TGF-β family signaling in endothelial cells and angiogenesis

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Chapter 1

General introduction

Endothelial cells and angiogenesis

The endothelial cells (ECs) constitute the inner layer of the blood vessels, where they function not only as supportive tubular structures for the distribution of blood, containing nutrients and oxygen, and the removal of waste products in the whole body, but also as a barrier for blood coagulation [1]. ECs and hematopoietic cells originate from mesodermal precursors during embryonic development, as shown in Figure 1 [2]. In brief, splanchnopleuric mesoderm transforms into mesenchymal cells that can differentiate into hemangioblasts. The hemangioblasts can further differentiate into hematopoietic cells or ECs [3]. Through the *de novo* differentiation of ECs and further morphogenesis of the vascular plexus different types of vascular structures can be formed, including arteries, veins, and capillaries [4]. The ECs are involved in and regulate many physiological processes, like immune responses and angiogenesis [5, 6]. Importantly, a large variety of hormones, paracrine and endocrine cytokines, metabolites (and also therapeutic drugs) are circulating in the blood, and may thereby exert effects on ECs. The EC function is therefore critical for tissue homeostasis and its dysfunction may trigger pathologic states.

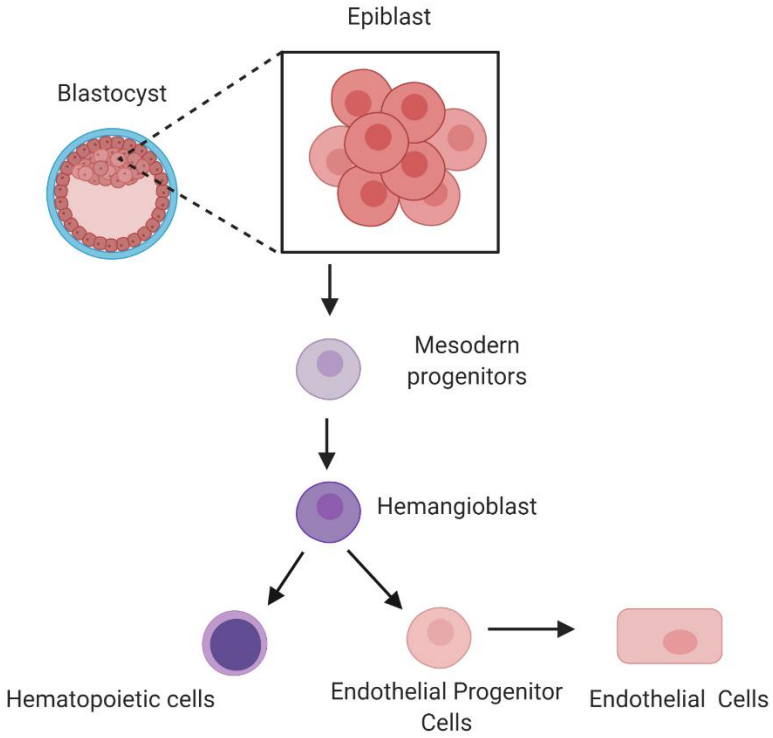

Figure 1. The origin of ECs.

Angiogenesis, the process by which new blood vessels are formed from preexisting vessels, is a phenomenon that is of key importance during embryogenesis [7]. In adults, angiogenesis is mostly quiescent, except in some particular cases, for example, during wound healing and the formation of the corpus luteum and endometrium of the reproductive system in females [8]. In some pathological processes, such as tumor growth and metastasis, cardiovascular diseases and ocular disorders, the process of angiogenesis is disrupted [9-11]. Manipulating angiogenesis, for example, to promote it in conditions in which there is a need of vascularization, and to

inhibit it when there is an excess of irregular vessels, is of key therapeutic interest for vessel related diseases. ECs line the lumen of the blood vessels, and their behavior and response to the microenvironment strongly determines the angiogenic process. Vessel ECs receive multiple angiogenic signals, and signaling that is initiated upon binding of vascular endothelial growth factor (VEGF) to its receptor plays a key role; the endothelial tip cells start to sprout and thereafter guide the talk cells to extend to establish cord and lumen [12]. The formed lumen is the basic structure for further vessel network. Following the proliferation, migration and differentiation of ECs, the vascular loops are formed and the new vessels are established (Figure 2).

