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Dissecting hematopoietic differentiation using the embryonic stem cell differentiation model

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ABSTRACT Embryonic stem cells (ESCs) have been successfully used to study the generation of the hematopoietic lineage. The ESC differentiation model provides access to distinct developmental stages during hematopoietic differentiation enabling us to study developmental transitions in a manner that is difficult to do with embryos. The identification of the bipotential hemangioblast/blast-colony forming cell (BL-CFC) which represents the earliest stage of hematopoietic commitment in ESC cultures has enabled the study of signalling pathways, transcription factors and enzymes at the level of this developmental stage. Reporter ESC lines, flow cytometry and serum-free culture reagents are helping the field to transition from serum-containing protocols to stepwise serum-free differentiation strategies that attempt to mimic the developmental processes in the embryo. This serves as a framework with which to approach directed differentiation of human ESCs for the purposes of regenerative medicine. This review is focused on the contributions that the ESC differentiation system has made to understanding hematopoiesis and will highlight the strengths of this model of development and the challenges it still faces.

KEY WORDS: *embryonic stem cell, hemangioblast, hemogenic endothelium, differentiation, hematopoiesis*

Introduction

Developmental biology is the study of cellular decisions and molecular programs that lead to the formation of the whole organism. This body of knowledge is comprised of discoveries from a variety of model systems such as the chick, *Xenopus*, zebrafish and mouse. One can add to this group of vertebrate models the embryonic stem cell (ESC) differentiation system where development is studied as it occurs *in vitro*. ESCs are the undifferentiated, self-renewing cell lines derived from the inner cell mass of the blastocyst stage embryo (Evans and Kaufman, 1981; Martin, 1981). They have the potential to give rise to the various cell types of the organism when cultured in appropriate tissue culture conditions. The ability to access cells at different stages of development and to use a combination of genetic, molecular biology and tissue culture tools has led to a wide range of perturbations that help us understand developmental processes. This is particularly helpful to the study of human development as human ESC (hESC) differentiation cultures can serve as a powerful surrogate for human embryos.

The study of the hematopoietic system using ESCs shows the power of using this *in vitro* model to understand development. Tools such as *in vitro* and *in vivo* hematopoietic assays provide the functional and quantitative analysis to strengthen conclusions made from hematopoietic gene expression analysis. Flow cytometry has also been helpful not only to demonstrate the presence of hematopoietic lineages but also to follow and isolate early developmental stages. Interest in the ESC system has been greatly fuelled by its potential to supply cells for cell replacement therapy in the treatment of a wide range of diseases. This has also been heightened by the advent of induced pluripotent stem (iPS) cells where a somatic cell can be reprogrammed into an ESC-like state (Okita *et al.*, 2007; Park *et al.*, 2008; Takahashi *et al.*, 2007; Takahashi and Yamanaka, 2006; Wernig *et al.*, 2007; Yu *et al.*, 2007). This was first demonstrated through the forced expression of Oct4, Sox2, c-Myc, and Klf4 in murine fibroblasts and now can be performed with two factors (either Oct4/Klf4 or Oct4/Sox2) and the addition of small-molecule compounds (Huangfu *et al.*, 2008; Okita *et al.*, 2007; Shi *et al.*, 2008; Takahashi and Yamanaka, 2006; Wernig *et al.*, 2007). This raises the possibility of generating patient-specific iPS cells that as with ESCs would then be differentiated towards the cell type of therapeutic interest. The hemato-

Abbreviations used in this paper: BL-CFC, blast-colony forming cell; ESC, embryonic stem cell; iPS induced pluripotent stem cell; YS, yolk sac.

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poietic system offers us a clear indication of the potential for this with the established example of hematopoietic stem cell (HSC) transplantations for the treatment of hematopoietic disorders. There is therefore an intense effort to direct ESCs towards the HSC fate. Key to the success of this is our ability to recapitulate in culture the developmental processes involved in generating functional HSCs.

From gastrulation to the embryonic sites of hematopoiesis

Hematopoietic cells originate in multiple sites during embryogenesis: the yolk sac (YS), aorta-gonad-mesonephros (AGM) region, placenta and the vitelline and umbilical arteries (Dzierzak and Speck, 2008). These cells are derived from mesoderm, one of the three primary germ layers of the embryo. This layer along with endoderm and ectoderm is established during a developmental process called gastrulation. In the mouse, cells in the posterior region of the cup-shaped epiblast layer start the process by moving through a transient structure called the primitive streak (PS). As development proceeds the PS extends towards the anterior part of the embryo with mesoderm and endoderm exiting in a temporally and spatially regulated manner (Lawson *et al.*, 1991). Ectoderm is formed by cells from the epiblast that do not traverse the PS.

The posterior PS gives rise to the extraembryonic mesoderm which contributes to the hematopoietic cells and vascular network of the YS, the embryonic contribution to the placenta, the umbilical artery and vein, and the amnion (Kinder *et al.*, 1999; Lawson *et al.*, 1991). The first hematopoietic cells to be made are the primitive erythroid progenitors which appear between embryonic day 7.0-9.0 (E7.0-9.0). Macrophage progenitors appear at about the same time as primitive erythroid progenitors reaching a peak at E9.5. The definite erythroid progenitors follow at E8.25 and definitive progenitors such as mast colony-forming cell (Mast-CFC) and bipotential granulocyte/macrophage (GM-CFC) progenitors are detected at E8.5 (Palis *et al.*, 1999). These progenitors enter circulation and begin to colonize the fetal liver by late E9.0 (Johnson and Moore, 1975; Lux *et al.*, 2008). By E9.0 a multilineage repopulating stem cell from the YS can be detected using a neonatal reconstitution assay (Yoder *et al.*, 1997). These cells when injected into the liver of conditioned neonates give multilineage engraftment; however they will not do so in an adult repopulating assay which suggests that they are deficient in their ability to home, expand and/or survive in the bone marrow. It is qualitatively different from the adult repopulating HSC that appears in the YS at around E11.0 (Moore and Metcalf, 1970; Muller *et al.*, 1994). Since other sites in the embryo contain HSCs prior to the YS, it has been assumed that they arrive there by circulation (Medvinsky and Dzierzak, 1996).

