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Endothelial-mesenchymal transition and its contribution to the emergence of stem cell phenotype

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Abstract

Vascular endothelial cells can demonstrate considerable plasticity to generate other cell types during embryonic development and disease progression. This process occurs through a cell differentiation mechanism known as endothelial-mesenchymal transition (EndMT). The generation of mesenchymal cells from endothelium is a crucial step in endothelial cell differentiation to several lineages including fibroblasts, myofibroblasts, mural cells, osteoblasts, chondrocytes, and adipocytes. Such differentiation patterns have been observed in systems of cardiac development, fibrosis, diabetic nephropathy, heterotopic ossification and cancer. Here we describe the EndMT program and discuss the current evidence of EndMT-mediated acquisition of stem cell characteristics and multipotent differentiation capabilities.

Keywords

Endothelial-mesenchymal transition; EMT; EndMT; stem cells; TGF-beta

The epithelial-mesenchymal transition (EMT) program is the primary mechanism of cancer metastasis, inducing carcinoma cells to lose their adhesion, acquire mesenchymal properties and migrate away from the primary tumor [1,2]. This process has also been shown to regulate several phases of embryonic development including gastrulation, somite dissociation, primitive streak and neural crest migration, and craniofacial development [3,4]. EMT has also been suggested to promote fibrosis and wound healing [5,6].

More recently, endothelial cells have been shown to undergo a similar process termed endothelial-mesenchymal transition (EndMT), which is of fundamental importance in mediating similar physiological and pathological processes [7]. Here we review the systems

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where EndMT has been identified and the molecular mechanisms that control this change in cellular phenotype.

The Molecular and Cellular Basis of EndMT

Endothelial cells compose the inner lining of blood vessels and lymphatic vessels [7]. These cells, which are anatomically similar to squamous epithelium, demonstrate apical-basal polarity and are tightly bound by adherens junctions and tight junctions. These cells express a distinct set of biomarkers that allow investigators to distinguish them from other cell types including VE-cadherin, CD31, TIE1, TIE2, von Willebrand Factor (vWF), and cytokeratins. During EndMT, expression of these markers is dramatically reduced, although some minimal levels of expression are usually maintained. Furthermore, mesenchymal-specific genes are expressed which include fibroblast-specific protein-1 (FSP-1), alpha-smooth muscle actin (α-SMA), vimentin, and N-cadherin. These changes in gene and protein expression cause the endothelial cells to lose their adhesion and stimulate alterations in cytoskeletal composition and organization to induce a striking change in cell morphology that forms elongated, spindle-shaped cells. These newly formed mesenchymal cells are highly invaisive and migratory [1,7] (Figure 1).

The EndMT program can be stimulated by many factors. The most common of these are the Transforming Growth Factor-beta (TGF-β)/Bone Morphogenetic Protein (BMP) family of growth factors [8]. All three TGF-β isoforms (TGF-β1, TGF-β2 and TGF-β3) have been shown to induce EMT [9, 10], however EndMT appears to be stimulated primarily by the TGF-β2 isoform [11–18]. Genetic ablation of TGF-β2 in mice prevents embryonic EndMT, whereas TGF-β1 or TGF-β3 knockout mice show no significant effects on EndMT during development [16]. TGF-β2 has also been shown to stimulate EndMT in cultured endothelial cells [12,13,17,18].

TGF-β3 has been shown to have a role in post-EndMT invasion and migration in the chick embryo, but not in EndMT itself [19,20]. TGF-β3 has no significant role in embryonic EndMT in mice [21]. Some studies have suggested that TGF-β1 may induce EndMT [22,23], although the primary effect of TGF-β1 on endothelial cells is to induce cell proliferation [24,25].

TGF-β1 is perhaps the most common inducer of EMT [1,2,9]. In epithelial cells, TGF-β1 binds to a complex of receptors that include the TGF-β type III receptor β-glycan, type II receptor (TβRII) and type I receptor activin-like kinase 5 (ALK5), which promotes signaling through Smad2/3 [26]. In endothelial cells, TGF-β1 promotes signaling through ALK1 rather than ALK5, which causes phosphorylation of Smad1/5/8. When ALK1 is involved, TGF-β1 signaling induces cell proliferation rather than EndMT [24,25]. This occurs because endothelial cells express another TGF-β type III receptor called endoglin. siRNA-mediated knockdown of endoglin in endothelial cells allows TGF-β1 to signal through β-glycan and ALK5 and phosphorylate Smad2/3 [24]. Inhibition of ALK5, TβRII, β-glycan and endoglin in the endothelium of mice have been shown to prevent embryonic EndMT [19, 21, 27]. SB-431542, a chemical inhibitor of ALK5, was found to be sufficient to inhibit EndMT of cultured endothelial cells [23]. Reducing ALK5 expression with siRNA showed similar inhibitory results [17].

