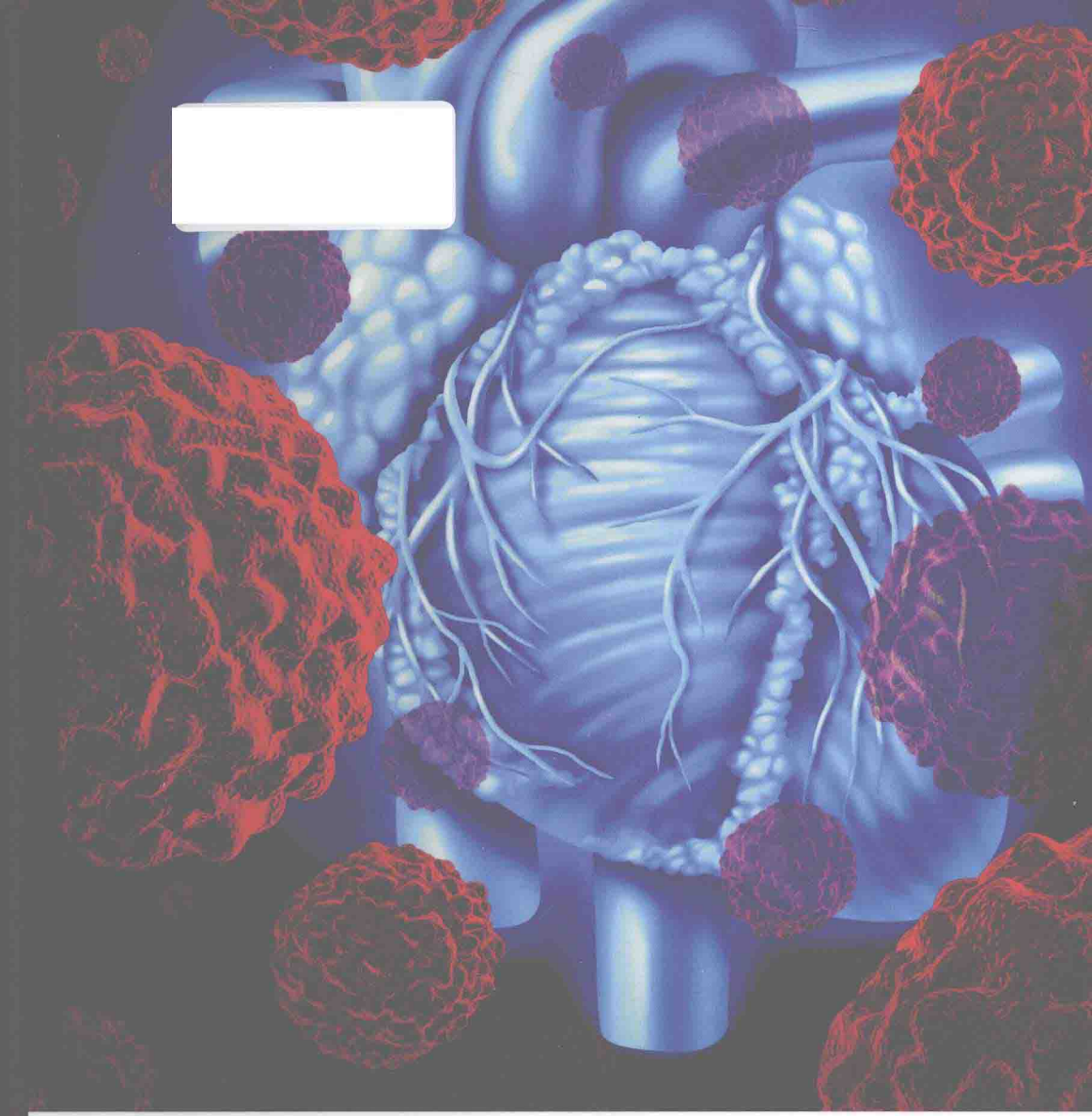

Global Researches in Tumor Angiogenesis

Vince O'Riely

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Edited by **Vince O'Riely**

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Preface

In my initial years as a student, I used to run to the library at every possible instance to grab a book and learn something new. Books were my primary source of knowledge and I would not have come such a long way without all that I learnt from them. Thus, when I was approached to edit this book; I became understandably nostalgic. It was an absolute honor to be considered worthy of guiding the current generation as well as those to come. I put all my knowledge and hard work into making this book most beneficial for its readers.

