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Hematopoietic stem cell emergence in the conceptus and the role of Runx1

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ABSTRACT Hematopoietic stem cells (HSCs) are functionally defined as cells that upon transplantation into irradiated or otherwise immunocompromised adult organisms provide long-term reconstitution of the entire hematopoietic system. They emerge in the vertebrate conceptus around midgestation. Genetic studies have identified a number of transcription factors and signaling molecules that act at the onset of hematopoiesis, and have begun to delineate the molecular mechanisms underlying the formation of HSCs. One molecule that has been a particularly useful marker of this developmental event in multiple species is Runx1 (also known as AML1, Pebp2α). Runx1 is a sequence-specific DNA-binding protein, that along with its homologues Runx2 and Runx3 and their shared non-DNA binding subunit CBFβ, constitute a small family of transcription factors called core-binding factors (CBFs). Runx1 is famous for its role in HSC emergence, and notorious for its involvement in leukemia, as chromosomal rearrangements and inactivating mutations in the human *RUNX1* gene are some of the most common events in *de novo* and therapy-related acute myelogenous leukemia, myelodysplastic syndrome and acute lymphocytic leukemia. Here we will review the role of *Runx1* in HSC emergence in the mouse conceptus and describe some of the genetic pathways that operate upstream and downstream of this gene. Where relevant, we will include data obtained from other species and embryonic stem (ES) cell differentiation cultures.

KEY WORDS: *Runx1, HSC, hemogenic endothelium, transcription, ontogeny*

Mouse developmental hematopoiesis

Hematopoiesis in the conceptus (we use the word conceptus to include all the products of conception, including the embryo and extra-embryonic tissues) is generally divided into primitive and definitive hematopoiesis. Primitive hematopoiesis is restricted to the extra-embryonic yolk sac and starts at 7.5 days post coitus (dpc) in the mouse. It gives rise to primitive erythrocytes, macrophages, and megakaryocytes (Haar and Ackerman, 1971; Moore and Metcalf, 1970; Palis *et al.*, 1999; Tober *et al.*, 2007). Definitive hematopoiesis occurs asynchronously at distinct locations in the mouse conceptus. These include the extra-embryonic yolk sac and placenta, the vitelline and umbilical arteries (which connect the yolk sac and placenta, respectively, to the embryo), and within the embryo proper in the area of the dorsal aorta where it is flanked by the urogenital ridges, the so-called aorta-gonad-mesonephros (AGM) region, and its predecessor the para-aortic splanchopleura (p-Sp) (Alvarez-Silva *et al.*, 2003; Cumano *et al.*, 1996; de Bruijn *et al.*, 2000; Garcia-Porrero *et al.*, 1995; Godin *et al.*, 1995; Medvinsky and Dzierzak, 1996; Medvinsky *et al.*, 1993; Müller *et al.*, 1994; Palis *et al.*, 1999). Definitive hematopoiesis gives rise to erythrocytes expressing βmajor and not embryonic globin (Brotherton *et al.*, 1979; Palis *et al.*, 1999; Wong *et al.*,

Abbreviations used in this paper: AGM, aorta-gonad-mesonephros region; dpc, days post coitus; ES, embryonic stem cell; HSC, hematopoietic stem cell; pSp, para-aortic splanchn opleura

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1983), cells of all myeloid and lymphoid lineages and ultimately to the definitive HSCs which maintain lifelong hematopoiesis. The first definitive blood cells appear in the yolk sac and are committed progenitors of various types, whilst functional definitive HSCs are first and autonomously generated in the >34 somite pair stage (sp) AGM (Medvinsky and Dzierzak, 1996; Müller *et al.*, 1994). For a more comprehensive description of the types of blood cell progenitors that emerge over the several day period between 7.5 and 11.0 dpc, and the types of assays used to detect them, we refer the reader to Table 1 in the review by Speck and Dzierzak (Dzierzak and Speck, 2008).

Runx1 deficiency blocks the formation of definitive hematopoietic cells

The phenotype of Runx1 deficient mice is unique, phenocopied only by knockout of the gene encoding for its heterodimeric partner CBFβ (*Cbfb*) (Okuda *et al.*, 1996; Sasaki *et al.*, 1996; Wang *et al.*, 1996a; Wang *et al.*, 1996b). Runx1 and CBFβ deficient embryos undergo a dramatic death, often heralded by profound hemorrhaging in relatively specific areas including the central nervous system and VII/VIII cranial nerve. Blood cells in the sites of hemorrhaging consist solely of primitive erythrocytes, while definitive blood cells (definitive hematopoietic progenitors and HSCs), are virtually absent from Runx1 deficient conceptuses, and severely reduced in CBFβ mutants (Cai *et al.*, 2000; Li *et al.*, 2006; Okuda *et al.*, 1996; Sasaki *et al.*, 1996; Wang *et al.*, 1996a; Wang *et al.*, 1996b). This lack of definitive hematopoietic cells is observed in all hematopoietic sites of the mouse conceptus (yolk sac, vitelline and umbilical arteries, AGM region, allantois/placenta, fetal liver, thymus) (Mukouyama *et al.*, 2000; North *et al.*, 1999; Okuda *et al.*, 1996; Rhodes *et al.*, 2008; Sasaki *et al.*, 1996; Wang *et al.*, 1996a; Wang *et al.*, 1996b; Yokomizo *et al.*, 2001; Zeigler *et al.*, 2006). This is unlike defects in Notch signaling that affect hematopoiesis in some sites (AGM region) but not others (yolk sac) (Hadland *et al.*, 2004; Kumano *et al.*, 2003; Robert-Moreno *et al.*, 2005; Robert-Moreno *et al.*, 2008).

Of the primitive lineages, primitive macrophages and their progenitors are also absent from Runx1-deficient mouse conceptuses and Runx1-null ES cell cultures (Lacaud *et al.*, 2002; Li *et al.*, 2006) (K. Liddiard and MdB, unpublished observations). Although primitive megakaryocyte development in Runx1-deficient conceptuses has not been examined directly, megakaryocytes and platelets are absent (Okuda *et al.*, 1996; Wang *et al.*, 1996a), indicative of a block somewhere during the differentiation of this lineage. Primitive erythrocytes, in contrast, were present in both Runx1- and CBFβ-deficient mouse conceptuses, and are generated from mouse ES cells with either *Runx1* or *Cbfb* mutations (Lacaud *et al.*, 2002; Miller *et al.*, 2001; Okuda *et al.*, 1996; Sasaki *et al.*, 1996; Wang *et al.*, 1996a; Wang *et al.*, 1996b). However, some defects in Runx1-deficient primitive erythrocytes (or in those expressing a dominant negative CBFβ protein) have been reported, including abnormal morphology, delayed maturation, and altered expression of cell surface markers (Ter119, CD41) and transcription factors (EKLF, KLF1, GATA1) (Castilla *et al.*, 1996; Yokomizo *et al.*, 2008). Knock down of Runx1 in zebrafish by morpholino injection similarly affects definitive hematopoiesis but not/marginally primitive erythropoiesis (Burns *et al.*, 2005; Gering and Patient, 2005; Kalev-Zhylinska *et al.*, 2002). Runx1 was the first gene that upon mutation so neatly segregated primitive erythropoiesis from definitive hematopoiesis, and for that reason it became a focal point for studying the origins of definitive (adult) blood.

