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Conditional Cre/LoxP strategies for the study of hematopoietic stem cell formation

Nancy A. Speck¹ and M. Luisa Iruela-Arispe²

¹Abramson Family Cancer Research Institute and Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia PA 19104

²Department of Molecular, Cell and Developmental Biology, UCLA, Los Angeles CA 90095

Abstract

Some of the questions that have intrigued developmental biologists studying blood cell formation are: where do blood cells form, what are their precursors, and what signals are required for their emergence. Elegant embryonic grafting experiments in non-mammalian vertebrates, transplantation assays in mouse, and genetic analyses in zebrafish and mouse have been brought to bear on these problems, with enormous success. More recently investigators have applied conditional gene deletion and replacement strategies to refine our knowledge of this process in mammals. Here we describe several studies that have used the Cre/LoxP system to study blood cell formation, and what has been learned as a result.

Blood cell formation in the embryo is a highly dynamic and complex process involving the coordinated activity of many signaling molecules and transcription factors. The importance of understanding how it occurs has been heightened by the potential promise of embryonic stem cell therapy. Describing the path that leads from the embryonic stem cell to the hematopoietic stem cell will guide our efforts to recapitulate this process *ex vivo* for the treatment of hematological disease.

Anatomical organs of hematopoietic stem cells

Hematopoietic stem cells (HSCs) reside in the bone marrow of the adult mammal, where they give rise to differentiated progeny while maintaining themselves throughout the life of the organism. Paradoxically, however, the HSC does not emerge in its ultimate site of residency, but instead appears in the midgestation conceptus before bone or bone marrow forms (49). Defining precisely where HSCs come from has proven to be a controversial area of investigation, due in large part to the inherent difficulty in studying an organ system that is by nature migratory. Thus HSCs that form in one site in the conceptus can colonize others, making it challenging to discover from whence they hail.

Important insights into the anatomical origins of HSCs were first provided by studies of non-mammalian vertebrates that lent themselves well to *in vivo* grafting experiments. Studies by Turpen and colleagues demonstrated that lateral plate mesoderm harbors the precursors of all

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Corresponding author: Nancy A. Speck, Ph.D., 215-898-0247 (office), 215-573-2486 (fax), nancyas@exchange.upenn.edu.

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adult blood (and hence hematopoietic stem cells) in the frog embryo (77). Dieterlen-Lievre and colleagues determined that a region in the avian embryo containing the dorsal aorta, and also an extra-embryonic tissue, the allantois, were potent sources of adult blood (7,13,14). The yolk sac is also a source of blood - in fact in both chicks and frogs it provides most of the blood in the embryo. However the yolk sac contribution to blood is transient, and only negligible numbers of yolk sac derived blood cells can be found in the adult HSC pool in these organisms.

In mouse conceptuses, which develop entirely in utero and thus cannot be easily engrafted, explant cultures combined with adoptive transfer of cells into irradiated or immunocompromised hosts identified several sites that harbored HSCs. These included the dorsal aorta where it is flanked by the developing urogenital ridges – the so-called aorta/gonad/mesonephros (AGM) region, the vitelline and umbilical arteries, the yolk sac, placenta, and fetal liver (11,12,23,47–49,60,83). Of the afore-mentioned sites the fetal liver is widely accepted to be a site of colonization, while all other sites have been shown or suggested to be places from which HSCs emerge. An oft-debated topic in mammalian hematopoiesis concerns which of these sites generates the most HSCs, with a particularly contentious issue revolving around the extent to which the yolk sac contributes to the adult bone marrow HSC population. Early experiments suggested that the murine yolk sac was the primary source of HSCs in mice (48,80), and very recent results suggest that at least 10% of adult bone marrow cells are yolk sac-derived (70). However a significant yolk sac contribution to adult HSCs in mice is difficult to reconcile with the very convincing grafting experiments performed in non-mammalian vertebrates that clearly identified the lateral plate mesoderm, the region surrounding the dorsal aorta, and the allantois as the most important sources (7,13,14,77).

Relationship of blood cell to blood vessel development

Another topic of interest is the long-noted developmental relationship between blood and its conduit, blood vessels. A common precursor of both blood and endothelium called the hemangioblast was postulated many years ago based on the synchronous appearance and close physical proximity of these two lineages in the yolk sac (51). A precursor that fits this description, that can directly give rise to both endothelial cells and primitive blood cells (primitive erythrocytes) was later identified in both embryonic stem cell cultures, and in the posterior region of the primitive streak of the mouse conceptus where gastrulation occurs (10,30).

