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# **Transcriptional Modulation of Tumour Induced Angiogenesis**

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Additional information is available at the end of the chapter

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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

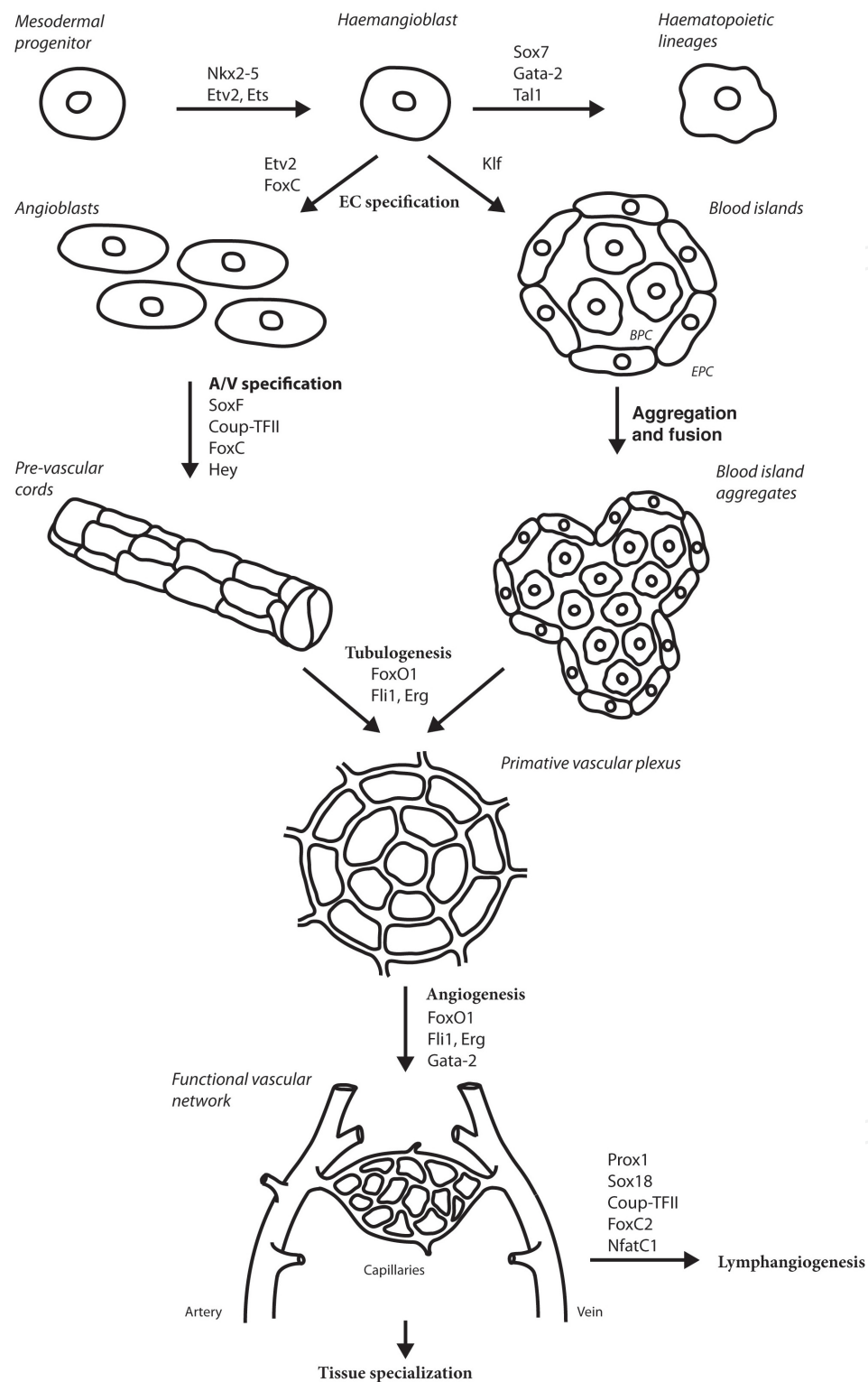
### 2.1.1. Endothelial specification and initial blood vessel formation

*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 ultimately 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 suppress 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].