A role for Runx1 in the formation of definitive blood from hemogenic endothelium in the embryo

The sites and cells in which Runx1 is expressed provided profound insights into the process by which definitive hematopoietic progenitors and HSCs are formed. Runx1 expression is found in all sites of hematopoiesis in the conceptus and precedes the emergence of definitive hematopoietic progenitors and HSCs. Quite strikingly, expression is seen in a subset of endothelial cells in the vitelline and umbilical arteries, the yolk sac, the placenta, and the ventral aspect of the dorsal aorta in the AGM region, but not in endothelial cells elsewhere (North *et al.*, 1999; Ottersbach and Dzierzak, 2005; Rhodes *et al.*, 2008). Runx1 is also expressed in mesenchymal cells in some of these sites, specifically those underlying the dorsal aorta in the AGM region, in the placenta, and in hematopoietic cell clusters attached to the luminal wall of the aorta and vitelline and umbilical arteries. The presence of Runx1 transcripts in the ventral aspect of the dorsal aorta is conserved in all vertebrate species that have been examined (Bollerot *et al.*, 2005; Burns *et al.*, 2002; Ciau-Uitz *et al.*, 2000; Gering and Patient, 2005; Kalev-Zhylinska *et al.*, 2002; North *et al.*, 1999).

The observation that Runx1 was expressed in endothelial cells prior to the onset of definitive hematopoiesis, supported an articulated, but at the time, not widely accepted hypothesis that blood developed from a “hemogenic endothelium” (Jaffredo *et al.*, 1998; Jordan, 1916; Nishikawa *et al.*, 1998; Smith and Glomski, 1982). The hemogenic endothelium was proposed to line the lumens of certain arteries in the conceptus and give rise to clusters of hematopoietic cells on the luminal side of the artery (or in the case of chick embryos and zebrafish embryos, also in the ventral mesenchyme (Jaffredo *et al.*, 2005b; Kissa *et al.*, 2008)) that expressed markers such as CD45, αIIb integrin (CD41), c-myb, Mpl, and c-kit (Bernex *et al.*, 1996; Burns *et al.*, 2005; Corbel, 2002; Gering and Patient, 2005; Jaffredo *et al.*, 2005a; Jaffredo *et al.*, 1998; Labastie *et al.*, 1998; Manaia *et al.*, 2000; Marshall *et al.*, 1999; Ody *et al.*, 1999; Pardanaud *et al.*, 1996; Petit-Cocault *et al.*, 2007; Tavian *et al.*, 1996; Thompson *et al.*, 1998). The clusters were found closely associated with aortic endothelium in the ventral aspect of the dorsal aorta, and the vitelline and umbilical arteries, suggesting an intimate relationship between the two lineages (Dieterlen-Lièvre and Martin, 1981; Garcia-Porrero *et al.*, 1995; Sabin, 1920).

The first direct experimental evidence for a hemogenic endothelial precursor for blood was provided by Dieterlen-Lievre and colleagues, who performed lineage-tracing analyses in the chick embryo using vital dyes (acetylated low density lipoprotein labeled with DiI) and retroviruses. She and her colleagues demonstrated that labeling of the entire vasculature prior to the formation of intra-arterial clusters, resulted in labeled $CD45^+$ cells in the clusters that subsequently formed, as well as in the mesenchyme ventral to the chick dorsal aorta (Jaffredo *et al.*, 2000; Jaffredo *et al.*, 1998). Further support for the differentiation of blood from endothelium was provided by the Nishikawa lab, who showed that cells isolated from mouse embryos on the basis of their expression of vascular

endothelial cadherin (VE-cadherin) and the absence of the hematopoietic markers Ter119 and CD45, could give rise to blood following *in vitro* culture (Nishikawa *et al.*, 1998). However, there was still some doubt that blood is borne directly from endothelium, and alternative models exist. For example, it was proposed that in the mouse the immediate precursors to the intra-arterial clusters originate in the mesenchyme ventral to the dorsal aorta in so called sub-aortic patches, and migrate towards the arteries and squeeze between endothelial cells to enter the lumen (Bertrand *et al.*, 2005; Godin and Cumano, 2002; Manaia *et al.*, 2000; Yoon *et al.*, 2008).

Runx1 deficiency blocked the formation of the intra-arterial clusters *in vivo*, and the formation of hematopoietic cells from purified endothelial cells *ex vivo*, suggesting a role for Runx1 in the hemogenic endothelium to hematopoietic cell transition (North *et al.*, 1999; Yokomizo *et al.*, 2001). Indeed, arterial endothelial cells in Runx1 deficient embryos initiated reporter gene expression from endogenous *Runx1* regulatory elements at the proper time of development (although expression was lost later on), indicating that the observed defect in hematopoiesis did not result from impaired migration/incorporation of endothelial progenitors into the aortic endothelium, or impaired specification of hemogenic endothelium, but rather from impaired differentiation of blood from endothelium (North *et al.*, 1999). Morpholino knockdown of Runx1 in zebrafish, also impedes hematopoietic cluster formation without affecting the aortic endothelium (Burns *et al.*, 2005; Gering and Patient, 2005; Kalev-Zhylinska *et al.*, 2002), in line with Runx1 being responsible for a hemogenic endothelium to hematopoietic cell transition. Runx1 was the first gene specifically implicated to function at this step.

