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BMP and Hedgehog Regulate Distinct AGM Hematopoietic Stem Cells Ex Vivo

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SUMMARY

Hematopoietic stem cells (HSC), the self-renewing cells of the adult blood differentiation hierarchy, are generated during embryonic stages. The first HSCs are produced in the aorta-gonad-mesonephros (AGM) region of the embryo through endothelial to a hematopoietic transition. BMP4 and Hedgehog affect their production and expansion, but it is unknown whether they act to affect the same HSCs. In this study using the *BRE GFP* reporter mouse strain that identifies BMP/Smad-activated cells, we find that the AGM harbors two types of adult-repopulating HSCs upon explant culture: One type is BMP-activated and the other is a non-BMP-activated HSC type that is indirectly controlled by Hedgehog signaling through the VEGF pathway. Transcriptomic analyses demonstrate that the two HSC types express distinct but overlapping genetic programs. These results revealing the bifurcation in HSC types at early embryonic stages in the AGM explant model suggest that their development is dependent upon the signaling molecules in the microenvironment.

INTRODUCTION

The first definitive long-term repopulating hematopoietic stem cells (HSCs) originate in the aorta-gonad-mesonephros (AGM) region at mouse embryonic day 10.5 (E10.5) (Medvinsky and Dzierzak, 1996) and emerge from hemogenic endothelial cells lining the aorta and other arteries through endothelial to hematopoietic transition (Jaffredo et al., 1998; de Bruijn et al., 2002; North et al., 2002; Zovein et al., 2008; Chen et al., 2009; Boisset et al., 2010). HSCs are found in hematopoietic clusters closely associated with the vasculature and are in an exclusively ventral position in the aorta (Taoudi and Medvinsky, 2007), highlighting the importance of positional information within the growing embryo. Indeed, in avian embryos ventralizing factors such as vascular endothelial growth factor (VEGF), bone morphogenetic protein 4 (BMP4), basic fibroblast growth factor (bFGF), and transforming growth factor β (TGF-β) are hematopoietic cell inductive, whereas dorsalizing factors such as epidermal growth factor and TGF-α are inhibitors (Pardanaud and Dieterlen-Lievre, 1999). After their generation, HSCs colonize other hematopoietic sites including the fetal liver (FL), where they are greatly expanded (Medvinsky and Dzierzak, 1996; Ema and Nakauchi, 2000; Kumaravelu et al., 2002; Gekas et al., 2005). HSCs migrate again just before birth and colonize the bone marrow (BM) where they reside throughout adult life in endothelial and osteoblastic niches (Mendelson and Frenette, 2014). Thus, the establishment of the vertebrate hematopoietic system is a temporally and spatially controlled ontogenic process that depends on inducing factors and/or growth factors in the different developmental niches.

A key factor in hematopoietic development is BMP4, which is required during different embryonic stages, beginning at the time of gastrulation and mesoderm formation (Winnier et al., 1995) and playing a central role in the hematopoietic specification of mesodermal cells (Zhao, 2003; Pearson et al., 2008). Mice lacking *Bmp4* die in utero before the onset of blood formation. Loss of *Bmp4* endows the embryo with "dorsalized" characteristics and decreases the ventral lineages including hematopoietic cells, vessels, and the pronephric kidney. In contrast, an increase in the ventral lineages is observed when *Bmp4* is overexpressed (Gupta et al., 2006). Similar effects of BMP4 are observed in *Xenopus*, and in zebrafish ventrally localized Bmp4 induces the blood stem cell program in the dorsal aorta (Wilkinson et al., 2009; Huber et al., 1998). In the mouse and human AGM region, ventrally localized BMP4 expression in the endothelial and mesenchymal cells underlying the emerging hematopoietic cluster cells (Marshall et al., 2000; Durand et al., 2007) is thought to influence HSC generation. Indeed, BMP4 increases HSC activity in mouse AGM explants and reaggregates (Durand et al., 2007; Kim

et al., 2015). Moreover, all AGM HSCs in vivo are BMP activated (Crisan et al., 2015).

Another developmental regulator, Hedgehog (Hh), acts as a morphogen in many developing tissues. Visceral endoderm is instructive to the development of endothelial and hematopoietic cells through Hh signaling early in mouse gastrulation (Belaoussoff et al., 1998; Dyer et al., 2001). Hh protein can replace endodermal tissue (gut) to induce HSCs in AGM explant cultures before the normal onset of HSC generation (Peeters et al., 2009). Zebrafish Hh pathway mutants display significant defects in HSC formation, and Hh factors act upstream of VEGF to regulate definitive hematopoiesis in the embryo (Gering and Patient, 2005).