However the hemangioblast has not been shown to directly give rise to HSCs. Instead a somewhat different precursor/progeny relationship has been suggested for HSCs, namely that endothelial cells form first, and they in turn give rise to HSCs (Figure 1) (34,54).

The differentiation of blood from endothelial cells was first demonstrated in intact chick embryos by lineage tracing experiments, in which endothelium was labeled via intra-cardiac injection with fluorescent dye or retroviruses, and was shown to give rise to clusters of labeled CD45⁺ blood cells that were closely associated with the endothelium (33,34). In a parallel line of investigation Nishikawa and colleagues demonstrated that CD45⁺ blood cells could develop in vitro from flow-purified vascular endothelial cadherin (VEC) positive, CD45[−] cells (53, 54). Runx1, a transcription factor essential for HSC emergence is expressed in endothelial cells in the conceptus specifically in sites where HSCs are found (56). Germline Runx1 deletions result in loss of the intra-aortic hematopoietic clusters and all functional HSCs, which lent considerable support to the notion that HSCs and intra-aortic clusters are derived from a Runx1⁺ “hemogenic endothelium” (6,56,84). However this idea was not universally accepted. For example, it has been suggested that HSCs are born in sub-aortic patches (SAPs) located in the mesenchyme underlying the endothelium and squeeze between endothelial cells en route to forming intra-aortic hematopoietic clusters that are released into the circulation (2,44). This

is supported by studies in zebrafish, where it was shown that HSCs (visualized as CD41⁺ or c-myb⁺ cells) first appear in the space between the dorsal aorta and cardinal vein, and enter the cardinal vein by intravasation to seed the thymus, or migrate along pronephric tubules to colonize the pronephros (3,35,38,50).

Are mouse endothelial cells precursors of blood cells?

The conditional Cre/LoxP system (4) was recently employed to confirm that endothelial cells are the direct precursors of most HSCs, at least in the mouse. Two lineage tracing experiments using an endothelial cell specific promoter from the VEC (*Cdh5*) gene to drive Cre expression, in conjunction with a Rosa26 marker allele (*R26R-yfp* or *R26R-lacZ*) to mark all VEC expressing cells and their progeny demonstrated that many, and perhaps almost all adult HSCs are derived from VEC-expressing cells (8,87). The extent to which VEC-Cre marked cells contributed to adult bone marrow was different in the two studies, probably for technical reasons. One study, which used the *R26R-lacZ* marker allele to identify the VEC-Cre expressing cells and their progeny, demonstrated that on average 50%, and in some animals as many as 80% of adult blood cells were derived from a VEC⁺ cell (87). An even greater number was obtained with an independently generated VEC-Cre transgene in conjunction with the *R26R-yfp* allele, which marked approximately 96% of adult bone marrow cells (8). Whether the remaining blood in these two studies was unlabeled because VEC-Cre is not 100% efficient, or if a small number of HSCs are derived from VEC⁻ cells (e.g. SAPs) remains to be established. It is likely that at least a portion of the unmarked cells result from inefficient excision, as the 86% excision efficiency in cell surface VEC⁺ cells in the fetus reported by one study was similar to the 86% and 96% marking efficiencies observed in fetal liver and adult blood cells, respectively (8).