Although the histological evidence for Runx1 expression and activity in the endothelium of the major arteries (dorsal aorta, vitelline, umbilical) was compelling, and was observed in multiple organisms, expression in the yolk sac and placenta were less easy to interpret. The yolk sac is a source of both primitive and definitive blood progenitors, which appear in separate waves and anatomical locations (Ferkowicz *et al.*, 2003; Ferkowicz and Yoder, 2005; Palis *et al.*, 1999). Progenitors to primitive erythrocytes, which are $CD41^{dim}$, develop in a band of prospective blood islands that encircle the proximal yolk sac at 7.5 dpc (Ferkowicz *et al.*, 2003; Li *et al.*, 2005). Slightly later (8.25 dpc) definitive progenitors appear as small clusters of $CD41^{bright}$ cells located at the proximal border of the blood islands (Ferkowicz *et al.*, 2003; Li *et al.*, 2005). Runx1 is expressed in the mesoderm of the prospective blood islands in mid to late primitive streak conceptuses (~7.5 dpc), and in the primitive erythrocytes as they begin to differentiate from mesoderm in the yolk sac (Lacaud *et al.*, 2002; North *et al.*, 1999). This "background" of Runx1 expression during the formation of the blood islands and the initial stages of primitive erythropoiesis makes identifying the emergence of $Runx1^{+}$ definitive precursors in the yolk sac difficult to visualize. Nevertheless Runx1 expression was observed in yolk sac endothelial cells and putative definitive blood cells associated with them, after expression in erythrocytes is extinguished by 8.5 dpc as Ter119 levels become elevated and maturation progresses (North *et al.*, 1999; North *et al.*, 2004). In addition, it was noted that in Runx1 deficient conceptuses, expression of reporter gene knock-ins in the yolk sac blood islands was normal, but later expression in endothelial cells scattered around the yolk sac was absent, as were clusters (North *et al.*, 1999; Yokomizo *et al.*, 2001). Studies in the ES cell differentiation model of yolk sac hematopoiesis showed that Runx1 expression in embryoid bodies (EBs) is upregulated contemporaneously with that of Flk1, and blast colony-forming cells (BL-CFCs), thought to represent hemangioblasts that differentiated from EBs were uniformly Runx1 positive (Lacaud *et al.*, 2002). In the absence of Runx1, the generation of definitive type hematopoietic progenitors in the blast colonies was completely blocked, with just the core of the colony, which has endothelial characteristics, remaining (Lacaud *et al.*, 2002; Lancrin *et al.*, 2009). Thus, similar to the situation in the dorsal aorta, Runx1 is an early marker of hematopoiesis in the yolk sac and plays a role in the generation of definitive hematopoietic cells, despite the fact that it is not required in the hemangioblast or for the onset of primitive erythropoiesis.

In the placenta, which recently was shown to harbor hematopoietic progenitors and stem cells (Alvarez-Silva *et al.*, 2003; Gekas *et al.*, 2005; Ottersbach and Dzierzak, 2005; Rhodes *et al.*, 2008), Runx1 positive endothelium in the labyrinth is similarly overshadowed by a larger population of Runx1 positive mesenchymal cells (Ottersbach and Dzierzak, 2005; Rhodes *et al.*, 2008; Zeigler *et al.*, 2006), and intra-luminal clusters of hematopoietic cells are relatively infrequent (Ottersbach and Dzierzak, 2005; Rhodes *et al.*, 2008). One potential explanation for the difficulty in finding $Runx1^{+}$ clusters is that the *Runx1-lacZ* allele that many investigators have used to track expression is nonfunctional (North *et al.*, 1999), and it is well-documented that Runx1 haploinsufficiency depresses definitive hematopoietic progenitor numbers and cluster formation (Cai *et al.*, 2000; Mukouyama *et al.*, 2000; Wang *et al.*, 1996a).

Most recently, the transition of cells with an endothelial phenotype and morphology into free-floating hematopoietic cells was directly visualized by time-lapse microscopy in culture (Eilken *et al.*, 2009), in live zebrafish embryos, and in thick sections of the mouse aorta cultured ex vivo (Bertrand *et al.*, 2010; Boisset *et al.*, 2010; Kissa and Herbomel, 2010). Runx1 mutants or morpholinos were used to demonstrate the specificity of the budding process, as they completely impaired hematopoietic cell formation in all systems (Boisset *et al.*, 2010; Kissa and Herbomel, 2010; Lam *et al.,* 2010). In a study using zebrafish embryos it was shown that very few cells budded from the endothelium of Runx1 morphants, and those that tried died immediately (Kissa and Herbomel, 2010). These data suggest that signals other than Runx1 may initiate the budding process, but that Runx1 is absolutely required for it to progress normally.

The vast majority of adult blood is derived from endothelium

Although intra-aortic clusters were clearly observed at developmental times when definitive hematopoietic progenitors and HSCs appeared, a question that remained was to what extent do the endothelial cells and intra-aortic clusters that appear so briefly in the midgestation conceptus contribute to the HSCs that are ultimately found in adult marrow? Two groups addressed this question in mice by labeling all cells expressing, or that at one time had expressed the endothelial marker VE-cadherin, by crossing VE-cadherin-Cre recombinase transgenic mice to Rosa26 reporter mice (Chen *et al.*, 2009; Zovein *et al.*, 2008). Both groups showed that cells in the adult bone marrow, which do not express cell surface VE-cadherin (Kim *et al.*, 2005; Taoudi *et al.*, 2005), were indeed derived from a precursor that had expressed VE-cadherin.

The percentage of labeled cells in the adult marrow was >95% in one study (Chen *et al.*, 2009), which, given the less than 100% efficiency of VE-cadherin/Cre excision suggests that almost all adult blood cells are born from VE-cadherin expressing cells. VE-cadherin is also transiently expressed in 7.5 dpc yolk sac mesoderm, raising the possibility that blood is not derived from VE-cadherin positive endothelium (Yokomizo *et al.*, 2007). However one group took advantage of an estrogen regulated form of Cre expressed from the VE-cadherin promoter, and activated Cre after the transient wave of VE-cadherin expression in yolk sac mesoderm had ended. They showed that blood was labeled, and was therefore derived from VE-cadherin positive endothelium (Zovein *et al.*, 2008). Together with the finding that deletion of Runx1 by VE-cadherin-Cre blocked the formation of intra-aortic clusters, HSCs, and definitive hematopoietic progenitors (in essence phenocopying germline Runx1 deficiency (Chen *et al.*, 2009)), these data provide compelling *in vivo* evidence that definitive hematopoietic progenitors and HSCs differentiate from VE-cadherin$^+$ cells, most of which are endothelial cells in a Runx1-dependent manner. Conversely, restricted expression of Runx1 or CBFβ only in Tie2$^+$ cells or their progeny allowed for the formation of HSCs and/or hematopoietic progenitors, consistent with the above-mentioned results (Liakhovitskaia *et al.*, 2009; Miller *et al.*, 2002). However, since Tie2 is not only expressed on endothelial cells but also on some mesenchymal cells and on quiescent adult HSCs (Arai *et al.*, 2004; Li *et al.*, 2006), these data were not as conclusive as those generated by conditional knockout using VE-cadherin-Cre. Recent experiments in zebrafish, using an endothelial cell-associated *kdrl:Cre* to activate a reporter gene, are in line with an endothelial origin of most blood cells (Bertrand *et al.*, 2010), although the spatiotemporal window during which the kdrl:Cre is active is not entirely clear (Bertrand *et al.*, 2010; Kissa and Herbomel, 2010).