The intraembryonic AGM develops from the para-aortic splanchnopleura (P-Sp) which when isolated prior to circulation and cultured as an explant for a few days reveals the presence of a cell that gives low but multilineage repopulation in irradiated immune-deficient Rag2$\gamma c^{-/-}$ recipients (Cumano *et al.*, 2001). This study demonstrates that the P-Sp/AGM comprises an intraembryonic site of hematopoiesis. At E9.0 neonatal repopulating cells can be detected in the P-Sp (Yoder *et al.*, 1997). Then prior to HSC appearance in the YS, they can be found in the AGM at E10.5 (de Bruijn *et al.*, 2000; Medvinsky and Dzierzak, 1996; Muller *et al.*, 1994). Taken together with the observation that the AGM is not a site for hematopoietic progenitor differentiation (Godin *et al.*, 1999) the P-Sp/AGM can be considered a site that generates cells with repopulating potential (Dzierzak and Speck, 2008).

The placenta, which was initially identified as a niche for HSCs appears to be yet another site of hematopoietic generation (Alvarez-Silva *et al.*, 2003; Gekas *et al.*, 2005; Ottersbach and Dzierzak, 2005; Rhodes *et al.*, 2008; Zeigler *et al.*, 2006). The placenta along with the vitelline and umbilical arteries, which connect it to the embryo, harbor HSCs at the same time that they appear in the AGM (de Bruijn *et al.*, 2000; Gekas *et al.*, 2005). By using a mouse that lacks circulation it could be shown that the placenta can give rise to erythroid, myeloid and lymphoid lineages, a hallmark of the multipotential HSCs (Rhodes *et al.*, 2008). Further *in vivo* studies on these placental cells will address their true HSC potential. The mesodermal component of the placenta is comprised of both chorionic and allantoic mesoderm, while that of the vitelline and umbilical arteries is from allantoic mesoderm (Downs, 2002). When isolated from embryos prior to the chorioallantoic fusion necessary to form the placenta, both the chorion and allantois showed hematopoietic potential (Corbel *et al.*, 2007; Zeigler *et al.*, 2006). Together this data point to the placenta as another site of hematopoietic cell generation.

If we consider the PS origins of the different hematopoietic sites we see that the chorion, YS blood islands and allantois are populated by the extraembryonic mesoderm which migrates early from the posterior PS and could contribute to these structures in an overlapping time window (Kinder *et al.*, 1999). The P-Sp/AGM is generated from lateral plate mesoderm which migrates from the PS later during gastrulation and tends to emanate from more anterior positions in the PS than the extraembryonic mesoderm (Kinder *et al.*, 1999). The spatial and temporal generation of mesoderm in the PS may thus play an important role in determining the kinds of hematopoietic cells that develop. The subsequent migration of the mesoderm into different embryonic locations provides addition environmental cues that could influence the timing of hematopoietic cell appearance and the type of hematopoietic cell that develops. For example, the mesoderm that gives rise to the YS blood islands and placenta are potentially exiting the streak at the same time however the environment in the YS versus the allantois or chorion may influence further hematopoietic differentiation (Fig. 1).

To understand the transition of mesoderm to hematopoietic cells, intermediate stages need to be identified and characterized. Many years ago, a cell called the hemangioblast was hypothesized to exist as a precursor to the hematopoietic and endothelial lineages on observation of the close physical proximity of these lineages in the chick YS (Sabin, 1920). That hematopoietic and endothelial cells share markers in common and are both affected by the deletion of certain genes lent further support to this hypothesis (Carmeliet *et al.*, 1996; Ferrara *et al.*, 1996; Fong *et al.*, 1996; Kabrun *et al.*, 1997; Kallianpur *et al.*, 1994; Millauer *et al.*, 1993; Shalaby *et al.*, 1997; Shalaby *et al.*, 1995; Watt *et al.*, 1995; Yamaguchi *et al.*, 1993; Young *et al.*, 1995). The appearance of hematopoietic cell clusters juxtaposed to endothelium in the AGM, the vitelline and umbilical arteries and most recently in the placenta has also suggested a close relationship between

these lineages (de Bruijn *et al.*, 2000; Jaffredo *et al.*, 2005; Rhodes *et al.*, 2008). These observations have been used to propose the existence of specialized endothelial cells with hematopoietic potential called hemogenic endothelium. The hematopoietic cell clusters of the AGM and underlying endothelium express some common markers (CD31, VE-cadherin, Sca-1, Runx1) (de Bruijn *et al.*, 2002; Garcia-Porrero *et al.*, 1998; Nishikawa *et al.*, 1998a; North *et al.*, 1999). Recently, using an inducible VE-cadherin Cre mouse line crossed to a Rosa26R Cre reporter mouse line it was shown that VE-cadherin+ endothelium is the source of HSCs in the AGM (Zovein *et al.*, 2008). Indeed when *Runx1* is deleted in this population, HSCs do not develop (Chen *et al.*, 2009). In contrast, when Runx1 is deleted in hematopoietic cells in the embryo, using the Vav-Cre mouse, HSC generation occurs. This indicates that Runx1 activity essential to hematopoietic development is absolutely required in an endothelial cell (Chen *et al.*, 2009).

An area termed the subaortic patch (SAP) that lies under the dorsal aorta has also been proposed as a source of the HSCs (Bertrand *et al.*, 2005). To address the question of a subaortic origin of the HSCs, SM22α Cre and myocardin Cre mouse lines were used where the former enabled the marking of an early and late mesodermal population while the latter led to the labelling only of the late mesodermal population. Only the early labelled mesodermal population, which could give rise to aortic endothelium, contributed cells to the adult bone marrow (Zovein *et al.*, 2008). For subaortic patches to contribute to AGM HSCs, given the findings above, they would have to exist in the early labelled mesodermal population and give rise to HSCs that upregulate VE-cadherin as they emerge through the endothelium of the aorta (Chen *et al.*, 2009; Zovein *et al.*, 2008).

