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Review

Transcriptional regulation and signalling pathways involved in the hemangioblast development

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ABSTRACT

The initial steps in hemangioblast specification occur in the primitive streak during gastrulation and result in the generation of mesodermal precursors committed to the hematopoietic or endothelial lineages. The differentiation process involves the acquisition of a specialized phenotype that depends on the expression of lineage-specific genes and the repression of genes characteristic of multipotent progenitors and alternative lineages. This genetic program to coordinate hemangioblast development is controlled by the complex and dynamic interplay between signalling pathways and transcription factors. In this review, we focus on recent studies that have revealed transcription factors and signalling pathways that progressively direct cell fate choices during hemangioblast specification and commitment.

KEYWORDS: hemangioblast, hemogenic endothelium, hematopoietic cells, endothelial cells, embryonic stem cell, differentiation

ABBREVIATIONS

BL-CFC, blast-colony forming cell; ESC, embryonic stem cell; YS, yolk sac; AGM, aorta gonad-mesonephros; HSC, hematopoietic stem cells; TF, transcription factor; EBs, embryoid bodies; CVP, cardiovascular progenitor cells

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INTRODUCTION

Ontogeny of the mammalian hematopoietic system

The ontogeny of the mammalian hematopoietic system is characterized by two waves of hematopoietic progenitors emerging from the yolk sac (YS) during embryonic development. The first 'primitive' wave takes place in the YS blood islands at around embryonic day (E)7.5 of gestation in the mouse conceptus and produces primitive erythrocytes, megakaryocytes and macrophages. Around E10 of gestation, hematopoiesis shifts to an intraembryonic site, the aorta gonad-mesonephros (AGM) region, where definitive hematopoietic precursors and the first long-term hematopoietic stem cells (HSC) are generated (Figure 1, top panel). Definitive hematopoietic precursors migrate to the liver to further develop and mature in all blood lineages. From E12 until birth, the liver becomes the main site of hematopoietic development, and support the growing midgestation foetus. After birth and during all adult life, hematopoiesis occurs in the bone marrow [1].

Hemangioblasts and hemogenic endothelium

In the mouse conceptus YS, mesodermal cells, which have migrated from the primitive streak, aggregate and form the blood islands. The central part within the blood islands generates primitive blood cells while the peripheral cells differentiate into endothelial cells. The close temporal and

spatial development of the hematopoietic and endothelial cells in these blood islands provided the basis for the hypothesis that these lineages share a common ancestor, a progenitor known as the hemangioblast [2, 3].

Several expression analyses and gene targeting experiments support this concept: endothelial and hematopoietic lineages share, indeed, a number of common cell surface markers and transcription factors (TFs), furthermore, both gain- and loss-of-function studies for several genes result in phenotypes affecting both lineages [4-6]. The first evidence for the existence of the hemangioblast comes from *in vitro* studies. Early embryonic events can be faithfully and fully recapitulated with the *in vitro* differentiation of embryonic stem cells (ESCs), able to generate three-dimensional, differentiated cell masses called embryoid bodies (EBs) [1, 7]. EBs have the ability to undergo differentiation and cell specification along the three germ lineages, endoderm, ectoderm, and mesoderm, which comprise all somatic cell types [8, 9] and can respond to similar cues that direct embryonic development [10]. The development of hematopoietic and endothelial cells within EBs mimics *in vivo* events with the emergence of YS blood island-like structures made of vascular channels containing hematopoietic cells [8, 11]. Studies using this *in vitro* system have led to the identification of a precursor, termed the blast colony forming cell (BL-CFC), which generates a blast colony containing both hematopoietic and endothelial cells, and as such represents the *in vitro* equivalent of the hemangioblast [12, 13]. The BL-CFC expresses the mesodermal marker Brachyury (Bry) and the receptor tyrosine kinase Flk-1 [14], indicating that it represents a population undergoing mesoderm specification towards the hematopoietic and vascular lineages (both expressing Flk-1) (Figure 1, lower panel). The *in vivo* evidence for the existence of the hemangioblast was the identification of BL-CFCs (displaying haematopoietic and vascular potential) in gastrulating mouse [15] and in zebrafish embryos [16], displaying clonal potential as assessed by cell mixing and limiting dilution analyses. These cells co-express Bry and Flk-1 and arise within the posterior region of the primitive streak, indicating that initial stages of haematopoietic and vascular commitment occur before blood island development in the YS [15].

In definitive hematopoiesis, blood cells of the AGM also develop in close association with the endothelium of the dorsal aorta. Indeed, immunohistological analysis of fixed embryo sections has revealed the presence of haematopoietic cell clusters attached to the aortic endothelium [17-19]. However, unlike what happens in the YS where endothelial and hematopoietic cells appear simultaneously, the aorta and associated endothelium is formed at least 1 day before the emergence of the hematopoietic clusters, leading to the emergence of a new hypothesis: blood cells and endothelial cells don't emerge from a common precursor, the hemangioblast, but rather blood cells are derived from a subset of already differentiated endothelial cells known as hemogenic endothelium [20, 21]. In support of this theory, *in vitro* culture system of ESC conditional gene targeting in mouse, cell fate mapping, *ex vivo* embryo and organ culture techniques, proved the endothelial origin of hematopoietic clusters in the aorta with the direct visualization of blood cell clusters "budding" from the aortic wall [22-27]. The controversy between the hemangioblast and hemogenic endothelium theories has been solved by a unifying theory. Blood cells are indeed derived from a hemogenic endothelium which in turn originates from the hemangioblast, presenting therefore hemangioblasts and hematogenic endothelial cells as two consecutive developmental stages leading to formation of blood (Figure 1, top panel). In the *in vitro* model the hemangioblast generates 2 types of endothelial cells, fully differentiated endothelial cells and hemogenic endothelial cells, which have all the characteristic of endothelial cells, but are able to give rise to blood cells through a process termed endothelial to hematopoietic transition (EHT) [22, 23]. *In vitro* observations, suggest that blood cells emerging in the YS and in the AGM derived from hemangioblast cells through a hemogenic endothelium, however *in vivo* proof of the existence of hemogenic endothelial cells in YS, and hemangioblast progenitors in the AGM region is still missing.