This book discusses the global researches in the field of tumor angiogenesis. Angiogenesis is an extension process of the cardiovascular system within human body. It is mostly instigated by the need for oxygen and nutrients by the fast growing tissue and uncontrollably dividing cells, as observed during wound healing and tumor progression. This book emphasizes on tumor angiogenesis and presents topics written by highly experienced scholars from different countries. The objective of this book is to provide readers with an insight into the molecular and cellular mechanisms of this biological process and to present new research directions for future therapeutic endeavors.

I wish to thank my publisher for supporting me at every step. I would also like to thank all the authors who have contributed their researches in this book. I hope this book will be a valuable contribution to the progress of the field.

Editor

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Chapter 1

Transcriptional Modulation of Tumour Induced Angiogenesis

Jeroen Overman and Mathias François

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1. Introduction

This chapter provides a summary of the current literature addressing key processes and transcriptional regulators of endothelial cell fate during embryonic blood vascular and lymphatic vascular development, and discusses the implications of these processes/regulators during tumour vascularization. First, we will address normal embryonic development of the vascular systems at the molecular and cellular level. With these fundamental processes recognized, the second part the chapter will focus on how these regulators face dysregulation during tumorigenesis and how they consequently facilitate abnormal vessel growth.

2. Blood vessel development in the embryo

During embryogenesis, the development of the vasculature occurs prior to the onset of blood circulation, and is initiated by *de novo* formation of endothelial cells (EC) from mesoderm derived precursor cells. In a succession of morphogenic events, intricate transcriptional programs orchestrate the further differentiation, proliferation and migration of blood endothelial cells (BECs) to establish the vascular systems (fig. 1). This includes assembly of individual ECs into linear structures and the formation of lumen to facilitate the flow of blood; the designation of arterial, venous, capillary and later lymphatic endothelial cell identity; and the remodelling, coalescence and maturation of the primary vascular plexus to form large heterogeneous interlaced structures, that warrants a contiguous and fully functional blood- and lymphatic vascular system.

2.1. Embryonic blood vessel morphogenesis

2.1.1. Endothelial specification and initial blood vessel formation

De novo generation of the first EC precursors in mammals occurs in the extra-embryonic mesoderm. The mesoderm is a hotbed for cell specification in the embryo, and the pluripotent haemangioblast ancestor of EC precursors (angioblasts) also gives rise to haematopoietic lineages and ostensibly even smooth muscle cells (SMC)[1-5]. In addition, ECs have been shown to share a common precursor with mesenchymal stem/stromal cells (MSC), the so-called mesenchymoangioblast[6], and it has been suggested that other precursors can propagate endothelial cell lineages in the yolk sac. Together these observations signify the differentiation potential of these precursor cells, and impending consequences for plasticity during later remodelling and pathologies[7-9]. During vasculogenesis, defined as *de novo* generation of embryonic blood vessels, these pluripotent mesodermal progenitor cells acquire an endothelial cell (EC) precursor- or blood cell (BC) precursor- phenotype, and subsequently co-localize and aggregate in the mesoderm to form blood islands[10-12], with the EC precursors flattened around the edges and the BC precursors in the centre to generate the haematopoietic lineages[11-13].

2.1.2. Blood vascular lumen formation

To initiate the formation of actual vessel-like structures, the angioblasts assemble into arterial and venous cords, and in doing so form the primitive vascular plexus. These nascent rope-like threads have a solid core and are consequently not yet able to facilitate the flow of blood. This functional feature requires the heart of the cord to be tunnelled out, to give way to a central continuous lumen along the length of the nascent vessel. The transition of EC cords into vascular tubes is a process that necessitates defined EC-polarity, and a delicate interplay between adhesion and contractility. Polarity is essential for the distribution of membrane junction proteins and the definition of apical/luminal (inside) and basal/abluminal (outside) surfaces. This is harmonized by the interplay between adhesion and contractility, through the regulating of physical force propensity that accounts for the EC-flattening against the extracellular matrix[14-16].

