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The roles of BMP and IL-3 signaling pathways in the control of hematopoietic stem cells in the mouse embryo

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ABSTRACT During mouse ontogeny, the first adult-type hematopoietic stem cells (HSC) are autonomously generated at mid-gestation in the AGM (Aorta-Gonad-Mesonephros) region. Successively present in different anatomical sites where they will expand, HSCs will finally colonize the bone marrow (BM) where they will reside during the entire adult life. In the bone marrow, both HSC self-renewal and differentiation are controlled at cellular and molecular levels by interactions with the stromal microenvironment. So far, very little is known about the extracellular factors involved in the regulation of embryonic HSC emergence, survival and expansion. In the present review, we outline the BMP and IL-3 signaling pathways that are critical for the growth and potential of embryonic HSCs. We will also discuss how these pathways might be integrated with the ones of Notch and Mpl/thrombopoietin, also identified as important key regulators of AGM HSC activity.

KEY WORDS: *AGM, hematopoietic stem cell, BMP, IL-3, transcriptional network*

Introduction

Hematopoietic stem cells (HSC) are extensively studied and constitute a good model for the study of other kinds of stem cells. HSCs and their progeny are now well identified phenotypically and functionally with the development of *in vitro* and *in vivo* assays. HSCs have been used for more than 50 years in transplantations because of their key role in blood regenerative medicine. However, the use of HSCs for therapeutic purposes requires an amplification of this rare population and thus an *ex vivo* reconstruction of the appropriate microenvironment necessary for HSC proliferation. So far no *in vitro* culture conditions (e.g. on the top of stromal cell lines and/or in the presence of growth factors) support optimally the expansion of HSCs. Instead, they progressively differentiate and commit into more mature hematopoietic cells and/or enter apoptosis (Weissman, 2000). Thus, it is crucial to precisely characterize the cellular composition of the HSC surrounding microenvironment and to identify the key factors/signals that support HSC survival/maintenance and self-renewal. The identification and molecular/cellular characterization of HSC regulators has been a long-lasting challenge. The study of embryonic development, where the first HSCs are generated and will phenomenally expand during the course of development, has become essential these past decades. In this review, we will present an overview of two regulators, BMP4 and IL-3, that we recently identified in the embryo as important key players of the HSC fate and we will discuss their relative importance within the complex network of already known HSC regulators.

Adult versus embryonic HSCs

HSCs are at the foundation of the adult hematopoietic system. They are defined by both their ability to self-renew, thus maintaining a constant pool of HSCs, and to extensively proliferate and differentiate to replenish all blood cell types. This homeostasis is maintained during the entire life of an individual. In the adult, HSCs are mainly in G_0 phase (Cheshier *et al.*, 1999) and are known to be slow-cycling cells. They reside in the BM in close contact with stromal cells, which provide a supportive microenvironment for HSCs via direct cell-cell contact or via the production of soluble regulatory molecules. Adult HSCs are found in at least

Abbreviations used in this paper: AGM, aorta-gonad-mesonephros; BM, bone marrow; BMP, bone morphogenetic protein; HSC, hematopoietic stem cell.

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two specialized areas called stem cell niches. HSCs are localized proximal to the endosteal surface of the bone in close contact to osteoblasts (endosteal niche), and to the sinusoids of the bone (vascular niche). They are probably also in contact with other stromal and perivascular cells that have yet to be defined (Calvi *et al.*, 2003, Kiel *et al.*, 2005, Zhang *et al.*, 2003) (Fig. 1). HSC fate is highly controlled by a long and non-exhaustive list of intrinsic regulators such as transcription factors (including GATA2, Runx1, Cbfβ, Lmo2 and SCL) and cell cycle regulators (e.g. p27kip1, p21cip1/waf1) (de Bruijn and Speck, 2004, Gering and Patient, 2005, Hadland *et al.*, 2004, Kumano *et al.*, 2003, Lecuyer and Hoang, 2004, Ling *et al.*, 2004, Porcher *et al.*, 1996, Robert-Moreno *et al.*, 2005, Robertson *et al.*, 2000, Shivdasani *et al.*, 1995, Cheng *et al.*, 2000a, Cheng *et al.*, 2000b, Hock *et al.*, 2004, Passegue *et al.*, 2005, Yuan *et al.*, 2004). Extrinsic regulatory factors of HSCs have also been identified (including TPO, Flt3/Flk2 Ligand, SCF, IL-11, IL-3). The fate of HSCs in the adult BM niches is under the control of several signaling pathways known to play a critical role. This includes hematopoietic cytokines and developmental regulators such as BMP-4 (Zhang *et al.*, 2003), Tie2/Angiopoietin-1 (Arai *et al.*, 2004), Wnt/β-catenin (Cobas *et al.*, 2004, Duncan *et al.*, 2005, Reya *et al.*, 2003, Willert *et al.*, 2003), TGF-β/p21 (Cheng *et al.*, 2000b, Fortunel *et al.*, 2000), Hedgehog (Trowbridge *et al.*, 2006, Bhardwaj *et al.*, 2001) and Notch/Jagged 1 (Calvi *et al.*, 2003, Mancini *et al.*, 2005, Duncan *et al.*, 2005) signaling pathways.

HSCs found in the adult are not produced in the BM but earlier during embryonic development (Cumano and Godin, 2007, Dzierzak and Speck, 2008). In contrast to the adult, HSCs reside in different anatomical sites and are found at different time points during development. The first adult-type HSCs, strictly defined experimentally by their ability to highly reconstitute all hematopoietic cell lineages of a normal adult irradiated recipient, are autonomously generated in the AGM (Aorta-Gonad-Mesonephros) region starting at precisely embryonic day (E)10.5 of mouse gestation (Medvinsky and Dzierzak, 1996, Muller *et al.*, 1994). HSCs are also found at that time in the vitelline and umbilical vessels (de Bruijn *et al.*, 2000). One day later HSCs are present in other highly vascularized organs, the extra-embryonic yolk sac (YS) and the vascular labyrinth of the placenta (Gekas *et al.*, 2005, Medvinsky and Dzierzak, 1996, Muller *et al.*, 1994, Ottersbach and Dzierzak, 2005). The presence of HSCs in the circulation between E11 and E13 suggests that HSCs from these three sites migrate via the circulation to colonize the fetal liver (FL), which becomes the main site of hematopoiesis until BM colonization starts just before birth (Kumaravelu *et al.*, 2002) (Fig. 1). Using an organ explant culture approach and long term transplantation experiments (Fig. 2), it was demonstrated that the AGM is the only tissue able to autonomously generate, maintain and expand HSCs independently of influx of cells from other embryonic sites (Medvinsky and Dzierzak, 1996). Although it is still unclear if the YS and placenta share similar properties, it was reported that the allantois and the chorionic mesoderm (two components of the developing placenta) are both able to generate *de novo* multipotent myeloid progenitors when dissected before the onset of blood circulation (which begins at E8.5) (Corbel *et al.*, 2007, Rhodes *et al.*, 2008, Zeigler *et al.*, 2006). The recent use of Ncx1$^{-/-}$ embryos that lack a heartbeat due to a Na/Ca exchanger 1 defect (Koushik *et al.*, 2001), reveals that the placenta can generate multipotent lympho-myeloid progenitors (Rhodes *et al.*, 2008) and also that most of the progenitors found in the embryo might be generated in the YS (Lux *et al.*, 2008). At E11, approximately one HSC is found each in the AGM, YS and liver. By E12, this number doubles in AGM and YS, and increases up to 50 times in the placenta. By E15, while the placenta retains only few HSCs, the pool in the liver continues to expand (more than 1000 times). In contrast to adult HSCs, fetal HSCs are largely cycling in the placenta and liver that provide suitable niches for HSC self-renewal and become HSC reservoirs at mid-gestation (Ema and Nakauchi, 2000, Kumaravelu *et al.*, 2002, Morrison *et al.*, 1995).