Once the cellular basis underlying the formation of the first blood cells from the mesoderm has been characterized, the following step is to determine the molecular mechanisms driving this developmental process. The emergence of hemangioblasts from the mesoderm and their successive differentiation towards hematopoietic and endothelial lineages is controlled by many signalling pathways. The resulting integrated action of these signals leads to the activation of specific TFs and therefore specific genetic programs that ultimately determine hemangioblast fate. These developmental processes are now known to occur in both the YS and AGM regions. However, these sites are separated in developmental space and time and generate different hematopoietic precursors and lineages. Therefore, it is likely that both common and region-distinct mechanisms direct the emergence of hematopoietic cells during development. In this review we will examine signalling pathways and TFs controlling three different stages of hemangioblast development: 1) the emergence of posterior primitive streak 2) the commitment of primitive streak population to the hemangioblast and the following hemogenic endothelium and finally 3) the specification of hemogenic endothelium towards the hematopoietic lineage.

1. Molecular regulation of the generation of posterior primitive streak-like population

a. Signalling Pathways involved in the generation of the primitive streak

Emergence of the primitive streak is a complex process regulated by multiple pathways, among them the Wnt, Activin/Nodal, Bmp and Notch pathways seem to play a decisive role [28-30]. The related factors and their inhibitors are produced from diverse locations within the embryonic and extra-embryonic tissues and integration of their graded actions results in spatially appropriate cellular responses resulting in the emergence of the primitive streak. The schematic representation of all the known pathways involved in the hemangioblast emergence and differentiation process is described in Figure 2.

The essential role of canonical Wnt signalling is evident in the mouse knock-out (KO) for β-catenin which fails to generate mesodermal tissue [31]. The early inhibition of the canonical Wnt pathway in *in vitro* differentiating murine ESC blocks the expression of mesoderm-specific marker genes and the generation of typical mesodermal colonies [28, 32, 33]. Interestingly, the T-box TF Bry, a direct target of Wnt/β-catenin pathway, is also required for posterior mesoderm formation [34, 35]. All these data indicate a strong requirement of canonical Wnt signalling during posterior mesoderm induction in the mouse early embryo, a prerequisite for the formation of hemangioblasts. It is worth noting that in human ESC the positive influence of canonical Wnt signalling on mesoderm specification is not present. Instead a short pulse of non-canonical signalling is necessary and sufficient to control the exit of human ESC from the pluripotent state and subsequent entry into the mesoderm lineage [36].

Activin A (a factor that binds the same receptor as nodal and likely mimics nodal signalling) induces either an anterior or posterior mesodermal population, depending on the concentration of factor used. Bry, which autoregulates its own production, is activated by low levels and repressed by high levels of activin signalling, leading to different developmental outcomes [29, 30, 37].

In BMP4 KO embryos, severe mesoderm deficiencies are observed and expression of Bry, normally found adjacent to the domain of BMP4 expression, is absent [38]. However, in studies using ESCs, BMP signalling seems not to be directly responsible for the induction of primitive streak-like population. It rather exerts its mesoderm-inducing role indirectly, through the endogenous activation of the Nodal and Wnt pathways [29, 30, 32]. A similar scenario may take place *in vivo* where BMP signalling from the extraembryonic ectoderm may be required to initiate the Wnt and/or Nodal pathways. Once Nodal and Wnt3 are induced, however, gastrulation could proceed in the absence of BMP, as observed in the epiblast-specific *bmpr1a*-deficient animals [39]. Although BMP is not directly required for the development of the primitive streak-like population *in vitro*, it does exert a “dominant posteriorizing” effect on this population. Inhibitors of BMP signalling are indeed expressed in the

region of the anterior primitive streak in the mouse embryo [40]. BMP induction during early gastrulation causes excess ventral-posterior mesoderm and enhanced hematopoiesis [41]. Therefore BMPs promote blood formation by specifying mesoderm during early dorso-ventral patterning, regulating the Wnt and Nodal pathways.