Runx1 is transiently required for hematopoietic progenitor and HSC emergence

Loss of Runx1 function in the adult causes not a reduction, but rather an expansion of phenotypic HSCs and progenitors in the bone marrow (Chen *et al.*, 2009; Growney *et al.*, 2005; Ichikawa *et al.*, 2004; Putz *et al.*, 2006; Schindler *et al.*, 2009), thus at some point hematopoietic stem and progenitor cells no longer absolutely require Runx1. Several studies have attempted to define the temporal window during which Runx1 is absolutely required (Fig. 1). Chen *et al.* showed that the transition to relative Runx1 "independence" occurs in the fetus, as deletion of Runx1 with Vav1-Cre, which is active in fetal liver hematopoietic progenitors and HSCs starting at approximately 11.5 dpc, does not block HSC or progenitor formation (Chen *et al.*, 2009). In line with this, retroviral transduction of Runx1 into cells derived from the AGM of 11.5 dpc Runx1 deficient embryos could not rescue hematopoietic progenitor formation, while transduction of cells derived from the 9.5 dpc p-Sp could do so (Goyama *et al.*, 2004; Mukouyama *et al.*, 2000). However, as the extent to which hematopoiesis was rescued was not addressed, it is not clear whether or not optimal progenitor and/or HSC production requires Runx1 during the entire window of its expression in the 8.5 dpc to 11.5 dpc p-Sp/AGM. Whether it is required only in the endothelium and not once the intra-arterial clusters have formed, is another outstanding question. Finally, given that hematopoiesis unfolds gradually in multiple anatomical locations, it is likely that the time at which Runx1 is required will differ in each hemogenic site.

Transcriptional regulation of Runx1 expression: promoters and cis-elements

The pivotal role for Runx1 at the onset of definitive hematopoiesis raises the question how the expression of this master regulator itself is controlled. The large size of the *Runx1* locus (224 Kb in the mouse) has made defining the *cis*-regulatory elements and *trans*-acting factors that govern its spatiotemporal expression a challenge. *Runx1*, like the other vertebrate *RUNX* genes, is transcribed from two alternative promoters, a distal P1 and a proximal P2 (Bee *et al.*, 2009b; Ghozi *et al.*, 1996; Levanon *et al.*, 2001; Levanon and Groner, 2004; Telfer and Rothenberg, 2001) (Fig. 2A). Alternative gene promoters are frequently found in the mammalian genome, and are believed to contribute to its greater regulatory complexity (Davuluri *et al.*, 2008). For *Runx1*, alternative promoter usage is known to result in the generation of a series of transcripts that differ in their untranslated regions and/or protein-coding exons, influencing mRNA stability, efficiency of translation by the use of alternative translation initiation mechanisms and micro-RNAs, and/or Runx1 protein structure (reviewed in (Bee *et al.*, 2009b; Levanon *et al.*, 2001; Levanon and Groner, 2004). In the mouse, both promoters are active at and required for the normal onset of definitive hematopoiesis, in a partially overlapping but non-redundant fashion (Bee *et al.*, 2009b; Bee *et al.*, 2010; Pozner *et al.*, 2007). In general, transcripts from the P2 promoter are produced earlier than those from the P1 promoter, in hemogenic endothelium as well as in the

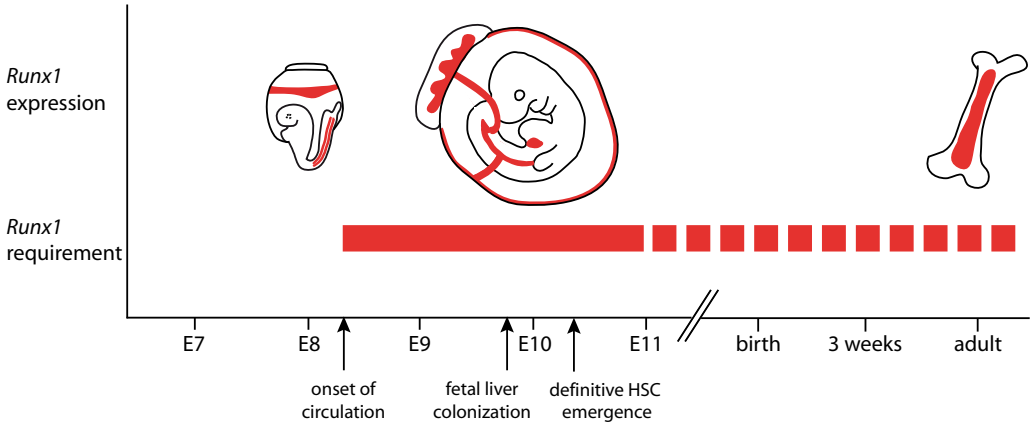

Fig. 1. The outer boundaries of the proposed developmental window of the absolute Runx1 requirement in definitive blood cell generation (solid red bar). *A continued role for Runx1 in specific hematopoietic cell types and lineages is represented by the dotted red bar. See text for additional explanation.*

very first emerging hematopoietic cells in the mouse embryo and ES cell cultures, while P1 is the dominant *Runx1* promoter in fetal liver and adult HSCs (Bee *et al.*, 2009b; Challen and Goodell, 2010; Fujita *et al.*, 2001; Sroczynska *et al.*, 2009; Telfer and Rothenberg, 2001).

Maintenance of Runx1 expression in hemogenic endothelial cells requires continued Runx1 function, raising the possibility that Runx1 positively regulates its own expression (North *et al.*, 1999). Negative regulation of Runx gene expression has also been observed; overexpression of Runx3 downregulated Runx1 expression in human B cell lines (Spender *et al.*, 2005), Runx2 repressed Runx3 in tooth development (Wang *et al.*, 2005), and Runx2 has been shown to repress its own expression *in vitro* (Drissi *et al.*, 2000). Several Runx binding sites are located in/nearby the mammalian P1 and P2 core promoters suggesting that both promoters may be auto- and cross-regulated by Runx proteins. The two adjacent Runx binding sites in the P1, which are located in the P1 5'UTR, are conserved between all three Runx genes and in multiple species extending to frog; and a positive autoregulatory loop was shown to act on the P1 promoter in a myeloid progenitor cell line (Bee *et al.*, 2009a; Levanon and Groner, 2004; Pimanda *et al.*, 2007a). However, eliminating both of these sites did not affect the activity of a P1 promoter fragment in transgenic mice (Bee *et al.*, 2009a). Presumably sequences at the P2, or more distal to either promoter confer autoregulation.