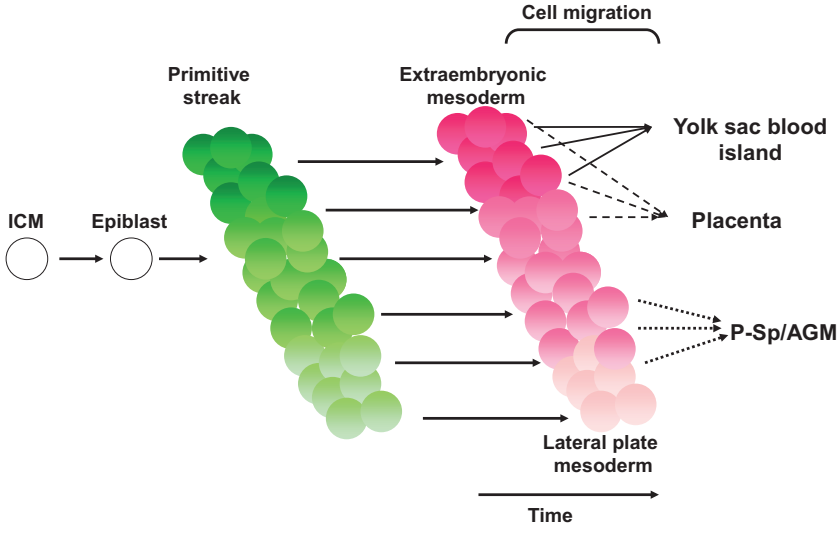

Fig. 1. The generation of embryonic hematopoietic sites from mesoderm. *The yolk sac blood islands, placenta and P-Sp/AGM are derived from mesoderm that develops from the PS. The first mesoderm is extraembryonic and migrates away from the posterior primitive streak to populate the chorion, YS, allantois and amnion. The mesoderm that contributes to the YS and placenta migrate in an overlapping time window (as indicated by overlapping arrows). The P-Sp/AGM is derived from lateral plate mesoderm that migrates later in time from the PS. How these mesoderm populations differ from each other is not know and may be important to the hematopoietic programs that develop from the different hematopoietic sites.*

The strong genetic evidence described here for the existence of hemogenic endothelium is complemented by studies using the ESC differentiation system that support the existence of the hemangioblast and hemogenic endothelium (see later). The identification of these developmental intermediates has enabled researchers to address the mechanism of hematopoietic commitment.

ESC differentiation to hematopoietic lineages

ESCs self-renew or differentiate based on the culture conditions they are placed in. They can be maintained in their pluripotent state when grown on a layer of feeder cells in the presence of leukemia inhibitory factor (LIF) and serum (Chambers and Smith, 2004; Smith *et al.*, 1988; Williams *et al.*, 1988). Differentiation protocols have typically involved the removal of ESCs from this environment and the subsequent culture of these cells in suspension as aggregates known as embryoid bodies (EBs) or as adherent monolayers on either extracellular matrices or stromal cell lines. All of these culturing approaches, sometimes in combination have been used in protocols to generate hematopoietic cells. The popularity of the EB method can be attributed to the observation that development proceeds well when cells are in a three-dimensional structure, presumably as it offers an environment akin to embryonic development. Culturing ESCs as adherent layers on extracellular matrices such as collagen IV offers better access to the cells, not only to expose them to more consistent culture conditions but also to visualize them as they develop. Supportive stroma derived from adult bone marrow, fetal liver and AGM are used in the hematopoietic field to promote the differentiation, proliferation and maintenance of *ex vivo* hematopoietic cells (Moore *et al.*, 1997; Nakano *et al.*, 1994; Oostendorp *et al.*, 2002; Weisel *et al.*, 2006; Xu *et al.*, 1998). Some of these stromal cell lines have been successfully used to promote ES cell differentiation to hematopoietic cells.

Protocols that allow for the production of murine and human primitive and definite hematopoietic cell lineages have been reviewed recently and will not be discussed in detail here (Murry and Keller, 2008; Olsen *et al.*, 2006; Orlovskaya *et al.*, 2008; Tian and Kaufman, 2008). ESCs are generally differentiated as EBs or on stroma in the presence of cytokines that promote specific lineages. For example, ESCs will develop into T lymphoid cells on coculture with OP9 cells modified to express the Notch ligand Delta-like 1 and in the presence of IL7 and Flt3 ligand (Schmitt *et al.*, 2004). The ability to generate definitive erythroid, myeloid and lymphoid lineages from ESC cultures suggests that HSCs are being induced; however protocols describing HSC differentiations have not gained wide usage (Burt *et al.*, 2004; Tian *et al.*, 2006; Wang *et al.*, 2005a). Recently, stroma derived from mouse AGMs was able to support the development of hematopoietic cells from human ESCs that could repopulate the immune compromised NOD/LtSz-*Scid*/IL2$\gamma^{-/-}$ mouse (Ledran *et al.*, 2008). The cells showed serial repopulation activity; however they

display an embryonic and fetal globin profile in culture. Further studies are needed to study the multilineage potential of these cells. Common to this and other studies on ESC-derived repopulating cells is the direct injection of cells into the femur when performing mouse transplantations. This highlights the concern that differentiation protocols still have to provide the appropriate maturation of cells for successful engraftment and bypass problems such as cell aggregation based emboli formation. While it is helpful to work with stroma that mimic the embryonic HSC niche, efforts to identify the stromal components that support the maintenance and differentiation of hematopoietic cells will aid in developing defined differentiation protocols that do not require cell coculture steps. The study by Ledran and colleagues identified TGFβ1 and TGFβ3 as factors involved in positively regulating the hematopoietic differentiation from hESCs. Thus far multilineage reconstitution in the mouse has been achieved with ESC-derived hematopoietic cells generated by overexpression of the *Hox* family member, *HoxB4*, and caudal-related transcription factor *Cdx4* (Wang *et al.*, 2005b). In brief, there are protocols available to generate different hematopoietic lineages. However defined, serum-free and coculture-free protocols to reproducibly generate robust levels of adult repopulating HSCs from genetically unmanipulated ESCs are still lacking.

Tools of ESC differentiation

As a developmental system the ESC differentiation model has some clear advantages. The high proliferative potential of ESCs allows for experiments on a scale that is not feasible with embryonic tissues. In order to establish the role of a particular gene or signalling pathway, the investigator has various ways to adjust gene expression or modulate the activity of a signalling pathway. Of particular help are ESC lines that allow for the inducible expression of a gene of interest by the addition of doxycycline, a tetracycline analog (Kyba *et al.*, 2002). This is a powerful resource as gene overexpression can be limited to the time window of interest. Loss of function experiments are performed using standard knockout cell lines, siRNA transfection for knock down of RNA transcripts and inducible siRNA cell lines to control the timing of RNA knockdown (Lohmann and Bieker, 2008; Wang *et al.*, 2007b). Signalling pathways are stimulated through the addition of growth factors or agonists and blocked by addition of natural-occurring inhibitors, chimeric receptors, or small inhibitory compounds. When these tools are used together they can establish how factors mediate their effects and whether they act through the induction of other signalling pathways.