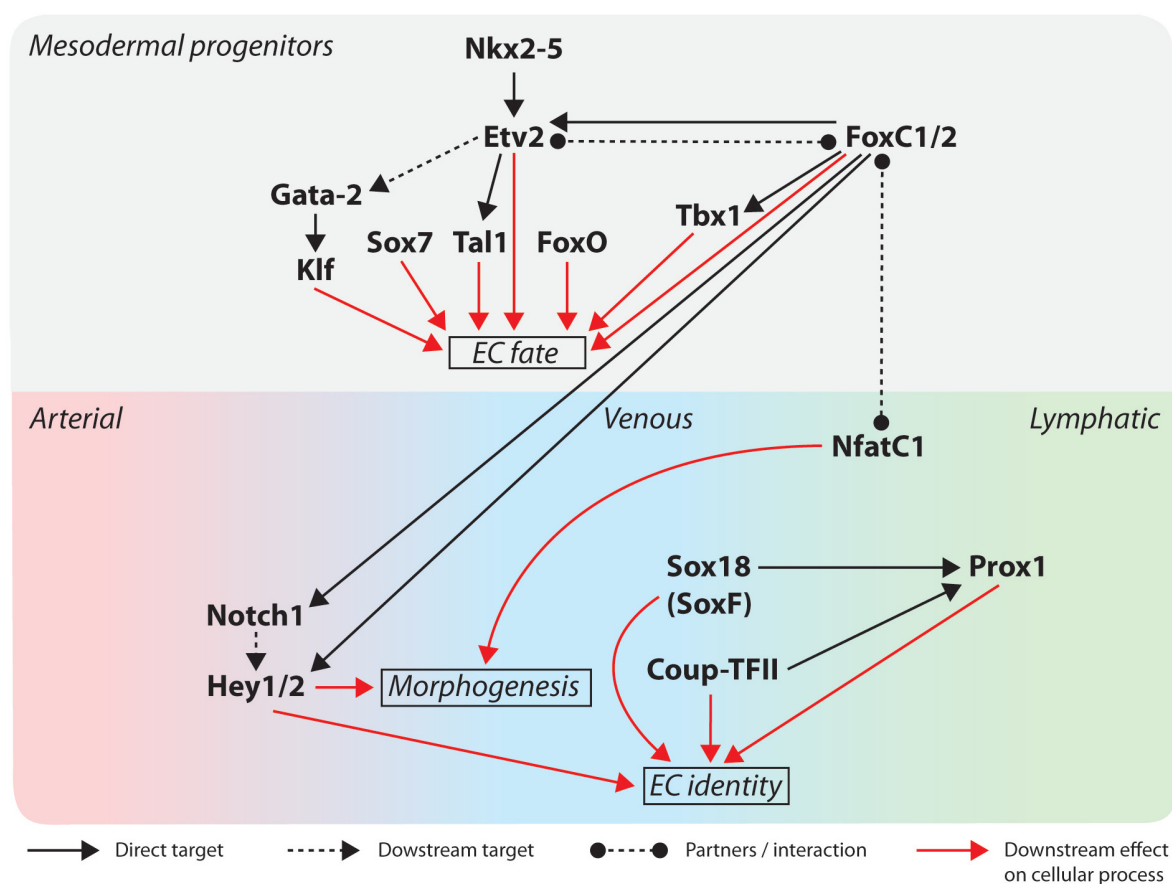
Etv2 has in recent years arisen as the master transcriptional regulator of endothelial cell fate in mouse and zebrafish, because its function is absolutely critical for endothelial specification, with Etv2-null embryos failing to express vital endothelial markers and being devoid of ECs. Expression patterns 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 indispensable 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 contrast, 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 immunoglobulin-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 thought 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), PDGFR $\beta$ , 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<sup>+</sup> 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 Ephrin-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 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  $\alpha$ -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

vascular stability during vascular development in zebrafish, with FoxC1 morphants having severe basement membrane defects similar to that reported for lama1[46].

The divergent roles of FoxC1/2 are not limited to orchestration blood vascular development, and concomitantly also control the development of the lymphatic vascular system. Naturally occurring mutations in the human FoxC2 gene are associated with hereditary lymphedema-distichiasis (LD) syndrome, an autosomal dominant disorder which is characterized by accumulation of interstitial fluid leading to swelling (lymphedema), and aberrant eyelash growth (distichiasis)[47]. Clinical studies have revealed that patient with LD have impaired lymphatic valve function[48], and *in vivo* mouse studies have shown that lymphatic valves do not form properly in FoxC2-nul mutants[49]. Also, the smooth muscle coverage of lymphatic collector vessels is increased in FoxC2 heterozygous mice, which is inherent to LD, owing to an increased expression of platelet derived growth factor  $\beta$  (Pdgf $\beta$ ) *in vivo*[49]. Hence, it has been suggested that FoxC2 regulates lymphatic vessel maturation, and possibly lymphatic sprouting, by interacting with growth factors and transcription factors that regulate lymphatic development. Notably, the lymphatic endothelial cell (LEC) receptor VEGFR3 is thought to be upstream of FoxC2, linking pro-lymphangiogenic VEGF signalling to FoxC2 activity[49], which supports the observation that FoxC2 mutants have increased vSMC-mediated LEC maturation. FoxC2 has since been shown to cooperate with the master regulator of LEC commitment prospero homeobox protein 1 (Prox1) during lymphatic valve formation in controlling the activity of gap junction protein connexin37 (Cx37) and nuclear factor of activated T-cells cytoplasmic-1 (NFATc1)[50]. In this context, NFATc1 activity is controlled by VEGF-C that leads to FoxC2 interaction[51]. Compound FoxC1 heterozygous; FoxC2 homozygous mice further have lymphatic sprouting defects during the earliest stages of lymphangiogenesis[43].