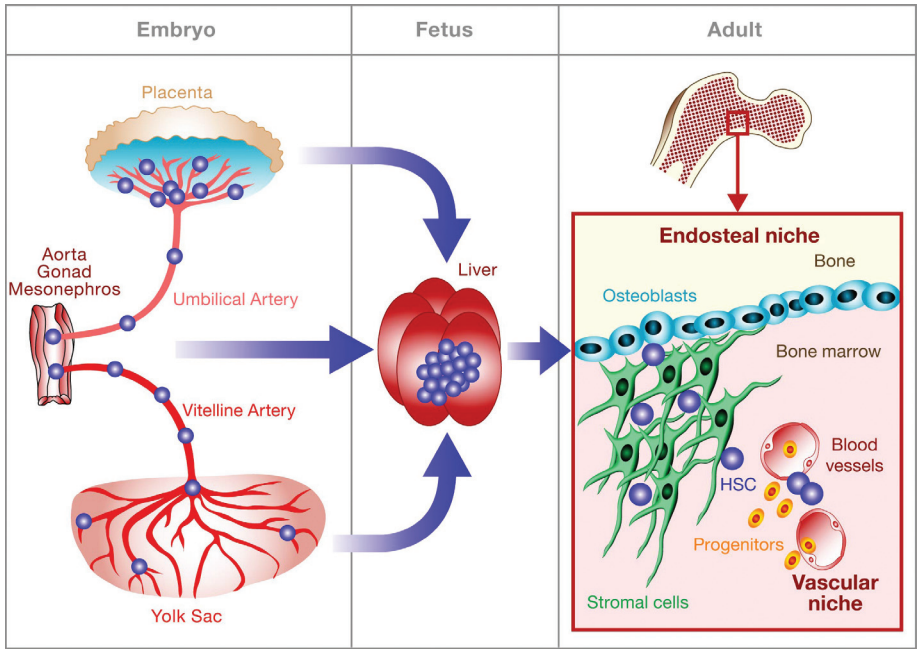

Fig. 1. Development of the hematopoietic system. *In the adult, all blood cell types are produced from hematopoietic stem cells (HSCs) that reside in the bone marrow (BM). The pool of HSCs is maintained in specific niches (endosteal and perivascular) where they are in close contact with the surrounding microenvironment. Adult HSCs are generated during embryonic development. First detected in the aorta-gonad-mesonephros (AGM) region and in the vitelline/umbilical vessels, HSCs are then found in other highly vascularized tissues including the yolk sac, placenta and fetal liver. At mid-gestation, HSCs massively expand in the placenta and fetal liver the latter of which becomes the main hematopoietic tissue until BM colonization at birth. Although many extrinsic and intrinsic regulators of BM HSCs have been indentified, very little is known about the factors involved in the regulation of embryonic and fetal HSCs.*

The composition of stem cell niches during embryonic HSC development is unclear. Interestingly, HSCs are always found in highly vascularized tissues. In several vertebrate embryos (e.g. avian, amphibian, human) (Ciau-Uitz *et al.*, 2000, Dieterlen-Lievre and Martin, 1981, Tavian *et al.*, 1999, Thompson

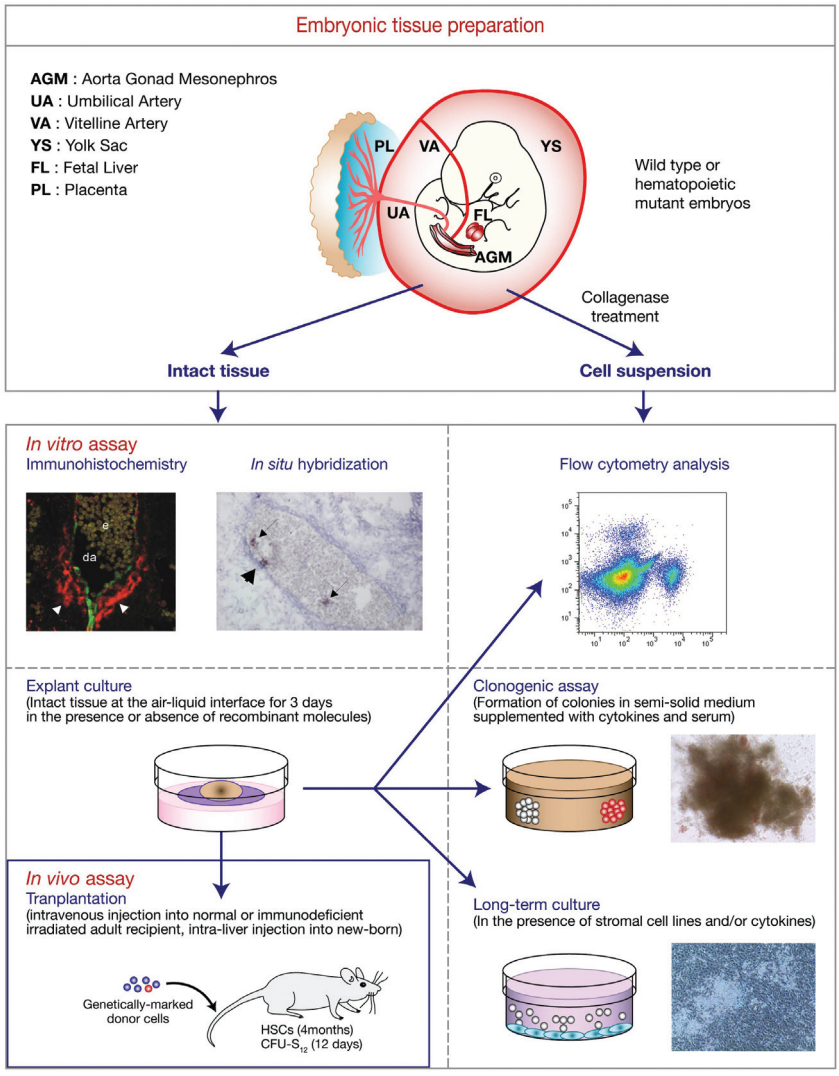

Fig. 2. Experimental design. *The study of hematopoiesis requires the use of a combination of* in vitro *and* in vivo *assays. Hematopoietic tissues are first dissected from wild-type or hematopoietic mutant embryos isolated at different time points of development. Intact tissues are analysed histologically by immunohistochemistry and* in situ *hybridization. Tissues can be cultured as explants for 3 days to induce/influence HSC activity or dissociated by enzymatic treatment. Cells are tested for surface and intracellular markers (by flow cytometry after staining with specific antibodies) and for hematopoietic potential* in vitro *in clonogenic assays or long-term culture in the presence of stroma. HSC activity is tested* in vivo *in transplantation experiments.*