BMP2 and BMP4 have been shown to induce both EMT [28,29] and EndMT [30,31] programs. These ligands primarily signal through the ALK2 receptor [32]. Interestingly, conditional knockouts of BMP2, BMP4 or ALK2 all inhibit embryonic EndMT [30,31,33]. Also, siRNA mediated knockdown or chemical inhibition of ALK2 with dorsomorphin blocked EndMT in endothelial cultures [17].

BMP7 is another isoform that binds and activates ALK2 [32], yet it is known to be an extremely potent inhibitor of EndMT [22]. Recent studies have suggested that TGF-β2 and BMP4 induce EndMT by activating both ALK2 and ALK5, which function together in a complex of signaling receptors to activate both Smad1/5/8 and Smad2/3 signaling pathways [17]. On the other hand, BMP7 activates only ALK2 and the Smad1/5/8 pathway [17], suggesting that this may play a role in the inhibitory effects of this isoform on EndMT. Vascular endothelial growth factor (VEGF) signaling has also been shown to inhibit EndMT [17,34], although the downstream signaling mechanisms of this inhibition are currently unknown (Figure 2).

Smad-independent signaling pathways have also been shown to have a role in regulating EndMT. Inhibitors of Smad4, MEK, PI3K and p38 MAPK all sufficiently blocked TGF-β2-induced EndMT in endothelial cultures, demonstrating critical roles for Smad-dependent and Smad-indepenent TGF-β signals [18]. All of these pathways were shown to be necessary for TGF-β2-induced expression of the EMT/EndMT-promoting transcription factor Snail [15,18].

Loss of cell-cell adhesion associated with EndMT is mediated by transcription factors such as Snail, Slug, ZEB-1, SIP-1, Twist, and LEF-1 that suppress transcription of genes encoding proteins involved in formation of adherens junctions and tight junctions [15, 17,18, 35–38]. All of these transcription factors are up-regulated during EndMT induced by TGF-β2 or BMP4 [17]. siRNA-mediated knockdown of Snail expression was sufficient to inhibit TGF-β2-induced EndMT in cultured endothelial cells. However, over-expression of Snail was insufficient to induce EndMT. Blocking the Snail inhibitor GSK-3β [39] with lithium chloride was sufficient to allow EndMT by Snail over-expression [18].

Other signaling pathways including FGF-2 [40]. Notch [41] and Wnt [12] have also been linked to EndMT. During EMT/EndMT, the basal lamina is degraded by matrix metalloproteinases (MMP) such as MMP-2 and MMP-9 and is replaced by new matrix molecules like type I collagen, type III collagen and fibronectin [2,7]. Type I collagen has long been associated with induction of EMT/EndMT by activating the signaling receptors α2β1 integrin [42,43], discoidin domain receptor 1 (DDR1) [44], and DDR2 [45]. Fibronectin induces post-EndMT cell migration through α5β1 integrin signaling, which promotes cytoskeletal reorganization [46].

EndMT in Embryonic Development

EndMT was initially discovered as an essential mechanism of heart development [47]. Vascular endothelial cells surrounding in atriventricular canal and the outflow tract undergo EndMT and invade surrounding tissues to form the valves and septa of the heart [21]. Several mechanistic studies have shown the crucial importance of TGF-β/BMP ligands and receptors in embryonic EndMT. Targeted inhibition of TGF-β2, BMP2, BMP4, ALK2, ALK5, endoglin, or β-glycan in mice show defective heart development due to a lack of EndMT [21]. Interestingly, genetic knockout of TGF-β1 or TGF-β3 in the endothelium of mice showed no detrimental effects on heart development [16], suggesting strong isoform specificity for TGF-β2 in the induction of EndMT. However, in the avian heart, both TGF-β2 and TGF-β3 play critical roles in development, with TGF-β2 inducing EndMT and TGF-β3 promoting post-EndMT invasion and migration into the underlying tissue [19]. Notch [41] and Wnt [12] signaling have also been shown to have an important role in regulating EndMT in the developing heart.

EndMT in Fibrosis

Although EndMT is an embryonic mechanism that is normally dormant in the adult organism, pathological conditions can arise that awaken this phenomenon. One such condition that effects most organs as a result of injury, inflammation or aging is fibrosis [2,48]. EMT is known to be essential for the formation of fibrotic tissues [2], but more recent evidence suggests that EndMT also contributes to fibrosis [7,22] (Figure 3).