Taken together, this suggests that FoxC signalling has critical roles during lymphangiogenesis and lymphatic maturation in addition to A/V specification and angiogenesis, through co-operation with lymphatic specific transcription factors.

### 2.2.3. Members SOXF transcription factors determine A/V specification and lymphangiogenic switch

The three members of the SOXF group – SOX7, SOX17 and SOX18 – are all endogenously expressed in ECs during vascular development[52], and several key functions of these transcription factors have been described over the years. This includes regulation of A/V specification, angiogenesis, lymphangiogenesis and red blood cell specification, but also other roles perceivably not associated with the blood or lymphatic vasculature, such as hair follicle development and endoderm differentiation.

SOXF transcription factors belong to the SRY-box (SOX) family that is comprised of 20 members. SOX members are all characterized and identified by their highly homologous 79 amino acid high-mobility group (HMG) domain, which was first discovered in their founding member sex-determining region Y (SRY)[53]. This typical SOX element binds the heptameric consensus sequence 5'-(A/T)(A/T)CAA(A/T)G-3'[54], to induce DNA bending and regulate the expression of a broad collection of genes during embryonic development[55]. Specificity

and functional differentiation between SOX-groups and individual members is accomplished by additional operative elements on the SOX transcription factors, and through association with partner proteins[54, 56, 57]. Their coexpression and HMG domain homology, however, does suggest that functional redundancies or cooperative roles apply for members within the same SOX group. However, of the SOXF group only SOX18 is endogenously expressed during lymphatic vascular development in LEC precursors[58].

SOX18 function in vascular development has received considerable attention since the naturally occurring *ragged* mouse mutation, the mural counterpart of the human syndrome hypotrichosis-lymphedema-telangiectasia (HLT) and underlying cause of severe cardiovascular and hair follicle defects, was identified in the *Sox18* gene (*Sox18<sup>Ra</sup>*)[59]. This mutation produces a truncated form of SOX18 that acts in a dominant negative fashion and fails to recruit essential co-factors, and is therefore unable to induce target gene transcription[56, 59]. The defects in the *ragged* mice are much more severe than the observed phenotype of *Sox18*-null mice[59], as truncated SOX18 competes with redundant SOXF members to occupy the same site on the DNA. This supports the notion that redundancies exist amongst SOXF transcription factors, and in fact it has been shown that SOX7 and SOX17 can activate SOX18 targets by binding to SOX18 promoter elements[58].

In the zebrafish embryo, individual knockdown of either SOX7 or SOX18 causes no obvious vascular defects, while the SOX7/18 double knockdown is characterized by partial loss of circulation, ectopic shunts between the main artery and vein, cardiac oedema, blood pooling, and a general loss of A/V specification[60, 61]. Indeed, SOX7 and SOX18 were found to be coexpressed in ECs and their precursors, and their combined loss of function resulted in reduction of arterial markers Ephrin-B2, notch3 and Dll4 and ectopic expression of the venous endothelial marker VEGFR3 in the dorsal aorta (DA)[60, 61].

Several direct SOX18 vascular target genes have been described, notably the genes encoding the tight junction component claudin-5[62] and the vascular adhesion molecule VCAM-1[63], which are both essential for vascular integrity and endothelial activation during angiogenesis. SOX18 also directly activates the expression of MMP7, EphrinB2, interleukin receptor 7 (IL-7R)[64] and Robo4[65] *in vitro*. Robo4 expression *in vivo* is correspondingly under control of *Sox7/18* activity in the mouse caudal vein, and in the intersegmental vessels (ISV) of zebrafish embryos[65]. Archotypically, Robo4 functions in axon guidance, but has more recently been identified as an important coordinator of EC migration during sprouting angiogenesis in zebrafish[66]. *In vitro* assays have further shown that compound SOX17 heterozygous; SOX18-null primary ECs have a sprouting and vascular remodelling defect[67].