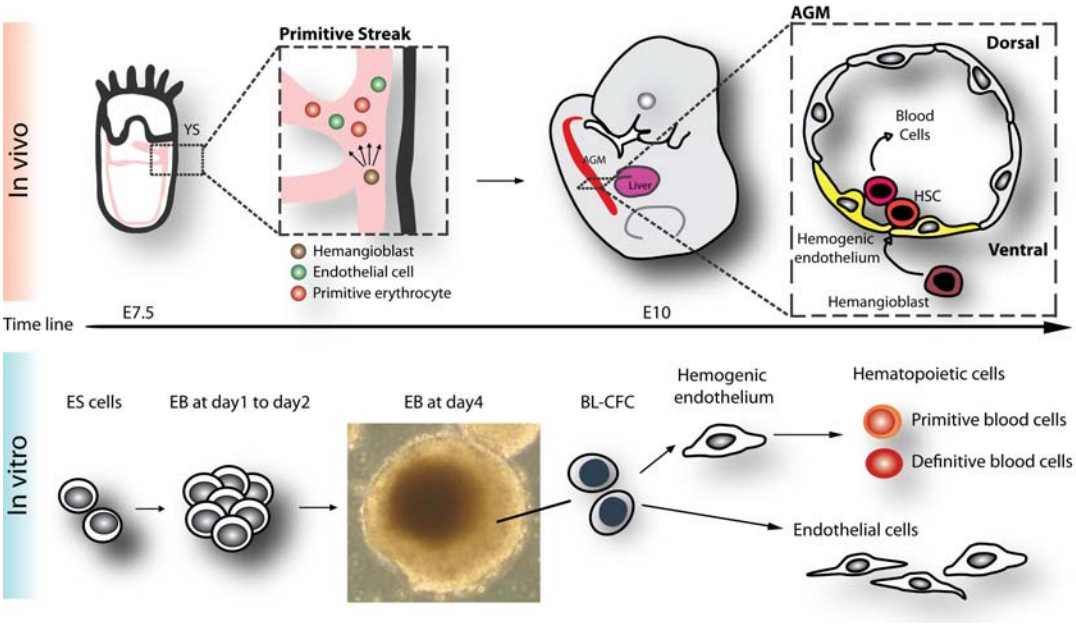

Figure 1

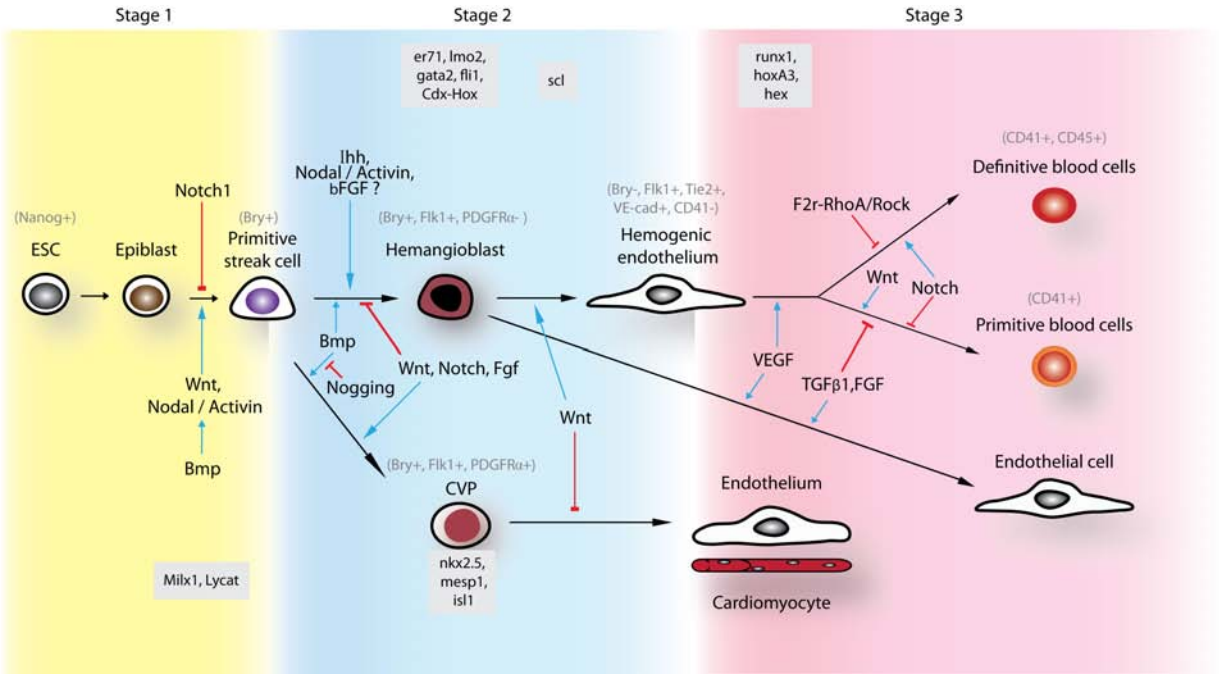

Figure 2

Notch1 signalling is also active during mouse gastrulation in nascent mesoderm and YS blood islands [42], suggesting that it may have a physiological role in early stages of mesodermal commitment and, as discussed later (see paragraph 2b and 3a), in the generation of early hematopoietic precursors. Activation of Notch1 signalling using an *in vitro* ESC differentiation system reduces the generation of mesodermal cells [43]. This result is in line with other findings: removal of Notch signalling in ESC by disruption of RPB-J, a critical downstream effector of all Notch receptors, increases the generation of mesodermal cell [44]; while activated Notch1 has been shown to promote neuronal commitment of ESC at the expense of the mesodermal lineage [45, 46]. However, it should be noted that mouse and human have 4 different Notch receptors (Notch1 to 4) and 5 different ligands: Jagged1, Jagged2, Delta-like 1 (Dll-1), Dll-3, and Dll-4 [47, 48]. The outcome of Notch signalling regulated processes can thus be the results of different actions exerted by distinct Notch ligands. The positive effects of Notch1 might, therefore, be counteracted by other receptors and/or ligands giving rise to contradictory results. Indeed, contrary to Notch1, Dll4 is able to promote mesoderm formation and negatively regulate the emergence of neuroectoderm [49].

b. Transcription factors involved in the generation of primitive streak

The generation of the primitive streak is the first essential step towards the generation of the hemangioblast, and genes involved in this process are located on the top of the hierarchy of TF cascade which leads to the emergence of hemangioblasts and ultimately the generation of blood cells. These genes activate other genes which in turn drive the emergence and differentiation of hemangioblasts. Among these factors, Mixl1 and Lycat seem to be indispensable.

The homeobox protein Mixl1 plays a central role in the formation of primitive streak during gastrulation, subsequent generation of mesoderm and endoderm, and their proper axial morphogenesis. Expression of Mixl1 is restricted to the primitive visceral endoderm of the pre-gastrulation embryo and to the primitive streak later on [50, 51]. Homozygous *Mixl1* mutant embryos show severe mesodermal and endodermal defects and arrest at E9.5 [52]. *In vitro* studies also suggest that mesoderm is directly affected by the loss of this TF, because differentiating *Mixl1*-null ESC indeed show a reduced frequency of hemangioblast formation and successive impairment in the differentiation of BL-CFC, placing the function of Mixl1 upstream of Flk-1^+ hematopoietic precursors [53]. Analysis of the differentiation of ESC in which GFP was targeted to one or both alleles of the mouse or human *Mixl1* locus, made it possible to identify and isolate a GFP^+ primitive streak-like population and demonstrate that the majority of ESC-derived BL-CFCs arose from $Mixl1^+Flk\text{-}1^+$ mesodermal progenitors [54]. Gain of function studies support the previous findings,

Legend to Figure 1. *In vivo* and *in vitro* model of hemangioblast differentiation.
In the early stages of development (E7.5), hemangioblasts are transiently observed in the primitive streak *in vivo* before they migrate to the YS. Segregation of hematopoietic and endothelial lineages from hemangioblasts occurs before these progenitors reach their final destination in the YS. At later stages (E10), hematopoietic clusters appear in close association with the ventral aortic hemogenic endothelial cells in the AGM region (top panel). *In vitro* differentiation of ESCs involves generation of EBs and emergence of BL-CFCs, the *in vitro* equivalent of hemangioblast. BL-CFCs give rise to endothelial and hematopoietic cells. The latter differentiate through an intermediate hemogenic endothelium stage. AGM, aorta-gonad-mesonephros region; BL-CFCs, blast colony forming cells; EBs, embryoid bodies; ESC, embryonic stem cells; YS, yolk sac.

Legend to Figure 2. Schematic representation of hemangioblast/hemogenic endothelium emergence and differentiation.
Schematic figure describing the molecular regulation of ESCs differentiation into primitive streak-like cells (Stage 1), the competitive induction of hemangioblast versus CVP from mesodermal progenitor cells and the hemogenic endothelium emergence (Stage 2), and the later stages of hemangioblast/hemogenic endothelium differentiation towards endothelial and hematopoietic lineages (Stage 3). Markers for the identification of cell populations (grey letters), key TF regulators (grey boxes), and signaling pathways (blue or red arrows), are indicated. ESC, embryonic stem cells; CVP, cardiovascular progenitor cell; TF, transcription factor.

indeed overexpression of *Mixl1* in EBs increases the formation of mesodermal, hemangioblast, and hematopoietic lineages [55]. Pereira *et al.* showed that most of the potential target genes of Mix11 are involved in cell migration and adhesion, and among them the promoter of the *Flk-1* and *PDGFR-α* genes were directly bound by Mixl1 [56]. *In vivo* and *in vitro* experiments identified Mixl1 as a direct target of BMP4 and activin A [53, 57], identifying Mixl1 as a mediator of BMP4 signalling pathway [58]. Thus, BMP4-induced *Mixl1* plays essential roles in both mesodermal induction and the formation of hemangioblasts.