Despite the specific activity pattern of mouse P1 and P2 *Runx1* promoters in developmental hematopoiesis, neither core promoter confers Runx1-specific expression to transgenic reporters *in vivo* or *in vitro* (Bee *et al.*, 2009a; Ghozi *et al.*, 1996), indicating that tissue-specific *cis*-regulatory elements are located elsewhere in the locus. Recently, a deeply conserved hematopoietic-specific enhancer was identified that is located in the first intron of *Runx1*, 23.5 kb downstream of the translation start site in exon 1 (Fig. 2 A,B). This +23 enhancer was found to act with either of the *Runx1* promoters or an exogenous promoter to confer specific expression of a reporter gene in hematopoietic sites in the conceptus, in a subset of the cells in which endogenous Runx1 is expressed (Bee *et al.*, 2009a; Nottingham *et al.*, 2007). Specifically, the +23 enhancer was active in the intra-arterial clusters, including the emerging HSCs, but in only a subset of endothelial cells and mesenchymal cells ventral to the dorsal aorta (Fig. 2B), probably reflecting the absence of other *cis*-acting sequences necessary to drive the full spectrum of *Runx1* expression at this site. It is of interest to note that in zebrafish, *cis*-acting sequences sufficient for *runx1* expression in the dorsal aorta are contained within 8 kb upstream of the P2 promoter (Lam *et al.*, 2009), whilst the +23 enhancer is not conserved in this species. Thus, there appears to be a divergence in the *cis*-regulation of *Runx1* between mammals and fish, although upstream factors appear to be conserved (discussed below and in (Bee *et al.*, 2009a; Bee *et al.*, 2009b)).

Direct transcriptional regulators of Runx1 in developmental hematopoiesis

The +23 enhancer contains several conserved motifs corresponding to putative binding sites for hematopoietic transcription factors, with GATA, ETS, and RUNX motifs critical for *in vitro* enhancer activity (Nottingham *et al.*, 2007). During the onset of hematopoiesis in the conceptus, enhancer activity was dependent on the GATA and ETS motifs, but not on the RUNX motif, indicating that *in vivo* the +23 enhancer acts during the initiation of Runx1 expression (Nottingham *et al.*, 2007) rather than during

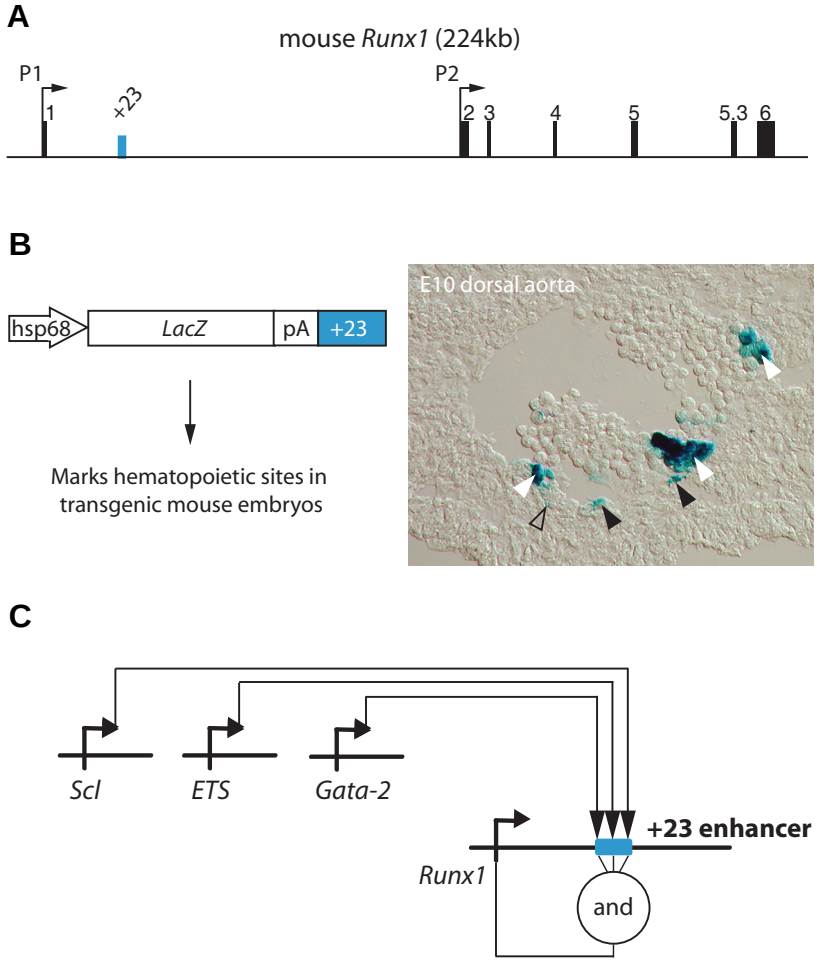

Fig. 2. The *Runx1* +23 enhancer recapitulates the hematopoietic specific expression pattern of *Runx1*. (A) *Schematic of the* Runx1 *locus. Vertebrate Runx1 is transcribed from two promoters, the P1 and the P2. A 531 bp mouse-frog conserved enhancer was identified (Nottingham* et al.*, 2007) and is located 23 kb downstream of the ATG in exon 1.* **(B)** *This +23 enhancer targets reporter gene expression to hematopoietic sites in the developing embryos, including all emerging HSCs. A transverse section through the dorsal aorta of an E10 transient transgenic embryo shows Xgal staining in emerging hematopoietic clusters (white arrowheads), in scattered cells of the endothelial wall (black arrowheads), and in a few mesenchymal cells (open arrowhead). Identical Xgal staining is seen in established mouse lines carrying the hsp68LacZ+23 transgene (not shown).* **(C)** *Targeted mutagenesis of putative transcription factor binding sites and chromatin IP (Nottingham* et al.*, 2007), and trans-activation assays (Landry* et al.*, 2008) placed the Runx1 +23 enhancer directly downstream of the ETS/GATA/SCL kernel (Liu* et al.*, 2008; Pimanda* et al.*, 2007b) that is active at the onset of developmental hematopoiesis.*

maintenance through autoregulation (Ferjoux *et al.*, 2007; Gajewski *et al.*, 2007). Chromatin immunoprecipitation analyses showed specific enrichment of Gata2, the ETS factors PU.1, Fli-1 and Elf1, and the SCL complex (presumably through GATA factors) on the +23 enhancer (Landry *et al.*, 2008; Nottingham *et al.*, 2007). Taken together, this places *Runx1* directly downstream of GATA, ETS and SCL transcription factors (Fig. 2C), which are themselves well known players in the generation of the vasculature and the hematopoietic system in the mouse and zebrafish, ((De Val and Black, 2009) and references therein).

The dorsal aorta is initially formed by endothelial precursors derived from lateral plate mesoderm (Esner *et al.*, 2006; Pouget *et al.*, 2006; Wasteson *et al.*, 2008). In the dorsal lateral plate mesoderm of *Xenopus* embryos, cells expressing Flk1, the endothelial maker Fli1 (XFli-1, encoding an ETS family protein), Scl (encoded by *Tal1*) and Gata2, but not Runx1, are found before the dorsal aorta forms (Ciau-Uitz *et al.*, 2000). A subset of these cells migrates towards a VEGF signal at the midline of the embryo that is secreted by the hypochord. Although Flk1 and XFli-1 expression are maintained as these endothelial precursors move towards the midline, Scl and Gata2 expression are extinguished in the actively migrating cells (Ciau-Uitz *et al.*, 2000). After the endothelial precursors reach the midline and form the (unpaired) dorsal aorta they activate Runx1 expression, and reactivate the expression of Scl and Gata2 (Ciau-Uitz *et al.*, 2000). This sequence of events suggests that a local signal in the vicinity of the ventral aspect of the dorsal aorta is responsible for activating Runx1, and for reactivating Scl and Gata2 expression.

