

# Endothelial Cell Differentiation and Hemogenic Specification

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Formation of the vasculature is a critical step within the developing embryo and its disruption causes early embryonic lethality. This complex process is driven by a cascade of signaling events that controls differentiation of mesodermal progenitors into primordial endothelial cells and their further specification into distinct subtypes (arterial, venous, hemogenic) that are needed to generate a blood circulatory network. Hemogenic endothelial cells give rise to hematopoietic stem and progenitor cells that generate all blood cells in the body during embryogenesis and postnatally. We focus our discussion on the regulation of endothelial cell differentiation, and subsequent hemogenic specification, and highlight many of the signaling pathways involved in these processes, which are conserved across vertebrates. Gaining a better understanding of the regulation of these processes will yield insights needed to optimize the treatment of vascular and hematopoietic disease and generate human stem cell-derived vascular and hematopoietic cells for tissue engineering and regenerative medicine.

Endothelial cells (ECs) form the thin luminal layer that lines blood vessels and enable efficient delivery of immune cells, oxygen, and nutrients to all tissues in the body. Formation of the vasculature occurs early in development and is a crucial step for the survival of the embryo. Primordial ECs are generated from mesodermal progenitors and form primitive capillary plexi that are then remodeled into a circulatory network of arteries, veins, and capillaries that are structurally distinct and perform different functions (Marcelo et al. 2013a). A small set of pri-

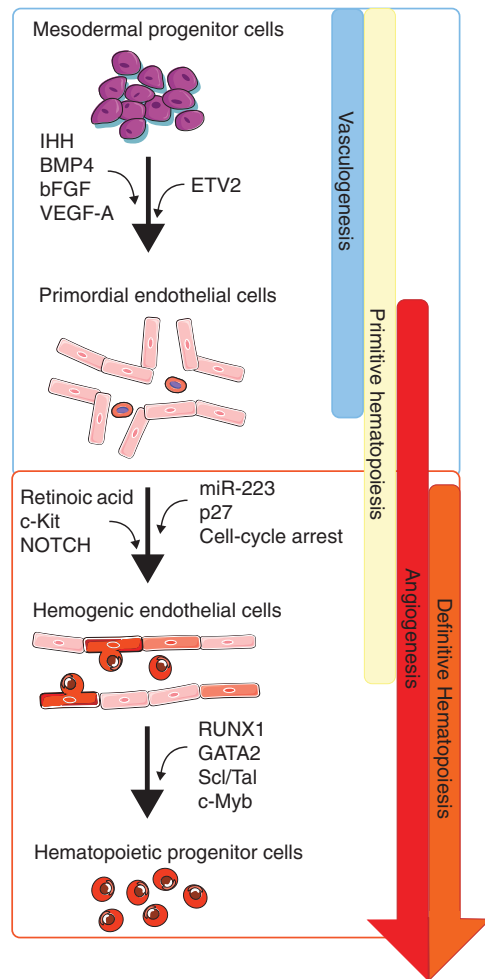
mordial ECs in the mammalian extraembryonic yolk sac and placenta are also specified to become hemogenic endothelial cells (HECs), as are a subset of arterial ECs in the aorta-gonad-mesonephros (AGM) region of the embryo (Marcelo et al. 2013b). Specification of these blood-producing HECs is also essential for embryonic survival, as they give rise to hematopoietic stem and progenitor cells (HSPCs) that serve as the foundation of the embryonic and postnatal hematopoietic system (Marcelo et al. 2013a).

Blood generation and blood vessel formation and remodeling are regulated by similar pathways in embryos and adults (Wu and Hirschi 2021); thus, gaining further insights into the molecular regulation of embryonic vascular and hematopoietic development can be applied to the generation of new therapies for vascular and hematopoietic diseases. In addition, further information would improve the currently complicated and inefficient methods used to generate purified EC populations, including HECs that produce HSPCs, from human stem cells in vitro. Herein, we review the current knowledge of the regulation of EC differentiation from mesodermal progenitors and their specification into blood-forming HECs (overview in Fig. 1).