Flow cytometry has been used both in the hematopoietic and ESC differentiation systems to determine cell diversity in cultures, track differentiation progress, and isolate and identify distinct cell populations. The ability to assess early stages of ESC differentiation is of particular use to the understanding of lineage commitment decisions. When tracking mesoderm development one can use the expression of the VEGF receptor, Flk-1, and/or the PDGF receptor alpha, PDGFRα. Nascent mesoderm expresses both markers but on further commitment lateral plate and extraembryonic mesoderm down-regulate PDGFRα and continue to express Flk-1, while paraxial mesoderm down-regulate Flk-1 and maintain PDGFRα expression (Ema *et al.*, 2006; Sakurai *et al.*, 2006; Takakura *et al.*, 1997). Lineage tracing experiments confirm that hematopoietic, cardiac, somatic and smooth muscle all transition through cells that expressed Flk-1 at some point during development (Ema *et al.*, 2006; Lugus *et al.*, 2008). To use a cell-type specific marker that is not on the cell surface one can use a reporter ESC line where a fluorescent protein (e.g. eGFP) or a cell-surface molecule from a different species (e.g. truncated human CD4) is expressed from the marker's promoter (Chung *et al.*, 2002; Fehling *et al.*, 2003; Gadue *et al.*, 2006; Ng *et al.*, 2005; Tada *et al.*, 2005). The development of PS-like cells in ESC differentiation cultures has been tracked using eGFP driven from the promoters of PS markers *brachyury* and *mixl.1* (Fehling *et al.*, 2003; Ng *et al.*, 2005). Both groups observed a GFP+ population by day 2.5 of EB differentiation that increased and peaked by day 4 where they comprise 85% of cells. The levels of GFP then fall on further EB differentiation which is consistent with cells transiting out of the PS stage as observed in the mouse embryo. The PS streak population can be further separated into anterior and posterior PS based on the expression of the anterior PS gene, Foxa2. Human CD4 cDNA was targeted to the *foxa2* locus in the GFP-Bry ESC line which allows for the identification of anterior PS (GFP-Bry^{+}CD4-Foxa2^{hi}) and posterior PS (GFP-Bry^{+}CD4-Foxa2$^{lo/-}$) populations in EB cultures (Gadue *et al.*, 2006).

Recently efforts have moved towards using defined and optimized culture conditions for maintaining and differentiating ESCs. The use of serum-free media and growth factors and/or small molecules removes the need to work with serum, a component that is prone to variation and can pose a challenge for labs when they attempt to replicate other groups' protocols. The self-renewal of ESCs can be sustained in the absence of feeders and serum, by the addition of BMP4 and LIF to serum-free media (Qi *et al.*, 2004; Ying *et al.*, 2003). And more recently a combination of three inhibitors termed 3i that inhibit FGF signalling and glycogen synthase kinase 3 (GSK3) activity were found to support ESC maintenance (Ying *et al.*, 2008). FGF4 is produced by ESCs and is needed for cells to respond to differentiation signals while GSK3 activity in ESCs appears to negatively influence their metabolic state (Kunath *et al.*, 2007; Ying *et al.*, 2008). Serum-free differentiation protocols for specific tissue types are being developed by incorporating what is known from studies in various developmental systems. This developmental biology approach has been successfully applied to the differentiation of the YS hematopoietic program from ESCs (see later in this review). By providing the appropriate signals at levels and with the timing reflective of the embryonic environment through combinations of growth factors/small molecules, and extracellular matrices one can theoretically promote the hematopoietic programs of the YS, P-Sp/AGM, placenta and vitelline/umbilical arteries. This approach is relevant to the efforts to generate HSCs from ESC cultures.

Characterizing the hemangioblast and hemogenic endothelium

The ESC differentiation system provided the first functional evidence for the existence of the bipotential hemangioblast. A progenitor with both endothelial and hematopoietic potential arises when ESCs are differentiated as EBs or as a monolayer on collagen IV (Choi *et al.*, 1998; Nishikawa *et al.*, 1998b). This cell appears earlier than hematopoietic progenitors which are typically detected after day 5 of ESC differentiation. A methycellulose-

based colony assay supplemented with VEGF, IL6 and the supernatant of an endothelial cell line called D4T, allows for the culture and quantification of hemangioblasts, which form a morphologically distinct colony called the blast colony (Choi *et al.*, 1998; Kennedy *et al.*, 1997). Clonal analysis demonstrated that the colonies were derived from a single cell termed the Blast-Colony Forming Cell (BL-CFC) and represents the *in vitro* hemangioblast. Recently, a serum-free blast colony assay was developed using ten cytokines (KL, IL3, BMP4, IL11, EPO, VEGF, LIF, IL6, bFGF, TGFβ1) which replaced the need for serum and D4T supernatant (Cheng *et al.*, 2008). The ability to capture this developmental stage provides a means to characterize it and determine the factors needed for its generation and further differentiation.