Another important factor acting at the gastrulation stage is lysocardiolipin acyltransferase (*lycat*) gene, identified as the responsible gene for the zebrafish *cloche* mutant [59, 60]. This mutant is characterized by reduced blood cells and vascular endothelial cells [61]. Expression analyses have shown that Lycat acts upstream of other important hematopoietic and endothelial TF genes like *Etsrp*, *Scl*, *Lmo2*, *Gata2*, *Runx1*, *Fli1* etc. [61-71], identifying it as the earliest gene acting in hemangioblast development. Loss-of-function and gain-of-function studies using the mouse ESC differentiation system confirmed the essential role of *lycat* in hemangioblast, hematopoietic, and endothelial lineage development [59]. *Lycat* is expressed in the Flk-1^+ BL-CFC-containing populations within the EBs and overexpression in a transgenic cell line leads to increased BL-CFCs and hematopoietic progenitors [59]. Acyltransferases have been shown to regulate signalling in the Hedgehog and Wnt pathways by modification of the respective ligands [59, 72-75], therefore it would be interesting to investigate the possible interaction between Lycat and these pathway in regulating hemangioblast development.

2. Molecular regulation from mesoderm to hemangioblast and hemogenic endothelium

a. Antagonistic relationship between hemangioblast and cardiovascular progenitor cell commitment

Mesoderm precursors within the primitive streak become progressively committed, in accordance with the timing and the site of ingression into the primitive streak. The first identified step toward hematopoietic commitment occurs when Bry-expressing mesodermal cells up-regulate Flk-1, thereby marking the formation of the hemangioblast [76]. Using a lineage tracing system, Lugus *et al.* proposed that all blood cells are the progeny of Flk-1^+ mesoderm [77].

In primitive streak stage embryos, Flk-1 is first detectable in the extraembryonic mesoderm and in the paraxial-lateral embryonic mesoderm, which is destined to become heart [78]. Disruption of Flk-1 *in vivo* results in embryonic lethality between E8.5 to E9.5 with an absence of blood islands and organized blood vessels [79]. However, Flk-1-/- ESCs can still differentiate into both lineages *in vitro* [80], indicating that Flk-1 is not required for the development of hemangioblast, but for the migration of these progenitors into the proper microenvironment during embryogenesis.

At the gastrulation stage the hemangioblasts are not the only mesodermal cells to express Flk-1 [81, 82]. The cardiac lineages develop from mesodermal cells that are specified early in the embryonic life, shortly after the formation of hematopoietic mesoderm [83, 84]. Studies with ESC model have successfully reproduced this temporal pattern of hematopoietic and cardiac development. Cells expressing Flk-1 early during *in vitro* ESC differentiation are fated to become hemangioblasts, a second wave of Flk-1 expression slightly later results in cardiovascular progenitor cells (CVP) [10, 85-90]. The hematopoietic subpopulation of Flk-1^+ cells can be distinguished from Flk-1^+ cells exhibiting cardiac potential by the co-expression of PDGFR-α in the latter [91] and for the different and specific set of TFs that drive the differentiation process of these two different lineages: *Scl* expression in hemangioblast, *Mesp1*, *Isl1* and *Nkx2.5* expression in the CVP [87, 92-95]. Accumulating evidence suggests that these two lineages develop from a common progenitor and that the specification of these lineages is inversely regulated, with an antagonistic relationship to achieve a proper and balanced development of the circulatory system [28, 96-104]. There are several studies confirming at the genetic level that this antagonism between cardiac and hemangioblast programmes is indeed mutual. For instance, hematopoietic program is inhibited by enforced expression of cardiac regulators such as

Mesp1 and *Nkx2.5*, which enhanced cardiac differentiation [99, 100]. Conversely *Scl* and *etsrp* (*Er71*) expression in zebrafish embryos expanded hematopoietic and endothelial cell specification, while concurrently reducing the myocardial field [97].

b. Pathways involved in hemangioblast commitment

Early *in vivo* studies suggested that pathways involved in mesoderm formation could be important in the following differentiation steps towards the production of blood and endothelial cells [105-109]. However it is difficult to determine their real involvement in hemangioblast development *in vivo* without affecting the previous step of mesoderm formation. However taking advantage of the stepwise nature of ESC-EB system, it has been possible to test multiple pathways on isolated population representing distinct stages of development, and to define stage-specific roles for these pathways without interfering with previous stages. Several *in vitro* experiments showed that during the transition of the primitive streak to Flk-1$^+$ hematopoietic mesoderm/hemangioblasts, BMP, activin/Nodal, Hedgehog, Wnt and Notch1 signalling have non-redundant functions [29, 30, 32].

BMP plays two distinct and sequential roles during blood formation. As previously mentioned, BMP mediates induction of ventral-posterior mesoderm through activation of the Wnt and Nodal pathways, in a second successive stage BMP directly promotes blood specification from preformed mesodermal cells. Although *Bmp4* deficient mice die between E7.5 and E9.5 with defects in mesoderm formation and patterning, those who survive up to E9.5 display severe defects in blood islands [38]. Using *Scl*$^+$/*CD4* knock-in ESCs, Park *et al.* showed that BMP4 induces Flk-1$^+$ and Scl$^+$ cells under serum-free conditions [110]. Inhibition of the Smad1/5 pathway, downstream signalling molecules of BMP4, resulted in a reduction of Flk-1$^+$ cell generation [110], while ectopic expression of Smad1 increases the hemangioblast population derived from murine ESC [111]. These results support the previous findings in mice deficient in *Smad1* or *Smad5*, which display varying degrees of defects in hematopoietic and vascular development [112, 113]. Conditional Smad4 inactivation in Flk-1$^+$ cells significantly compromised murine blood formation [114], further implicating the BMP/TGF-β/activin pathway in murine blood specification. BMP signalling directly activates important hematopoietic TFs such as Gata2 and Runx1 [115, 116] and the Cdx-Hox pathway which is essential for hemangioblast commitment [117]. Finally, in a recent report, using a time-lapse video microscopy following the *in vitro* differentiation of ESC, Chiang and colleagues showed that the presence of BMP4 dramatically enhances formation of hemogenic endothelium [32]. The deterministic effect of BMP4 during mesoderm differentiation is consistent with hemangioblast commitment in the posterior region of the primitive streak [15] where BMP4 activity is highest at the gastrula stage [118]. The essential role of BMP signalling is also supported by several recent studies on Endoglin, an accessory receptor which can complex with TGF-β superfamily ligands (e.g. BMPs, activin A) and their receptor to activate downstream signalling proteins like Smad1/5/8 [119]. Endoglin seems to be essential for the hemangioblast development [120-122].