Two principal cellular mechanisms have been described to explain for the formation of *de novo* blood vascular lumen: cord hollowing and cell hollowing[13, 16, 17]. Both mechanisms rely on the accumulation of vacuoles, but a fundamental difference between them is revealed in the distinct nature and location of vacuole accumulation, which is usually determined by vessel type and size. Cord hollowing is characterized by the creation of an extracellular luminal space within a cylindrical EC-cord. This involves the loss of apical cell adhesion between the central- but not peripheral- ECs, and results in a lumen diameter that is enclosed by multiple ECs[14-16, 18, 19]. Cell hollowing on the other hand involves the intracellular fusion of vacuoles within a single EC to give rise to a cytoplasmic lumen that spans the length of the cell, and typically results in vessels that have single-EC lining[17, 20]. The aorta in the mouse embryo for example relies on extracellular lumen formation as do most major vessels[15], while intracellular lumen formation is generally the designated mechanism for smaller vessels.

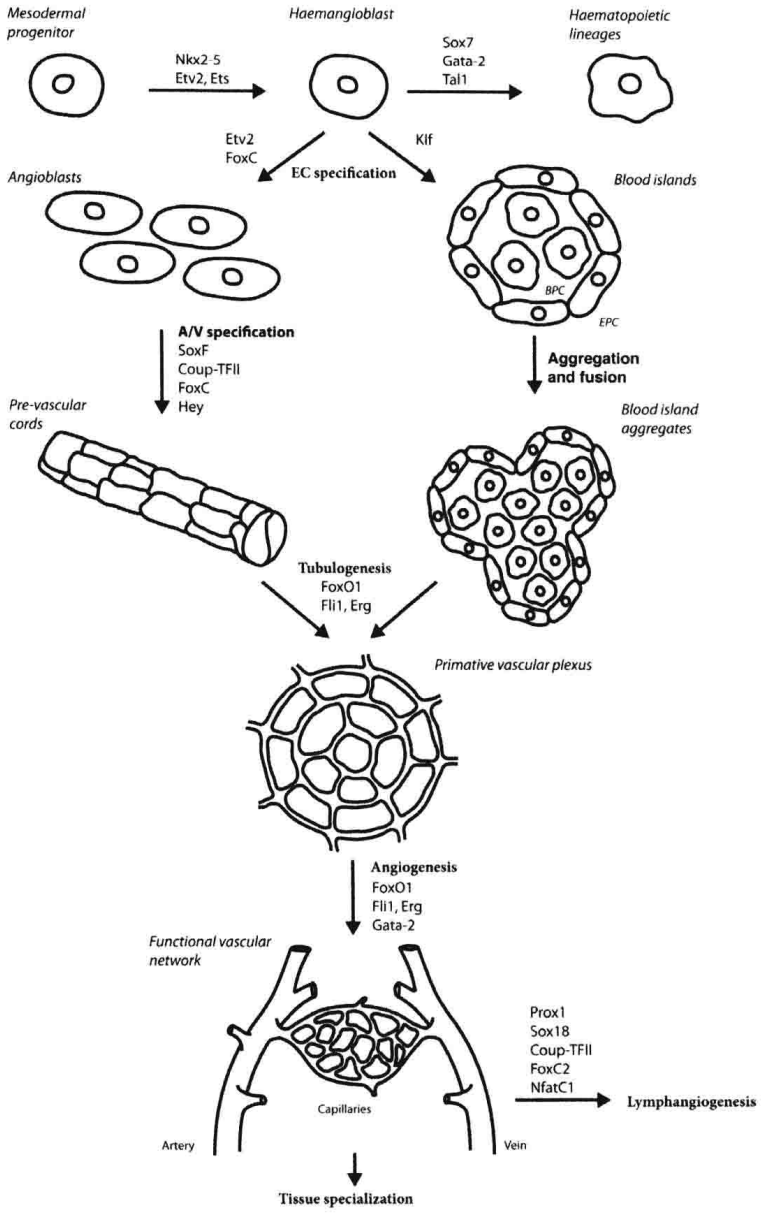

Figure 1. *Embryonic morphogenesis of the blood vasculature.* Mesodermal progenitor cells give rise the vascular endothelium through a series of steps that progressively specify ECs. In the mesoderm, angioblasts (EC-precursors) are formed and aggregate into cords or blood island, which later arrange into the primitive vascular plexus. Angiogenic remodelling of the primary plexus gives rise to a functional vascular network, from where the lymphatic vascular system eventually develops.