## ENDOTHELIAL CELL DIFFERENTIATION

At mouse embryonic day 6 (E6), gastrulation occurs and gives rise to three different germ layers: mesoderm, ectoderm, and endoderm (Tam and Meier 1982). The mesoderm is the main source of multipotent progenitors that will form the cardiovascular and hematopoietic systems during embryonic development. One factor important for the specification of the mesoderm is the T-box transcription factor (T) encoded by the *Brachyury* (*Bra*) gene (Papaioannou 2014). In vitro, T interacts with SMAD1 in the presence of bone morphogenetic protein 4 (BMP4) to promote the formation of mesodermal cells. In the absence of BMP4, T interacts with SMAD2/3 and influences the expression of endodermal genes (Faial et al. 2015). T binds to a variety of genes that are necessary for the differentiation of ECs from mesodermal progenitors (Faial et al. 2015; Beisaw et al. 2018), and embryos deficient for *Bra* and T exhibit fatal defects in vascular development (Inman 2006).

At E7–7.5, vasculogenesis occurs, which is the de novo differentiation of mesodermal progenitors into ECs and their formation of primitive capillary plexi. The initial site of EC and primitive blood cell formation is the mesodermal layer of the extraembryonic yolk sac in structures called blood islands (Wang and Zhao 2010). EC precursors, or angioblasts, in the outer layer of blood islands migrate to form long chords, eventually form lu-



**Figure 1.** Major factors that regulate endothelial cell differentiation and hemogenic specification through embryonic vascular development.

mens, and create a complex network of primitive blood vessels (Bautch et al. 1996). Angioblasts express vascular EC growth factor receptor 2 (VEGFR2 or Flk1) and Tal1 before EC-enriched proteins such as CD31 (*Pecam1*) (Drake and Fleming 2000), and they form vessels via an integrin  $\alpha1\beta1$ -collagen-mediated process that promotes vacuole and lumen formation in the newly differentiating ECs (Davis and Camarillo 1996). New blood vessels then sprout from the primitive plexi (Gama Sosa et al. 2021), guided by oxygen concentration and nutrient levels (Blatchley and Gerech 2020) to form a vascular network.

Just after the onset of blood island formation, mesodermal cells migrate along the embryonic midline to form the heart tube. While the heart tube is forming, vasculogenesis initiates in the paraaortic splanchnopleure, developing into the AGM region, along the aortae primordia (Dzierzak 2003). This wave of intraembryonic vasculogenesis forms the primary vascular structures in the embryo proper: the cardinal vein and the dorsal aortae (de Bruijn et al. 2002). Later in development (~E9.5), a subset of ECs in the AGM is specified to become HECs that give rise to HSPCs (Gritz and Hirschi 2016). Both *Xenopus* and zebrafish have similar and conserved molecular mechanisms that control vasculogenesis (Kabrun et al. 1997; Ny et al. 2006), providing researchers with a combination of methods to study the molecular and genetic regulators of this critical process.

## IMPORTANT FACTORS GUIDING ENDOTHELIAL CELL DIFFERENTIATION

### BMP4

Like T, BMP4 is critical for gastrulation and mesoderm development; thus, it is essential for all mesodermal-derived lineages (Mishina et al. 1995; Winnier et al. 1995). BMP4 signaling occurs through the BMP receptor, a heterotetrameric mix of two types of complexes; type 1, activin receptor-like kinases (ALKs) and type 2, BMPRII/ACVRL1/ENG2. Binding of the type 2 complex to BMP promotes phosphorylation in the type 1 complex. This promotes recruitment and phosphorylation of various SMAD (short for SMA, small worm phenotype, and MAD [mothers against decapentaplegic]) proteins (Lowery and de Caestecker 2010). BMP4-deficient mice do not form mesoderm, and stem cell studies show its role and necessity in EC development via regulation of key genes, including *Notch* and *eNos* (*nos3*) (Kelly and Hirschi 2009). Inactivation of multiple SMAD proteins that function downstream of BMP4 leads to defective vascular development and lethality (Yang et al. 1999; Lechleider et al. 2001), revealing the importance of BMP4 signal transduction for EC development. In further support of this, studies in zebra-