SCL, Gata2 and Fli1 are expressed in the mouse pSp/AGM in endothelial cells and the hematopoietic clusters, and are required for normal definitive hematopoiesis (Elefanty *et al.*, 1999; Hart *et al.*, 2000; Khandekar *et al.*, 2007; Kobayashi-Osaki *et al.*, 2005; Ling *et al.*, 2004; Melet *et al.*, 1996; Minegishi *et al.*, 1999; Minegishi *et al.*, 2003; Pimanda *et al.*, 2007b; Porcher *et al.*, 1996; Sanchez *et al.*, 1999; Sinclair *et al.*, 1999; Spyropoulos *et al.*, 2000; Tsai *et al.*, 1994). In addition, SCL and Gata2 were shown to be expressed in and play a role in HSC function in the embryo and/or the adult (Elefanty *et al.*, 1999; Lacombe *et al.*, 2009; Ling *et al.*, 2004; Rodrigues *et al.*, 2005; Sanchez *et al.*, 1999). In HSC emergence, Scl and its interaction partner, the LIM domain only 2 protein (Lmo2) function earlier than Runx1, prior to expression of the endothelial markers VE-Cadherin and Tie2 (Drake and Fleming, 2000; Endoh *et al.*, 2002; Gering *et al.*, 2003; Li *et al.*, 2006; Patterson *et al.*, 2007; Schlaeger *et al.*, 2005). Scl is required in the lateral plate mesoderm for hematopoietic specification, and unlike Runx1, is no longer required once the hemogenic endothelium forms (Chen *et al.*, 2009; Endoh *et al.*, 2002; Lancrin *et al.*, 2009; Li *et al.*, 2006; Schlaeger *et al.*, 2005). The ETS transcription factor Fli1 is at the top of the genetic hierarchy that leads to Gata2, Scl, Flk1, Tie2, and ultimately Runx1 expression (Liu *et al.*, 2008). Morpholinos against *Xenopus* Fli1 dramatically decreased the expression of all of these genes. Gata2 morphants, on the other hand, expressed Fli1 normally but not Scl, Lmo2, Tie2, or Runx1, placing Gata2 downstream of Fli1 but upstream of these other genes (Liu *et al.*, 2008). The +23 hematopoietic-specific enhancer in *Runx1* contains binding sites for all of the transcription factors in this early hematopoietic "kernel" (Gata2, Scl/Lmo2, Fli-1), and thus may integrate the information coming from all three signals (Landry *et al.*, 2008; Liu *et al.*, 2008; Nottingham *et al.*, 2007; Pimanda *et al.*, 2007b) (Fig. 2C).

Signaling pathways acting upstream of Runx1

Hedgehog signaling is required at multiple stages of blood cell formation in zebrafish (Gering and Patient, 2005). It is required first for the migration of endothelial precursors from the dorsal lateral plate towards the midline, and slightly later for arterial specification. Inhibition of hedgehog signaling at either of these two steps severely reduces the number of Runx1$^+$ cells in the dorsal aorta. Hedgehog signaling, however, is not thought to activate Runx1 expression directly (Wilkinson *et al.*, 2009). VEGF and Notch signaling are also upstream of Runx1 expression (Burns *et al.*, 2005; Gering and Patient, 2005). Inhibition of either signaling pathway prevents arterial specification (monitored by the expression of ephrinB2a) and the formation of Runx1$^+$ cells. The Notch signaling hematopoietic defect can be overcome in both zebrafish and mice by ectopic expression of Runx1, indicating that Runx1 is genetically downstream of Notch (Burns *et al.*, 2005; Nakagawa *et al.*, 2006). However, hematopoietic markers including Runx1, are detectable in the dorsal aorta prior to the requirement for Notch activity (Burns *et al.*, 2005; Gering and Patient, 2005; Kalev-Zhylinska *et al.*, 2002; Wilkinson *et al.*, 2009), suggesting that Notch may be necessary to maintain but not to initiate Runx1 expression. Whether Runx1 is a direct or indirect target of Notch signaling, and whether it also works in parallel with Notch is not known. No binding sites for the CSL transcription factor (CBF1; recombinant binding protein-J kappa (RBPjÍ); Suppressor of Hairless (Su[H]); Lag-1), which transmits the Notch signal to downstream target genes, have been reported within the *Runx1* gene. It has been shown that Hes1, a downstream effector of Notch1, augments the transcriptional activity of Runx1 (McLarren *et al.*, 2000), and thus the two pathways may work in parallel, as they do during the early stages of T cell development (Guo *et al.*, 2008; Nakagawa *et al.*, 2006).

One mode by which Notch signaling regulates cell fate is through lateral inhibition, in which a cell expressing Notch ligands activates signaling in adjacent cells, and the cells delivering and receiving the signal adopt different identities (Bray, 2006). Since not all Runx1$^+$ endothelial cells appear to give rise to hematopoietic clusters, Notch signaling could potentially be involved in selecting the Runx1$^+$ cells that do form clusters. Notch signaling in both zebrafish and mice is required only for the formation of definitive hematopoietic cells in the AGM region, but surprisingly not in the yolk sac (Burns *et al.*, 2005; Gering and Patient, 2005; Hadland *et al.*, 2004; Kumano *et al.*, 2003; Robert-Moreno *et al.*, 2005; Robert-Moreno *et al.*, 2008). Differences in signaling pathways like these could underlie the differences in definitive blood cell types in the various anatomical sites of blood cell formation.

The signaling pathway activated by bone morphogenic protein 4 (Bmp4) is also essential for blood cell formation, and may be one of the local signals that induce *Runx1* expression in the ventral aspect of the dorsal aorta. Bmp4 expression in mesenchyme underlying intra-arterial clusters in the dorsal aorta has been documented in multiple species (Durand *et al.*, 2007; Marshall *et al.*, 2000; Pimanda *et al.*, 2007a; Suonpaa *et al.*, 2005), and inhibition of Bmp4 signaling in zebrafish blocked both the initiation and maintenance of Runx1 expression (McReynolds *et al.*, 2007; Wilkinson *et al.*, 2009). Bmp4 appears to act later than Hedgehog

and Notch, in that specification of arterial endothelium, which requires the latter two signals, does not require Bmp4 signaling (Wilkinson *et al.*, 2009). Runx1 may be a direct target of Bmp4 signaling, as one of its downstream effectors, Smad1, was shown to occupy the distal P1 promoter of Runx1 in a hematopoietic cell line *in vivo* (Pimanda *et al.*, 2006). A schematic representing the main steps in HSC formation as discussed above is shown in Fig. 3.