The interaction of ECs with pericytes and smooth muscle cells, generally known as mural cells, is also critical for the vessel architecture [13, 14]. On the one hand, mural cells secrete diffusible factors, including fibroblast growth factor (FGF-2) and hepatocyte growth factor (HGF), to activate ECs and facilitate angiogenesis [15]. The latent transforming growth factor (TGF)-β form can be activated after these mural cells contact with ECs, thereby affecting cell differentiation and angiogenesis [16]. On the other hand, the extracellular matrix produced by these mural cells provides a scaffold to maintain the elasticity and stability of the newly formed lumen.

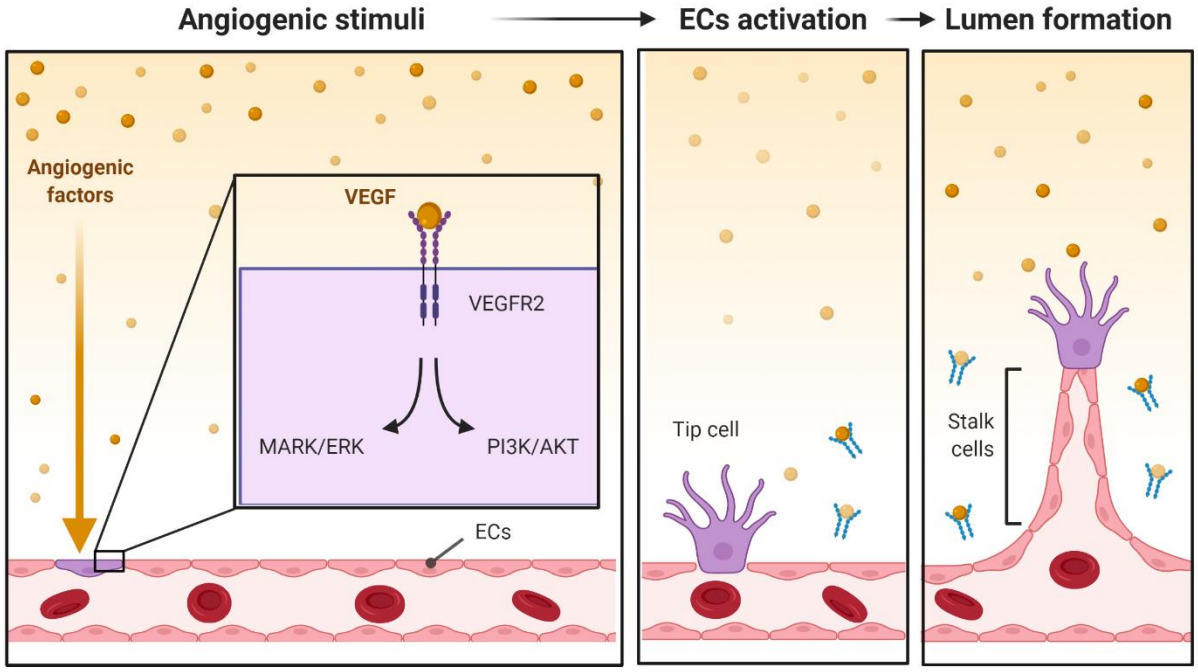

Figure 2. The role of angiogenic factors in angiogenesis. Pro-angiogenic factors, such as VEGF, activate signal transduction in ECs leading to the migration of tip cells. The tip cells sprout and drive the movement of the ECs forefront. Subsequently, the proliferating stalk cells elongate the sprouts and form a new lumen.

TGF-β signaling in ECs

The TGF-β is the prototypic cytokine of a large family of structurally and functionally related proteins that exert a large plethora of functions on many different cell types. Mis-regulation of