2.1.3. Angiogenesis and blood vessel maturation

The institution of a continuous blood vascular lumen is a milestone for the developing vascular system and paramount for further vascular development, as it permits the flow of blood. The nascent blood vessels that constitute this primitive vascular network will subsequently expand, and then functionalize, into an extensive and more intricate systemic vasculature, in two processes respectively known as angiogenesis and vessel maturation. Angiogenesis describes the processes of branching, expansion and remodelling of the primitive vasculature in response to pro-angiogenic signals. This is different from vasculogenesis in that the ECs are not generated by *de novo* differentiation of stem cells, but rather depend on the proliferation and migration of pre-existing vascular ECs. Vessel maturation on the other hand describes the functionalization of nascent blood vessels, and is characterized by mural cell ensheathment of the vessel walls. The continuous mêlée between angiogenesis and vessel maturation – wherein vessel maturation blocks angiogenic growth, and visa versa – ensures optimal systemic blood vascular performance.

Vascular remodelling conventionally occurs through sprouting- and intussusception angiogenesis, and together with vessel maturation gives rise to organ specific vascular beds. Intussusception angiogenesis is a process of vessel invagination wherein vessels ultimate divide and split – which requires appreciably high levels of polarization and localized en masse loss of cell junctions. Sprouting angiogenesis is visibly distinct from intussusception, and unsurprisingly involves the sprouting of a subset of ECs from the vascular wall to protrude into a primed ECM. In this discrete set of ECs, the cell-cell contacts are loosened to promote a motile phenotype. The actual stromal invasion requires enzymatic degradation of the basement membrane and ECM. There is a remarkably strict hierarchy amongst the distinct EC-types in angiogenic sprouts, as a single tip-cell (TC) leads the way, and a host of stalk-cells (SC) follow[21]. Filopodia protrude from the TC that sense the microenvironment for attractive and repulsive signals to guide their migration, and to eventually fuse with adjacent vessels (anastomosis), while SCs contribute principally to the recruitment of pericytes and lumen preservation, while at the same time maintaining the connection between the TC and parent vessel.

Once the newly formed blood vasculature has extended and webbed to an appropriate level, the temporal pro-angiogenic signal will fade and the nascent vessel will be disposed to maturation. Blood vessels maturation primarily requires the recruitment of pericytes and SMCs, to ensheath and stabilize the vessel wall. This mural cell coverage strengthens the cell-cell contacts, decreases vessel permeability, and assures control over vessel diameter and therefore blood flow. Also, pericytes supress EC proliferation and promote EC survival, resulting in a long EC life and a quiescent state, which is typical for mature and functional vessels. Pericytes also subsidize the construction of the vessel basement membrane and deposit various ECM components into the stroma, to generate an angiogenesis incompetent milieu.

The whole process of vessel maturation is strikingly dynamic and intermittently reversible. Mature ECs can, conversely to quiescence, be activated by pro-angiogenic signals, upon which pericytes detach, cell-junctions are loosened, and the ECM is primed for angiogenic growth. In the adult, these processes are recapitulated during pathophysiological conditions

as a means to maintain vessel perfusion and tissue oxygenation in a dynamic milieu. Pro-angiogenic signals can, for example, originate from inflammation and hypoxia as a transient cue, or from a more broadly encompassing and tenacious source such as a neoplasm. The latter type of molecular (dys-) regulation results in abnormal vessel formation, and will be discussed later in this chapter, once the transcriptional basis for EC specification and angiogenesis has been established.

2.2. Transcriptional basis of blood vascular endothelial cell differentiation

The complexity and significance of the numerous morphological events contributing to blood vessel formation, as are highlighted above, underline the necessity for scrupulous regulation to ensure that these processes occur in a spatiotemporally controlled fashion with a high level of precision over EC behaviour (fig. 2). Copious amounts of transcription factors are at the foundation of these coordinating programs, to guide the dynamic gene expression profiles at different stages of embryonic EC fate determination and vascular development (fig. 1), which are later – at least partially – recapitulated during vessel growth in the adult.