Although both lineage tracing experiments lent considerable support to the notion that HSCs are derived from endothelium, there was an important caveat, in that other investigators had previously demonstrated that not only endothelial cells, but also fetal liver HSCs express cell surface VEC (37,76). Therefore it could not be definitively concluded that the R26R marker was only being activated in VEC⁺ endothelial cells and not also in VEC⁺ fetal liver HSCs. To examine this more directly, Zovein et al. (87) utilized a VEC-CreERT mouse, in which a tamoxifen-regulated form of Cre (22) was expressed from the VEC promoter sequences, enabling them to activate the R26R-driven marker gene in VEC⁺ cells at specific times. When AGM regions and fetal livers from 11.5 days post-coitus (dpc) conceptuses were isolated and cultured ex vivo in the presence of tamoxifen, only AGM explant cultures could produce labeled blood cells, whereas in fetal liver explants only endothelium and not blood was labeled. Zovein and colleagues concluded that, despite the fact that cell surface VEC (which is present on AGM HSCs) (55) perdures on fetal liver HSCs (37,76), the VEC promoter used in their transgene (and hence Cre) is no longer active once HSCs reached the fetal liver (87). This was consistent with studies in chick embryos that showed VEC mRNA, which is easily detected in endothelium, is already downregulated dramatically in intra-aortic hematopoietic clusters in the AGM region (32) (although in mouse VEC mRNA is still detectable in fetal liver HSCs) (37). In a separate study Chen et al. (8) similarly concluded that VEC-Cre is not active in fetal liver HSCs. They further showed that Runx1, a transcription factor that is necessary for HSC formation, is required in VEC⁺ cells for HSCs to emerge, but it is no longer needed once HSCs reach the fetal liver. This was determined using a combination of VEC-Cre and Vav-Cre to inactivate Runx1 in the endothelium and in fetal liver HSCs, respectively. Only Runx1 inactivation with VEC-Cre blocked HSC formation, whereas excision by Vav-Cre affected HSC homeostasis, but not emergence.

The role of Scl transcription factor

The Scl transcription factor is also required for blood formation. Germline deletion of Scl imposed a profound block in all aspects of hematopoietic development (primitive and adult) (67,75). Although both Scl and Runx1 are expressed in many of the same cells during embryonic hematopoiesis including yolk sac mesoderm, endothelial cells, and HSCs (18,55, 56,71,86), conditional targeting strategies confirmed earlier conclusions that the two transcription factors are required at different times and in distinct HSC precursors (19,25,46, 56,67,75,84). Scl is required in the mesoderm for HSC specification, and unlike Runx1, is no longer required once the hemogenic endothelium forms. This was determined by conditionally deleting Scl in endothelial cells with Tie2 (Tek)-Cre, which had no effect on HSC formation (72). It was also shown, by restoring Scl expression at specific times during the culture of *Scl*^{-/-} embryonic stem cells using a tamoxifen activated Cre, that Scl must be provided before VEC expression (20). Finally, it was established that Scl is required for the differentiation of VEC⁺ cells that could in turn give rise to hematopoietic cells (20). In summary, Scl is required for mesoderm to acquire the competence to produce HSCs, which includes the formation of hemogenic endothelium, but it is not required in the hemogenic endothelium per se. Runx1, on the other hand, is not required for the formation of endothelium, but is necessary in order for endothelium to have hemogenic potential and hence produce HSCs (Figure 1).

The use of the Cre/LoxP system to relate adult to fetal blood cells

The Cre/LoxP system has also been used to query whether all cells in the adult bone marrow are born in the mouse conceptus. This was addressed by activating a Rosa26 marker in the midgestation fetus with a brief pulse (a single, or in one case two injections) of tamoxifen, to activate CreERT. CreERT expression was driven by several different regulatory sequences, including those from the *Runx1*, *Scl*, and *Cdh5* (VEC) genes (27,70,87). In all cases a certain percentage of adult blood cells was labeled when pregnant mice were injected with tamoxifen at midgestation; approximately 10% using Scl-CreERT and up to 24% with VEC-CreERT (the results with VEC-ERT being equivalent to approximately 50% of the labeling that could be achieved by the same group with the constitutively expressed VEC-Cre) (27,87). The Runx1-CreERT mice yielded the most remarkable, and in some ways the most difficult to understand results, with 100% of adult bone marrow being labeled by a single tamoxifen injection of pregnant females at 9.5 dpc, but on average less than 10% when tamoxifen was injected one day later, at 10.5 dpc (70). The ~10% marking at 10.5 dpc by Runx1-CreERT is in line with the data obtained with Scl-CreERT (10.5 and 11.5 dpc injections) and VEC-CreERT (9.5 dpc injection). But the drop in labeling efficiency obtained with 10.5 dpc versus 9.5 dpc injections using Runx1-CreERT is perplexing given that Runx1 expression marks all HSCs at 10.5 dpc (in fact Runx1 expression marks all HSCs in both the embryo and the adult at any time that has been examined) (43,55,57). Perhaps a decline in the level of Runx1-driven CreERT protein between 9.5 and 10.5 dpc could explain such a result, but this is merely conjecture.