TGF-β family signaling has been implicated in multitude of human diseases, including fibrosis, cancer and cardiovascular diseases [17, 18]. TGF-β family proteins include TGF-βs (i.e. TGF-β1, -β2, -β3), activins and BMPs, among others. Whereas TGF-βs interact with TGF-β type I and type II receptors and induce the phosphorylation of receptor regulated (R-)SMAD2/3, BMPs induce heteromeric complexes with BMP type II and type I receptors of which the latter are also termed activin receptor-like kinase (ALK)1/2/3/6 to mediate the phosphorylation of R-SMAD1/5/8. These R-SMADs form heteromeric complexes with SMAD4, which act as transcription factor complexes to regulate target gene expression. By controlling gene expression, TGF-β family members exert important responses in ECs and thereby affect EC proliferation, migration, sprouting and/or differentiation. By doing so, they regulate EC function and angiogenesis (Figure 3 and Figure 4) [19-21]. The different effects of the TGF-β family members on ECs, like also on other cell types, are highly dependent on the cellular context, particular TGF-β isoform or ligand that is used in the assay, concentration of ligand and EC subtype [22, 23]. As a result, different and even opposite effects of TGF-β family members on EC responses *in vitro* have been reported [24].

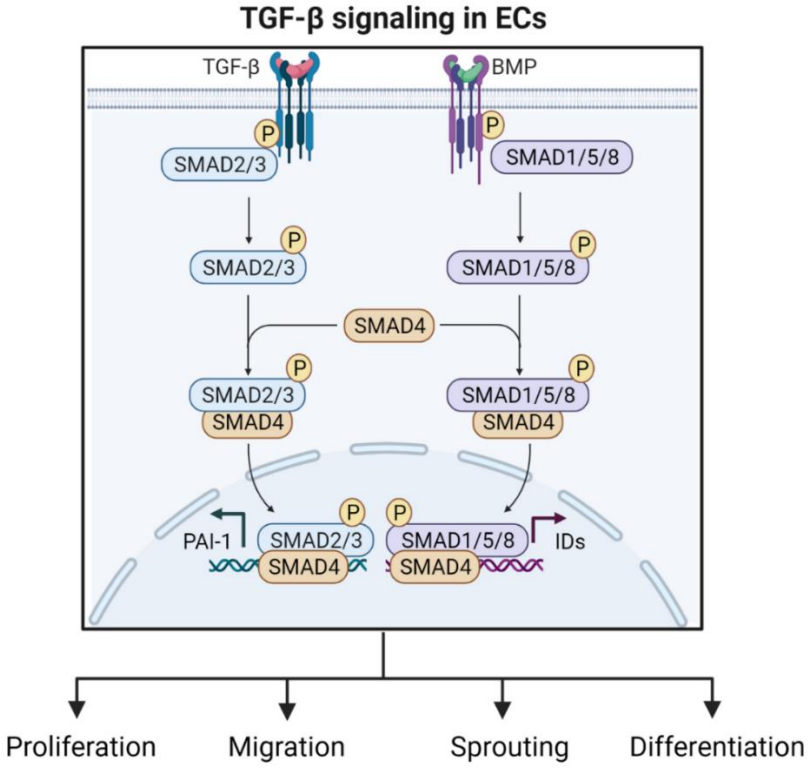

Figure 3. The role of TGF-β signaling in ECs. The TGF-β and BMP pathways are activated upon binding of the extracellular ligands to the cell surface TGF-β/BMP receptors, thereby phosphorylating TGF-β/BMP type I receptors. The activation of TGF-β/BMP type I receptors induces the phosphorylation of SMAD2/3 and SMAD1/5/8. Together with SMAD4, the phosphorylated SMAD2/3 and SMAD1/5/8 are translocated into the nucleus to regulate gene expression, including genes of which the encoded proteins mediate EC proliferation, migration, sprouting and differentiation.

TGF-β can trigger the differentiation of ECs into mesenchymal cells, through a process termed endothelial to mesenchymal transition (EndMT), which is comprehensively introduced in

Chapter 2 (Figure 4**)** [25, 26]. During EndMT, ECs lose their endothelial properties and differentiate into a mesenchymal cell type; this process is characterized by cell morphological variations and changes in endothelial and mesenchymal marker gene/protein expression [27, 28]. Emerging evidence demonstrates that EndMT is critical for cardiovascular system development in early embryonic stages. Recapitulating this process in adult ECs may be of advantage for tissue engineering. EndMT also contributes to the occurrence and progression of several diseases, such as fibrotic diseases and cancer [29]. The effects of TGF-β family proteins in EndMT is not understood in depth and more detailed insights from the underlying mechanisms may provide benefit for the precise control of tissue regeneration and therapeutic targeting of EndMT associated diseases.