BL-CFCs/hemangioblasts are found in the Flk-1+ mesoderm population that develops in EBs (Faloon *et al.*, 2000; Fehling *et al.*, 2003; Nishikawa *et al.*, 1998b). This is consistent with the findings that the cells of the YS blood island go through a Flk-1+ cell stage during development (Ema *et al.*, 2006; Lugus *et al.*, 2008). The BMP signalling pathway has been implicated in the generation of BL-CFCs and the regulation of key hematopoietic transcription factors. Its downstream effector Smad1 is more highly expressed in the BL-CFC-enriched population (Bry-GFP$^+$Flk-1$^+$) compared to PS (Bry-GFP$^+$Flk-1$^-$) and non-PS (Bry-GFP$^-$Flk-1$^-$) EB cells (Zafonte *et al.*, 2007). Overexpression of Smad1 in the presence of serum during PS induction and patterning (EB day 2-2.25) led to a statistically significant upregulation of the hematopoietic transcription factors GATA2, Runx1 and SCL by day 2.25, a subsequent increase in Flk-1 expression by day 3 and a 5-fold increase in BL-CFCs by day 3.75 compared to uninduced cells. GATA2 is expressed in a BL-CFC enriched population (Flk-1+Scl+ cells from day 2.75 EBs) and is downregulated in the progeny of the BL-CFCs, the blast colonies. When overexpressed in serum-free cultures during PS induction and patterning, GATA2 can increase the Flk-1+ population and lead to increased primitive erythroid progenitors (Lugus *et al.*, 2007). The basic helix-loop-helix transcription factor, SCL is essential for the commitment of BL-CFCs to the hematopoietic fate. In its absence, blast colonies do not develop in the blast assay; this is not because BL-CFCs are absent but rather that they can not give rise to hematopoietic progeny and thus do not form recognizable blast colonies (D'Souza *et al.*, 2005). Overexpression of SCL from EB day 2-4 leads to an increase in the Flk-1+ population and from EB day 3-4 gives rise to higher levels of the hematopoietic markers CD41 and CD45 at EB day 6 (Ismailoglu *et al.*, 2008). The Ets family member ER71, is also expressed in BL-CFC enriched populations and on over-expression in serum-free conditions from EB day 2-4 will lead to induction of a Flk-1+ cell population and from EB day 3 will lead to the generation of hematopoietic and endothelial cells(Lee *et al.*, 2008; Lugus *et al.*, 2007).

GATA2, SCL and ER71 appear to have overlapping effects when overexpressed as EBs during PS induction and patterning. Interestingly, GATA2, SCL, and the Ets family member, Fli1, and their enhancers *Gata2*-3, *Scl+19* and *Fli1+12* comprise a hemangioblast and hematopoietic stem cell gene regulatory network (GRN) kernel (Liu *et al.*, 2008; Pimanda *et al.*, 2007b). GRN kernels are conserved subcircuits comprised of regulatory genes and their target enhancer elements, and can serve key instructive functions during development (Davidson and Erwin, 2006; Hinman *et al.*, 2003). The expression levels of *gata2*, *scl* and *fli1* are higher in the BL-CFC-enriched population (Bry-GFP$^+$Flk-1$^+$) compared to PS (Bry-GFP$^+$Flk-1$^-$) and non-PS (Bry-GFP$^-$Flk-1$^-$) EB cells (Pimanda *et al.*, 2007b). Chromatin immunoprecipitation experiments show that the three transcription factors bind to their respective enhancers in day 3 EBs indicating that these sites are bound at a time BL-CFCs are present. The loss-of-function phenotype for ER71 shows that it is essential for both hematopoietic and endothelial cell development in the YS; however a similar result was not found for Fli1 (Hart *et al.*, 2000; Lee *et al.*, 2008; Spyropoulos *et al.*, 2000). This suggests that ER71 is a critical Ets family member and may be part of the hemangioblast GRN kernel. This is in contrast to findings in zebrafish and *Xenopus* where Fli1 appears to be a master regulator for hematopoietic and endothelial development (Liu *et al.*, 2008). Analysis of the role of Fli1 in BL-CFC development would expand our understanding of its involvement in the hemangioblast GRN kernel.

Other transcription factors can be integrated into the hemangioblast GRN. Runx1 which is regulated by BMP signalling is essential for definitive hematopoiesis (Lacaud *et al.*, 2002; Pimanda *et al.*, 2007a). It starts to be expressed in the blast colony after 24 hours of colony growth; over the next 24 hours of blast colony development there is a great increase in the appearance of definitive progenitors (Cheng *et al.*, 2008). The homeobox transcription factor Hex is expressed in BL-CFCs and acts to negatively regulate the levels of this progenitor (Kubo *et al.*, 2005). In addition, it appears dispensable for primitive erythroid formation but required for differentiation of definitive hematopoietic and endothelial cells (Guo *et al.*, 2003; Kubo *et al.*, 2005). The *Cdx* genes, *Cdx1* and *Cdx4*, are critical to the development of blood in the zebrafish system through their regulation of *Hox* genes that promote posterior/hematopoietic fate (Davidson *et al.*, 2003; Davidson and Zon, 2006). The mouse *Cdx* genes, *Cdx1* and *Cdx4*, show redundant functions in promoting posterior patterning through the regulation of posterior *Hox* genes (Lohnes, 2003; Subramanian *et al.*, 1995; van den Akker *et al.*, 2002; van Nes *et al.*, 2006). They are expressed during EB differentiation in overlapping time windows which include the period of BL-CFC generation (Lengerke *et al.*, 2008; McKinney-Freeman *et al.*, 2008). When *Cdx4* is overexpressed from day 2-4 of serum differentiation and during the blast colony assay it leads to an increase in BL-CFCs (Wang *et al.*, 2005b). While its effect on BL-CFC numbers was not reported, overexpression of *Cdx1* during the same time window leads to an increase in hematopoietic progenitors (McKinney-Freeman *et al.*, 2008). *Cdx1* or *Cdx4* homozygous null mice do not appear to have altered hematopoietic development, however, ESCs deficient for either of these genes show reduced hematopoietic potential that can be further abrogated when the remaining *Cdx* genes including family member *Cdx2* are reduced (Wang *et al.*, 2008). These results point to the importance of the *Cdx-hox* pathway in regulating commitment and proliferation of the hematopoietic lineage.

Supplying additional levels of regulation in BL-CFC fate determination are factors that may act by modulating signalling pathways. Endoglin (CD105), an accessory receptor for TGFβ superfamily members, is expressed on BL-CFCs and when absent leads to a significant reduction in blast colony numbers (Perlingeiro, 2007). It can complex with TGFβ superfamily ligands (e.g. ActivinA, TGFβ1, BMP2) and their receptors (e.g. type 1 receptors ALK3

and ALK6, and type II receptors TβRII, ActRIIA and ActRIIB) (Bernabeu *et al.*, 2007). This association may regulate the activity of BMPs, Activin/Nodal and TGFβ in the BL-CFC formation process and warrants further dissection. The lysocardiolipin acyltransferase (Lycat) catalyzes the addition of palmitate groups to proteins such as cardiolipin (Cao *et al.*, 2004). It is expressed in the Flk-1+ BL-CFC-containing populations of day 4 EBs and on overexpression in a transgenic cell line leads to increased BL-CFCs and hematopoietic progenitors (Wang *et al.*, 2007a). Acyltransferases have been shown to affect signalling in the Hedgehog and Wnt pathways by acting upon the ligands (Buglino and Resh, 2008; Chamoun *et al.*, 2001; Kadowaki *et al.*, 1996; Micchelli *et al.*, 2002). It would be interesting to determine what substrates, particularly factors in signalling pathways Lycat might act upon to impact BL-CFC commitment and development.