et al., 1998), clusters of cells are found attached to the ventral aspect of the endothelial layer of the main vessels, including the aorta (Taoudi and Medvinsky, 2007). Because some of these cells express hematopoietic markers (e.g. CD45, c-kit, CD41) and the fact that the clusters are totally absent in hematopoietic mutants (e.g. Runx1 and GATA2 knock out (KO)) (Ling *et al.*, 2004, North *et al.*, 1999), it is believed that hematopoietic progenitor and stem cells are localized in these intra-aortic clusters. The development of hematopoietic cells in such a close contact with the endothelium led to the suggestion that endothelial cells possessing hemogenic potential might be the direct precursors of AGM HSCs (de Bruijn *et al.*, 2002, Jaffredo *et al.*, 1998, Oberlin *et al.*, 2002). *In vivo* and *in vitro* experiments performed with mouse, chicken and human embryos support this idea of specialized endothelial cells that are still able to produce hematopoietic cells. Dye and retroviral labelling of endothelial cells from the chick aorta (Jaffredo *et al.*, 2000, Jaffredo *et al.*, 1998) and the *in vitro* differentiation of phenotypically defined endothelial cells (i.e. $Flk1^{+}$VE-cadherin^{+} or $CD34^{+}45^{-}$ cells) into both endothelial and hematopoietic cells (Fraser *et al.*, 2003, Fujimoto *et al.*, 2001, Nishikawa *et al.*, 1998a, Nishikawa *et al.*, 1998b, Oberlin *et al.*, 2002, Yokomizo *et al.*, 2001) demonstrate the existence of the hemogenic endothelium. It was recently demonstrated that in the mouse embryo, hematopoietic clusters are also found attached to the dorsal part of the aorta (Taoudi and Medvinsky, 2007). While the dorsal subdissected part of the aorta contains only progenitor cells, the ventral part of the aorta contains both progenitors and HSCs, revealing the functional heterogeneity of the intra-aortic clusters (Taoudi and Medvinsky, 2007) and suggesting that hemogenic endothelial cells would localize exclusively ventrally. Consistent with this idea, different developmental origins of ventral and dorsal aortic endothelial cells have been described in the avian model (Pouget *et al.*, 2006). It has been also suggested that HSC precursors might also be present in the underlying aortic mesenchyme. Such areas called sub-aortic patches were reported to express an early hematopoietic signature (Bertrand *et al.*, 2005). The birth of HSCs, from specialized hemogenic endothelial cells located to the ventral aspect of the dorsal aorta, has been recently imaged by real-time confocal imaging in live zebrafish embryos (Bertrand *et al.*, 2010, Kissa and Herbomel, 2010) and live mouse embryo slices (Boisset *et al.*, 2010). These three studies provide the ultimate proof of the aortic endothelial origin of adult-type HSCs.

During embryonic development, HSCs always develop in highly vascularized tissues and hematopoietic clusters are found mainly in arteries. Cells in the clusters are in close contact with the blood stream and all the factors produced by the circulating blood cells. They are also exposed to factors produced by all the surrounding tissues such as the aortic endothelium and the mesenchymal tissues underneath. Although not well characterized yet, HSCs can be regulated extrinsically by many factors from many different origins (see below). It becomes evident that a better knowledge of the embryonic microenvironment that provides all the signals required for HSC generation, survival and expansion will contribute to improving the fate manipulation and *ex vivo* expansion of adult HSCs. By using two different experimental approaches, we recently demonstrated the critical role of BMP and IL-3 signaling pathways in the growth and potential of embryonic HSCs.

Embryonic HSC regulation by the BMP pathway

To study and characterize the hematopoietic microenvironment of the AGM region, the site of emergence of the first HSCs, a large panel of stromal cell clones has been generated from the two subdissected AGM regions (aorta mesenchyme (AM) and urogenital ridges (UG)), the gastro-intestinal region (GI) and the embryonic liver (EL) (Oostendorp *et al.*, 2002a, Oostendorp *et al.*, 2002b). Phenotypic characterization of these stromal cell lines reveals that they follow the vascular smooth muscle cell differentiation pathway (VSMC) (Charbord *et al.*, 2002). When cultured in appropriate conditions known to support osteogenic, adipogenic, chondrogenic or endothelial differentiation, most clones possess uni/bilineage osteogenic, adipogenic and/or endothelial potential (Durand *et al.*, 2006) indicating that they are mesenchymal cell lines. Interestingly, the differentiation potential of the stromal clones seems to relate to their site of origin but not to their ability to support hematopoiesis. *In vivo* and *in vitro* assays clearly show that some embryonic stromal clones are potent supporters of hematopoietic stem/progenitor cells and thus are likely to provide important signals for the maintenance and growth of the first emerging HSCs. They might therefore serve as representatives of the AGM HSC-supportive microenvironment. To study the molecular signature of the AGM hematopoietic microenvironment, we compared the expression profile of two closely-related stromal clones derived from the UG region and that differentially support HSC activity. By using a cytokine array, three putative HSC regulators have been identified: MIP-1γ (chemokine of the C-C family), β-NGF (neurotrophic factor) and BMP-4, a well known developmental signaling molecule that we particularly focused on (Durand *et al.*, 2007).

The BMP signaling pathway plays a role in both embryo and adult

BMP factors are members of the TGF-β superfamily. They bind as dimers to cell surface receptors consisting of typeI and typeII serine/threonine kinase subunits. Once a BMP typeI (such as Alk3, Alk6)/typeII complex is formed, the serine/threonine kinase activity of the typeII subunit transphosphorylates a consensus intracellular domain (a GC box) of the typeI receptor which leads to kinase domain activation. Further downstream, the binding of BMP factors to their receptors also results in the phosphorylation of receptor-regulated Smad proteins (R-Smad1, 5 and 8 for BMP factors and R-Smad2 and 3 for TGF-β molecules). Once phosphorylated, R-Smads associate with Smad4. These complexes translocate to the nucleus and in combination with other transcription factors regulate the expression of target genes such as *Runx1* and *GATA2* (Maeno *et al.*, 1996, Pimanda *et al.*, 2007). Negative regulation of BMP signaling involves both extracellular and intracellular molecules. For example, the BMP antagonists gremlin, noggin, chordin and cerberus bind BMP factors to prevent their interaction with BMP receptors. Intracellular regulation involves Smad6 and Smad7 proteins that prevent the phosphorylation of R-Smads and their association with Smad4 (Fig. 3A).