SOX18-null mice, although devoid of any obvious blood vascular defects, are characterized by the lack of lymphatic vasculature. This is inherent to the *Ragged* mouse, and describes a nonredundant role for SOX18 in mouse lymphatic endothelial differentiation[68]. At the onset of lymphangiogenesis, SOX18 is coexpressed with COUP-TFII and drives the expression of *Prox1* in a subset of endothelial cells lining the wall of the CV. These LECs form the basis of the lymphatic vasculature, and absolutely require transient SOX18 and COUP-TFII activity to induce *Prox1* transcription[68, 69]. SOX18-null and COUP-TFII-null mice do not express *Prox1* in the embryonic CV, are devoid of LECs, and consequently have a total lack of

lymph sacs and lymphatic vasculature[68, 69]. However, after the initial LEC specification, Prox1 expression becomes independent of SOX18, and later COUP-TFII, but itself remains critical for lymphatic remodelling and maintenance of LEC identity[68, 69].

### 3. Blood vessel development in solid tumours

Tumour cells are characterized by chronic proliferation and immortality, due to mutations in genes that regulate cell cycle, homeostasis and cell death[70]. As a solid tumour grows, it is evident that the need for oxygen and nutrients increases correspondingly, and waste materials need to be carried off in escalating amounts, which rationalizes the commonly observed tumour-induced neo-vascularisation. To accomplish this remarkable feat, tumour cells exploit many of the vascular signalling pathways that are activated during embryogenesis, but without tight spatiotemporal control (fig. 3). Vascular architecture and integrity is therefore often compromised, promoting malign features of progressive tumours, such as metastatic behaviour.

#### 3.1. Characteristics of the tumour vasculature

Due to the high oxygen demand and great metabolic activity of tumour cells, the peritumoral region usually becomes hypervascularised. However, this does not truly solve the problem for tumour cells, as in their gluttony they induce constitutive pro-angiogenic signalling that fails to generate a functional vascular network (fig. 3ab). The balance between pro-angiogenic signalling and the subsequent maturation of the newly formed nascent vessels is key for proper circulation and perfusion. Typically, vessel maturation is inadequate in tumour tissue, owing to persistent presence of pro-angiogenic factors. The overabundance of pro-angiogenic signalling originates in part from the tumour directly, but is also a result of the chronic hypoxic and acidic state of the tumour microenvironment. In addition, tumours often trigger and maintain a chronic inflammatory response, wherein cells of the innate and adaptive immune system – mostly macrophages, neutrophils, mast cells and lymphocytes – infiltrate the tumour stroma and crosstalk with ECs to activate quiescent ECs and sustain pro-angiogenic signalling. Although an immune response can in fact reject certain tumours, malignant tumours and their microenvironment can generally evade immune cell mediated destruction, and instead recruit them to their angiogenic campaign[70, 71].

However, tumour angiogenesis proceeds in an unorganized tempest of random sprouting because the guiding signals in the stroma are disorganized, and sprouting cells are unable to filter out any consistent cues. Abnormal shunts, including arteriovenous anastomoses, are commonly observed due to abrogated intervascular communication leading to bi-directional blood flow and impaired perfusion[72]. Tumours are highly diverse due to their tissue of origin and the heterogeneity of the mutations underlying their tumorigenic state. The type and degree of tumour vessel abnormality is correspondingly context dependent, but there are some general traits that tumour vessels share. These regard to overall vascular organiza-



tion and hierarchy as a network, immediate manifestation of maturation deficiencies, and morphology of vascular ECs.

While the dysregulation of angiogenesis causes overall hypervascularization, vessels are distributed unevenly throughout the peritumoral region, with very low vascular density in some areas. Moreover, large tumours instigate high tissue pressure that can compress and constrict vessels, and vessel diameter thus becomes independent of blood flow rate[73]. Normally, high interstitial pressure is an important queue for lymphatic vessel to drain off the excess fluid, but this function is perturbed in tumour tissue and extravasated fluid is not the sole cause of pressure rise[74, 75]. Where larger blood vessels in normal tissue branch into gradually decreasing size vessels and eventually thin-walled capillaries, this obvious hierarchy is often lost in tumour vasculature, and heterogeneous vessel subtypes are randomly distributed throughout the tumour vascular bed[76, 77]. This affects, but not truly reflects, their functional status.

Where normal vascular endothelial cells line up in the vessel wall to create a continuous barrier to maintain tissue fluid homeostasis and allow the selective diffusion and transport of certain molecules, the tumour vasculature is characterized by loss of EC polarity and cell-cell adhesion that results in an incontinuous and leaky vessel wall. This is aggravated by the loosening of EC-associated mural cells, who fail attach tightly to ECs in the presence of constitutive pro-angiogenic signalling, which in turn leads to reduced vessel stability and incoherent deposition of basement membrane- and ECM components[78, 79]. These resultant vessels cannot maintain a trans-vascular pressure gradient, because excessive amounts of fluid leak into the interstitial space through the porous vessels. Furthermore, tumour cells can gain entrance to the vascular system, for either transport throughout the circulation, or incorporation into the vessel wall.