fish show that mutating or inhibiting the BMP4 receptor disrupts vascular development, but enhances hematopoiesis, while increased BMP4 signaling leads to less *Gata1* expression and reduced hematopoiesis (Gupta 2006). In mice, inhibition of BMP4 down-regulates other important EC differentiation factors including ETS variant transcription factor 2 (ETV2) and Flk1 (Lee et al. 2008), causing a lack of, or defective, vasculature, respectively. In vitro, BMP4 is used to direct embryonic stem cells toward a mesodermal state and increases the commitment of mesodermal progenitors into ECs. Importantly, BMP4 in vitro decreases the expression of *Gata2* and *Runx1* (Goldman et al. 2009), similar to its effects in vivo, directing mesodermal cells to an endothelial fate, rather than hematopoietic.

### Hedgehog Signaling

An important regulator of BMP4 signaling and EC differentiation is hedgehog (HH) signaling. HH ligands interact with the Patched-1 receptor (Ptch1), which activates Smoothened (Smo), a transmembrane protein, leading to initiation of Gli transcription factors (Farzan et al. 2008; Robbins et al. 2012), which promote proliferation and migration. In vitro, Indian hedgehog (IHH) drives BMP4 expression to promote EC commitment of human embryonic stem cells (Kelly and Hirschi 2009). Similarly, in vivo, IHH up-regulates BMP4 and its ablation inhibits hematopoiesis and vasculogenesis (Dyer et al. 2001). Interestingly, another study found that the initial BMP4 expression in the mesoderm during gastrulation is independent of HH; however, as the cells migrate down the primitive streak, BMP4 expression becomes controlled by sonic hedgehog (SHH) and IHH produced by the endoderm (Astorga and Carlsson 2007). Forkhead transcription factor *Foxf1* also controls BMP4, and in vitro explant studies show that HH signaling up-regulates *Foxf1*, which promotes vasculogenesis (Astorga and Carlsson 2007).

### ETV2

ETV2 is the essential ETS domain transcription factor that regulates endothelial, hematopoietic,

and cardiac development. ETV2 expression is reactivated in mice during vessel regeneration and injury-induced neovascularization (Park et al. 2016). This observation, along with its essential role in vascular development, makes it a key factor to study, but its regulation is not well defined. ETV2 is expressed early in development at E7.0 with expression subsiding by E10.5 (Lee et al. 2008). ETV2 expression drives mesodermal progenitor cells to an EC fate, and *Etv2*-deficient mouse embryos die early in development due to failed vascular and hematopoietic development (Hollenhorst 2004; Ferdous et al. 2009). In zebrafish, *Etv2*-null mutants have severe vasculogenic defects with a major reduction in EC markers (Sumanas and Lin 2006). ETV2 has been shown to bind in conjunction with FoxC2 (De Val et al. 2008) to promote the expression of many genes important for vascular development, including *Tie2*, *Vegfr2*, *Cdh5*, *Nrp1*, *Nrp2*, *Dll4*, and *Notch1* (Liu et al. 2015; Zhao and Choi 2017; Lee et al. 2019). Further studies show that ETV2 may also work in conjunction with Sox17 to promote *Ece-1* expression to enhance vascular development and vessel tone during embryogenesis (Robinson et al. 2014). Epigenetic studies in human-induced pluripotent stem cells show that ETV2 also binds to *Robo4*, a roundabout transmembrane receptor that is exclusively expressed in ECs due to demethylation of the promoter by TET1/2 during EC differentiation (Tanaka et al. 2018). This raises the question of what other epigenetic factors could also contribute to the regulation of the mesodermal-to-endothelial fate transition.