BMP is also critical in cardiomyocyte differentiation. Like the hemangioblast BMP signalling is essential for at least two steps in the cardiomyocyte induction process: mesodermal induction and cardiomyocyte differentiation [38]. However, between these two steps, a transient block of intrinsic BMP signalling by Noggin seems to be the most important step for determining cardiomyogenic differentiation [123]. It would be interesting to investigate if this blocking action of Noggin can determine the boundary between hemangioblast and CVP cells during mesodermal commitment.

Both activin/Nodal and bFGF play also a promoting role for Flk-1$^+$ hematopoietic mesoderm/hemangioblasts. The addition of activin and bFGF to EBs, 2.25 days after induction BMP4, resulted in a strong induction of BL-CFC numbers compared to just addition of BMP4 alone [30]. Activin and bFGF, in the presence of BMP4 led to an induction of *Runx1*, *Hex*, *Scl*, *Fli1*, and *Lmo2* within the first 6 hours of stimulation [32, 110, 124].

Among the Hedgehog family, only *Indian Hedgehog* (*Ihh*) is expressed in the visceral endoderm of the YS and its receptor-downstream molecules are expressed in the posterior epiblast, which is destined to form blood and endothelial cells during early gastrulation [109, 125]. EBs derived from ESC mutant for *Ihh* cannot form blood islands or produce endothelial cells while ectopic expression of Hedgehog protein is sufficient to induce the formation of hematopoietic and endothelial cells in explant cultures of early streak epiblast and more mature anterior epiblast [109, 126]. Hedgehog signalling was shown to induce *Scl* expression during early hemangioblast specification through the activation of the TF Gli which directly binds to *Scl* 3' enhancer [127].

The Wnt pathway is one of the regulators in the competitive relationship between CVP and hemangioblast. *In vivo* experiments show that Wnt-β catenin signalling support hematopoietic development at the expense of cardiac lineages. In chick and frog embryos Wnt-β catenin inhibition in posterior mesoderm, which normally gives rise to blood, leads to induction of cardiac markers and simultaneous inhibition of hematopoietic marker gene expression, furthermore forced expression of Wnt ligands in precardiac mesoderm leads to inhibition of cardiogenesis and ectopic expression of hematopoietic markers [128, 129]. However Wnt-β catenin signalling in the ESC differentiation system exhibits a biphasic and antagonistic effects on cardiomyogenesis and hematopoiesis depending on the stage of EB development: in the early stages Wnt suppresses hematopoiesis-endothelial development and increases CVP commitment while the opposite effect is evident in the late stages of EB formation [130]. Using the ESC/EB system, Liu *et al.* recently showed that Wnt signalling inhibition is a key mechanism by which ER71, a TF essential for the emergence of hemangioblast (see paragraph 2c), promotes hemangiogenic differentiation of mesoderm at the expense of cardiogenic fate supporting a negative role for Wnt-β catenin pathway in the early stage of hemangioblast commitment [104]. Also the non-canonical Wnt pathway is involved in this competition, supporting the cardiac lineage while blocking hemangioblast commitment and differentiation [98, 131].

Induced expression of Notch1 inhibits the emergence and successive differentiation of hemangioblast [43]. An *in vitro* study shows that Notch activity promotes the degradation of Scl by facilitating its ubiquitination [132]. An opposite stimulating effect on cardiomyocytes development is evident; activation of Notch4 in the $Bry^{+}Flk\text{-}1^{-}$ population after 3-3.5 days of ESC differentiation increases differentiation towards cardiac lineage. Activation of Notch signalling is also able to re-specify $Flk\text{-}1^{+}$ hemangioblasts to a cardiac cell fate [98]. This respecification is mediated in part through upregulation of molecules inhibiting Wnt-β catenin pathway. Notch mediated inhibition of canonical Wnt signalling is a recurrent motive during cardiomyocyte differentiation [133].

In zebrafish, the ratio of cardiac to blood/ endothelial cells during gastrulation is determined in part by the magnitude of fibroblast growth factor (FGF) signalling. FGF signalling indeed inhibits hemangioblast and favours myocardial development, through repression of the hemangioblast regulator *Lycat* and induction of *Nhx2.5* [103].

c. Transcription factors involved in hemangioblast commitment

A newly identified player in hematopoietic specification, the Ets TF ER71 (ETV2), appears to be critical for the specification of endothelial and hematopoietic lineages. Recent studies have placed ER71 at the top of the hierarchy in specification of these lineages and have identified some of the upstream regulators and downstream effectors of ER71 function using multiple model organisms and experimental systems [28, 70, 101, 102, 104, 134-138]. ER71 is expressed transiently prior to or at the time of $Flk\text{-}1^{+}$ expression within the mesoderm. Furthermore genetic fate map studies revealed that the ER71-expressing cells give rise to the hematopoietic and endothelial lineages through the generation of hemogenic endothelium [28, 115, 137]. *ER71*-deficient mice lack hematopoietic and endothelial lineages and display an upregulation of cardiac genes [28]. It functions downstream of the BMP, Notch and Wnt pathways activating the expression of several important TFs like *Scl*, *Gata2*, *Fli1* and *Lmo2* that are essential for hemangioblast development [28, 101, 135, 136]. In an inducible ESC/EB system

overexpression of *ER71* repressed cardiogenesis [101, 102, 104]. These studies identify ER71 as a critical regulator of mesodermal fate decisions that act to specify the hematopoietic and endothelial lineages at the expense of cardiac lineages.