Studies performed on amphibian embryos have shown that BMP-4 plays a critical role in mesoderm patterning and commit-

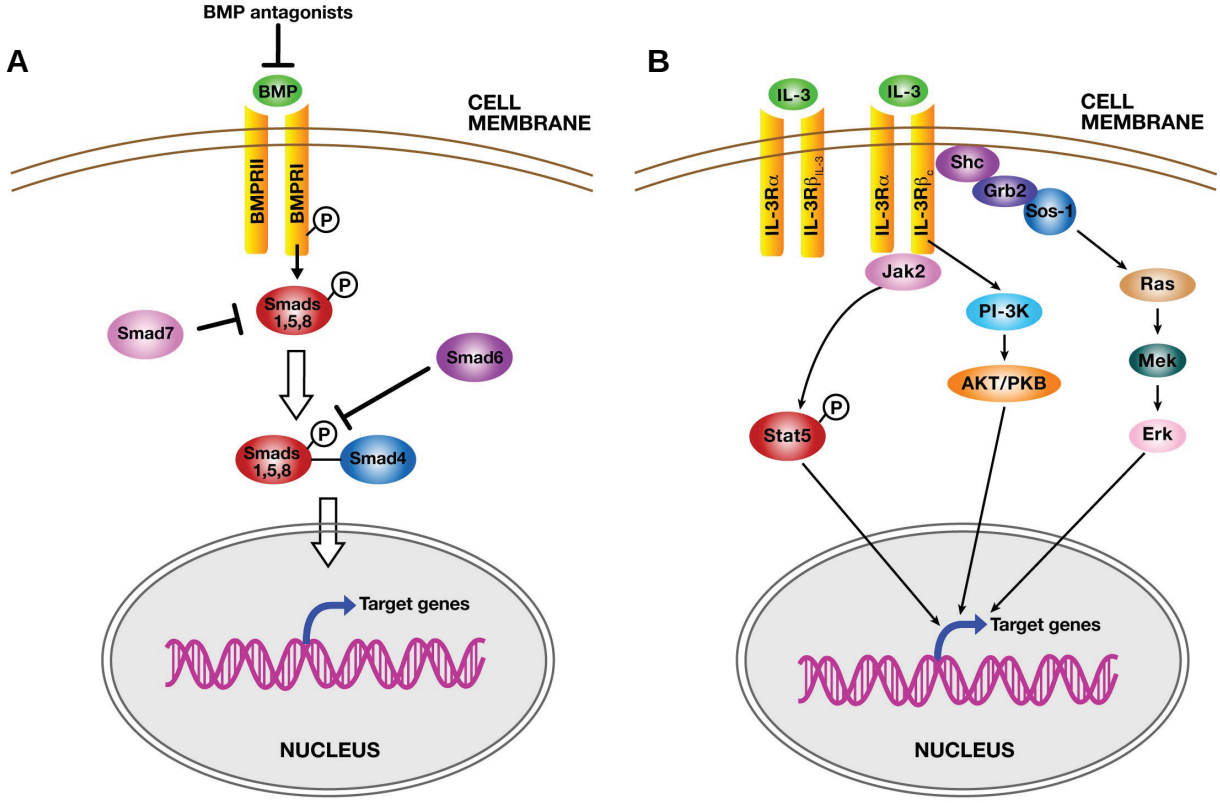

Fig. 3. BMP and IL-3 signal transduction pathways. *Interaction of BMP factors with their receptors* **(A)** *leads to the activation of the canonical Smad cascade. For the IL-3 signaling* **(B)**, *the JAK-STAT, Ras-MAP kinase and PI-3K pathways are represented.*

ment of mesodermal tissue to a hematopoietic fate (Huber and Zon, 1998). In the blastula, BMP-4, which acts as a morphogen, is produced by cells of the ventral marginal zone. The ventral BMP-4 activity is blocked (along the marginal zone) by the production of BMP antagonists, such as Noggin and Chordin, from the dorsal marginal zone (the Spemann organizer). Functional experiments have shown that BMP-4 prevents the formation of dorsal tissues whereas ectopic expression of BMP-4 in the ectodermal animal cap of *Xenopus* embryos results in the expression of hematopoietic genes such as *Scl/Tal1* or *GATA2* (Maeno *et al.*, 1996, Mead *et al.*, 1998).

During mammalian development, BMP-4 is involved in the induction of the YS hematopoietic program. Signals from the visceral endoderm, such as Indian hedgehog, are critical for the development of the YS hematopoietic program and the regulation of BMP-4 expression (Dyer *et al.*, 2001). The implication of BMP-4 in the production of hematopoietic cells was clearly demonstrated by using the embryonic stem (ES) cell differentiation system (Chadwick *et al.*, 2003, Sadlon *et al.*, 2004, Snyder *et al.*, 2004). BMP-4 also controls the self-renewal of human immature hematopoietic progenitors from cord blood (Bhatia *et al.*, 1999). More recently, using *in vivo* transgenic analysis, Zhang *et al.,* and Calvi *et al.,* have shown, respectively, that the BMP and Notch pathways play an important role in the regulation of the BM HSC niche (Calvi *et al.*, 2003, Zhang *et al.*, 2003). These two studies also strongly support the role of osteoblastic cells as a key component of the BM HSC niche.

The BMP signaling pathway is active in the AGM

BMP-4 protein is expressed in the underlying mesenchyme of the dorsal aorta at week 5 of human gestation (Marshall *et al.*, 2000). *BMP-4* transcripts are also expressed in the same area in E10.5 mouse embryos (Pimanda *et al.*, 2007). By immunohistochemistry, we confirmed the presence of the BMP-4 protein in mesenchymal cells underlying the dorsal aorta of E11 wild-type embryos. We also performed immunostaining for BMP-4 on *Ly-6A GFP* transgenic embryos (Fig. 4) (Durand *et al.*, 2007), which have GFP inserted into the first untranslated exon of Ly6A that codes for Sca1, a well known HSC marker (Ma *et al.*, 2002). We have previously described the Ly-6A GFP transgene expression in hematopoietic clusters as well as some cells incorporated in the aortic endothelial layer (de Bruijn *et al.*, 2002) and have shown by transplantation studies that it marks all embryonic and adult HSCs (de Bruijn *et al.*, 2002, Ma *et al.*, 2002, Ottersbach and Dzierzak, 2005). A strong expression of BMP-4 protein was observed in the mesenchyme underlying the GFP^+ hematopoietic clusters attached to the ventral wall of the aorta (Fig. 4A). Interestingly, foci of BMP-4 were also observed on the surface of the GFP^+ cells suggesting that BMP-4 proteins produced by mesenchymal cells may interact directly with the BMP receptors present on the surface of HSCs (Fig. 4B). To test this hypothesis, GFP^+ and GFP^- cell fractions were sorted from AGM and tested by RT-PCR for the expression of three BMP receptors: *Alk3*, *Alk6* and *BMPRII*. GFP^- cells express all *BMP* receptors mRNAs whereas the GFP^+ fraction (enriched in HSCs) expresses predominantly *Alk3* and *BMPRII* transcripts. Thus AGM HSCs could directly respond to BMP-4. To definitively confirm this hypothesis, it would be interesting to look at the expression of BMP receptor proteins on AGM sections and to perform *in vivo* transplantations with cells sorted based on BMP receptor expression.