Current investigations into epigenetic regulation of ETV2 found that lysine-specific histone demethylase 1A (LSD1) mutant zebrafish show up-regulation of ETV2 and other EC regulators, including *gata1* and *fli1a*. Morpholino-mediated knockdown of *Etv2* rescues this phenotype and ChIP assays show a significant increase in H3-K4 histone methylation at the int2 enhancer of the *Etv2* locus (Takeuchi et al. 2015). These data show that ETV2 may be negatively regulated by LSD1 in zebrafish and suggests that ETV2 may be controlled epigenetically, but this remains to be shown in mammals.

In embryonic stem cells, a CRISPR screen was done to identify genes that regulate ETV2 and revealed that *Foxh1* is required for the generation of Flk1-expressing mesodermal progenitors. In addition, doxycycline-induced *Foxh1* deletion in mice leads to a reduced ETV2 expression, suggesting it may be a regulator (Zhao and Choi 2017). ETV2 and Flk1 are not direct gene targets of Foxh1, but its exogenous expression in  $\Delta$ *Foxh1* embryonic stem cells does partially rescue ETV2 expression (Zhao and Choi 2017), suggesting complex control by multiple factors. In vitro, ETV2 was found to be regulated by BMP, Notch, Flk1, and Wnt signaling, but in vivo regulators have yet to be determined (Lee et al. 2008; Zhao and Choi 2017). MESP-1 has been shown to work through Creb-1 to be a coactivator of ETV2 (Shi et al. 2015). An interesting connection is that MESP-1 is also not expressed in  $\Delta$ *Foxh1* embryonic stem cells (Zhao and Choi 2017), but more work is needed to determine the role of MESP-1 in ETV2 regulation.

In zebrafish, *cloche* mutants do not express ETV2 (Liao et al. 1997). *Cloche* has a loose human homolog called neuronal PAS domain protein 4 (NPAS4), which is a transcription factor important for inhibitory neuron synapse development. NPAS4 does not have a known role in EC development, as *NPAS4*-deficient mice develop normal vasculature. Nonetheless, despite the limited homology, human NPAS4 can rescue zebrafish *cloche* mutants. More recent studies have shown that cultured ECs overexpressing NPAS4 have increased angiogenic sprouting potential (Esser et al. 2017). More studies are needed to determine the role of NPAS4 in vasculogenesis and whether other NPAS genes regulate ETV2 in mammals.

### Fli1a/b

Fli1a and Fli1b are also ETS domain transcription factors that are induced by ETV2. Fli1b has similar gene targets as ETV2 and provides a positive feedback mechanism to sustain EC differentiation and maintenance after ETV2 expression subsides (Abedin et al. 2014). Disturbance of ETS factors other than ETV2 does not

disrupt vascular formation in mice, but a combination of ETS factor knockouts (excluding ETV2) does cause vascular defects in zebrafish (Pham et al. 2007). These data suggest that ETS transcription factors work synergistically to promote vasculogenesis, but exactly how this occurs and is regulated remains to be determined.

### Flk1 and VEGF-A

Flk1/Vegfr2 (KDR in humans) is a major receptor for VEGF-A and is required for mesodermal-to-endothelial differentiation (Lugus et al. 2009). There are three VEGF receptors in mammals, all have similar tyrosine kinase receptors with strong intracellular binding domains for PI3 kinase activation and signaling (Takahashi et al. 1999). Flk1 expression, in conjunction with VE-cadherin (Cdh5) and ETV2 expression, appear to commit mesodermal progenitor differentiation toward an EC lineage rather than hematopoietic (Nishikawa et al. 1998). VEGF-A does this by controlling the expression of Delta-like ligand 4 (Dll4), which is important for propagation of ETV2 and other ETS transcription factor activity (Liu et al. 2003). Flk1 has been shown in vitro to regulate ETV2 expression and keep ETV2 levels high in differentiating ECs, but it does not promote the initiation of ETV2 expression (Zhao and Choi 2017). ETV2 modulates VEGF-A signaling, as well (Liu et al. 2015), showing a feedback loop between VEGF-A and ETV2 expression for successful EC differentiation.