Cardiac fibrosis is a common result of heart failure. Myocardial infarction and ischemia can induce scarring of the heart tissue [49]. Lineage tracing studies using Tie1-Cre reporter mice have shown that many of the fibroblast formed in fibrotic lesions of the heart are of endothelial origin and arise through EndMT. Further evidence suggests that this process occurs in a TGF-β- and Smad3-dependent manner, similar to developmental EndMT. Furthermore, treatment with recombinant BMP7 was sufficient to inhibit EndMT and the incidence of cardiac fibrosis in mice [22].

Fibrosis has been shown to be prominent in renal diseases such as Alport syndrome and diabetic nephropathy [50]. Studies in mouse models of Alport syndrome and nephropathy induced by streptozotocin or unilateral urethral obstruction showed that up to half of all fibroblasts formed in the kidneys under these conditions expressed the endothelial marker CD31, suggesting that they could arise by EndMT [51]. This was confirmed by recent evidence using lineage tracing with Tie2-Cre reporter mice showing that many of the myofibroblasts in kidneys of mice with diabetic nephropathy were indeed of endothelial origin [51,52]. Similar to cardiac fibrosis, EndMT in kidney fibrosis was found to be Smad3-dependent [53].

Capillary endothelial cells in the lung have been shown to undergo EndMT to contribute to pulmonary fibrosis [54]. EndMT may also contribute in wound healing [7,40].

EndMT in Cancer

The tumor microenvironment plays an important role in cancer growth, angiogenesis and metastasis [55]. One component of this microenvironment that regulates these processes is cancer-associated fibroblasts (CAFs), which are part of the tumor stroma. In an elegant study, Zeisberg et al. [56] showed that up to 40% of CAFs were formed by EndMT in two distinct mouse models of cancer. CAFs in these models co-expressed the endothelial marker CD31 along with mesenchymal markers FSP-1 or α-SMA. Moreover, lineage using Tie2-Cre reporter mice confirmed the endothelial origin of these CAFs [56]. It has been suggested that CAFs regulate metastasis by secreting cytokines such as TGF-β1, which induces EMT of the cancer cells [7].

Hypoxia is another condition in which EndMT-dependent formation of fibroblasts may occur [57,58]. As tumors increase in size, they naturally become hypoxic. This lack of oxygen activates Hypoxia Inducible Factor-1 (HIF-1), a transcription factor responsible for stimulating expression of endothelial growth factors to induce tumor angiogenesis; an important mechanism for tumor growth and metastasis [59]. Hypoxia has previously been linked to EMT and metastasis [60]. Therefore, it is likely that as tumors grow larger and become more hypoxic, a higher incidence CAF formation through EndMT will occur (Figure 4).

EndMT may also play a direct role in regulating tumor angiogenesis. It has been suggested that vessel branching may be a result of EndMT forming the tip cells, which have characteristics of mesenchymal cells [7,61]. At the angiogenic tip, these cells are highly migratory and are exposed to matrix proteins like type I collagen [61], a known inducer of

EMT/EndMT [43,45], rather than type IV collagen in the standard endothelial basal lamina [7]. These data suggest that EndMT might occur in the leading edge of branching vessels.

EndMT and the Stem Cell Phenotype

In a recent study of a rare bone disease called Fibrodysplasia Ossificans Progressiva (FOP), vascular endothelial cells were shown differentiate into chondrocytes and osteoblasts through EndMT [17,62]. In patients with FOP, acute inflammation triggers heterotopic ossification in soft tissues [63,64]. FOP patients carry a heterozygous germ-line mutation (R206H) in the TGF-β/BMP receptor ALK2 [65]. Studies have shown that R206H is a gain of function mutation that causes the receptor to be constitutively active in the absence of ligands [66].

Tissue sections of ectopic lesions acquired from FOP patients showed positive staining of cartilage and bone cells for the endothelial biomarkers TIE2 and vWF. Bone and cartilage cells from normal skeletal tissue did not express such markers. Furthermore, heterotopic lesions from a transgenic mouse model of FOP, which express the mutant ALK2 gene found in FOP patients in a Cre-dependent manner, showed similar positive expression of endothelial markers in the cartilage and bone cells [17]. Lineage tracing with Tie2-Cre reporter mice also suggested an endothelial origin to heterotopic cartilage and bone cells, with approximately 50% of these cells showing expression of the GFP reporter [17]. The origin of the remaining 50% is currently unknown. Also, in the early stages of heterotopic ossification, a condensation of mesenchymal cells appears prior to chondorgenesis or osteogenesis [63,67], many of which are of endothelial origin [17] providing *in vivo* evidence for EndMT as a critical mechanism mediating this disease.