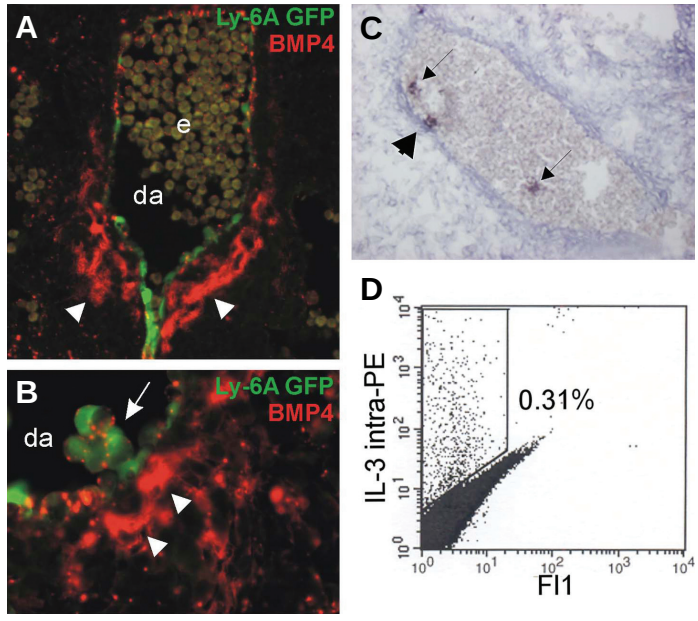

Fig. 4. Expression of IL-3 and BMP-4 in the E11 AGM region. (A,B) *Immunohistochemistry for BMP-4 expression (in red) performed on AGM sections from Ly-6A GFP transgenic embryo. A high expression of BMP-4 is observed in the underlying mesenchyme in the ventral region of the aorta (A, arrow head) and also at the surface of the GFP⁺ cells localized in the intra-aortic hematopoietic clusters (B, arrow) (Durand* et al.*, 2007).* **(C)** *IL-3 transcripts detected in the aorta by* in situ *hybridization on wild type embryo sections. IL-3 expressing cells are present in the lumen of the aorta (arrow) or attached to the aortic endothelium (arrow head).* **(D)** *IL-3 protein detected by flow cytometry after intra-cytoplasmic staining of wild type AGM cells with an antibody against IL-3 (Robin* et al.*, 2006). da: dorsal aorta, e: erythrocytes. With copyright authorizations from Proc. Natl. Acad. Sci. USA and Developmental Cell.*

The BMP signaling pathway is involved in the control of AGM HSC activity

To examine if BMP-4 influences AGM HSC activity *in vivo*, AGM explant cultures were performed in the presence or absence of recombinant BMP-4. At the end of the culture period, AGM cells were collected and transplanted (Fig. 2). We observed that the addition of 20 ng/ml of BMP-4 to the explant medium slightly increases AGM HSC activity while a lower dose (2 ng/ml) has no effect. To evaluate the role of BMP-4 endogenously produced in the AGM, *in vivo* transplantations were performed after AGM explant cultures in presence or absence of the BMP inhibitor, gremlin. In those conditions no reconstituted recipients were obtained indicating that the HSC activity was abolished by gremlin. The negative effect of gremlin was also observed on other immature hematopoietic populations such as the CFU-S (short-term *in vivo* Colony Forming Unit in the Spleen) and the CFU-GEMM (Colony Forming Unit- Granulocyte, Erythrocyte, Macrophage, Megakaryocyte), the most immature progenitor type identified in the *in vitro* Colony Forming Unit-Culture. Thus, BMP signaling positively regulates the potential and/or growth of the most immature progenitors as well as the HSCs found in the AGM region.

To understand how BMP-4 mediates its effect on AGM he-

matopoiesis, we analyzed different hematopoietic populations as well as pre-apoptotic/apoptotic cells present in AGM explants after culture in the presence or absence of gremlin. No significant differences in the level of apoptosis or hematopoietic cell composition were observed. According to our data, it is now clear that the BMP signaling pathway plays a critical role in the maintenance of AGM HSC potential. This was also recently confirmed by Marshall *et al.,* who showed that BMP-4 increases the growth/survival of AGM CD34^{+}c-kit^{high} cells in long-term culture (Marshall *et al.*, 2007).

Embryonic HSC regulation by interleukin-3, a well-known adult hematopoietic growth factor

Many soluble hematopoietic growth factors are produced *in vivo* by the stromal cells that compose the adult BM niches as well as by circulating hematopoietic cells. Such factors are known to positively (e.g. colony stimulating factors, interleukins) or negatively (e.g. TGFβ, Tumor Necrosis Factor) control HSCs at different functional levels (e.g. for self-renewal, cell cycle progression, survival/apoptosis, proliferation, and differentiation processes). In adult, a wide variety of growth factors are implicated in the proliferation and differentiation of hematopoietic stem/progenitor cells into committed lineages (e.g. Epo for erythroid lineage, IL-3/G-CSF/GM-CSF for granulocyte/macrophage differentiation). In comparison, very little is known concerning the factors able to maintain/expand HSCs *in vitro* (e.g. TPO, SCF, Flt3-L). Many cytokines implicated in adult hematopoiesis are functionally overlapping and/or act in synergy. Very often contradictory effects of these molecules are reported in the literature most likely influenced by the different culture conditions and the cell populations used. The possible implication of well-known adult growth factors on embryonic HSC regulation is as yet poorly documented. We tested the potential role of soluble growth factors including well-known adult hematopoietic cytokines on embryonic HSCs.

Growth factors and embryonic HSCs

So far very little is known concerning the soluble factors that control HSCs in the different sites through which they progress during development. Most of the studies are either performed *in vitro*, focusing on cell lines or on already committed progenitor cells, or *in vivo* by the use of knock out mice. SCF and bFGF are known as co-regulators acting mainly synergistically with other cytokines. Both were shown to induce the *in vitro* endothelial/multipotent hematopoietic differentiation of AGM cells when used in combination with oncostatin M (OSM) (Mukouyama *et al.*, 1998). OSM is an interesting factor because it is expressed in the AGM together with its receptor OSMRβ (Tamura *et al.*, 2002). Nevertheless, OSMR$^{-/-}$ mice display only a defect in erythroid and megakaryocytic lineages (Tanaka *et al.*, 2003) and it seems that OSM does not directly affect HSCs, but instead acts on the hematopoietic microenvironment. OSM acts via the signal transducing receptor (gp130) which is common to the IL-6 family members (IL-6, IL-11, LIF, ciliary neurotrophic factor, cardiotrophin-1 and cardiotrophin-like cytokine (Taga and Kishimoto, 1997)). gp130 is required for the *in vitro* expansion of AGM immature hematopoietic precursor population defined by a c-kit^{+}Sca-1^{+}Lin^{-} phenotype (Takizawa *et al.*, 2003). gp130$^{-/-}$ embryos die progressively from E12.5 to soon after birth, with a decrease in the number of hematopoietic progenitors (Yoshida *et al.*, 1996). Thus, this may suggest that members of the IL-6 family, other than OSM, might have an effect on embryonic HSCs. Several other factors have been reported to be potential players in embryonic hematopoiesis. The vascular endothelial growth factor (VEGF) and its receptor Flk1 (VEGF-R2) control the development of hematopoietic and endothelial lineages in the splanchnopleural mesoderm (Eichmann *et al.*, 1997). Indeed a severe defect is observed in both lineages in the Flk1 ko mice (Carmeliet *et al.*, 1996, Ferrara *et al.*, 1996, Shalaby *et al.*, 1997, Shalaby *et al.*, 1995). Another interesting factor is TGFβ1. It was also reported to have a critical impact on both lineages as shown in the chick embryo and knock-out mice (Dickson *et al.*, 1995). A role for M-CSF in the development of hematopoietic and endothelial cells in AGM culture has been reported (Minehata *et al.*, 2002). It was shown to negatively modulate the development of hematopoiesis by stimulating the differentiation of PCLP^{+}CD45^{-} cells to endothelial cells in the AGM region. Recently, the Thrombopoietin/Mpl signaling pathway has been shown to play a role in the regulation of HSCs in the mouse embryo (Petit-Cocault *et al.*, 2007). Nevertheless, so far very few studies have clearly demonstrated an *in vivo* role of adult cytokines in embryonic HSC regulation.