VEGF-A signaling is a major regulator of EC propagation and vessel growth in the embryo. As the embryo grows, the necessity to efficiently disperse oxygen across tissues increases as well. In hypoxic cells, oxygen needs outweigh available oxygen supply, and hypoxia-inducible factors (HIFs) become stabilized when HIF-prolyl hydroxylases are unable to hydroxylate the HIF-1 $\alpha$  subunits to mark them for degradation (Strowitzki et al. 2019). This enables HIF-1 $\alpha$  subunits to form dimers with HIF-1 $\beta$  (ARNT) subunits, leading to the transcription of glycolytic genes and VEGF-A in oxygen-starved tissues. VEGF-A up-regulation forms a signaling gradient from the oxygen-starved cells and promotes EC mi-

gration and vessel formation (Greijer et al. 2005; Potente and Carmeliet 2017). *ARNT*<sup>-/-</sup> mouse embryos die at E9.5–10 due to lack of blood vessel formation while blood island formation appears normal (Maltepe et al. 1997), suggesting the need for hypoxia-induced VEGF-A signaling during blood vessel development and maturation, but not initial formation. In *Xenopus*, VEGF-A is needed for angioblast development and migration from the midline by acting as a chemoattractant, guiding ECs to form major vessels (Cleaver and Krieg 1998); a similar mechanism is proposed in zebrafish (Poole et al. 2001). It is important to note that VEGF-A signaling is important for EC survival, proliferation, and migration, but a combination of signaling events is needed for the formation of functional vessels.

### FGF

In vitro, induced pluripotent stem cells can be differentiated into a mesodermal phenotype using BMP4 and then an EC phenotype using VEGF-A, BMP4, and fibroblast growth factor 2 (FGF2 or bFGF). Like BMP4, FGF2 is an important factor in mesoderm development. FGF2 and VEGF-A induce vasculogenesis in quail mesodermal tissues by promoting angioblast migration to sites of FGF2 expression (Poole et al. 2001). Further cell studies show that, in BMP4-supplemented media, FGF2 alone cannot induce an endothelial fate, VEGF-A can promote expression of Pecam1 and Cdh5. FGF2, VEGF-A, and BMP4 together increase expression of Cdh5 and Pecam1, indicating FGF2 enhances their vasculogenic activity (Harding et al. 2017). In vitro, cells derived from the intraembryonic mesoderm can be treated with FGF2 along with leukemia inhibitory factor (Lif) to change morphology and cause a vasculogenic phenotype (Gendron et al. 1996). Later studies show FGF2 alone can be used on coronary explants in a matrix to cause angioblast migration and tube formation. Addition of FGF2, FGF1, or FGF18 to the media causes increased tubule length, and VEGF-A-controlled migration was found to be enhanced with FGF signaling (Tomanek et al. 2001, 2010). Together, this evidence

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shows that FGFs may not induce vasculogenesis, but enhance mesoderm differentiation, vascular plexus formation, and angiogenesis.

### Extracellular Matrix

In vitro studies show that stiffness of the extracellular matrix (ECM) may play a role in EC differentiation from mesodermal progenitor cells. A more compliant, softer matrix promotes the formation of Flk1-expressing mesodermal cells from pluripotent stem cells by enabling the release of stabilized  $\beta$ -catenin to induce Wnt signaling, leading to expression of *Brachyury* (Smith et al. 2017). Laminin is an important ECM component, and when Laminin 411 is added to the growth matrix of pluripotent stem cells treated with BMP4 and VEGF-A, commitment to ECs is increased (Ohta et al. 2016). Fibronectin, via interactions with integrins  $\alpha 5$  and  $\alpha v$ , also mediates EC migration and remodeling (Bohnsack et al. 2004). Integrin  $\alpha 5$  (*Itga5*)-deficient mice are embryonic lethal and exhibit vascular development defects (Francis et al. 2002).