Although BMP4 and Hh, when studied individually, have been shown to influence HSC growth, it is unknown whether these signaling pathways intersect in the same HSCs. In this study, we make use of BMP Responsive Element (*BRE*) *GFP* transgenic mice to study the BMP signaling pathway and the effects of Hh simultaneously on AGM HSC development. We show in explant cultures that the AGM contains two types of HSCs, BMP-activated and non-BMP-activated HSCs, with distinct but overlapping genetic programs. The non-BMP-activated HSC type is lost when the Hh signaling pathway is inhibited, but can be partially rescued by VEGF. We reveal here the signaling pathway regulation involved in the bifurcation of HSC types during development.

RESULTS

BMP and Hedgehog Factors Affect HSC Activity in Serum-Free AGM Explants

Although BMP4 and Hedgehog factors individually influence HSC growth, it is unknown whether these signaling pathways intersect to control HSCs. To address this question, we used AGM explant culture (AGM^{ex}) as a tractable system by which the specific effect of BMP4 or Shh individually, or in combination, on HSCs could be examined. E11 AGM explants were cultured for 3 days in serum-free medium to eliminate the contribution of growth factors known to be present in serum. When tested by transplantation into irradiated adult recipients, no HSCs were found in the AGM^{ex} in the absence of serum (none repopulated of six transplanted recipients) compared with 40% of recipients repopulated (two of five) with HSCs from AGM^{ex} in medium containing serum (Figure 1A). When BMP4 or Shh were added to serum-free AGM^{ex}, 33% of transplanted mice (two repopulated of six transplanted) were high-level, long-term reconstituted (Figure 1A), thus suggesting that individually, BMP4 and Shh have a positive effect on AGM HSC activity. When BMP4 and Shh were added together, 83% of transplanted mice were reconstituted (five repopulated of six transplanted), with the average level of donor chimerism (36%). In combination, BMP4 and Shh significantly improve HSC activity ($p = 0.0005$) compared with no factors in serum-free AGM^{ex}, and the level of HSC activity is similar to that obtained in recipients transplanted with AGM^{ex} in serum-containing medium (40%). Although the combined addition of factors did not yield a significant increase in HSC activity when compared with the single factor additions, this trend suggests that they may control different HSCs.

The AGM Contains Two HSC Types in Explant Culture

To more specifically investigate the distinct or combined effects of BMP and Hh factors on HSC activity in AGM^{ex}, we used the *BRE GFP* transgenic reporter mouse model (Monteiro et al., 2008). In these mice GFP expression reports those cells that, at the time of isolation, are activated by BMP. Recently we showed that this model allows the isolation of HSCs based on their BMP-activation status (Crisan et al., 2015). Our data showed that all AGM HSCs in vivo (AGM^{in}) are BMP activated whereas at later ontogenic stages in vivo (in the E14 FL and adult BM), two distinct HSC types exist: BMP activated and non-BMP activated (Crisan et al., 2015).

Surprisingly, when E11 AGM explants from *BRE GFP* transgenic embryos were cultured for 3 days in serum-containing medium followed by transplantation of GFP^+ and GFP^- sorted cells into irradiated adult mice, HSCs were found in both fractions (Figure 1B). Six out of seven recipients receiving GFP^+ and three out of five recipients receiving GFP^- AGM^{ex} cells were high-level, multilineage engrafted at 4 months post transplantation. These HSCs were self-renewing, as shown by secondary transplantations (Figure S1). Thus, in contrast to AGM^{in}, the explant culture of the AGM reveals the existence of two HSC types: BMP activated and non-BMP activated.

Non-BMP-Activated AGM^{ex} HSCs Are Controlled by Hh/VEGF

We sought to examine whether Hh influences both of the AGM^{ex} HSC types. To test this, we added the Hh pathway inhibitor cyclopamine to *BRE GFP* AGM explants. Following 3 days of culture, GFP^+ and GFP^- cells were sorted and transplanted (Figure 1B). No effect was observed on long-term repopulation by GFP^+ HSCs from AGM^{ex} in the presence of cyclopamine. These HSCs provided the same high-level, multilineage engraftment as in the absence of cyclopamine. In contrast, all HSC activity was lost (none of four) in the GFP^- fraction and almost reached significance ($p = 0.06$) when the Hh pathway was inhibited, compared with the AGM^{ex} GFP^- control (three of five). Since HSCs in zebrafish embryos are controlled by VEGF downstream of the Hh pathway (Gering and Patient,