IL-3 positively regulates embryonic HSCs

To identify soluble embryonic HSC regulators, we performed a set of experiments where we used as a model embryos haploinsufficient for Runx1. Because of the lower HSC number in the *Runx1*$^{+/-}$ AGMs (Cai *et al.*, 2000, Robin *et al.*, 2006) they constitute a good model to study positive effects of hematopoietic growth factors on HSC survival and/or expansion. Explant cultures of *Runx1*$^{+/-}$ AGMs were performed in the presence of different concentrations of a selection of potentially interesting candidate growth factors: IL-3, GM-CSF, SCF, bFGF and OSM, which were added alone or in combination to the explant medium (Fig. 2). As tested by *in vivo* transplantations, the addition of GM-CSF, SCF, OSM and/or bFGF had no positive effect on AGM HSC activity when compared to the control in which the explant was performed in the absence of added recombinant factors. Surprisingly the addition of IL-3 alone (20 ng/ml) completely rescues the low hematopoietic activity in the *Runx1*$^{+/-}$ AGMs and allows the repopulation of 100% of the mice injected. By performing cell limiting dilution transplantations of *Runx1*$^{+/-}$ E11 AGM explant cells cultured with increasing concentrations of IL-3, it was clear that IL-3 acts in a dose dependant manner to increase the absolute number of HSCs per AGM (till 35-fold increase). Interestingly IL-3 acts similarly on HSCs from the YS and placenta indicating that IL-3 can positively regulate HSCs found in the different hematopoietic sites during development. It promotes the increase in HSC numbers by both proliferation and survival effects. This was shown by the fact that the absolute number of two phenotypically enriched HSC populations (CD34^{+}c-kit^{+} and Ly6A-GFP^{+}c-kit^{+}) was significantly increased in the AGM cultured in the presence of IL-3 while the number of pre-apoptotic cells (AnnexinV^{+}7AAD^{-}) in several hematopoietic populations (CD45^{+}, c-kit^{+}, CD34^{+}) was decreased.

In the adult, IL-3 (Ihle *et al.*, 1981) influences both myeloid and lymphoid lineages by stimulating proliferation/differentiation as well as cell survival. On HSCs, contradictory effects of IL-3 have been reported including enhancement of differentiation and self-

renewal. This can probably be explained by the use of different cell populations (with different levels of heterogeneity) and differences in culture conditions with respect to the use of serum and different combinations and concentrations of cytokines (Ivanovic, 2004). IL-3 was also recently shown to promote the expansion of hemopoietic-derived $CD45^+$ angiogenic cells in the adult with a further involvement in promoting arterial specification (Zeoli *et al.*, 2008). In the embryo, the unique role of IL-3 was reinforced when we performed explant cultures of *Runx1*$^{+/-}$ AGMs in non-contact co-cultures with AGM stromal cell lines. As mentioned previously, several stromal lines (UG26-2D3, UG26-1B6 and AM20-1B4) can maintain/expand adult and embryonic HSCs when cells from dissociated tissues are cultured in direct contact with these cell lines (Oostendorp *et al.*, 2002a, Oostendorp *et al.*, 2002b). These embryonic cell lines express a wide variety of hematopoietic factors that may influence the hematopoietic fate of HSCs during the culture, including TPO, SCF, Flt3L, G-CSF, IL-1β, IL-6, IL-11, LIF, Weche, and TGFβ. Importantly, these cell lines do not produce IL-3 or oncostatin M (Ohneda *et al.*, 1998, Ohneda *et al.*, 2000, Oostendorp *et al.*, 2002b). After 3 days of co-culture with AGM stromal cell lines, *Runx1*$^{+/-}$ AGM explant cells were tested for long term transplantation. Interestingly, none of the cell lines tested was able to rescue the HSC defect observed in *Runx1*$^{+/-}$ AGM explants. The fact that the stromal cell lines tested do not express IL-3 confirmed the powerful role of IL-3 *in vitro* (Robin and Durand, personal data). The important role of IL-3 in HSC amplification has been recently confirmed in a reaggregate AGM explant culture system where IL-3 was used in combination with SCF and Flt3-L (Taoudi and Medvinsky, 2007).

The presence of IL-3 expressing cells in the embryo was tested by *in situ* hybridization on transverse embryo sections and by intra-cellular flow cytometry (Fig. 2). A limited number of IL-3 expressing cells was found circulating in the major vessels of the embryo such as the dorsal aorta, the vitelline artery and the cardinal veins, where they are occasionally attached to the endothelial layer lining the vessels (Fig. 4C). Most IL-3 expressing cells were found in the stomach rudiment which develops close to the AGM region. The presence of IL-3 expressing cells was confirmed by flow cytometry after intra-cytoplasmic staining for IL-3 (Fig. 4D). In peripheral adult blood, several cell types including activated T cells, Natural Killer cells, mast cells and some megakaryocytes produce IL-3. In contrast, IL-3-producing cells in the embryo express neither endothelial nor hematopoietic/lymphoid markers ($CD45^-CD31^-IL\text{-}7R^-$) and their identity remains to be determined (Robin *et al.*, 2006).