Further investigations into the role of mutant (R206H) ALK2 in normal vascular endothelial cells showed that over-expression of this mutant gene induced EndMT. These cells also expressed biomarkers of mesenchymal stem cells such as STRO-1, CD10, CD44, CD71, CD90, and CD117. Furthermore, EndMT also induced physiological properties of stem cells, as they could be stimulated to differentiate into osteoblasts, chondrocytes or adipocytes both *in vitro* and *in vivo* [17]. These results provide evidence that EndMT can generate cell types other than fibroblasts or myofibroblasts. These data demonstrated that EndMT is a mechanism for generating mesenchymal stem cells that can subsequently differentiate into other cell types (Figure 5). These results suggest that EndMT may not be a direct transformation to fibroblasts as originally thought, but rather a dedifferentiation to mesenchymal stem cells.

In a study of tumor calcification, prostate carcinomas showed bone-forming cells in the tumors that induce ossification. Interestingly, these osteoblasts stained positive for the endothelial biomarker CD31 [68]. Since EndMT is a prominent mechanism for the formation of cancer-associated fibroblasts in the tumor microenvironment [56], it is likely that the same mechanism that induces heterotopic ossification in FOP could be happening to induce tumor calcifications by generating endothelial-derived osteoblasts.

Others have suggested that mural cells (pericytes and smooth muscle cells) may arise by EndMT [7,23,69]. Pericytes are mesenchymal cells that have been described to have stem-like properties and differentiate into other cell types [70]. Furthermore, circulating endothelial progenitor cells can undergo EndMT and have been suggested to give rise to smooth muscle cell progeny [23].

Cancer stem cells are generated by EMT [71,72]; the same mechanism that induces metastasis [1,2]. Although EndMT has not yet been shown to produce cancer stem cells, the ability of endothelial cells to convert into mesenchymal stem cells has been demonstrated in

FOP [17]. Considering that EndMT is known to generate cancer-associated fibroblasts in the tumor microenvironment [56], it is reasonable to think that some cancer stem cells found in tumors might be of endothelial origin.

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Abbreviations

EndMT	endothelial-mesenchymal transition
EMT	epithelial-mesenchymal transition
TGF-β	transforming growth factor-beta
BMP	bone morphogenetic protein
TβRII	transforming growth factor-beta receptor 2
ALK	activin-like kinase
FSP-1	fibroblast-specific protein-1
α-SMA	alpha-smooth muscle actin
DDR	discoidin domain receptor
vWF	von Willebrand factor
MMP	matrix metaloproteinase
CAF	cancer-associated fibroblast
HIF-1	hypoxia-inducible factor-1
FOP	fibrodysplasia ossificans progressiva
VEGF	vascular endothelial growth factor

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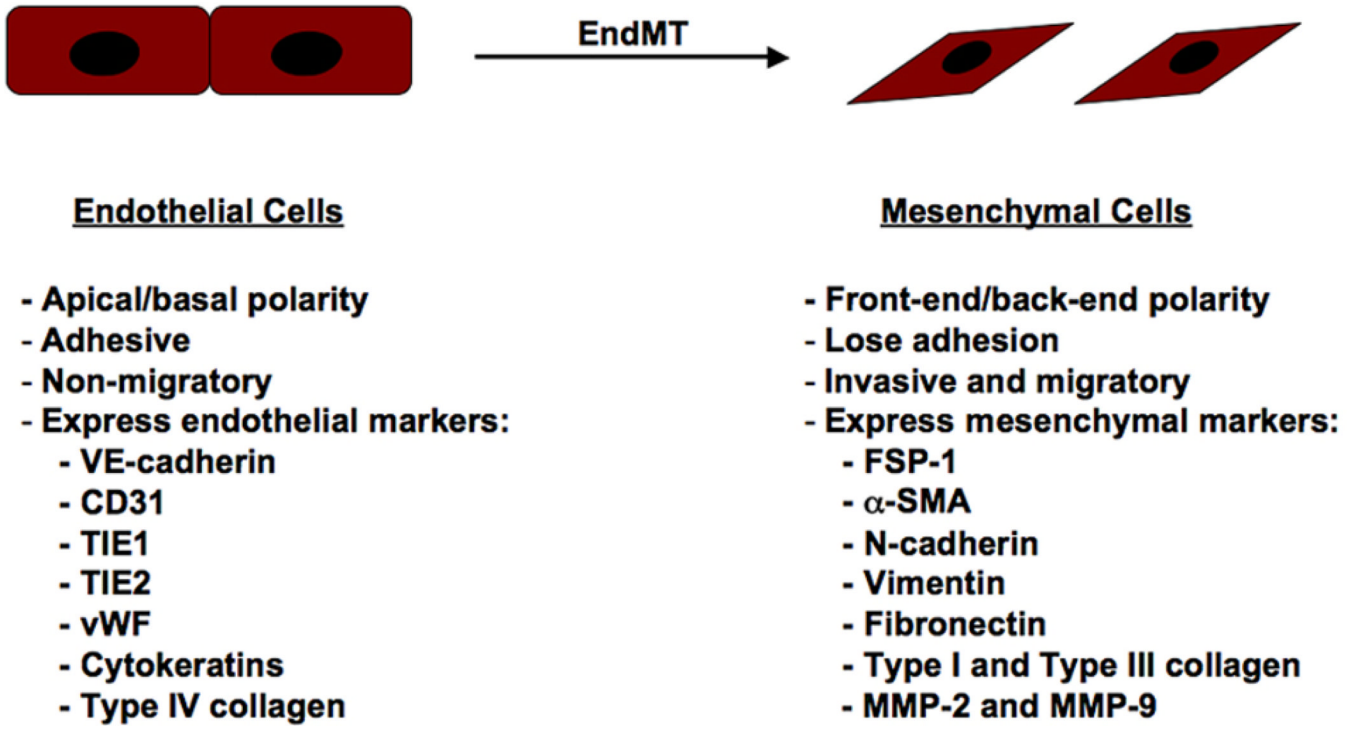