The entry of tumour cells into the vasculature is a primary facilitator of distant metastasis formation, and is importantly applicable for both blood vessel and lymphatic vessels (fig 3b). It is of note that the lymphatic system is specifically designed to not only transport immune cells, but also to absorb, and drain off, fluid and larger molecules. Therefore, lymphatic capillaries are inadvertently effective in the uptake of tumour cells, and regional lymph node metastasis is a common indication of malignant tumour progression that is used a prognostic tool in human cancer patients[80, 81].

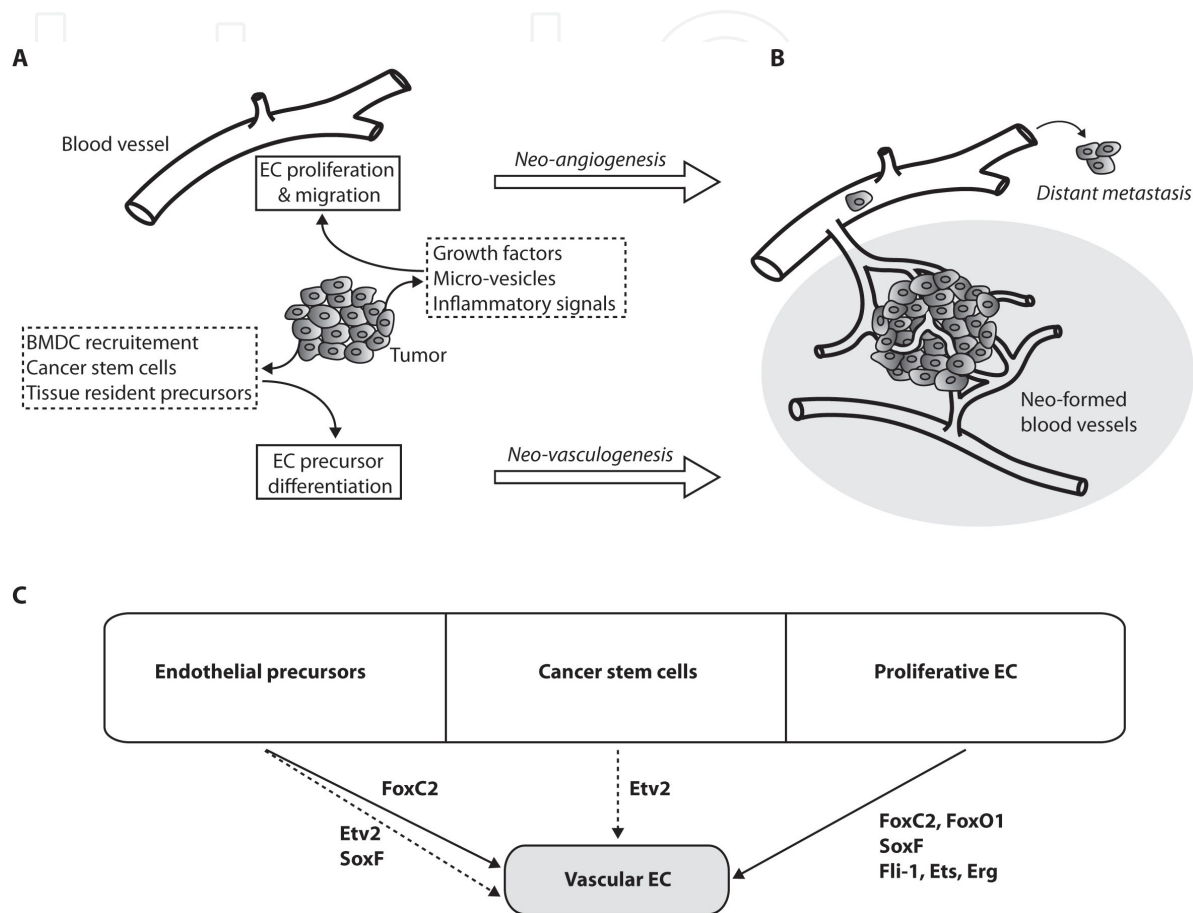
Overall, tumour cells seem to be able to initiate a chronic state of angiogenesis and lymphangiogenesis, but in doing so fail to create normal functional vascular networks. The signalling programmes that underlie these tumour-induced malformations may often have their foundation at a transcription level, with balance in transcriptional networks tipped towards proliferation of both tumour- and vascular EC proliferation and migration.

### **3.2. Cellular origin of the tumour derived endothelium**

The vascular expansion that rapid growing tumours induce requires great numbers of vascular EC to form these structures. Tumours engage in three distinct strategies to wheel in these recruits and promote angiogenesis. The most obvious pro-angiogenic signalling path-



way is that which leads to proliferation of a pre-existing vasculature, as it occurs in embryonic remodelling and normal vascularization in the adult. However, tumours also promote the mobilization and specification of bone marrow derived cells (BMDCs). In addition, tumour cells themselves can transdifferentiate into ECs to be incorporated into the tumour vasculature (fig. 3)[82].