2.2.1. Ets transcription factors regulate mesodermal specification of endothelial and haematopoietic lineages

The E-twenty-six (ETS) family is a large group of proteins, with close to thirty members in human and mouse, that achieves transcriptional regulation by binding clusters of ETS binding motifs on gene enhancers and promoters[22]. In itself, this conserved core DNA sequence, 5'-GGA(A/T)-3', offers little binding specificity between Ets members, and is by no means exclusive to endothelial-associated genes. Similarly, Ets expression extends beyond the vascular endothelium. Even so, multiple Ets members are of crucial importance for vascular development by regulating endothelial gene transcription. The way this is accomplished despite these seemingly ubiquitous features, is illustrated by the presence of multiple ETS motifs in large number of enhancers and promoters that regulate specific EC gene transcription. There is also a combination of distinct Ets members being expressed in cells that are programmed to attain or maintain an EC phenotype. It is thus proposed that the combinatorial effort of these transcription factors accounts for the tight control over EC differentiation[23, 24]. Complementary to interaction within the Ets family, recent studies indicate that Ets members also affiliate with other partner proteins to this end, and that multiple Ets members form a transcriptional network with associated partner proteins such as Tal1 and GATA-2 to regulate EC differentiation[25]. Another method by which specificity and function is thought to be regulated is post-translational modification, such as phosphorylation, sumoylation and acetylation[26], while regions flanking the ETS motif on the DNA have also been shown to affect the binding specificity of some Ets members[22].

The exact mechanisms by which the individual or combinatorial Ets expression profiles achieve endothelial gene regulation remain largely unknown, but several Ets members have been identified in recent years to be critical at different stages during EC specification, vasculogenesis and angiogenic remodelling. For example, mouse null-embryos for the ETS translocation variant 2 (Etv2/Er71/Etsrp71) transcription factor do not form blood island due

to lack of EC and HPC specification, and are embryonic lethal with severe blood and vascular defects[27, 28]. Friend leukemia integration 1 (Fli-1), another Ets member, has alternatively been shown to be essential during the establishment of the vascular plexus but not for endothelial specification[29]. Phylogenetically and functionally close to Fli-1 is ETS related gene (Erg)[30]. This particular Ets member acts slightly later during vascular development and is associated predominantly with angiogenesis, by controlling a host of processes such as EC junction dynamics and migration[31, 32].

Etv2 has in recent years arisen as the master transcriptional regulator of endothelial cell fate in mouse and zebrafish, because its function is absolutely critical for endothelial specification, with Etv2-null embryos failing to express vital endothelial markers and being devoid of ECs. Expression patterns have shown that Etv2 mainly functions in the embryonic mesoderm and blood islands at around 7.5 dpc (days post coitum) in mice, and is transiently present in larger vessels until at least 9.5 dpc[28, 33]. Mesodermally expressed Etv2 does not only direct specification towards EC lineages, but is also indispensible for the development of haematopoietic cells. In support of this, the endodermal stem cell precursors common to HPCs and ECs, halt differentiating towards haematopoietic or EC lineages prematurely in Etv2-null mice, in vascular endothelial growth factor (VEGF) receptor-2 (VEGFR2)-positive cells [28]. The vascular endothelial growth factor receptor-2 (VEGFR-2/Flk1), receptor to VEGF-A and considered to be one of the most potent transducers of pro-angiogenic signalling, is thus not regulated by Etv2 in the mouse embryo. By contras, it has previously been reported that the zebrafish orthologue of Etv2, Etsrp, is required for the expression of the zebrafish VEGFR-2 orthologue, kdr[33], and the VEGFR-2 enhancer contains an ETS motif[34].