Runx1 expression has been used as a convenient marker to screen for genes, compound libraries, or conditions that affect its expression and thus definitive hematopoiesis (Burns *et al.*, 2009; North *et al.*, 2009; North *et al.*, 2007). North *et al.* (North *et al.*, 2007) used a combination of probes for Runx1 and c-Myb expression to monitor the effects of small molecules on AGM hematopoiesis in zebrafish (North *et al.*, 2007). This resulted in the identification of compounds that modulate the prostaglandin E2 pathway as regulators of HSC formation in the embryo. This finding was quickly translated into a clinical trial (http://clinicaltrials.gov/ct2/show/NCT00890500) designed to test the ability of a stabilized derivative of prostaglandin E2 1(6, 16 Dimethyl-Prostaglandin E2) to improve the engraftment of patients receiving allogeneic umbilical cord blood stem cell transplants for the treatment of their leukemia.

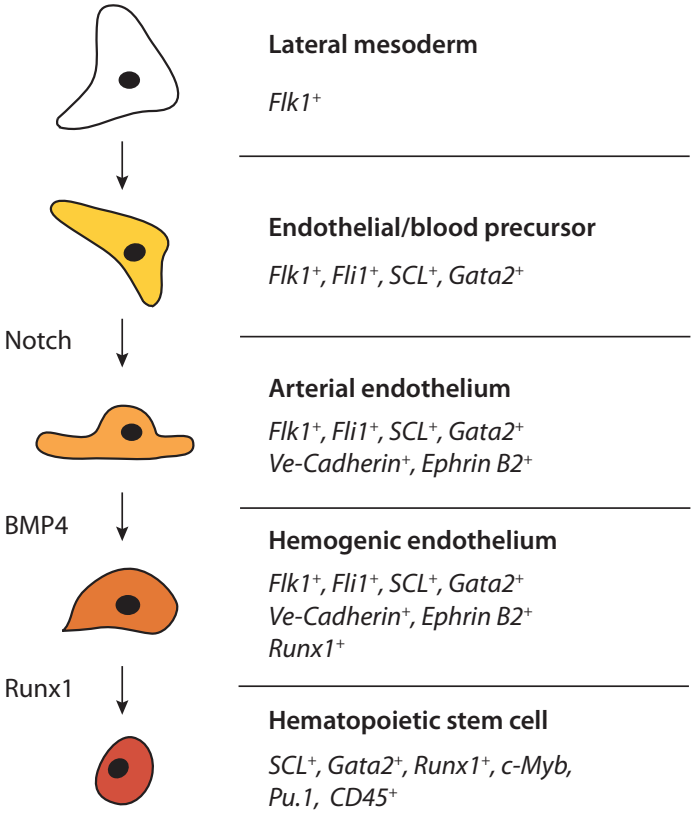

Fig. 3. Steps in hematopoietic stem cell formation, based on the model in which definitive blood is derived from so-called hemogenic endothelium. *Arrows indicate the order of events only and should not be taken to represent direct transitions in all instances. Transcription factors, signaling pathways, and cell type specific genes active during this process are shown. This list is not exhaustive and is based on data available on mRNA and/or gene enhancer-mediated expression in mouse, chick, zebrafish and/or* Xenopus *embryos. In some instances there is a discrepancy in mRNA and protein expression. For example, VE-Cadherin protein is expressed on the cell membrane of mouse HSCs isolated from the AGM region, while in the chicken embryo aortic clusters do not express VE-Cadherin mRNA). This may be caused by the perdurance of protein following loss of mRNA synthesis. Pu.1 and c-Myb are expressed in pSp/AGM hematopoietic cells and play a role in fetal liver and adult bone marrow HSCs (Garcia* et al.*, 2009; Iwasaki* et al.*, 2005, Kim* et al.*, 2004, Lieu and Reddy, 2009; Mukouyama* et al.*, 1999; Sandberg* et al.*, 2005). Thus, these transcription factors are likely to be expressed in AGM HSCs, although to our knowledge this was not formally shown.*

The same screen for Runx1/c-Myb expression in zebrafish found that a group of compounds that regulate blood flow also affect blood cell formation in the AGM region (North *et al.*, 2009). Compounds that increased Runx1/c-Myb expression and blood formation included antagonists of the adrenergic signaling pathway, the $Ca2^{+}$-channel blocker nifedipine, and compounds that increased the production of nitric oxide (NO). All of these compounds increased vasodilation and subsequently the volume of blood flowing through the dorsal aorta. Genes associated with the three affected pathways (*adra2b, adra2, da, adra2c; ace2, agtrl1a, adt; nos1*) were expressed in endothelial and hematopoietic ($Lmo2^{+}$ and/or $CD41^{+}$) cells of zebrafish embryos, and the expression of several were upregulated at the onset of definitive hematopoiesis. The importance of blood flow in regulating adult blood cell formation from endothelium was confirmed genetically by demonstrating that zebrafish lacking a heartbeat due to a mutation in the gene encoding cardiac troponin T (*sih*) had dramatically decreased Runx1/c-myb expression in the AGM region.

Since HSC formation occurs subsequent to the establishment of the embryonic circulation, the mechanical stimulation of NO production, modulated by sheer stress and alterations in blood flow (Fukumura *et al.*, 2001), could play an important role in inducing HSC and intra-arterial cluster formation. Activation of NO signaling prior to the establishment of the circulation enhanced Runx1/c-myb expression and blood formation, and inhibition of NO signaling had the opposite effect in both zebrafish and mouse embryos (North *et al.*, 2009). Interestingly, chemicals that enhance NO signaling (S-nitroso-N-acetyl-penicillamine, or SNAP) could rescue Runx1/c-myb expression in zebrafish with defective Notch signaling, and correspondingly could block the enhanced expression of ephrinB2a and Runx1/c-myb in transgenic zebrafish expressing the activated intracellular component of the Notch receptor. These data indicate that the NO pathway, and by extension blood flowing through the vasculature, functions downstream of Notch to specify arterial identity and promote blood cell formation.

The effect of flow on blood cell formation from mouse embryonic stem cells was also monitored using Runx1 and c-Myb as markers (Adamo *et al.*, 2009). Using an apparatus that reproduced the biomechanical sheer stress experienced in the embryonic dorsal aorta from the flow of blood, Adamo *et al.* demonstrated that Runx1 and c-Myb expression, as well as the number committed hematopoietic progenitor numbers were upregulated by approximately 2-3 fold by sheer stress. Similar increases were also observed in explants of para-aortic splanchnopleuras or AGM regions exposed to the sheer stress caused by flow. Runx1 expression was reduced in the para-aortic splanchnopleuras of mice deficient for the Na+/Ca+ exchanger encoded by Ncx1

(Slc8a), which lack a heartbeat (Koushik *et al.*, 2001), providing genetic confirmation for the *ex vivo* effects that were observed (Adamo *et al.*, 2009).