Another essential factor for hemangioblast development is the bHLH TF Scl. In the developing mouse embryo *Scl* is expressed at E6.5 in the extra-embryonic mesoderm and continues to be expressed in the YS blood progenitors and endothelial cells [139]. At the stage of cluster formation and emergence of HSCs in the arteries, *Scl* is expressed in both the endothelium and the cluster cells [140]. *Scl* KO embryos die before E9.5, with a complete absence of YS primitive erythrocytes and myeloid progenitors [141] and $Scl^{-/-}$ ESCs fail to contribute to any of the hematopoietic lineages *in vitro* or *in vivo* [142]. In addition to the complete absence of hematopoietic cells, *Scl* null embryos fail to properly remodel the YS vasculature, suggesting a role for *Scl* in angiogenesis [5]. Several studies support the notion that Scl is critical for hemangioblast development. Using *Scl*/*hCD4* ESCs in which a non-functional human CD4 (hCD4) was knocked into the *Scl* locus, Chung *et al.* demonstrated that hemangioblasts can be identified as a Flk-$1^{+}Scl^{+}$ cell population [143]. Ema *et al.* [94] generated Flk-$1^{+}/^{Scl}$ ESCs and demonstrated that enhanced expression of *Scl* from the *Flk-1* locus produced an increased number of blast colonies as compared to wild-type ESCs. *Scl* deficient ESCs fail to generate blast colonies [76]. The absence of blast colonies is not due to the absence of BL-CFCs but rather to the inability of these cells to generate the hemogenic endothelium intermediate population [23]. Scl is therefore required for the maturation of hemangioblast into hemogenic endothelium [23, 141, 144]. Several studies also suggested an earlier role for Scl as ectopic expression of this TF has been shown to promote the patterning of mesoderm toward blood at the expense of other lineages, i.e. over-expression of *Scl* from EB day 2-4 from induction indeed leads to an increase in the Flk-1^{+} population [145, 146]. Scl can interact with various proteins, including E2A, the LIM-only protein Lmo2, the LIM domain-binding protein Ldb1 and Gata factors Gata1/Gata2 in multimeric complexes [147, 148]. Scl/Gata-containing complexes are known to bind to a composite E-box/GATA motif present in many erythroid-specific regulatory elements [149-153]. These complexes recruit cofactors such as p300, Gfi1b, Cbfa2t3 (Eto2), and Kdm1A [154-156] activating or repressing specific target genes. It is still not clear which factors are part of Scl-complexes in hemangioblast and what are the target genes of these complexes. Recent ChIP-seq experiments for Scl in progenitor cells have identified the cellular environment (chromatin accessibility and cell specific TFs) as an important factor in the genomic binding selectivity for Scl, with a complete different regulatory output for different cell types [152, 157].

BMP signalling cooperates with Wnt to promote blood fate through the activation of the *Cdx-Hox* genetic pathway giving rise to a BMP-Wnt-Cdx-Hox pathway which regulates hemangioblast commitment [117]. *Cdx* genes encode for caudal-related TFs that mediate anterior-posterior patterning through *Hox* genes regulation. Three *Cdx* genes (*Cdx1*, *Cdx2*, and *Cdx4*) have been identified to date in mammals with redundant functions [158-161]. Accumulating evidence suggests that the *Cdx* family members are involved in the proliferation and differentiation of hematopoietic cells. In the ESC/EBs system they are expressed during the emergence of BL-CFC [117, 162]. Overexpression of *Cdx4* or *Cdx1* from day 2-4 of differentiation and during the blast colony assay leads to an increase in BL-CFCs and hematopoietic progenitors [162, 163]. Using the same *in vitro* system Wang *et al.* demonstrated that deficiency of *Cdx* genes have only a transient negative effect during primitive hematopoiesis, which explains why *Cdx* KO mice have been reported not to have hematopoietic defects [164]. Ectopic expression and loss-of-function studies have implicated many *Hox* genes in normal hematopoiesis and leukemogenesis [165]. In a recent paper Oshima *et al.* performed a genome-wide analysis of target genes for one member of these family, *HoxB4*, showing that many of the genes essential for hemangioblast or HSC development, such as *Runx1*, *Scl* and *Gata2*, are indeed direct targets of HoxB4 [166].

Fli1 is an Ets family member expressed early during endothelial and hematopoietic

development [62, 167]. Enforced expression of *Fli1* in zebrafish induced expression of *Scl*, *Gata2*, and other hemangioblast markers but is not able to rescue the phenotype in *Scl* morphants. *Fli1* knockdown reduces the expression of *Gata2* along with *Scl*, *Lmo2*, while reduced expression of *Gata2* has no effect on *Fli1* expression. Furthermore the Fli1 morphant is rescued by a combination of Scl, Lmo2 and Gata2 mRNAs. These observations suggest that Fli1 acts upstream of these TFs in hemangioblast development [168]. Ets sites within gene enhancers for Gata2, Scl, and Fli1 itself are essential for their expression, and each of these enhancers is bound by Fli1 [168-170]. Analysis of the role of Fli1 in BL-CFC development would expand our understanding of its involvement in hemangioblast development.

Lmo2 is a zinc-finger, LIM-only protein. It does not directly bind DNA but interacts with TFs serving as a bridge protein within and between complexes [171]. Early hematopoietic development critically depends on a protein complex containing Scl and Lmo2 as *Lmo2* KO mice die due to failure of YS erythropoiesis [172] with a phenotype very similar to its interacting partner *Scl* KO mouse. Lmo2 and Scl function together at the hemangioblast level where they play a key role in the initiation of blood and vascular transcriptional programs [65]. The expression of *Lmo2*, like *Scl*, is under control of BMP [121] and FGF signalling pathways [173].

The zinc-finger TF Gata2 is also an important regulator of hemangioblast development. Gata2 is expressed at E7.5 in the extraembryonic YS. From E9, Gata2 expression is seen in the dorsal aorta, and at the time of hematopoietic cluster formation is found in the endothelium, the clusters, and in sub-aortic patches/para-aortic foci [174-177]. *Gata2*-null embryos die at E10.5 with severe anemia and with strong reduction in the number of primitive erythroid cells [178]. *Gata2* KO ESCs and YS suggest that Gata2 is required for expansion/ proliferation of the early, primitive hematopoietic compartment [179], and inhibits hematopoietic differentiation [176]. Forced expression of Gata2 suppressed expression of later hematopoietic TFs [180]. *Gata2*$^{-/-}$ ESCs in chimeric animals show no contribution to any hematopoietic compartments suggesting an essential role of Gata2 in definitive hematopoiesis. Several other papers confirm the role of Gata2 in the generation and maintenance of definitive HSCs [181, 182]. In EBs, *Gata2* expression reaches its maximum in the BL-CFC enriched population, and its expression is reduced during their differentiation into blast colonies. When overexpressed in serum-free cultures during primitive streak induction, Gata2 can increase the generation of the hemangioblast population. Enforced *Gata2* expression upregulates *Bmp4*, *Flk-1* and *Scl* [115]. Exactly opposite results are obtained using *Gata2*$^{-/-}$ ESCs, with a reduction in blast colonies and reduced expression of *Scl* and *Gata1*. These studies support the notion that Gata2 is involved in the specification of hemangioblast progenitors from early mesoderm. Gata2, Fli1 and Scl cross-regulate each other: Gata2 and Fli1 regulate *Scl* and *Fli1* expression by binding the Scl+19 and Fli+12 enhancer respectively [169, 183]. Gata2 is able to bind the Gata2-3 enhancer and positively autoregulates its own production [184]. The same enhancer is also bound by Fli1 and Scl [170]. This fully connected triad generates a positive feed-forward loop, that once activated is kept in a constant 'on' state due to the ability of these TFs to perpetuate the expression of each other. Therefore a transient activating signal is sufficient to initiate a specific transcriptional program that will be retained by the active Gata2/Fli1/Scl triad. BMP4 signalling might be the potential initiator of the circuit, as the *Gata2* promoter responds to BMP4 which is also able to initiate *Fli1* expression [185]. The possible scenario therefore is that BMP4 initiates *Gata2* and *Fli1* expression, which then combine to activate *Scl*. These three TFs can then maintain the expression of each other and activate a specific genetic program for the generation of hemangioblast and differentiation towards hemogenic endothelium [170]. One plausible way to disrupt this circuit and allow the cells to differentiate towards specific lineages is to diminish the concentration of these factors.