Other endothelial genes have been shown to be transcriptionally regulated by Etv2, confirming its essential role in early vasculogenesis (refer to table 1). For example, the angiopoietin (Ang) receptor tyrosine kinase with immunoglobin-like and EGF-like domains-1 (Tie2) gene is a direct target of Etv2, and is an important vascular marker that regulates angiogenesis[27]. Endothelial transcription factor GATA-2 is also a likely downstream target of Etv2[23, 28]. Similar to Etv2, GATA-2 is involved in both haemangioblast and endothelial development, and GATA-2 is severely downregulated in Etv2-null embryos[28]. Downstream targets of GATA-2 include VEGFR-2[35] and ANG-2[36], and several other genes that encode endothelial proteins, such as Kruppel-like factor-2 (KLF2), Ets variant- (Etv6) and myocyte enhancer factor-2 (MEF2C), have been identified to be occupied by transcription factor GATA-2[37], hence might be indirectly affected by Etv2 loss of function.

The bulk of transcriptional regulation by Etv2, however, is though to be achieved through recognition of the composite FOX:ETS motif, which is exclusive to endothelial-specific enhancers, and is present in approximately 23% of all endothelial genes[24]. Members of both the forkhead and Ets transcription factor families, in particular the forkhead box protein C2 (FoxC2) and Etv2, synergistically bind this motif to activate endothelial gene expression[24]. In vivo studies in Xenopus and zebrafish embryos have identified this motif within the enhancer of 11 important endothelial genes, being Mef2c, VEGFR-2, Tal1, Tie2, VE-cadherin (Cdh5), ECE1, VEGFR-3 (Flt-4), PDGFRβ, FoxP1, NRP1 and NOTCH4[24]. Not all of these molecular players are individually discussed in this chapter, but it is clear that the FOX:ETS

motif is prevalent in endothelial enhancers and appreciably regulate endothelial gene transcription. In support of this, forced activity of both Etv2 and Foxc2 induces ectopic expression of vascular markers VEGFR-2, Tie2, Tal1, NOTCH4 and VE-cadherin, while conversely, a mutation in the FOX:ETS motif disrupts Etv2/FoxC2 function and ablates endothelial specific LacZ expression in mice[24].

Upstream regulation of Etv2 has been an additional focus of recent studies, to further understand the mechanisms whereby endocardial and endothelial fate is determined and to trace back the transcriptional programs even further. In mice, the homeobox transcription factor Nkx2-5 has been shown to directly bind the Etv2 promoter and transactivate its expression in endothelial progenitor cells within the heart *in vitro* and *in vivo*[27]. In zebrafish, Etsrp was identified to be downstream of Foxc1a/b (FoxC1/C2 homologues found in zebrafish) in angioblast development[38]. These factors were shown to be able to bind the upstream Etsrp enhancer *up1*, and the knockdown of Foxc1a/b results in loss of *up1* enhancer activity to drive transcription[38]. This supports the collaborative role of forkhead transcription factors and Etv2 in endothelial gene expression, and adds a dimension to the transcriptional network.