**Figure 3.** Tumour vascularization strategies originating from TF-dysregulation. **(A)** As it grows, a tumour adapts several techniques to induce vascularization, either through proliferation of preexisting peritumoral vessels or by promoting differentiation of non-EC into vascular endothelium. **(B)** The peritumoral and intratumoral regions get hypervascularized by the pro-angiogenic and pro-vasculogenic signals that the tumour instigates, which facilitates vessel intravasation metastasis through the vasculature. **(C)** Transcriptional dysregulation underlies the angiogenic and vasculogenic signalling that tumour emanate.

Proliferation of the existing vasculature proceeds for a large part through VEGF signalling. The VEGF signalling axis controls angiogenic- and lymphangiogenic sprouting through regulation of cell proliferation and migration, with a set of several VEGF ligands and VEGFR receptors. VEGF-A is particularly angiogenic, while VEGF-C and VEGF-D are primarily lymphangiogenic. The downstream effect however is much dependant on the VEGFR they bind, with several possible combinations and dynamic receptor homodimerization, heterodimerization or co-receptor (NRPs) interaction adding to the complexity. In general, VEGF-A binds to VEGFR1 or VEGFR2 with the former interaction being anti-angiogenic to due high affinity but low downstream tyrosine kinase activity, and the latter being pro-angio-

genic. VEGF-C and VEGF-D on the other hand primarily bind the lymphangiogenic VEGFR3 receptor or VEGFR2-3 heterodimers to promote lymphangiogenesis. Hence, VEGFs, their receptors, and regulatory proteins upstream of VEGF – or signalling molecules that crosstalk with VEGF – are beguiling (lymph-)angiogenic players[83, 84].

Recently, light has been shed on tumour signalling to neighbouring endothelium, which convolutes this classical growth factor signalling. Microvesicles released from tumour cells can transport genetic material and signalling molecules directly into endothelial (progenitor) cells that can make epigenetic modification to regulatory genes and otherwise alter expression patterns[85-88]. These microvesicles can also originate from non-tumour cells, such as EPC, to activate angiogenic programmes in vascular ECs[89, 90]. This demonstrates that cells residing in the tumour stroma are altered at a more fundamental level to contribute to tumour vascularization.

Although angiogenesis is the prevailing concept that accounts for tumour vascularization, it is becoming ever more prevalent that vasculogenesis has a significant contribution to vessel formation in tumours. EPCs, and other BMDCs such as tumour associated macrophages (TAMs), mesenchymal progenitor cells (MPC), monocytes, are thought to participate in tumour vascularization in varying degrees, and are common components of the tumour stroma [91-95]. These cells can actively be recruited to the site of neovascularization [96], and reside there to promote angiogenesis or differentiate into vascular EC themselves. This process is further propagated by chronic inflammation of the tumour microenvironment[97]. Furthermore, tissue resident stem cells may contribute to angiogenesis as was shown to be the case in renal carcinoma's[98].

Adding to the mechanism of vasculogenesis and the role of stem cells, is an active role for tumour cells themselves. A heterogeneous malignant tumour is often characterized by subpopulations of cancer stem cells (CSCs) that have great self-renewal and differentiation capacity, similar to normal stem cells[99, 100]. These CMCs have the ability to acquire an endothelial progenitor phenotype, and function as vascular ECs, which benefits tumour vascularization and proliferation[101, 102]. This practise is generally dependent on conditions such as hypoxia, where tumour cells find themselves in acute need of supply and transdifferentiate in vascular progenitors[103-105]. Vascular mimicry is a remarkable demonstration of this CSC-trait. Tumour cells in this process align into channel-like structures, gain EC gene expression, acquire an EC phenotype, and roughly function as blood vessel (fig. 3B). Suggested mechanisms by which tumour cells can differentiate into vascular progenitor include signalling through VEGF and IKK $\beta$  [102, 106].

### 3.3. Dysregulation of transcriptional angiogenic pathways

#### 3.3.1. *Ets* transcription factors

Many *Ets* transcription factors have a suggested or confirmed role in tumour angiogenesis and progression. Probably the most obvious *Ets* members to be involved in tumorigenesis are Fli1 and ERG, which have been acknowledged for their role in embryonic angiogenesis and vasculogenesis in a previous section of this chapter, but also ETS1/2 and several mem-

bers of the ternary complex factor (TCF) subfamily. These transcription factors have been shown to be overexpressed in tumour cells of divergent cancer types, and to facilitate tumour progression, vascularization and invasion by regulation of growth factor responsiveness and MMP expression [107-112] (fig. 3C).