Gata1 has been shown to disrupt Gata2 positive auto-regulation by binding the Gata2-3 enhancer inducing chromatin changes that block *Gata2* expression [186]. Shutting down *Gata2* may be sufficient to disrupt the triad. At the erythroid stage Gata1 displaces Gata2 to become the dominant

factor to sustain *Scl* expression. *Gata1* and *Gata2* expression profiles are dynamic during erythroid differentiation and characterized by a reciprocal, although partially overlapping, pattern, responsible for the balance between proliferation and differentiation. *Gata2* gene is actively expressed in HSCs and early progenitors, but its downregulation is required for terminal differentiation [187, 188]. Gata1 is abundantly expressed during late stages of erythroid differentiation and is essential for primitive erythropoiesis in the YS [189]. Gata2 initiates *Gata1* gene expression [190], once activated, Gata1 turns off *Gata2* and accelerates its own expression. Furthermore Gata1 displaces Gata2 from chromatin sites, often (but not always) instigating a distinct transcriptional output [186, 191, 192], this "Gata switch" leads to terminal erythroid differentiation. A recent investigation on Gata switch using an erythromegakaryocytic progenitor cell line, showed that in both cases Gata1 and Gata2 control an overlapping genetic program [193]. It would therefore be interesting to investigate the Gata switch during hemangioblast development.

3. Molecular regulation of hemogenic endothelium specification towards hematopoietic lineage

a. Pathways involved in hemangioblast/hemogenic endothelium differentiation

The transition of hemangioblasts to hematopoietic progenitors requires the vascular endothelial growth factor (VEGF) as, in its absence, hematopoietic progenitors do not arise [29, 30, 114]. Heterozygous inactivation of the *Vegf* gene results in impaired development of the vascular and hematopoietic systems [194, 195]. VEGF production from the YS visceral endoderm is sufficient and necessary for blood island formation and for vascular development [196] and it appears to act by sustaining the expression of the hematopoietic genes *Scl*, *Fli1* and *Lmo2* that have been initiated during the generation of the Flk-1^{+} mesoderm/ hemangioblast [30]. Park *et al.* showed that VEGF function was required within a narrow window of time: the presence of VEGF between days 3 and 4 of EBs differentiation was sufficient for subsequent hematopoietic and endothelial cell differentiation. However activation of VEGF signalling between day 5 and 8 negatively influences blood development and promotes endothelial development [110]. This late negative function of VEGF on hematopoietic development is consistent with recent data showing that overexpression of VEGF in the erythroid lineage blocks the differentiation of both primitive and definitive erythroid progenitors through decreased expression of *Gata1* [197].

Another important player in hematopoiesis is *TGF-ß1*, which is expressed in YS blood islands, mesodermal cells of the allantois, and cardiogenic mesoderm of the embryo [198]. *TGF-ß1*$^{-/-}$ mice displayed YS anemia due to a severe reduction of erythrocytes and a defect in endothelial cell differentiation [199], while *TGF-ßrI* KO mice displayed increased number of erythroid progenitors [200]. Park *et al.* showed that TGF-ß1 inhibited BMP4 and VEGF-mediated hematopoietic induction in the ESC-EB system [110]. Therefore TGF-ß1 signalling seems to be necessary for normal vascular development, with an inhibitory role for the differentiation of hematopoietic progenitors.

The role of FGF and FGF receptor signalling on hematopoietic and endothelial cell differentiation is still controversial. Loss of FGFR1 function studies in ESC showed that FGFR1 signalling is required for hematopoietic but not endothelial development [201]. In contrast, in the chick, high FGF activity inhibits primitive hematopoiesis and promotes an endothelial cell fate, whereas inhibition of FGFR activity leads to ectopic blood formation and down-regulation of endothelial markers [202]. This controversial result could be partly explained by a feedback system activated by high level of FGF with subsequent inhibition of hematopoiesis [203].

The thrombin receptor (F2r) signalling pathway might be also important in keeping the balance between hematopoietic and vascular development. F2r receptor KO leads to vasculature damage and severe bleeding [204]. Through *in vitro* and *in vivo* experiments, Yue and colleagues showed that the F2r-RhoA/ROCK pathway negatively regulates the emergence of hematopoietic progenitors from hemogenic endothelium [205].

The antagonistic action between Wnt and Notch signalling pathways is interesting as a potential

regulator of choice between the generation of primitive and definitive hematopoietic precursors from the hemangioblast. Nostro *et al.* used the ESC differentiation system and demonstrated that activation of a Wnt/β-catenin signalling pathway is essential for the establishment of the primitive, but not the definitive, hematopoietic lineage [29]. Activation of Notch signalling has an opposite effect. Indeed, activation of Notch signalling at the hemangioblast stage leads to an increase in the expression of Wnt inhibitors and a consequent reduction in Wnt signalling thus blocking the production of primitive erythroid cells. In contrast, overexpression of *Numb* (an inhibitor of the Notch pathway) at the same stage, leads to reduced Wnt inhibitor expression, enhanced levels of Wnt signalling and therefore increased generation of primitive erythroid cells [206]. Several studies show that Notch1 is essential for the generation of definitive hematopoietic cells from hemogenic endothelium in the mouse embryo but not for the generation of primitive blood cells in the YS [207-211]. During the differentiation of hemangioblast towards blast colonies there is a transition from Wnt to Notch signalling and Notch seems to restrict the window of Wnt signalling and thereby regulating the primitive erythroid phase of hematopoiesis [206]. This antagonistic action between Wnt and Notch signalling pathways might determine the choice between the generation of primitive or definitive hematopoietic precursors from the hemangioblast.

b. Transcription factors involved in hemangioblast/hemogenic endothelium differentiation

Runx1, a Runt domain TF, is one of the most important factor regulating the endothelial-to-hematopoietic transition. Runx1 is first detectable in the extraembryonic mesodermal cells and then in both primitive erythrocytes and endothelial cells in the YS blood islands at E8.0. In the embryo proper, Runx1 is expressed at E9.5-10.5 in both endothelial and mesenchymal cells in the dorsal aorta and placental labyrinth [212-214]. Runx1 is expressed in all definitive HSCs [215, 216].