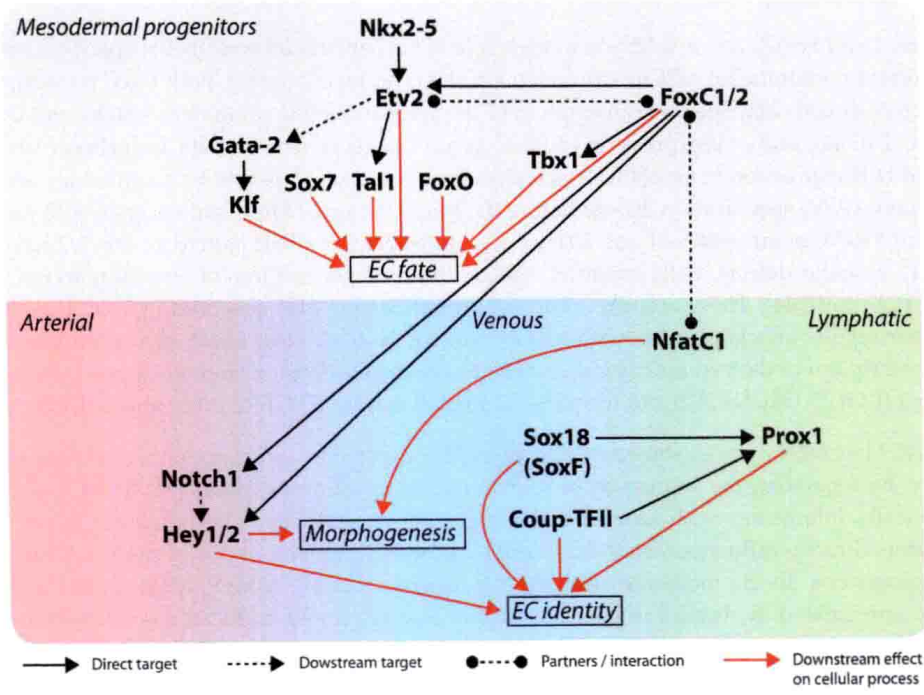

Figure 2. Transcriptional hierarchy orchestrating embryonic vascular development. Endothelial cell specification is an intricate process that relies on extensive crosstalk between transcription factors. Downstream of their transcriptional regulation are signalling molecules that shape the cells and define EC identity and morphogenesis.

2.2.2. Fox transcription factors regulate arteriovenous specification and angiogenesis

It is clear that forkhead transcription factor FoxC2 has an important role during EC specification, through the collaboration with Etv2 at early stages of embryogenesis. Notably, FoxO1 is also able to operate synergistically with Etv2 by binding the FOX:ETS motif[24]. However, not unlike Etv2, FoxO and FoxC transcription factors also direct FOX:ETS independent endothelial gene transcription, which is crucial for vascular development.

Endothelial cells are specified in FoxO1-null mice, and thus differentiate beyond the $VEGFR2^+$ stage of Etv2-null embryos. However, embryonic lethality occurs only slightly later due to a severe angiogenic defect, characterized by disorganized and few vessels by E9.5, with low expression of some crucial vascular markers[39]. Amongst those downregulated is the arterial marker Eprin-B2, a key regulator of VEGFR3 receptor internalization and transducer of VEGF-C/PI3K/Akt signalling, so it is hypothesized that FoxO1 regulates angiogenesis by controlling VEGF responsiveness[39-41]. What further underlines the importance of FoxO1 is the elaborate control over its the transcriptional activity, which is regulated on many levels by posttranscriptional modifications, interaction with co-activators or co-repressors, and absolute FoxO1 protein levels, to regulate localization, DNA-binding activity, and function[42].

FoxC1 and FoxC2 are, in addition to their role in Etv2-mediated endothelial specification, required for endothelial cells to acquire an arterial cell phenotype[43]. Both FoxC transcription factors directly activate the transcription of the arterial cell fate promoters Notch1 and Delta-like 4 (Dll4), and overexpression of FoxC genes results in concomitant induction of Notch and Dll4 expression *in vitro*[43]. Notch signalling has been shown to be essential for arteriovenous (A/V) specification, by mediating the transcription of Hairy/enhancer-of-split related with YRPW motif protein 1 and 2 (Hey1/2). Null-mice for either Notch1 or Hey1/2 have severe vascular defects, with impaired remodelling and general loss of arterial markers such as Ephrin-B2[44]. These arteriovenous malformations are also observed in FoxC1/2 double homozygous knockout mice, with loss of Notch1, Notch4, Dll4, Hey2 and ephrinB2, while transcription of the venous marker chicken ovalbumin upstream promoter transcription factor 2 (COUP-TFII/NR2F2) and the pan-endothelial marker VEGFR2 is not affected[43].

FoxC1 has recently been shown to control ECM composition and basement membrane integrity, by regulating the expression of several matrix metalloproteinases (MMPs)[45], and genetically interacting with laminin α-1(lama1)[46], respectively. The homeostasis of these factors directly influences the vasculature's microenvironment, and is of great relevance to angiogenesis. In the mouse corneal stroma, MMP1a, MMP3, MMP9, MMP12 and MMP12 are upregulated in absence of FoxC1, which is associated with induced angiogenesis by the excessive degradation of the ECM and increased bioavailability of VEGF[45]. The crosstalk between VEGF signalling and forkhead transcription factors is thus a recurring observation, although it is unclear if and how they physically interact. Expression levels of collagens Col1a1, Col3a1, Col4a1 seem unaffected by loss of FoxC1[45], suggesting that FoxC1 does not directly contribute so structural basement membrane or stromal components. However, as mentioned, FoxC1 does interact with lama1 to support basement membrane integrity and