When the blast colony culture conditions were used on dispersed E7.5 mouse embryos, hemangioblasts could be identified. They displayed many of the features of their *in vitro* counterparts; they possessed hematopoietic and endothelial potential and were a subpopulation of Flk-1+ mesoderm (Bry-GFP$^+$Flk-1$^+$) (Huber *et al.*, 2004). They were most abundantly found in the posterior PS indicating that commitment to the hemangioblast fate occurred in the PS prior to migration of mesoderm into the YS. Interestingly, primitive erythroid progenitors are observed near the PS (the distal YS) at E7.25 (Ferkowicz *et al.*, 2003). This finding fits with a model of YS blood island formation where the hemangioblast divides into endothelial and hematopoietic precursors, in particular primitive erythroid precursors, soon after it migrates from the PS into the YS. Besides hemangioblasts, angioblasts also arise from the posterior PS and contribute to the endothelial cells of the YS (Furuta *et al.*, 2006). Primitive erythroid precursors then migrate to the proximal YS and exist extravascularly as a band of cells until they are partially ensheathed by endothelial cells to form what has long been considered a YS blood island (Ferkowicz and Yoder, 2005). Rather than being surrounded in endothelium the blood cells are subdivided into endothelial channels that ultimately stream into the primary vascular plexus of the YS. This model may offer an explanation for why lineage tracing has thus far questioned the contribution of hemangioblasts to the YS blood island (Kinder *et al.*, 1999; Ueno and Weissman, 2006). If the existence of a hemangioblast is inferred from the presence of labelled hematopoietic and endothelial cells in the same section of a "blood island" then the presence of this cell may be underestimated. This is because the hematopoietic and endothelial lineages from the hemangioblasts appear to separate early and with the intermingling of cells in the YS the hematopoietic and endothelial progeny of a hemangioblast may not in fact be in close proximity to each other by the time blood islands are inspected.

BL-CFCs have been detected in hESC differentiation cultures. Depending on the culture conditions used they can appear as early as day 3-4 or later from day 5-12 of EB culture (Davis *et al.*, 2008; Kennedy *et al.*, 2007; Lu *et al.*, 2007; Wang *et al.*, 2004; Zambidis *et al.*, 2005). hBL-CFCs express the Flk-1 homologue, KDR as seen in the mouse system (Kennedy *et al.*, 2007; Wang *et al.*, 2004) and are found enriched in the MIXL1-GFP$^+$PDGRα$^+$ cells that represent an early mesoderm population (Davis *et al.*, 2008). They have also been found to express the angiotensin-converting enzyme (ACE, CD143) which has been identified as a marker of human HSCs (Jokubaitis *et al.*, 2008). hBL-CFCs exist as a transient population prior to the appearance of hematopoietic progenitors and they can give rise to both hematopoietic and endothelial progeny. Serum-free protocols used to generate EBs have used either a single culture condition (BMP4, VEGF, SCF) or a multi-step approach with BMP4 present since the initiation of EB culture and later addition of VEGF(Davis *et al.*, 2008; Kennedy *et al.*, 2007; Lu *et al.*, 2007). A serum-free human blast colony protocol was developed and includes BMP4, VEGF, FGF2, heparan sulphate and TPO (Zambidis *et al.*, 2008). Further discussions of human hemangioblasts are covered in the review by Zambidis *et al.* (2010).

Recently, two papers demonstrated the existence of hemogenic endothelium using the mouse ESC system (Eilken *et al.*, 2009; Lancrin *et al.*, 2009). When the hemangioblast-containing Flk-1$^+$ population is plated in liquid blast conditions and cultured for two days a population of endothelial cells, marked by the endothelial marker Tie2 and c-kit and lacking the hematopoietic marker CD41 (Tie2^{hi}c-kit$^+$CD41$^-$), arises (Lancrin *et al.*, 2009). This population contains clonogenic endothelial cells that can give rise to hematopoietic progeny. This hemogenic population can also be identified from mouse neural plate stage embryos and E10.5 AGMs, indicating that this intermediate exists *in vivo* during two periods when hematopoietic cells are generated. By using time-lapse microscopy and morphological and molecular markers, the emergence of hematopoietic cells was observed from endothelial cells that arise in cultures of Flk-1$^+$E-cadherin$^-$ mesoderm plated on OP9 stroma (Eilken *et al.*, 2009). Hemogenic endothelial cells were observed to express VE-cadherin, form tight junctions and take up acetylated low density lipoprotein (Ac-LDL) and reside in an endothelial sheet. They then divide into cells that upregulate the hematopoietic markers CD41 and CD45, and migrate out of the endothelial sheet. The first division of the hemogenic endothelial cells gives rise to two daughter hematopoietic cells. Thus the hemangioblast is a cell with hematopoietic and endothelial potential and hemogenic endothelium is a cell with endothelial identity that gives rise to hematopoietic progeny. The findings from these papers allow for the placement of the hemogenic endothelium stage after the hemangioblast stage in a single developmental process, reconciling what appeared to be two alternate theories of how hematopoietic cells arise.

The function of the hematopoietic transcription factors, SCL and Runx1 were studied with relation to the hemogenic endothelium developmental stage. SCL-/- ES cells do not generate the Tie2^{hi}c-kit$^+$CD41$^-$ population indicating that SCL is critical for the development of the hemogenic endothelial stage (Lancrin *et al.*, 2009). In contrast, from studies using a Runx1$^{-/-}$ ES cell line that can be induced to express Runx1 (iRunx1), the Tie2^{hi}c-kit$^+$CD41$^-$ hemogenic endothelium was shown to arise independently of Runx1 while the formation of definite hematopoietic cells from this population requires Runx1. This is consistent with the findings from the mouse embryo studies (Chen *et al.*, 2009). With the ability to access the hemogenic endothelium, studies can now focus on the cell intrinsic and extrinsic signals that lead to hematopoietic commitment at the level of this developmental stage.