Figure 1.
Changes in cellular characteristics during EndMT. The EndMT program causes decreased expression of endothelial markers VE-cadherin, CD31, TIE1, TIE2, and vWF, and a gain of mesenchymal markers FSP-1, α-SMA, N-cadherin, vimentin, fibronectin, type I and type III collagen, and MMP-2 and MMP-9. Distinct changes in cell polarity and morphology accompany EndMT, as well as loss of cell-cell junctions and increased motility.

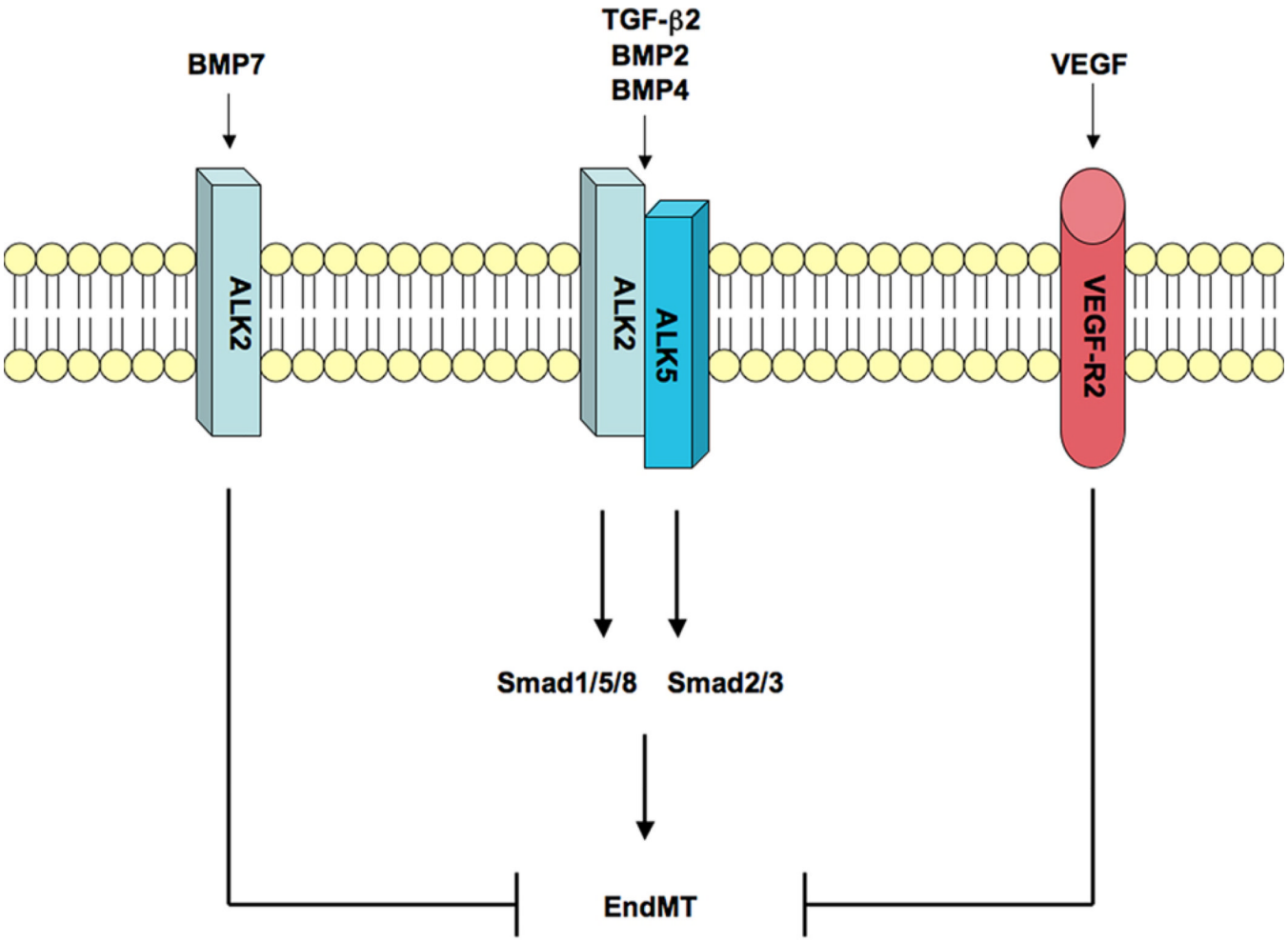

Figure 2.

Signaling mechanisms of EndMT. TGF-β/BMP receptors termed activin-like kinases (ALK) control EndMT. Ligands such as BMP2, BMP4, and TGF-β2 bind and activate ALK2 and ALK5, which stimulate Smad1/5/8 and Smad2/3 signaling to induce EndMT. BMP7 activates ALK2, but not ALK5, which inhibits EndMT. VEGF signaling through the VEGF receptor 2 (VEGF-R2) has also been described to block EndMT.