With the recently discovery of tumour associated vascular ECs, however, it is imminent that key players of cell fate determination contribute to tumour induced neo-vascularization. The master regulator of endothelial and haematopoietic cell specification, Etv2, is only transiently expressed during embryonic development, as further angiogenesis generally occurs through proliferation of pre-existing vasculature. As Etv2 activity is absolutely critical for the specification of ECs, it is conceivable that transdifferentiation of tumour cells and specification and/or mobilization of bone marrow derived progenitors, requires Etv2 activity in tumour angiogenesis[91] (fig. 3c).

Although little is known about the actual expression levels of Etv2 in tumour cells or their microenvironment, several direct target genes or other downstream Etv2 targets are upregulated in tumour tissue. The Ang-2/Tie-2 system, for example, is often strongly activated in endothelial cells of tumour associated remodelling vessels, leading to increased angiogenesis and proliferation[93, 113-115]. MMPs are known to facilitate a broad range of vascular events by ECM remodelling and paving the tumour stroma to promote angiogenesis, and MMP overexpression is instrumental to progression of distinct cancer types[116, 117]. Etv2 can also directly activate the MMP-1 promoter, and MMP-1 is often overexpressed in cancer as are many others[118-121].

Other Etv2 targets, many of which carry the FOX:ETS motif in their promoter, are ubiquitously dysregulated during tumour angiogenesis[122-126]. It is not clear whether this is Etv2-dependent, but it has been shown that Etv2 activity can induce ectopic expression of these genes in embryonic development, and it is conceivable that Etv2 function is recapitulated and exploited in tumour vasculogenesis and angiogenesis. This could explain the transdifferentiation capacity of tumour cells that contribute to the vascular progenitor population, and the recruitment of BMDCs as Etv2 activity specifies EC and haematopoietic lineages from stem cells in the mesoderm. In addition, putative Etv2 targets during tumour angiogenesis have extensive crosstalk with growth factor signalling, which further endorses the suggested role and significance of Etv2 in this process[127].

### 3.3.2. Forkhead transcription factors

The presence and role of FoxC2 in tumour angiogenesis has been fairly well characterized over the past few years, and it has been shown that the expression of FoxC2 in tumour endothelium coincides with neovascularization. This further supports the notion of Etv2 recurrence during tumour vascularization because of the synergistic function between these transcription factors in regulating endothelial genes expression through the FOX:ETS motif.

FoxC2 overexpression is associated with aggressive human cancers, and has been shown to be overexpressed in mammary breast cancer cells *in vitro* where it directly promotes a meta-

stasis phenotype[128]. More recently, FoxC2 was detected in the tumour ECs of human and mouse melanomas, and it therefore hypothesized that FoxC2 directly contributes to tumour angiogenesis[129]. In a B16 melanoma mouse model, the high expression level of FoxC2 in tumour cells and endothelium correlates with the induced expression of a set of angiogenic factors, such as Notch ligand Dll4, MMP-2, Pdgfr $\beta$  and VEGF. Deleting one copy of FoxC2 causes reduction of their expression levels, and these FoxC2 heterozygous mutants also display reduced angiogenesis and correspondingly perturbed tumour growth with signs of tumour necrosis [129]. This is in line with the roles of the suggested targets of FoxC2 in tumour neovascularization[127, 130], and the pro-migratory and angiogenic phenotype of FoxC2 overexpressing ECs[129, 131] (fig 3c).

Tumour-induced endothelial to mesenchymal transition can promote FoxC2 expression, which feeds back into further mesenchymal differentiation[128, 132]. This can for a part explain the pro-tumorigenic character of FoxC2, as it increases the ability of tumour ECs to migrate and proliferate, and prevents entry of tumour ECs into a quiescent state. Interestingly, FoxC2 heterozygous mutant mice indeed show a reduced amount of tumour-associated fibroblasts, corroborating this hypothesis[129]. FoxC2 may further contribute to tumour angiogenesis by recruiting mesenchymal stem cells[133], or endothelial progenitor cells [134], although this has yet to be determined.

Interestingly, FoxC1 is also upregulated in some tumour but its role in in tumour angiogenesis is unclear, as deletion of one copy of FoxC1 in mice does not seem to affect melanoma tumour growth or angiogenesis[129]. Also, neither FoxC1 nor FoxC2 explicitly affect tumour lymphangiogenesis as lymphatic marker Lyve-1 and Prox1 expression levels are independent of FoxC1/2 activity in melanoma tumours[129].