Dissecting the signalling requirements for the YS hematopoietic program

The process of hematopoietic differentiation can be expressed

as a progression through developmental stages (Fig. 2). ESCs differentiate into epiblast-like cells that are induced to become PS cells (step 1). These cells are patterned to a posterior fate and develop into a Flk-1-expressing extraembryonic/lateral plate mesoderm cell that has hematopoietic potential, known as a hemangioblast (step 2). As there are other fates for Flk-1 mesoderm this commitment to hematopoietic mesoderm/hemangioblast is a significant step. The hemangioblast gives rise to hematopoietic progenitors (primitive or definitive) and vascular cells. The roles of the BMP, Wnt, Activin/Nodal, Notch, FGF and VEGF signalling pathways have been assessed in this differentiation process (Cheng *et al.*, 2008; Faloon *et al.*, 2000; Lengerke *et al.*, 2008; Nostro *et al.*, 2008; Park *et al.*, 2004; Pearson *et al.*, 2008). This has identified distinct signalling requirements for the different transitions during hematopoietic development. When BMP4 is added to ES cultures under serum-free conditions it can induce the formation of the PS and Flk-1+ mesoderm but compared to cultures treated with serum, it promotes lower levels of hematopoietic commitment and differentiation as determined by hematopoietic colony assays and hematopoietic marker analysis (Johansson and Wiles, 1995; Ng *et al.*, 2005; Nostro *et al.*, 2008; Park *et al.*, 2005; Pearson *et al.*, 2008). This indicates that the addition of BMP4 alone is not enough to robustly promote the hematopoietic program.

ES cells treated with BMP4 generate a posterior PS-like population (GFP-Bry^{+}CD4-Foxa2^{lo}) (Nostro *et al.*, 2008). The addition of inhibitors of the Wnt and Activin/Nodal pathways however prevent the formation of this population, indicating that BMP4 induces the PS indirectly through the action of Wnt and Activin/Nodal (Fig. 2, Step 1). In turn, Wnt and Activin have been shown to individually be capable of inducing PS populations; however, they require the action of the other in order to do this (Gadue *et al.*, 2006). The addition of BMP4 to Wnt and Activin will shift the PS population induced from an anterior PS (GFP-Bry^{+}CD4-Foxa2$^{mid\text{-}high}$) to posterior PS fate (GFP-Bry^{+}CD4-Foxa2^{lo}) (Nostro *et al.*, 2008). This shows that BMP signalling can pattern the PS which is a finding consistent with the patterning roles of BMP4 observed in other developmental systems (Kishimoto *et al.*, 1997; Maeno *et al.*, 1994; Mullins *et al.*, 1996; Re'em-Kalma *et al.*, 1995).

The next transition of the PS to Flk-1+ hematopoietic mesoderm/hemangioblasts is when BMP, Activin/Nodal, and Wnt signalling serve nonredundant functions (Fig. 2, step 2) (Nostro *et al.*, 2008). As PS cells are not fully committed to a particular anterior-posterior fate this transition is sensitive to factors that regulate PS patterning (Gadue *et al.*, 2006; Nostro *et al.*, 2008). The patterning role of BMP signalling is important at this stage. It has recently been linked to the axis patterning *Cdx-hox* pathway through the action of Wnt (Lengerke *et al.*, 2008). In the absence of Wnt signalling, BMP4 in serum-free media can not promote hematopoietic induction from a serum-induced day 2.25 PS-containing EB population. The overexpression of *Cdx1* and *Cdx4* can substitute for BMP4 in the same time window to induce hematopoietic development. BMP4 appears to activate the expression of the *Cdx* genes through the activity of Wnt3a. It will be important to establish how the *Cdx-hox* pathway promote hematopoietic fate, particularly how it might influence the expression of hematopoietic transcription factors such as SCL.

A role for Activin/Nodal in combination with bFGF in promoting Flk-1+ hematopoietic mesoderm/hemangioblasts was also shown by Pearson at al. The addition of Activin and bFGF to unsorted cultures of day 2.25 BMP4-treated ESCs resulted in a strong induction of BL-CFC numbers compared to just addition of BMP-4 alone (Pearson *et al.*, 2008). 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. bFGF has previously been shown to stimulate the proliferation of Flk-1+ mesoderm rather than promote its formation so it is possible that the induction of hematopoietic transcription factors in this study may be stimulated by activin (Faloon *et al.*, 2000; Park *et al.*, 2004). Experiments without bFGF and with inhibitors to the FGF pathway would clarify the contribution of this growth factor.

The transition of Flk-1+ mesoderm/hemangioblast to hematopoietic progenitors requires VEGF as in its absence hematopoietic progenitors do not arise even when conditions to generate robust BL-CFCs are used (Fig. 2, Step 3) (Nostro *et al.*, 2008; Park *et al.*, 2005; Pearson *et al.*, 2008). VEGF appears to act by sustaining the expression of the hematopoietic transcription factors *Scl*, *Fli1* and *Lmo2* that have been initiated during the generation of the Flk-1+ mesoderm/hemangioblast (Pearson *et al.*, 2008). This effect can not be replicated by the