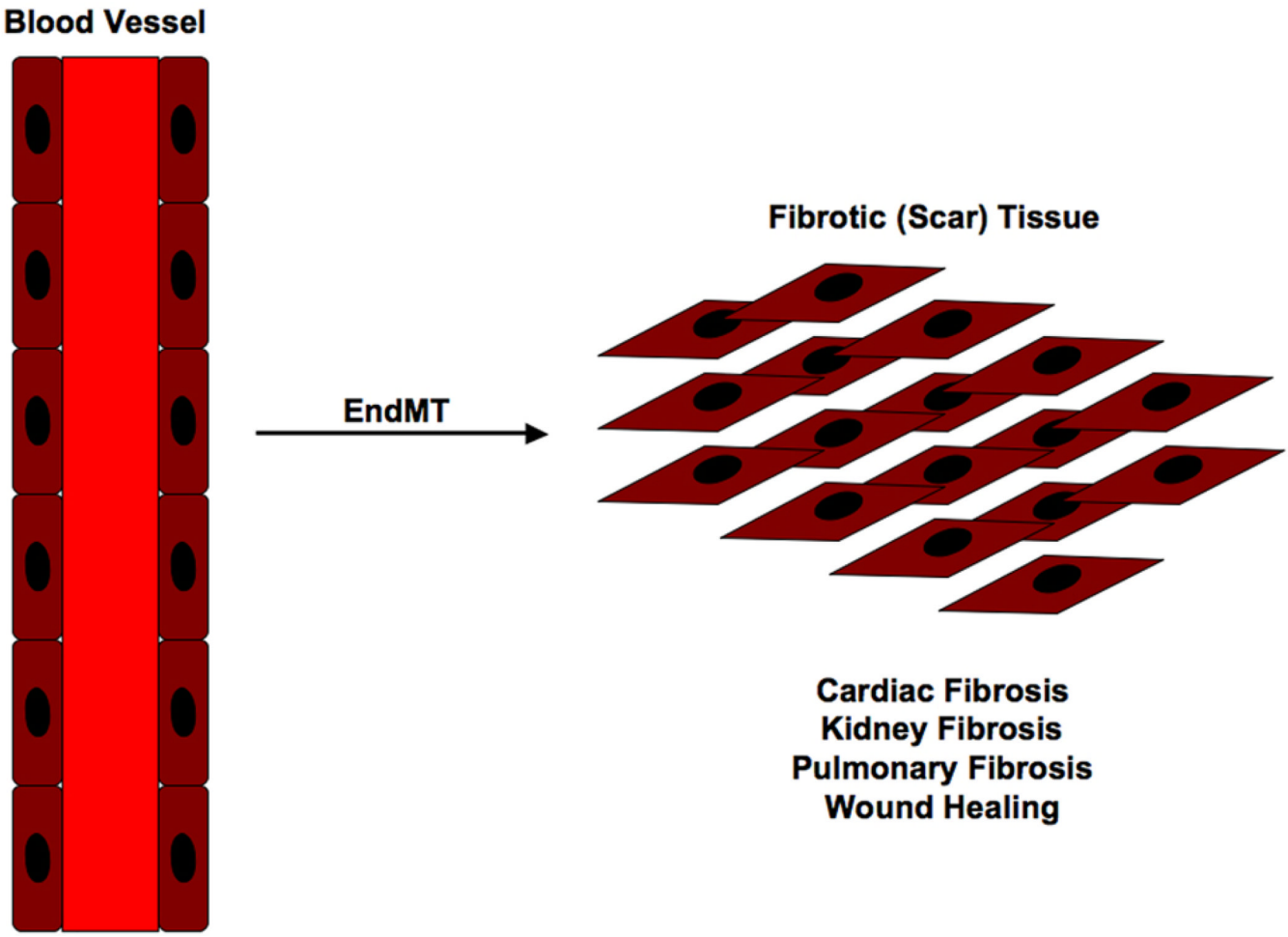

Figure 3.
EndMT generates fibrotic tissue. Endothelial cells from capillary blood vessels in organs have been shown to undergo EndMT and form fibrotic tissue. This mechanism causes fibrosis in organs such as kidney, lung and heart.