We also analyzed the expression of IL-3 receptors in the mouse embryo. Receptors for IL-3 (as well as for IL-5 and GM-CSF) are members of the gp140 family. Interestingly, two IL-3 receptors have been identified in the mouse as opposed to all other species tested that have only one receptor. They are formed by two closely related IL-3 receptor β chains (Fig. 3B). The β common chain (βc) associates to IL-3Rα, IL-5Rα or GM-CSFRα chains to form receptors that bind IL-3, IL-5 and GM-CSF respectively (Miyajima *et al.*, 1993). The $\beta_{IL\text{-}3}$ chain associates only with the IL-3Rα chain to form the murine specific IL-3 receptor (Hara and Miyajima, 1992). α and β chains belong to the cytokine receptor super family that is characterized by a conserved region homologous to the fibronectin type-III domain. The $\beta_{IL\text{-}3}$ chain is used in preference to the βc chain for IL-3 signal transduction. The IL-3Rα chain binds IL-3 with a low affinity and then dimerises with the β chain to form a high affinity and functional receptor that oligomerizes with other IL-3 receptors. The ligand binding induces many cellular events with the recruitment of multiple signal transduction cascades that involve members of distinct protein families (Blalock *et al.*, 1999, Reddy *et al.*, 2000). The JAK-STAT signaling pathway is involved after activation of JAK2 that leads to the tyrosine phosphorylation of the IL-3R β chain, which then serves as a docking site for other signal transducing proteins including STAT-5 and also STAT-1, -3 and -6. Tyrosine phosphorylation of STATs induces their activation and phosphorylation, before they translocate to the nucleus where they act as transcription factors (Ihle, 1995). Beside the JAK-STAT pathway, the Ras-mediated pathway is also employed. After IL-3 binding, the adaptor molecule Shc is phosphorylated and associates with the phosphorylated βc chain. Shc binds to Grb2, which then associates with mSos to activate Ras. The inositol phosphatase SHIP is also phosphorylated after IL-3 binding. It forms a complex with Shc, Grb2 and Sos which are members of the Ras pathway, followed by the activation of Ras and c-Raf1 and subsequently of MAP kinase family members. The PI-3K signaling pathway is also activated by the binding of IL-3 to its receptor and involves the recruitment of AKT/PKB proteins. These principal pathways lead to the transcription of c-myc, c-fos, Pim-1, OSM and/or c-jun that are implicated in the proliferation and survival of hematopoietic cells.

Both IL-3Rα and βc chains were detected by semi-quantitative RT-PCR and flow cytometry analyses after surface staining with antibodies specific for IL-3R chains on E11 wild type hematopoietic tissues (aorta-mesenchyme and urogenital ridge subregions of the AGM, YS and the V/U vessels) as well as on HSC-enriched $CD34^+c\text{-}kit^+$ populations sorted from AGM explants cultured in the presence or absence of IL-3. We only detect the $\beta_{IL\text{-}3}$ specific chain in $CD34^+c\text{-}kit^+$ cells sorted from AGM explant cultured in the presence of IL-3, indicating some degree of auto-regulation of these receptors. Because IL-3R chains are not expressed by endothelial ($CD45^-CD31^+$) cells but by a small fraction of phenotypically enriched AGM HSCs ($CD34^+c\text{-}kit^+$) cells, IL-3 might exert its effect directly on HSCs. This hypothesis could be confirmed by performing long-term transplantation of cells sorted based on IL-3R expression. Interestingly, we demonstrated that IL-3 also stimulates the growth of YS and placental HSCs (Robin *et al.*, 2006). It would be interesting to search for IL-3 and IL-3 receptors also in these two hematopoietic sites.

Gene targeting experiments (e.g. $\beta c^{-/-}$ (Nishinakamura *et al.*, 1995, Robb *et al.*, 1995), $\beta c^{-/-}IL\text{-}3^{-/-}$ (Nishinakamura *et al.*, 1996), $\beta_{IL\text{-}3}{}^{-/-}$ (Nishinakamura *et al.*, 1995, Nicola *et al.*, 1996)) have shown that in the absence of IL-3 signaling, the mice survive with very minor effects on hematopoiesis. These indicates that in the embryo as well as in adult mice the functions of IL-3 (and IL-5 and GM-CSF) are probably compensated for by other cytokines. Nevertheless, the presence of IL-3 and IL-3 receptors in the midgestation embryo strongly suggests that IL-3 might play an important *in vivo* role in AGM HSC regulation. To test this hypothesis, we examined whether an absence of IL-3 interferes with AGM HSC function. IL-3 present and/or produced in the AGM was neutralized by adding to the AGM explant culture a specific IL-3 blocking antibody prior to transplantation. In this condition a 6-fold decrease in the number of reconstituted recipients injected