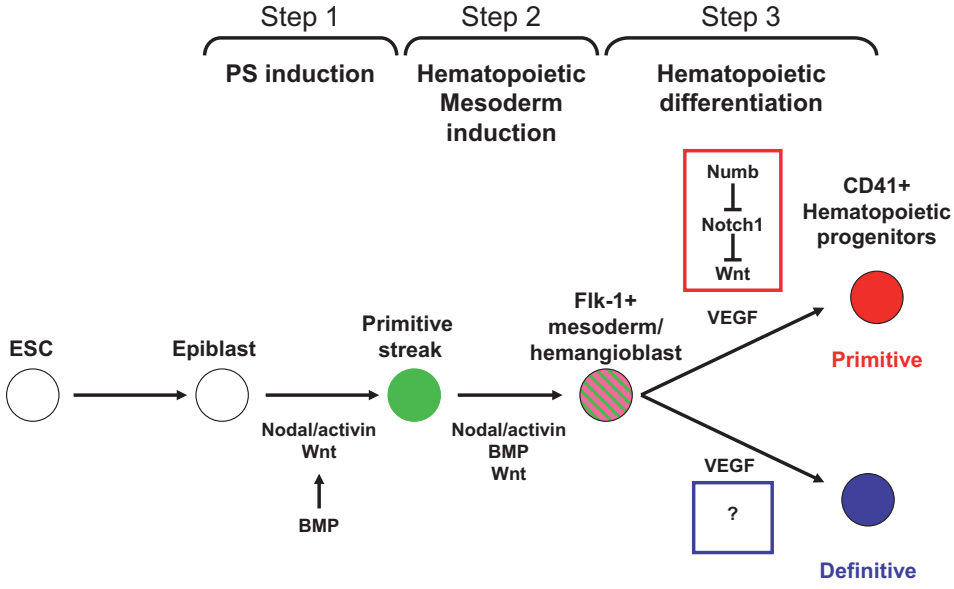

Fig. 2. Growth factor requirements in hematopoietic differentiation in ESC Cultures. *ESCs differentiate into epiblast that is induced to form the PS. Posterior patterning of the PS leads to Flk-1+ mesoderm/hemangioblast induction. Hematopoietic progenitors differentiate from the Flk-1+ mesoderm/hemangioblast in response to VEGF. The primitive hematopoietic lineage is dependent on Wnt signaling while the pathways responsible for definitive lineages are not known. Modified from Nostro* et al. *(2008).*

addition of BMP4, Activin or Wnt (Nostro *et al.*, 2008). However, when the Wnt pathway is inhibited in the presence of VEGF, the primitive erythroid lineage is suppressed indicating that Wnt signalling is required for the generation of these cells from Flk-1+ mesoderm/hemangioblasts (Fig. 2, Step 3, red box) (Nostro *et al.*, 2008). Wnt signalling functions early in this process as addition of the Wnt inhibitor DKK up to 6 hours into culture will reduce the number of primitive erythroid progenitors whereas addition at 12 hrs essentially does not have an effect. In addition Wnt signalling is modulated by the interaction between Notch1 and its negative regulator, Numb (Cheng *et al.*, 2008). The activation of Notch signalling in Flk-1+ mesoderm leads to an increase in the expression of Wnt inhibitors and a consequent reduction in Wnt signalling thus abrogating primitive erythroid formation. In contrast overexpression of Numb in Flk-1+ cells leads to reduced Wnt inhibitor expression, enhanced levels of Wnt signalling and hence increased primitive erythroid potential. The definitive hematopoietic potential from the Flk-1+ mesoderm/hemangioblast, at least as determined by total definitive colony counts in hematopoietic colony assays, appears unaffected by changes in Wnt and Notch1 signalling suggesting that other pathways are critical to the appearance of this program (Fig. 2, Step 3 blue box). However, this does not rule out effects that these pathways may have on the types of definitive progenitors that arise.

Efforts to establish the growth factor requirements for developmental transitions can lead to optimized serum-free directed differentiation protocols. This is borne out by the observation that serum-free conditions to generate hematopoietic progenitors and culture blast colonies performed better than the initial serum-containing protocols (Cheng *et al.*, 2008; Pearson *et al.*, 2008). In the hESC system, BMP4 and VEGF are common to serum-free protocols to generate hematopoietic progeny (Davis *et al.*, 2008; Kennedy *et al.*, 2007; Lu *et al.*, 2007; Pick *et al.*, 2007; Tian *et al.*, 2004; Zambidis *et al.*, 2008). Other growth factors that have been included in protocols are SCF, FGF1, FGF2, TPO and Flt3L (Davis *et al.*, 2008; Kennedy *et al.*, 2007; Lu *et al.*, 2007; Zambidis *et al.*, 2008). As in the mouse system, BMP4, Activin/Nodal and Wnt can individually induce the formation of EB populations that express PS markers (Davis *et al.*, 2008; Sumi *et al.*, 2008). However the assessment of the growth factor requirements for each step in human hematopoietic differentiation awaits analysis of the kind of detail performed in the mouse system.

Concluding remarks

Our ability to differentiate hematopoietic cells from mouse ESCs has advanced significantly over the last 20 years. Where initially hematopoietic cells were induced in a poorly defined serum-containing medium, differentiation can now be directed towards the YS hematopoietic program in a robust fashion using specific factors added in multi-step protocols derived from our understanding of development *in vivo*. This approach has been successfully applied to the differentiation of other tissue types such as pancreatic endocrine cells (D'Amour *et al.*, 2006) and certainly is the future for generating cell lineages for cell replacement therapy. Currently, the goal of deriving adult repopulating HSCs requires the ability to develop protocols that promote the AGM or possibly placental program over the YS program. This can benefit from a better understanding of the developmental steps that lead to the generation of the HSC and includes determining the factors that promote proper HSC maturation so that they will effectively home and repopulate in an adult reconstitution assay. The development of an *ex vivo* AGM culture system and the characterization of supportive stroma are directions that can yield information to apply to differentiation protocols (Ledran *et al.*, 2008; Taoudi *et al.*, 2008).

The ESC differentiation system will continue to yield new insights into hematopoietic development. It can help reveal the structure and behaviour of the transcriptional network that determines hematopoietic commitment and differentiation. The high proliferative capacity of ESCs make them ideal for global analyses such as transcriptome profiling and genome-wide mapping of chromatin marks as well as transcription factor binding sites. These approaches have been applied to undifferentiated ESCs and have greatly advanced our knowledge of the molecular regulation of pluripotency (Bibikova *et al.*, 2008; Jaenisch and Young, 2008; Stanton and Bakre, 2007). For these approaches to be fruitful the cell populations for study should be as homogeneous as possible. To this end appropriate reporter ESC lines and cell surface markers enable the isolation of developmentally distinct cell populations from ESC cultures. A comparison of the transcriptome of a hemangioblast-containing population (Flk-1+Scl+ cells from day 2.75 EBs) with that of its progeny (blast colonies) is one example where early stages of the hematopoietic commitment process have been analyzed in a global manner (Lugus *et al.*, 2007). By using high-throughput technologies to establish the transcriptional network and epigenetic regulation during hematopoietic differentiation, one may integrate the knowledge of the roles of key players into a more comprehensive molecular understanding of what governs hematopoietic lineage commitment.

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