FoxO transcription factors operate, in contrast to FoxC1, as tumour suppressors[135-137]. Their function in mediating PI3K-AKT and HIF signalling make them key regulators of cell cycle and apoptosis, and therefore, inactivation of FoxO's is frequently observed in cancer[136, 138-141]. Mouse studies have revealed that FoxOs display functional redundancy in tumour suppression and vascular homeostasis, and triple FoxO knockout (FoxO1, FoxO3, FoxO4) mice develop aggressive tumours with a poor survival rate, and have widely altered expression levels of EC-survival and vascular genes[137]. FoxO1 is required for embryonic vascular development, and its inactivation in cancer has repercussions on tumour vascularization, which is confirmed by vascular remodelling defects in FoxO1-null mice and their established crosstalk with VEGF-signalling[39]. This instigates a paradox wherein tumour cells gain 'immortality' through FoxO inactivation, and simultaneously seem to lose vessel functionalization via the same mechanism[39, 141-143].

On a particular note, FoxO3 depletion in tumour cells can attenuate migration due to reduction in MMP expression, leading to decreased tumour size[144]. Henceforth, the compound FoxO alterations in tumours, and modifications to specific Fox members, must be further explored to fully appreciate the contexts dependent roles of these transcription factors.



### 3.3.3. SoxF transcription factors

SOXF is expressed transiently in the developing endothelium and then again during pathological conditions, such as wound healing where SOX18 is reexpressed in the capillary endothelium[145], and in tumorigenesis where SOX18 is reexpressed in the tumour stroma[146], including the blood and lymphatic vasculature[52, 147]. Recently, SOXF transcription factors have emerged as novel prognostic markers during gastric cancer progression, as SOX7, SOX17 and particularly SOX18 are frequently overexpressed in gastric tumour tissue of human cancer patients, and survival rates are considerably lower for patients with SOX18 positive tumours [146].

The role of Sox18 in tumour angiogenesis has been studied in SOX18-null, and SOXF loss of function (SOX18 dominant negative mutant-) mice. These studies revealed that melanoma tumours grow more slowly in absence of SOX18 protein or function *in vivo*, with a corresponding reduction in tumour associated microvessel density[52] (fig. 3c). This was further illustrated *in vitro*, where ECs and human breast cancer cells with the dominant negative form of SOX18 proliferate poorly, and tube formation of ECs is impaired, which could be improved by overexpressing functional SOX18[52].

SOX18 has also been shown to directly facilitate the metastatic spread of tumour cells to the sentinel lymph node in mice[147]. This is likely to be achieved by promoting neolymphangiogenesis in the tumour microenvironment and thereby paving the way for tumour cell migration towards the draining lymph node. During tumour growth, SOX18 has been shown to be reexpressed in LECs and is suggested to promote lymphatic vascular expansion[147]. Indeed, SOX18 heterozygous mutant mice have reduced lymphatic vessel density, which is accompanied by a decrease in lymphatic drainage and sentinel lymph node metastasis[147].

Taken together, these observations allocate an important role to SOX18 and possibly other SOXF transcription factors in regulation tumour vascularization. A recent finding describes that SOX18 expression in tumour tissue is regulated on an epigenetic level by multiple states of promoter-methylation, which underlines the intricacy and divergency of transcriptional programmes in tumours[148]. With a role for SOXF members in arteriovenous specification, angiogenesis and lymphangiogenesis, their dysregulation in tumour settings might be a parameter influencing the heterogeneity and overabundance of tumour vasculature.

## 4. Concluding remarks

The blood and lymphatic vascular systems are crucial in higher vertebrates for the transport of fluids, oxygen, signalling molecules, immune cells, waste material and other components that maintain homeostasis in the body. These systems develop very early on during embryonic development and are orchestrated by a finely tuned combination of transcriptional regulators that can flick cell fate switches.

The transcriptional networks that underlie EC specification are usually transient or at least very well ordered in the embryo, but this all changes in tumour settings where they are dis-



torted and exploited to induce chronic angiogenesis and vasculogenesis. Although most attention in therapeutic cancer research over the years has gone to growth factor signalling or other downstream players of proliferation, migration and morphogenesis there seems to be an emerging paradigm shift in studying both prognostic and therapeutic potential of fundamental transcription factors. The ETS, Forkhead, and SOXF transcription factors discussed in this overview are in many ways associated with tumour proliferation and vascularization. Studies in developmental biology have laid the groundwork for further study of transcription factors dysregulation in tumours. Remarkably, there is a high level of crosstalk with traditional VEGF signalling either through increased VEGF bio-availability, transduction, or responsiveness within these transcriptional networks.

In the years to come, these transcription factors will expectantly further develop as prognostic tools for tumorigenesis and possibly arise as molecular targets for treatment of malignant tumours. At the very least, studying these fundamental regulators in cancer will add to our understanding of tumour origins and the tools they utilize to achieve proliferation, angiogenesis, and malignancy.

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