Mechanisms of Runx1 action

Although several target genes of Runx1 have been reported ((Otto *et al.*, 2003) and references therein, (Hug *et al.*, 2004; Michaud *et al.*, 2008; Sakai *et al.*, 2009; Wotton *et al.*, 2008)), critical direct targets at the onset of hematopoiesis remain to be identified. Given that Runx1 is a transcription factor required for an endothelial to hematopoietic cell transition, logic dictates that it should repress an endothelial program, while activating genes specifically expressed in blood. Indeed, expression of several endothelial markers, including Flk1 and VE-cadherin, are downregulated in cells after Runx1 is expressed, and before intra-arterial clusters expressing hematopoietic markers are apparent (Hirai *et al.*, 2003; Jaffredo *et al.*, 2005a; Jaffredo *et al.*, 1998). Corresponding changes in endothelial and hematopoietic gene expression were observed in ES cell differentiation cultures in which Runx1 cDNA was conditionally expressed (Sakai *et al.*, 2009). The dynamic expression of hematopoietic and endothelial markers has been most carefully described in chick, *Xenopus*, and zebrafish embryos. Expression of *Runx1* in the ventral endothelium of the dorsal aorta in chick embryos is followed by the progressive loss of *Flk1* and *VE-cadherin* transcripts, and the upregulation of the hematopoietic markers *Scl*, *Lmo2*, *Gata2*, and *Gata3* (Jaffredo *et al.*, 2005a). Intra-aortic clusters are conspicuously *VE-cadherin* and *Flk1* negative (although in the mouse cell surface VE-Cadherin perdures on HSCs in the clusters (North *et al.*, 2002; Taoudi *et al.*, 2005)), and express additional hematopoietic markers including *Pu.1*, *c-myb*, *CD41*, and *CD45* (Jaffredo *et al.*, 2005a; Jaffredo *et al.*, 1998; Manaia *et al.*, 2000; Ody *et al.*, 1999; Pardanaud *et al.*, 1996). Some of these genes may be Runx1 targets (Okada *et al.*, 1998). Runx1 was shown to down-regulate the expression of Flk1, but whether Flk1 is a direct or indirect target is not known (Hirai *et al.*, 2005). Pu.1 is a bona fide downstream target of Runx1 that contains an upstream regulatory element (URE) that is occupied by Runx1 *in vivo* (Hoogenkamp *et al.*, 2009; Huang *et al.*, 2008).

Runx1 is expressed in the hemangioblast, and although Runx1 deficient ES cells can generate hemangioblasts expressing Flk1, Scl, and Fli1, they cannot express the Runx1 downstream target Pu.1 (Hoogenkamp *et al.*, 2009). Runx1 binding to its target sites (the 3' URE and promoter) in the Pu.1 locus in hemangioblasts was weak and unstable, but nonetheless sufficient to induce local chromatin unfolding, as defined by increased sensitivity to DNaseI treatment and demethylation of CpGs. A twelve-hour exposure to Runx1 appeared sufficient to sustain Pu.1 expression and chromatin occupancy by other transcription factors (C/EBP, ETS proteins) on the 3' URE and promoter, even after Runx1 was withdrawn (Hoogenkamp *et al.*, 2009). No ATP-dependent chromatin-remodeling proteins that associate with Runx1 have been identified, so the mechanistic details by which Runx1 promotes this early chromatin unfolding is not known.

The Runx proteins are well-documented activators and repressors of transcription, and domains mediating those activities have been mapped to sequences N- and C-terminal to the DNA-binding Runt domain (Kanno *et al.*, 1998; Liu *et al.*, 2006). Transcriptional activation seems to be the predominant function during HSC emergence, as mutant forms of Runx1 lacking the C-terminal transactivation domain are unable to rescue hematopoiesis in Runx1 deficient ES cells (Nishimura *et al.*, 2004; Okuda *et al.*, 2000), or when reintroduced into cells derived from the p-Sp of Runx1 deficient embryos (Goyama *et al.*, 2004). Deletion of repressive sequences, on the other hand, did not affect definitive progenitor cell emergence, although it did seem to impair the contribution of ES cells carrying those mutations to the fetal liver and thymus of chimeric mice following injection into blastocysts (Goyama *et al.*, 2004; Nishimura *et al.*, 2004).

Several co-factors and co-activators that interact with the transactivation domain of Runx1 could potentially mediate its activity during HSC emergence. The C-terminal transactivation domain, which was mapped between amino acids 291-371 (Kanno *et al.*, 1998), interacts with the transcriptional co-activators MOZ (KAT6a, a member of the MYST family), p300, and the CREB-binding protein (CBP), all three of which have histone acetyltransferase activity (Kitabayashi *et al.*, 2001; Kitabayashi *et al.*, 1998). All three proteins co-purified with Runx1 as a complex that also contained the homeodomain interacting protein kinase 2 (HIPK2), and the promyelocytic leukemia protein (PML) (Kitabayashi *et al.*, 2001; Kitabayashi *et al.*, 1998). Germline deletion of MOZ caused a 5-10 fold decrease in the number of functional and phenotypic hematopoietic progenitors, and the fetal livers contained no transplantable HSCs (Katsumoto *et al.*, 2006). Deletion of p300 or CBP did not affect the emergence of HSCs in the fetus, although CBP was essential for HSC self-renewal (Rebel *et al.*, 2002). Mice doubly deficient for HIPK2, a nuclear serine/threonine kinase that phosphorylates Runx1, p300, and MOZ (Aikawa *et al.*, 2006), and its paralog HIPK1 (*Hipk1*$^{-/-}$ *Hipk2*$^{-/-}$) had a phenotype very similar to that caused by p300 or CBP deficiency (Isono *et al.*, 2006). The fact that progenitors or HSCs emerged at all would indicate that the function of Runx1 in HSC emergence is not entirely dependent on MOZ, p300, CBP, or HIPK1/2. Either there is functional redundancy between these proteins, or other co-activator proteins must be involved.

Conclusions and future directions

The current interest in stem cell-based therapies has emphasized the importance of understanding how tissue-specific stem cells are specified in development. Studies on the expression and role of Runx1 at the onset of definitive hematopoiesis have firmly established this transcription factor as a pivotal player in HSC emergence. The next challenge will be to establish the gene regulatory network in which Runx1 functions. This will provide an important mechanistic framework of HSC emergence and will open the way to future studies into how to modulate this network to promote stem cell maintenance and/or de novo generation. In addition, given the link between deregulation of Runx1 function and leukemia, such a framework will form a basis to explore the changes in the network associated with disease.

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