate EndMT.

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Interestingly, oxLDL can also bind and innervate alternative receptors, such as the scavenger receptor LOX-1[175] and RAGE.[176] OxLDL binding to LOX-1 induces TGF-β expression in arterial EC *in vivo*[175] and LOX-1 signalling is associated with increased collagen production in myofibroblasts,[177] suggesting that oxLDL-induced LOX-1 activity might play a role in EndMT. Binding of advanced oxidation protein products, such as oxLDL, to RAGE can induce mesenchymal transition in epithelial cells in a PKC-δ-mediated manner.[178] Of note, high-density lipoprotein (HDL), an established inhibitor of oxLDL signalling in EC,[179] abolished EndMT induction by yet unknown mechanisms.[180]

5.3 Hyperhomocystinuria and EndMT

Elevation of plasma homocysteine level is a risk factor for cardiovascular disease, including atherosclerosis.[181] Hyperhomocystinuria induces ER stress and a decrease in the production of the gasotransmitter H_2S.[182] Interestingly, exogenous H_2S supplementation protects against EndMT by the attenuation of ER stress and the resulting TGF-β signalling,[93] potentially through the activation of AMPK signalling,[182] resulting in the expression of inhibitory Smad-7.[183]

5.4 Oestrogen receptor signalling and EndMT

Atherosclerosis preferentially develops in male subjects, which might indicate a role for oestrogen signalling in atheroprotection[184] and the inhibition of EndMT. Indeed, in EMT, oestrogen signalling through its receptors ERα and ERβ maintains the epithelial phenotype by the direct repression of Slug expression and loss of oestrogen signalling induces EMT.[185] In ECs, oestrogen signalling induces endothelial adherens and tight junction formation.[186] These data suggest that oestrogen might be an inhibitor of EndMT, yet, this hypothesis remains to be investigated.

In summary, atherosclerosis risk factors can facilitate EndMT via multiple signalling cascades, all involving oxidative stress-induced expression and the activation of TGF-β signalling. Antagonizing the deranged signalling associated with atherosclerosis risk factors might inhibit EndMT and atherosclerosis progression.

6. Summary and conclusions

Atherosclerosis is a progressive pathological remodelling of the vessel wall, characterized by the accumulation of lipids and intimal mesenchymal cells, resulting in the creation of an atheromatous plaque. It is well-established that endothelial dysfunction plays a pivotal initiating role in atherosclerosis development.[187] Recent studies indicate another role for the endothelium in atherosclerosis development and progression that links the atherosclerosis initiating events to the subsequent vascular remodelling, i.e. EndMT.[19–21] EndMT is a process wherein the EC lose the expression of their characteristic marker proteins and cellular functions and acquire the expression of mesenchymal marker proteins and adopt mesenchymal cell functions, such as ECM production and contractile behaviour, thus adding to the pool of mesenchymal cells in the neointima. Here, we have reviewed the major biochemical and biomechanical signalling cascades that induce, modulate, or inhibit EndMT.

It should be emphasized that, for clarity reasons, the biochemical signalling and biomechanical signalling cascades were discussed as separate entities; however, in combination *they* form an intricate signalling network *in vivo*.[188,189] It becomes apparent that the native vessel architecture determines the areas at risk for atherosclerosis development through biomechanical signalling, either induced by LOSS or CS.[96,153] At these so-called atheroprone areas, biochemical signals originating from the systemic atherosclerosis risk factors (e.g. inflammation, hyperglycaemia, and dyslipidaemia) induce endothelial dysfunction[5] and, if persistent, start to induce EndMT which participates in intimal hyperplasia.[19,21] Vascular remodelling, originating from the increase in mesenchymal cells in the intima, results in an increase in vessel stiffness and cyclic wall strain, which might aggravate EndMT creating the vicious cycle that facilitates the progressive nature of atherosclerosis.

Endothelial lineage tracing studies have established the occurrence of EndMT during atherosclerosis.[21,96] Albeit that EndMT is implicated in atherosclerosis progression in humans,[22] the extent by which EndMT contributes to the initiation and progression of atherosclerosis needs further elucidation to provide a strong basis for anti-atherosclerosis therapies that specifically target EndMT. Although the endothelial-specific deletion of Erk5, an MAPK that protects against EndMT,[19] aggravates atherosclerosis development and pharmacological activation of Erk5 diminished atherosclerosis formation,[190] it is currently unknown whether the modulation of EndMT or the modulation of other antiatherosclerosis genes (i.e. KLF2, KLF4, and eNOS) underlies these observations. Concurrently, pharmacological activation of FGF signalling protects against EndMT-mediated atherosclerosis progression.[21,97] Although these data are encouraging, *proof-of-concept* studies wherein EndMT is therapeutically targeted in advanced stages of atherosclerosis are missing.

In summary, EndMT contributes to the initiation and progression of atherosclerosis. EndMT is modulated by multiple integrative pathways,

Box 1 Evidence suggesting a link between EndMT and atherosclerosis

- Endothelial-specific lineage tracing experiments showed contribution of endothelial-derived cells to atherosclerotic lesions.
- Expression of EndMT TF (Twist1, Snail, and Gata4) and activity of the potent EndMT inducer TGF-β (revealed by expression of p-Smad2) observed in luminal EC at atheroprone sites in healthy mice.
- TGF-β activity detected in the luminal endothelium overlying the plaque in human.
- Occurrence of EndMT in atherosclerotic plaque in mouse and human as suggested by co-expression of endothelial markers with EndMT TF or mesenchymal markers.
- Correlation between EndMT extent and plaque stability/disease severity in coronary artery disease patient.
- Hypoxia, oxidative stress, and inflammatory cytokines, all present in atherosclerotic plaque, constitute a favourable environment for EndMT
- EC-specific deletion of main EndMT TF, i.e. Twist1 or Gata4 reduces the lesion size in atherosclerotic mice.
- EC-specific disruption of FGF signalling (known inhibitor of EndMT) leads to an increase of atherosclerotic development.

Moonen et al., 2015;[19] Chen et al., 2015;[21] Evrard et al., 2016;[22] Mahmoud et al., 2016, 2017[27,20].

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including TGF-β, Wnt/β-catenin, and Notch, originating from both biochemical and biomechanical stimuli. Therapeutic strategies that reduce EndMT might have potential as anti-atherosclerosis therapies.

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