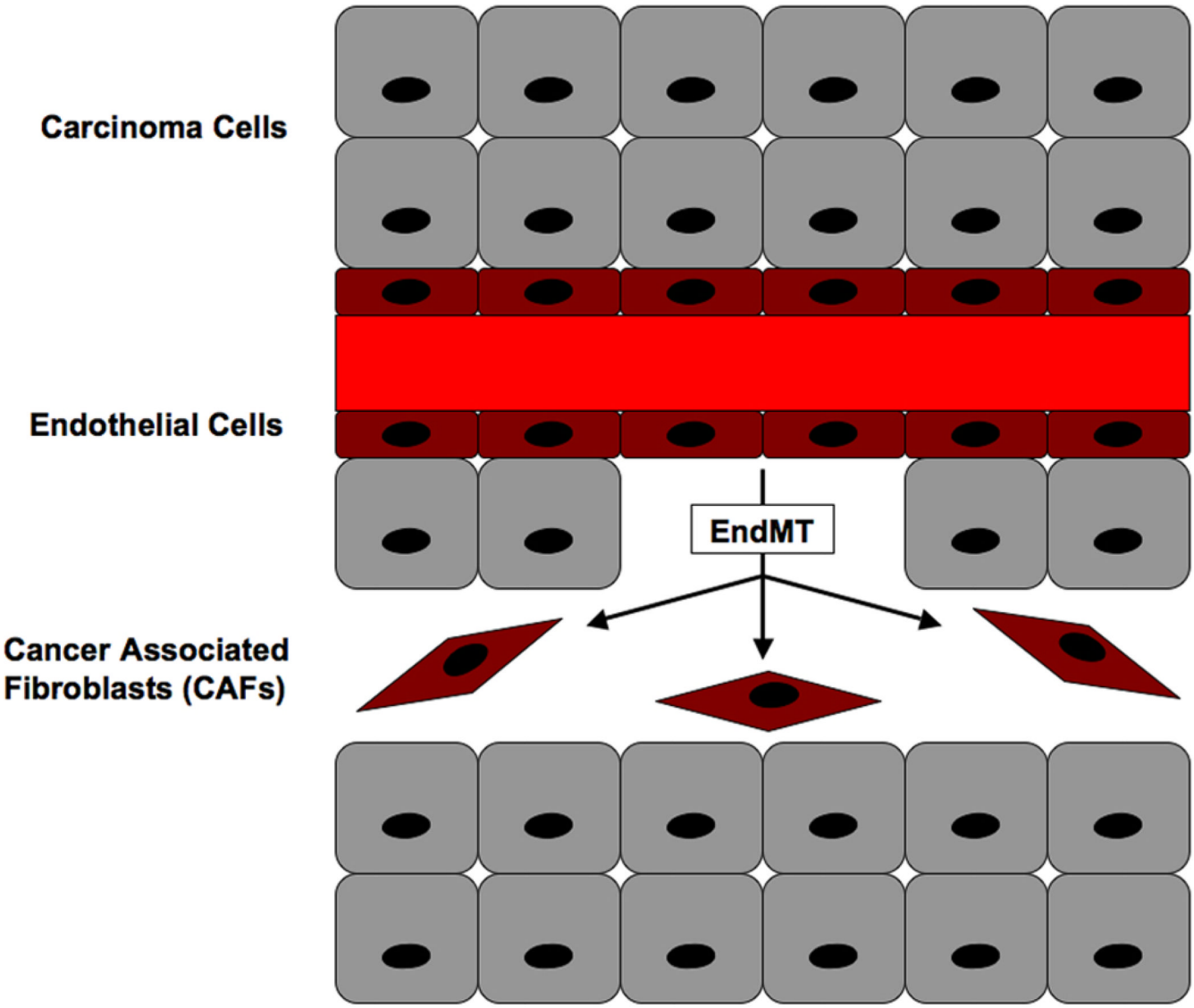

Figure 4.
EndMT generates cancer-associated fibroblasts (CAFs). Capillary blood vessels in angiogenic tumors undergo EndMT to form CAFs. These fibroblasts play an essential role in the tumor microenvironment and regulate cancer growth and metastasis.