ECM remodeling is also important for vascular plexi formation. Studies in vitro show that hypoxia can drive endothelial progenitor cell clustering via matrix metalloproteinase activation, leading to EC migration and tube formation (Blatchley et al. 2019). In cultured ECs, hypoxia promotes migration via VE-cadherin down-regulation, which causes reduced cellular adhesion, allowing for cellular movement. Hypoxic conditions also drive angioblast and EC migration by up-regulating VEGF-A expression (Tabata et al. 2019). Thus, hypoxia likely contributes in multiple ways to vascular plexus formation and remodeling during vasculogenesis and angiogenesis.

### HEMOGENIC ENDOTHELIAL CELL SPECIFICATION

#### Primitive and Definitive Hematopoiesis

During mammalian embryonic development, there are two waves of hematopoiesis. The first occurs in the murine extraembryonic yolk sac at

E7.5, in regions referred to as blood islands. This process is called primitive hematopoiesis and predominantly produces transient primitive erythrocytes (Palis et al. 1999); the cellular origin of these initial blood cells in vivo remains poorly defined (Baron et al. 2012). Primitive hematopoiesis is followed by a second wave of definitive hematopoiesis, starting at E8.25 in the yolk sac, E9.5 in the placenta, and E10.5 in the AGM (Gritz and Hirschi 2016). During this process, HECs are specified in all these tissues and give rise to HSPCs via endothelial-to-hematopoietic transition (EHT). The HSPCs subsequently differentiate into all blood cell types, migrate to and propagate within other hematopoietic tissues including the spleen, thymus, and fetal liver, as they develop in utero from E10.5 to E14.5 (Coşkun et al. 2014). HSPCs finally migrate into fetal bone marrow by E16.5 (Coşkun et al. 2014), where they remain postnatally and generate all of the blood cells needed throughout life.

HECs represent a small, transient population of ECs (1%–3%) that exhibit endothelial and hematopoietic characteristics and are defined as Flk1<sup>+</sup>c-Kit<sup>+</sup>CD45<sup>-</sup>SP<sup>+</sup> cells in the mouse (Marcelo et al. 2013b; Stefanska et al. 2014). There is still much to learn about the regulation of HEC specification, as well as their generation of HSPCs, but major pathways that have already been found to play a role in HEC specification include retinoic acid (RA) signaling, as well as Notch1, cKit, p27-mediated cell-cycle control, and miR-223, which are discussed below.

### IMPORTANT FACTORS GUIDING HEMOGENIC ENDOTHELIAL CELL SPECIFICATION

#### Retinoic Acid Signaling, p27, and Cell-Cycle Arrest

RA is derived from vitamin A, which is converted to retinol, retinal, and then finally active RA via retinal dehydrogenase (Raldh) (Zhao et al. 1996). Signaling occurs when RA is taken up by cells and binds to RA receptors embedded within the nuclear membrane causing a conformational change and receptor uptake by the nucle-

us, enabling modulation of gene expression (Ghyselinck and Duester 2019). RA signaling is essential for vascular development (Bohnsack et al. 2004) and the generation of HECs, and *Raldh2*<sup>-/-</sup> embryos, which lack active RA, fail to form HECs in the yolk sac and AGM (Goldie et al. 2008; Marcelo et al. 2013b). Interestingly, these RA-deficient embryos can be rescued by exogenous RA in utero or in embryo culture, and RA was found to function cell-autonomously to promote hemogenic specification of ECs (Bohnsack et al. 2004; Goldie et al. 2008; Marcelo et al. 2013b).