Homozygous null *Runx1* mutants died between E12.5 and E13.5. They still exhibit primitive hematopoiesis in the YS, however they do not develop definitive hematopoietic cells [4]. *In vivo* targeting studies in the mouse demonstrated that *Runx1* is indeed essential for the generation of definitive hematopoietic progenitors and hematopoietic stem cells, while primitive erythropoiesis is only slightly affected [217, 218]. *In vitro* experiments showed that, in the absence of Runx1, only few blast colonies are generated and the few hematopoietic cells produced are restricted to a primitive erythroid fate [213]. As a result of Runx1 deficiency the process of hemangioblast development is blocked at the stage of hemogenic endothelium. Reactivation of *Runx1* expression in this cell population leads to the generation of definitive hematopoietic progenitors and the concomitant down-regulated expression of endothelial markers [23]. *Runx1* overexpression in hemangioblasts indeed represses endothelial genes [219]. Therefore comprehensive *in vitro* and *in vivo* studies of Runx1 protein, show that Runx1 is indispensable for the development of blood stem/progenitor cells from hemogenic endothelial cells. It is also a direct target of BMP signalling [116, 213]. In a recent paper Tanaka *et al.* used ESC differentiation as a model system to define the transcriptional programme controlled by this TF during the emergence of blood progenitor cells from the hemogenic endothelium. Runx1 upregulates genes which drive hematopoietic development while simultaneously repressing genes associated with non-hematopoietic tissue development. They also showed that the majority of hematopoietic genes induced by Runx1 are also controlled by the Scl/Gata2/Fli1 triad. In the hemangioblast stage (see paragraph 2c) this triad is not sufficient to initiate their expression, but it probably recruits Runx1 to its gene targets as soon as it starts to be produced. Therefore hematopoietic genes induced by Runx1 will ultimately be controlled by both Runx1 and the Scl/Gata2/Fli1 triad [220].

Another important factor in the regulation between hematopoietic and endothelial fate is *HoxA3* a member of the Homeobox (Hox) gene family. A recent study by Iacovino *et al.* revealed that *HoxA3* and *Runx1* present a pattern of mutually exclusive expression at hematopoietic and vascular sites. In the early embryo HoxA3 expression is restricted in the developing embryonic aorta while Runx1 is expressed in the extra-embryonic YS.

In later stages, HoxA3 disappears from the aortic endothelium prior to Runx1 expression and the activation of the hemogenic program [221]. In the EB system enforced expression of HoxA3 at the hemogenic endothelium stage suppresses hematopoietic differentiation while retaining its endothelial nature. This block in hematopoietic differentiation is reversible upon Runx1 over-expression [221]. HoxA3 directly represses the expression of *Runx1* which otherwise would promote hematopoietic development. It raised the question why this antagonistic action is present only in the AGM region and not in the YS. In the YS mesoderm, where *HoxA3* is not expressed, hematopoietic and endothelial lineages emerge simultaneously giving rise to the blood islands. Hematopoietic differentiation from the lateral plate mesoderm is delayed and the HoxA3 expression in the early embryonic endothelium of the dorsal aorta restrains hematopoietic differentiation by maintaining the expression of endothelial-specific genes ensuring the right timing for the emergence of HSCs within the embryonic vessels. Accordingly, *HoxA3*-null mouse embryos show premature and increased formation of $Runx1^+$ cells in the dorsal aorta.

Another member of the Homeobox (Hox) gene family which might be involved in this stage of hemangioblast development is *Hex*. It is expressed in the BL-CFCs and acts as a negative regulator of the hemangioblast and its differentiation into definitive hematopoietic and endothelial cells [222]. However, Guo *et al.* reach a different conclusion: *Hex* is not expressed in the BL-CFCs and dispensable for the formation of the hemangioblast, but is required for its differentiation into hematopoietic and endothelial lineages [223]. Mouse *Hex* is expressed in extraembryonic YS mesoderm during gastrulation but in $Hex^{-/-}$ mouse endothelial and hematopoietic lineages appear not to be affected [224].

CONCLUSION

Development of the hemangioblast defines the onset of hematopoiesis. Recent exciting findings have begun to shed light on the molecular and cellular pathways leading from mesoderm to hemangioblast and hematopoietic/endothelial cell lineage differentiation. The ESC model, which accurately recapitulates the developmental events observed in the embryo, has been shown to be a useful tool for dissecting the different stages of this complex developmental process.

The commitment and differentiation of the hemangioblast is the result of the coordinated activity of multiple signalling pathways. Some of them are involved in several developmental stages, such as BMP, Wnt, activin/Nodal and Notch, sometimes with different or opposite outputs. Other pathways, such as VEGF, are instead stage-specific. We have highlighted how the effects of some signalling pathways are transient and restricted to a specific window of development and that most of them can interact with one or another, both synergistically and antagonistically, in order to activate a specific genetic program. The activation of the genetic program is mediated by specific TFs which form active and/or repressive complexes governing gene expression patterns in differentiated cells. Several TFs are already known to be involved in hemangioblast development and more will probably be discovered. The next important step will be to determine how these TFs interact together in transcriptional network to orchestrate the progressive specification of blood and vascular progenitors. Analysis of the composition of these molecular complexes and investigation of downstream target genes of TFs in specific stages of hemangioblast development might provide important insights on this issue.

All the different steps of the multi-level regulation of gene expression which are between the activation of specific signalling pathways and the downstream action of specific TFs remain to be investigated. Specificity of receptor/ligand interactions, negative or positive regulatory loops within pathways, inter-relationships between pathways, activation and post-translational modifications of TFs, which drive hemangioblast development, still represent question marks and provide future challenges in the attempt to fully understand the commitment to the hematopoietic and endothelial lineages.

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