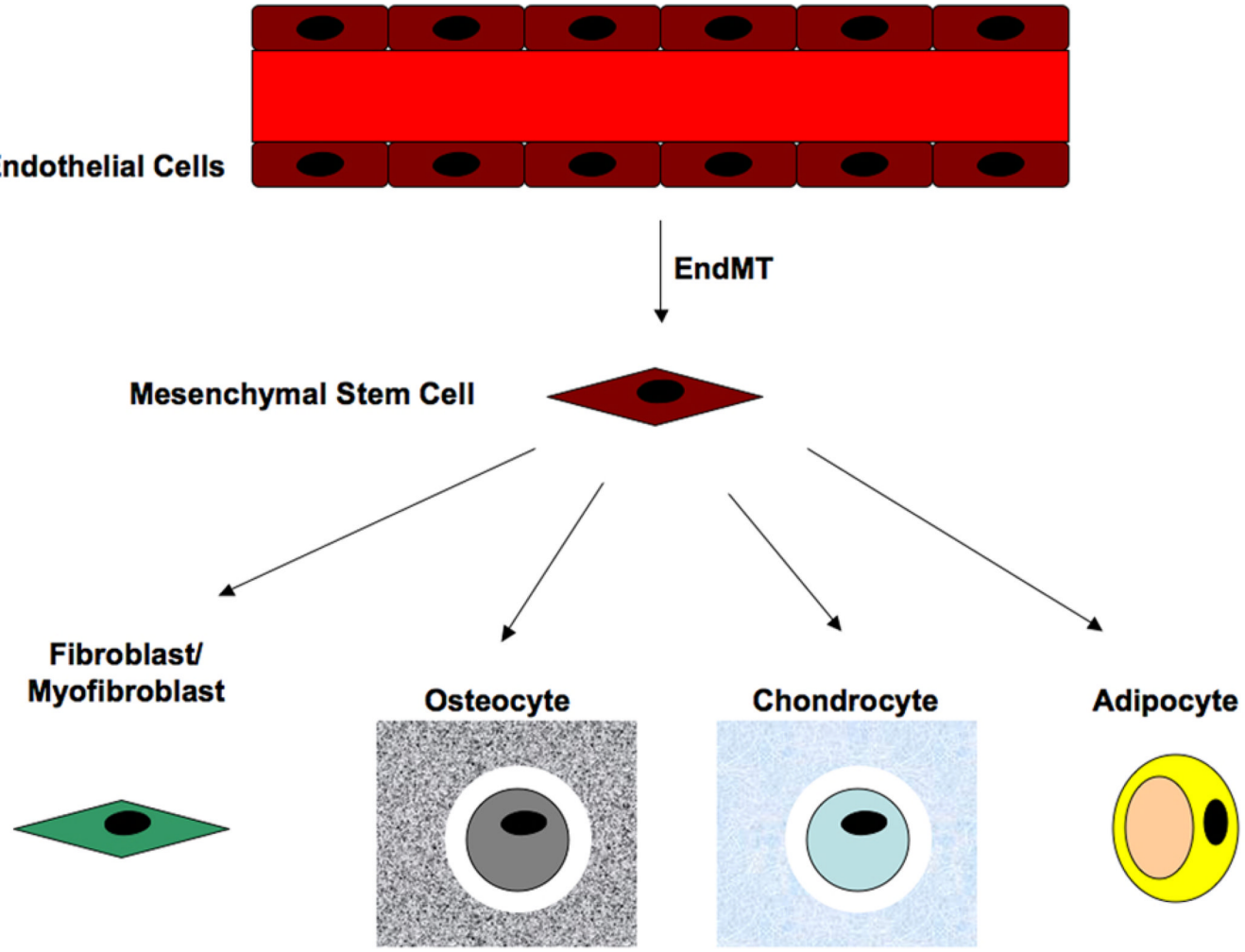

Figure 5.
EndMT generates mesenchymal stem cells. Endothelial cells that convert into mesenchyme acquire properties of stem cells including expression of stem cell markers and multipotent differentiation capabilities. EndMT has been show to induce formation of fibroblasts/ myofibroblasts, osteoblasts/osteocytes, chondrocytes and adipocytes.