*Raldh2*<sup>-/-</sup> embryos show reduced c-Kit (*Kit*) expression, and reexpression of *Kit* in *Raldh2*<sup>-/-</sup> embryos restores HEC development, as well as increases expression of *Runx1*, which is critical for HSPC generation from HECs (Marcelo et al. 2013b). *Raldh2*<sup>-/-</sup> embryos also exhibit decreased expression of cell-cycle inhibitor p27, and increased EC proliferation (Bohnsack et al. 2004). RA treatment of, as well as *Kit* or *Cdkn1b* reexpression in, *Raldh2*<sup>-/-</sup> embryos restores p27 expression (Marcelo et al. 2013b) and promotes EC cycle arrest, which was found to be required for HEC specification (Marcelo et al. 2013b). Further investigation is needed to understand the mechanisms by which EC cycle control enables hemogenic specification.

### c-Kit

c-Kit is a tyrosine kinase receptor that is expressed on a subset of ECs in both the yolk sac and AGM during embryonic development (Marcelo et al. 2013b). Upon binding of stem cell factor (Scf), its ligand, dimerization of c-Kit is initiated, causing autophosphorylation of its intracellular domain and a resulting signaling cascade (Van Handel et al. 2012). c-Kit is also expressed during endothelial tube formation during development (Matsui et al. 2004), and *Kit* mutants exhibit hematopoietic defects and prenatal fatality (Bernstein et al. 1990). *Scf*<sup>-/-</sup> mouse embryonic stem cells show lack of hemogenic phenotype and potential (Porcher et al. 1996; Rybtsov et al. 2011). c-Kit functions downstream of RA signaling, as *Raldh2*<sup>-/-</sup> mouse embryos show reduced c-Kit expression,

and its reexpression therein rescues their hematopoietic defects (Marcelo et al. 2013b).

### Notch Signaling

Notch receptors (1–4) are membrane-bound on the cell surface and have several membrane-bound ligands, including Delta-like ligands (DLLs) and serrated (Jagged in mammals). Binding of ligands to Notch receptors results in mechano-mediated conformational changes, allowing proteo-cleavage of the receptor and release of the active Notch intracellular domain (NICD). The NICD can then enter the nucleus and regulate gene expression (Bray 2006). Notch signaling, specifically through Notch1, is critical for developmental hematopoiesis, as Notch signaling within ECs sustains *Runx1* expression, which is critical for hematopoietic development (Gritz and Hirschi 2016). Mutants lacking Notch1 appear to have normal development within the first 9 days of embryonic development, and then exhibit massive cell death leading to fatality (Swiatek et al. 1994). Notch1 mutants within ECs exhibit vascular defects in the embryo proper and yolk sac at E9.5 (Limbourg et al. 2005). E7.5–8 mouse embryos treated with the Notch inhibitor DAPT exhibit reduced p27 expression and increased EC proliferation. Reexpression of p27 in DAPT-treated wild-type embryos leads to a restoration of EC cycle control and hemogenic specification (Marcelo et al. 2013b). It has been suggested that if Notch signaling remains elevated in HECs, they will be pushed toward a hematopoietic fate rather than an endothelial fate (Richard et al. 2013; Uenishi et al. 2018). These studies show the importance of Notch signaling, and the complex microenvironment, in establishing HEC phenotype and function.

### Runx1

*Runx1* is a transcription factor necessary for EHT and formation of HSPCs that give rise to all hematopoietic cell lineages. Inducible Cre-mediated deletion of the *Runx1* gene in mouse embryos causes embryonic lethality and a dramatic decrease in hematopoiesis (Chen et al.