Marker genes for hematopoietic sites

Conditional strategies have also been used in attempts to activate marker genes only in certain hematopoietic sites. This is theoretically possible because hematopoiesis in the various anatomical sites comes in successive waves, with primitive erythropoiesis in the yolk sac starting at the mid-primitive streak stage (7.25 dpc), followed by definitive activity (committed erythroid, myeloid, and B cell progenitors) in both the yolk sac and the precursor of the AGM region, the para-aortic splanchnopleura at the 10 somite pair (sp) stage (8.5 dpc), then the placenta at 20 sp (9.0 dpc), and the fetal liver at 40–43 sp (10.5 dpc) (1,26,61). These waves of hematopoietic activity are preceded by, or in the case of the fetal liver coincident with Runx1 expression in each of these sites (42,56,59,86). But there are several technical and biological

challenges in restricting CreERT activity to a particular hematopoietic location. First, there is a fairly wide range in the developmental age of conceptuses between, and even, within litters (15), which makes it very difficult to know at precisely what stage they were exposed to tamoxifen that has been injected into the black box of a pregnant mouse. An equally important variable is the timing of Cre activation – specifically when does it come on, and how long does it last? Various methods have been used to assess this. One approach has been to inject pregnant mice with tamoxifen and then monitor conceptuses for the appearance of marker gene activity over time (29,52,70). Other approaches were to determine how long CreERT remained in the nucleus following a single exposure to tamoxifen (29), or to measure the serum concentration of tamoxifen and its active metabolites over time (87). Although these different strategies have not resulted in a firm answer, a synthesis of the available data suggests that the first evidence of CreERT activity is at about 4–6 hours post tamoxifen injection based on expression of the marker and tamoxifen pharmacokinetics, and it peaks at about 12 hours.

Some investigators report that CreERT activity will decline somewhere between 24 to 48 hours (29,87), and low serum levels of tamoxifen are still detectable 72 hours after injection (87). The kinetics can be influenced by the concentration of both the CreERT protein and tamoxifen, which will be different for individual CreERT strains and studies, and may also depend on whether the injection was performed before or after the maternal/fetal circulation was established. A conservative interpretation of data would assume that CreERT is active from the time of injection and for 48 hours afterwards, particularly under circumstances where knowing when CreERT activity ends is crucial.

A single injection of tamoxifen into a pregnant mouse expressing Runx1-CreERT or VEC-CreERT at 10.5 dpc will miss the first wave of yolk sac hematopoiesis, while capturing a portion of the hematopoietic activity emerging from the AGM region, vitelline and umbilical arteries, and placenta. This strategy was used by Zovein et al. (87) to avoid activating VEC-CreERT in yolk sac mesoderm, enabling them to confine their marking to developmental times when VEC-CreERT activity is restricted to endothelium. On the other hand, a single tamoxifen injection at 7.5 dpc will result in Cre activity from VEC-CreERT or Runx1-CreERT until at least 8.5 dpc, and conservatively 9.5 dpc, and therefore should label definitive hematopoietic progenitors emerging from the yolk sac, dorsal aortae, vitelline and umbilical arteries, and placenta. Thus the experiments of Samakhvalov et al. (70), which demonstrated a yolk sac contribution to adult blood based on an injection of pregnant females expressing Runx1-CreERT at 7.5 dpc must be interpreted with caution, because Runx1 expression has spread to all hematopoietic sites by 8.5 dpc with the exception of fetal liver. We suspect that the labeling of endothelial cells and hematopoietic clusters seen in 10.5 dpc Runx1-CreERT conceptuses following tamoxifen injection at 7.5 dpc is not due to colonization of the dorsal aorta, umbilical and vitelline arteries by labeled yolk sac cells as these authors suggest, but rather by the independent initiation of Runx1 expression, and hence CreERT activity in cells with hemogenic endothelial capacity in these sites.