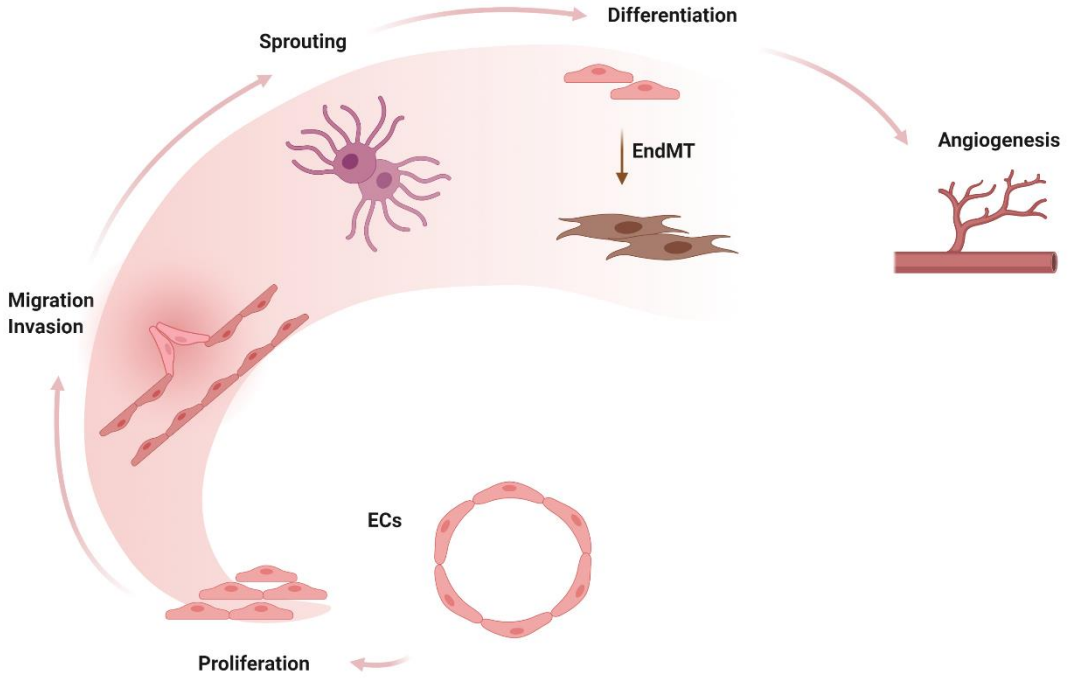

Figure 4. The activation of certain signaling pathways, for example TGF-β signaling, influences EC proliferation, migration, invasion, sprouting, differentiation (especially endothelial to mesenchymal transition (EndMT)) and EC associated angiogenic processes.

TGF-β signaling in angiogenesis

Angiogenesis is a complex multistep process, which is determined by the balance between the levels of angiogenic inducers relative to levels of inhibitors to which the ECs are exposed. The "angiogenic switch" refers to the shift from vascular quiescence to activation of angiogenic programs [30, 31]. These pro-angiogenic and anti-angiogenic factors can be produced by ECs themselves, neighboring cells and also can be systemic factors. If the balance is in favor of angiogenesis stimulators, then the ECs become activated and angiogenesis is induced [32]. The majority of the angiogenic inhibitors are polypeptide factors that inhibit the proliferation and migration of ECs, such as angiostatin and endostatin, while the pro-angiogenesis agents play an opposite function on ECs. Vascular endothelial growth factor (VEGF) is a major inducer of

angiogenesis, and exerts its effects by binding to vascular endothelial growth factor receptor 2 (VEGFR2) to activate downstream signaling and thereby elicit endothelial responses [33, 34]. It has been shown that VEGF enhanced the ECs' ability to divide, migrate and invade into collagen gels to form capillary-like tubules [35]. Besides VEGF, multiple growth factors are involved in the activation of angiogenesis, including FGF2, HGF, angiopoietin 1 (Ang1) [36-40].