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2009). Runx1 is aided by the non-DNA-binding partner, CBF- $\beta$ , that enhances Runx1-DNA binding (Wang et al. 1996a). Embryonic lethality is similarly seen in *Cbfb*<sup>-/-</sup> mouse embryos (Wang et al. 1996b). Regulators of Runx1 are well characterized; Gata-binding protein 2 (Gata2), LIM domain only 2 (Lmo2), Stem cell-like factor (Scl), and other Ets domain transcription factors have been identified to bind in a complex on the *Runx1* promoter (Nottingham et al. 2007). Expression of Runx1 seems to be temporally regulated as well. In ETV2-GFP-expressing ECs in the murine yolk sac, it has been observed that Runx1 expression is differentially regulated between E7.5 and E8.5, and BMI1 is thought to repress Runx1 expression at E8.5 (Eliades et al. 2016).

Runx1 has been studied in the context of EHT and later hematopoietic differentiation events. It has a variety of context-dependent targets (Tracey et al. 1998; Tober et al. 2007; Gao et al. 2018), but during EHT, Runx1 targets hematopoietic regulators, such as Sox17 (Lie-A-Ling et al. 2018) and Gata3. Runx1 targets also include important cell adhesion and motility genes such as *Itgb3* (Lie-A-Ling et al. 2014). Importantly, Runx1 targets GFI1a/b transcription factors, and ectopic expression of both GFI1a and b in *Runx1*<sup>-/-</sup> HECs restores the EHT processes. GFI1 expression has also been observed in ECs in the dorsal aorta, at sites of hemogenic activity (Thambyrajah et al. 2016b), and it is thought to repress the endothelial phenotype and promote hematopoietic cell morphology (Lancrin et al. 2012). Inhibition of GFI1a/b results in reduced release of hematopoietic cells into blood circulation (Lancrin et al. 2012; Thambyrajah et al. 2016a).

### miRNAs

MicroRNAs (miRNAs) are noncoding RNAs that control gene expression through a variety of complexes, but specific mechanisms (Cannell et al. 2008). Proper control of miRNAs is essential for vascular development, as dysregulation of miRNAs has been implicated in vascular plexus malformation (Henn et al. 2019). miR-223 was recently shown to play an important

role in the formation of HECs and, thus, definitive hematopoiesis (Kasper et al. 2020). Lack of miR-223 in zebrafish and mouse mutants results in increased HECs and hematopoietic cells in the AGM. In zebrafish, miR-223 was found to target *alg2* and *st3gal2* genes that are involved in regulating the *N*-glycosylation of proteins. The *N*-glycosylation process involves post-translational modification of proteins through the addition or cleavage of various sugar moieties to regulate protein stability and folding that affects the function of the final protein product. *miR-223*<sup>-/-</sup> zebrafish exhibit a lack of mannose modification of proteins involved in HEC specification and EHT, and these modifications lead to increased EHT and an overexpansion of hematopoietic cells (Kasper et al. 2020). The function of other miRNAs in these processes is under investigation.

### SUMMARY AND CONCLUSIONS

The regulation of EC differentiation and specification is a complex tug of war between signaling pathways. Our knowledge of the molecular mechanisms that control mesodermal-to-endothelial transition is just in its infancy, but new technology like CRISPR, next-generation sequencing, and proteomics are enabling more detailed study of this important turning point in differentiation. Discovering these mechanisms is critical for improving clinical therapies to treat vascular and hematopoietic diseases. For example, studies in vitro have been shown that endogenously expressed ETV2 is sufficient to differentiate human fibroblasts into ECs, whereas exogenous ETV2 can partially differentiate fibroblasts. Promisingly, these cells are even engraftable to form new vascular plexi in NOD SCID mice (Morita et al. 2015) and strategies like these could lead to new therapies.

There are many remaining questions about early EC development, and the recent discovery of the role of the EC *N*-glycome in hematopoiesis raises many more questions about the complex regulation of HEC specification and EHT. Thus, more studies are needed to better understand mechanisms that regulate these complex processes.



Despite the exhaustive amount of research that has been devoted to investigating the mechanisms and regulators of ECs, there is much yet to be discovered and new knowledge can be applied to develop better analytical tools and treatments for a wide variety of diseases.

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