Signaling pathways and hematopoiesis

Many signaling pathways program hematopoietic cell emergence, including the bone morphogenic protein (BMP), TGF β , Notch, Wnt, vascular endothelial growth factor (VEGF), and Hedgehog pathways (5,9,17,21,24,36,41,58,62,74,82). Conditional Cre/LoxP strategies are being used to dissect the temporal and spatial requirements for some of these signals. A number of studies in both mammalian and non-mammalian vertebrates demonstrated that activation of the BMP pathway is an important early step in hematopoiesis through its role in the formation of mesoderm (36,63,65,73,78). At later stages, high levels of BMP4 in the mesenchyme underlying the ventral aspect of the dorsal aorta may promote HSC formation or expansion in the AGM region (16,45). Bmp4 deficient mice die with early defects in mesoderm

formation (82), making it difficult to ascertain the contribution of BMP4 signaling in later stages of blood cell formation. Park and colleagues (64) assessed the temporal requirement of BMP4 signaling by deleting genes encoding the BMP4 receptor (Alk3) or the downstream signaling molecule Smad4 by Flk1-Cre. These deletions caused widespread defects in vascular remodeling and integrity, however they had relatively modest effects on yolk sac hematopoiesis. BMP4 is critical for the formation of Brachyury⁺ mesoderm from mouse embryonic stem cells, and of Flk1⁺ cells from Brachyury⁺ cells (63,65). Disruption of BMP4 signaling in Flk1⁺ cells bypassed its requirement at these earlier steps and revealed that the major contribution of Bmp4 to yolk sac hematopoiesis occurs prior to the acquisition of Flk1 expression by progenitor cells. Unfortunately Alk3 deletion by Flk1-Cre disrupted the organization of the dorsal aorta and its interaction with smooth muscle cells, making it difficult to evaluate the contribution of BMP4 signaling to HSC formation from the AGM region (63).

Notch signaling is specifically required for hematopoietic cell emergence from the AGM region, but not for either primitive or definitive yolk sac hematopoiesis (5,28,41,68,69). Two of the Notch signaling receptors, Notch1 and Notch4, and the Notch ligands Delta-like ligand 4 (Dll4), Jagged 1 (Jag1) and Jag2 (68,69,85) are expressed by aortic endothelial cells. However Jag1 and Dll1 are also expressed in the subaortic mesenchyme (85), and therefore Notch signaling from either the endothelium or the mesenchyme beneath it could presumably drive HSC formation in the AGM region.

To identify the relevant cell source of the Notch signal, Yoon et al. (85) compared a germline deletion of Mib1, which encodes an ubiquitin E3 ligase required for processing the Notch ligand in the signaling cell (31,39,40) with Mib1 deletion in the endothelium by Tie2-Cre. Mib1 deficient fetuses had no hematopoietic progenitors in the AGM region, but deletion of Mib1 in endothelium with Tie2-Cre did not affect hematopoiesis to nearly the same extent. This suggests that only a portion of the requisite Notch signal emanates from the aortic endothelial cells, and the remainder presumably originates from the subaortic mesenchyme.

Both lineage-tracing and conventional fate-mapping studies have provided clues into why HSC formation from the AGM region is transient, ending at about 12.5 dpc. Studies in both avian and mouse embryos revealed that the subaortic mesenchyme, which can also provide endothelial cells to the floor of the aorta, initially originates from lateral plate mesoderm but is later replaced by somite-derived mesenchyme (66,79). Lateral plate mesoderm specifically labeled with Hoxb4-Cre contributed to mesenchyme and to endothelial cells in the ventral aspect of the dorsal aorta up through 10.5 dpc (79). However at 11.5 dpc the Hoxb4-Cre labeled mesenchymal cells were replaced by somite-derived mesenchyme that could be labeled with Meox1-Cre. This replacement of lateral plate by somite-derived mesenchyme coincides with the disappearance of Intra-aortic hematopoietic clusters in the dorsal aorta (66,79,81). While additional studies are needed, these findings suggest that a specialized mesenchyme, presumably producing Notch ligands, Bmp4, and other signaling molecules are required to facilitate the production of HSCs, and that both the hemogenic endothelium and this mesenchyme are transient populations of cells.

Summary

There is much to be learned from conditional Cre/LoxP strategies, which are the mouse geneticist's answer to the temperature sensitive alleles that have been such powerful tools in the studies of nonvertebrate organisms. Hopefully these approaches will contribute significantly to our knowledge of HSC development, and help pave the way for exploiting the relevant pathways and transcription factors necessary for driving HSC formation *ex vivo*.

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Figure 1.

Requirements for Runx1, Scl, and BMP4 as determined by conditional strategies in mice. A role for BMP4 in hemogenic endothelium and intra-arterial clusters was not assessed (64). Conditional deletion and replacement strategies have, for the most part, defined the end of a required period.