The role of TGF-β family ligands in angiogenesis can be either stimulation or inhibition. Evidence demonstrated the indispensable role of TGF-β in formation and remodeling of networks during embryonic angiogenesis. Knockdown of TGF-β1 in mice leads to embryonic lethality (E10.5) due to severe vascular defects and defective hematopoiesis [41]. Emerging reports demonstrate that TGF-β signaling mediated through TβRI (also terms activin receptor-like kinase 5 (ALK5)) has anti-angiogenic properties. However, BMP and TGF-β signaling mediated through ALK1 activate ECs proliferation and migration, which are necessary for angiogenesis [42]. Thus, the vessel formation is dependent on the activation balance of the TGF-β signaling [20, 43, 44]. However, as more studies unveiled the opposite effects of BMP signaling mediated through ALK1/2/3 on ECs sprouting and angiogenesis, further investigation is required to dissect the specific effects of BMP signaling activation in ECs [45-48].

Angiogenesis inhibitors

Anti-angiogenic agents have been used to treat vessel-related diseases, for example, neoplasia and cardiovascular disorders. Endogenous inhibitors of angiogenesis, such as angiostatin and endostatin, are produced by the cleavage of extracellular matrix (ECM) proteins, and antagonize ECs proliferation and migration responses to angiogenesis inducers. Other anti-angiogenesis agents, including small molecules and antibodies, are designed by negative targeting of pro-angiogenic factors or their signaling. The U.S. Food and Drug Administration (FDA) has approved several anti-angiogenic drugs for clinical use and many of them are directed against VEGF signaling and used for cancer therapy [49]. For example, Bevacizumab, a monoclonal antibody that prevents VEGF-A from binding to its receptors, is a FDA approved anti-angiogenesis drug that is used in combination with standard chemotherapy for treatment of metastatic colorectal cancer [50]. Additional angiogenesis inhibitors have been developed in the past years, for example, ALK1 neutralizing antibodies and VEGFR kinase inhibitors. The angiogenesis inhibitors can not only antagonize blood vessel formation to block the supply of nutrients for cancer cells, but also enhance the normalization of immature tumor vessels to prevent cancer cells becoming more aggressive and provide more efficient delivery of chemotherapeutic agents [51-53]. Clinical studies have shown that the combination of chemotherapy/radiation therapy with anti-angiogenic drugs benefits cancer patients [54-57]. More recently, clinical trials evaluating the combination treatment of immune checkpoint inhibitors (ICIs) and anti-angiogenesis agents for cancer patients have shown improved anti-cancer efficiency and prolonged overall survival [58-61]. However, anti-angiogenic drug resistance is easily acquired [62, 63]. Therefore, identifying more inhibitors against other signaling pathways, for example, BMP or other pro-angiogenic pathways, are needed to achieve more efficient targeting of angiogenesis process.

On the contrary, agents that can stimulate angiogenesis are beneficial in the treatment of the diseases/cases that lack of vessels, such as coronary artery disease (CAD), cardiac failure, tissue injury, etc. In conclusion, we anticipate that the precise control of angiogenesis using different agents will contribute to the treatment of vessel related diseases.

Scope of the investigation

In this thesis, I start with a general introduction in **Chapter 1** to briefly present the relevance of EC behaviour in vascular morphogenesis and in angiogenesis. Moreover, I discuss how EC function is intricately regulated by positive and negative factors, and how their function can be manipulated for therapeutic gain in cancer and cardiovascular diseases. In **Chapter 2**, we discuss in detail the role of the TGF-β signaling pathway in EndMT and discuss the contribution of this process to disease development, as well as its potential applications in tissue engineering. In **Chapter 3**, we disclose detailed work protocols to investigate TGF-β-induced EndMT and how to assess the involvement of EndMT effectors using CRISPR/Cas9 gene editing. In **Chapter 4**, we investigated the function of EndMT transcription factors and elucidated their working mechanism. We found that the EndMT transcription factors (TFs) SNAIL and SLUG are critical for EndMT in murine endothelial cells and that the ID proteins counterbalance their function in EndMT. In **Chapter 5**, we provide a technical overview of embryonic zebrafish xenograft assays to investigate TGF-β family signaling in human breast cancer progression, including tumor cell intravasation/extravasation and tumor angiogenesis. In **Chapter 6**, we identify and investigate two novel BMP type I receptor macrocyclic kinase inhibitors with therapeutic potential to normalize angiogenesis in normal and tumour vessel formation in zebrafish. In **Chapter 7**, I summarize all the studies in the thesis and provide some future perspectives related to our results.

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