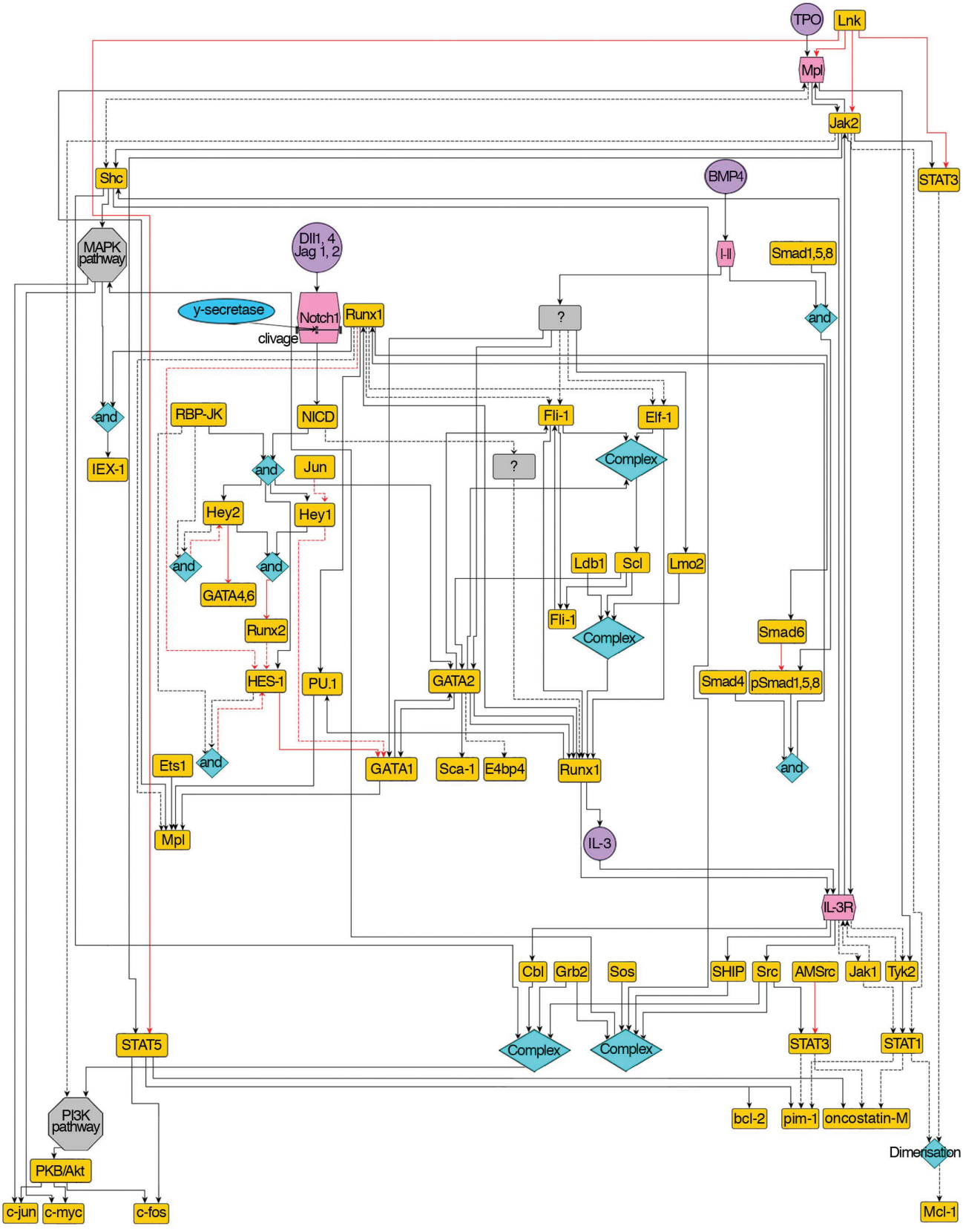

Fig. 5. Network involving BMP, IL-3, Notch and TPO signaling pathways. *No discrimination for protein-protein or protein-DNA interactions is made in the drawing. Full black arrows show activating effects while red arrows represent inhibiting effects. Dotted lines show possible interactions.*

with E11 *Runx1*$^{+/+}$ or *Runx1*$^{+/-}$ AGM explant cells was observed, while the addition of an IgG1 control antibody had no effect. The *in vivo* importance of IL-3 was confirmed by using *IL-3* mutant embryos. Transplantations of E11 *IL-3*$^{+/-}$ and *IL-3*$^{-/-}$ cells from AGM explants revealed a significant decrease in the number of reconstituted recipients when compared to the transplantation of *IL-3*$^{+/+}$ cells. The long-term multilineage reconstitution observed in primary and secondary repopulated recipients indicates that HSCs from these IL-3 deficient embryos are functionally normal, but that their number is dramatically decreased. The reduction of HSC activity in the IL-3 mutant embryos was also observed in freshly dissected AGM, YS and placenta. Thus, a wild type level of IL-3 is important and required to achieve a normal number of HSCs in the major HSC sites in the mouse embryo (Robin *et al.*, 2006).

IL-3 can also affect hematopoiesis at earlier time points of development. Indeed we found that IL-3 promotes HSC activity in early E10 AGM and YS at a time when normally no HSCs are yet detected (prior to the 35 somite pair stage) (Robin *et al.*, 2006). It would be very interesting to find out if IL-3 is also implicated in the regulation of the direct precursors of HSCs in the AGM, and if IL-3 could also influence the hemangioblast fate, the common precursor of hematopoietic and endothelial lineages, found in the YS at an earlier time point of mouse development.

Discussion

Identification of markers uniquely expressed by stem cells and dissection of the molecular mechanisms that control their function are of fundamental interest in the field of stem cell biology. In the adult BM, HSCs reside in specialized niches composed of endosteal and perivascular cells. It is actually unclear whether or not these two cell types are functionally equivalent or distinct. Extrinsic factors are known to play a critical role in the maintenance of adult BM HSCs. These include signaling factors (e.g. BMP, Notch, Shh and Wnt), cytokines (e.g. Tpo and SCF), angiopoietin, extracellular matrix molecules (e.g. osteopontin and fibronectin) and cell adhesion proteins (Wilson and Trumpp, 2006). According to the asymmetric cell division model, integration of these complex signals should regulate HSC functions. As soon as HSCs leave their niche, they are not exposed to these protective signals anymore and thus are more susceptible to undergo a differentiation program.

In the embryo, very little is known about the molecular and cellular complexity of the hematopoietic microenvironment and the possible existence of specialized HSC niches throughout embryonic development. We have shown that in the AGM, where the first adult-type HSCs emerge, the microenvironment is highly complex and contains stromal cells with various mesenchymal lineage potential (Durand *et al.*, 2006, Mendes *et al.*, 2005). Proximal to the underlying mesenchyme, clusters of hematopoietic cells are found closely associated to both the ventral and dorsal aspects of the aortic endothelium. However functional assays have revealed that HSCs reside exclusively in the subdissected ventral part of the aorta while both ventral and dorsal parts contain hematopoietic progenitors (Taoudi and Medvinsky, 2007). This observation strongly suggests that the ventral underlying mesenchyme of the aorta plays an important role in the regulation of AGM HSCs. But so far, very few extrinsic factors have been shown to be involved in the emergence, proliferation and survival of AGM HSCs. *Mpl* mRNA (which codes for the Tpo receptor) is found in the AGM hematopoietic clusters as tested by *in situ* hybridization (Petit-Cocault *et al.*, 2007). Interestingly, AGM HSCs from Mpl$^{-/-}$ embryos have normal long-term repopulating potential but show a defect in the self renewal capacity. Bigas and collaborators have also shown that the Notch pathway is critical for the formation of intra-embryonic hematopoietic cells and for the regulation of GATA-2 expression in the AGM (Robert-Moreno *et al.*, 2005, Robert-Moreno *et al.*, 2008). The comparison of the expression profile of two AGM stromal clones that differentially support HSCs (Durand *et al.*, 2007) and the analysis of the impaired hematopoiesis in Runx1$^{+/-}$ mice (Robin *et al.*, 2006) led to the identification of at least two other extrinsic regulators: BMP-4 and IL-3. The blocking of the activity of both endogenous BMP-4 and IL-3 in the AGM results in the loss of HSC potential.

There are two fundamental questions, 1) how does a single stem cell integrate extrinsic and intrinsic signals? 2) how do these complex molecular events interact and finally drive a stem cell to decide between self-renewal, proliferation, differentiation or apoptotic pathways? Identification and characterization of regulatory elements controlling the expression of key hematopoietic genes in combination with bio-informatic and transgenic analysis have successfully led to a description of complex gene networks (Pimanda *et al.*, 2007, Swiers *et al.*, 2006). Since BMP, IL-3, Notch and Tpo signaling is critical for the regulation of AGM HSCs, we have analyzed the connections between these four pathways. Based on the literature and by using computer modeling (Yed Software), we propose a preliminary model that integrates the pathways of IL-3, BMP-4, TPO and Notch (Fig. 5). For example, the IL-3 and Tpo pathways are integrated through the Jak/Stat and MAPK cascades. They presumably control the expression of hematopoietic genes such as *c-myc* and *oncostatin M*. In addition, both Notch and BMP pathways play a role in the regulation of *GATA-2* and *Runx1* expression, two master genes of the development of AGM HSCs. It is of particular interest to note that Pimanda *et al.,* have shown that the Runx1 and BMP pathways are integrated into the SCL transcriptional network. Indeed, Smad1 activates the *Runx1* promoter whereas Smad6 blocks the activity of Runx1 (Pimanda *et al.*, 2007).

Accumulating evidence suggests that the transcriptional networks controlling HSC fate in the embryo are highly complex. Future studies will attempt to understand how other molecules (i.e microRNAs) regulate the expression of target genes and how extracellular or intracellular antagonists such as gremlin/noggin for the BMP and numb for the Notch pathways may control the activation versus inhibition of signaling pathways at different time points during ontogeny.

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