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PROSPECTS

Bone Morphogenetic Proteins in Vertebrate Hematopoietic Development

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Abstract During embryonic development, the hematopoietic system is the first to generate terminally differentiated, functional cell types. The urgent necessity for the early formation of blood and blood vessels during embryogenesis means that the induction, expansion, and maturation of these systems must be rapidly and precisely controlled. Bone morphogenic proteins (BMPs) have been implicated in hematopoietic development in the vertebrate embryo and stimulate the proliferation and/or differentiation of human cord blood hematopoietic stem cells (HSC) and embryonic stem cells in vitro. Here we review the mechanisms of action and potential roles of these soluble signaling molecules in vertebrate hematopoiesis. J. Cell. Biochem. 93: 224–232, 2004. © 2004 Wiley-Liss, Inc.

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"Primitive" erythroblasts, nucleated red blood cells produced in the yolk sac (YS) (or, in amphibian embryos, the ventral blood island) during the earliest stages of hematopoiesis in the vertebrate embryo [Baron, 2003], are among the first mature, fully functional cell types generated during development. These cells, which recently have been shown to undergo enucleation during a window of time when "definitive" erythroid cells begin to expand within the circulation [reviewed in Baron, 2003], are assumed to function in the rapid and efficient transport of oxygen in the embryo. Somewhat later, "definitive" or adult type hematopoietic development initiates, generating a collection of lineages, including "definitive" erythroid, myeloid, and lymphoid cells, whose production persists through fetal and adult ontogeny. Definitive hematopoietic stem cells (HSCs) form in the YS, para-aortic splanchnopleura (P-Sp)/aorta-gonadal-mesonephros (AGM) region (or, in *Xenopus*, the dorsal lateral plate), and—at least for mouse—in the placenta [Alvarez-Silva et al., 2003]. HSCs are then believed to seed the fetal liver, which serves as the primary hematopoietic organ until around birth, when bone marrow takes over as the major hematopoietic tissue [reviewed by Baron, 2003]. The hematopoietic system is maintained by a stem cell population capable of both self-renewal and generation of more committed progeny (i.e., more restricted developmental potential). These HSCs constantly regenerate the vast numbers of blood cells that are turned over during the course of normal homeostasis.

Within the developing "blood islands" of the YS and in certain intraembryonic regions, endothelial and hematopoietic cells arise in close spatial and temporal association [for review, see Baron, 2003]. At least some of these

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hematopoietic and endothelial cells appear to arise from a common mesodermal progenitor, the "hemangioblast." Accumulating evidence also supports the existence of adult cells with hemangioblastic activity [reviewed by Bailey and Fleming, 2003].

Considerable attention has been focused on the mechanisms that regulate the induction, differentiation and maintenance of the hematopoietic system. Hematopoietic cytokines and their associated signal transduction pathways have been extremely well studied but much less is known about hematopoietic functions of other classes of signaling molecules. The Notch and Wnt pathways play important roles in a variety of developmental processes and have also been shown to control self-renewal or differentiation of HSCs and/or more committed progenitors [for review, see Pear and Radtke, 2003; Reya et al., 2003]. Experiments performed in vitro have implicated the hedgehog signaling pathway in hemato-vascular development [Baron, 2003].

In this review, we focus on bone morphogenetic protein (BMP) members of the transforming growth factor-β (TGF-β) superfamily of extracellular signaling molecules. Although BMPs were originally discovered on the basis of their ability to induce ectopic bone formation, it rapidly became evident that these peptides have much broader functions during development. Genetic studies have demonstrated that BMP4, in particular, plays critical roles in formation and patterning of mesoderm. Relevant aspects of the BMP signaling pathway will be presented first. We will then review evidence for hematopoietic activities for the BMP signaling pathway during development.

THE BMP SIGNALING CASCADE

The bone morphogenic proteins (BMPs) are soluble factors related to TGF-β and activin. They are synthesized as inactive precursors that undergo proteolytic processing [reviewed in de Caestecker, 2004], folding to form a "cysteine knot" motif and dimerizing via covalent (disulfide) bonds [de Caestecker, 2004]. Secreted BMP dimers bind to two type I and two type II serine–threonine kinase cell-surface receptors (Table I) to form a heterotetrameric signaling complex [reviewed in de Caestecker, 2004]. Upon binding BMP ligand, the type II receptor pairs with a type I receptor, which it phosphorylates within a glycine-serine rich juxtamembrane domain (the GS box), thereby activating the complex (Fig. 1). Other cell surface proteins, notably endoglin [see below, and Chen et al., 2003], also interact with the ligand–receptor complex and, at least in some cases, regulate signaling. The activated BMP-receptor complex phosphorylates the receptor Smad proteins (R-Smads) 1, 5, or 8 and initiates the signaling cascade [for review, see de Caestecker, 2004]. Phosphorylated R-Smads associate with the common Smad 4 (which is also a component of the TGFβ pathway), translocating into the nucleus as a complex (Fig. 1) whose stoichiometry remains unclear. The inhibitory Smads (6 and 7) antagonize signaling by preventing phosphorylation of R-Smads or their interaction with Smad 4 [Fig. 1 and de Caestecker, 2004]. R-Smads bind DNA weakly and are thought to function in concert with sequence-specific DNA binding transcription factors in vivo. Within the nucleus, the

TABLE I. BMP Pathway Ligands and Receptors

Ligand	Type II receptor	Type I receptor	Accessory receptor
BMP2	BMPRII	BMPR1A (Alk3)[a]	
	ActRII	BMPR1B (Alk6)	Endoglin
	ActRIIB		Endoglin
BMP4	BMPRII	BMPR1B (Alk6)	
		BMPR1A (Alk3)	
		Alk1,[b]ActR1A (Alk2)[b]	
BMP7 BMPRII		ActR1A (Alk2)	
	ActRII	BMPR1A (Alk3)	Endoglin
	ActRIIB	BMPR1B (Alk6)	Endoglin

Information is included here only for BMP2, BMP4, and BMP7, which are closely related to one another and have hematopoietic activities in various assays (see text). For reviews, see [Shi and Massague, 2003; de Caestecker, 2004].

[a]Alk, activin-like kinase.

[b]Note that Alk1 and Alk2 (but not Alk6) are upregulated in *Alk3*$^{(-/-)}$ ES cells, suggesting that ALK1 and/or ALK2 proteins may be able to function as BMP4 type I receptors in the absence of ALK3 [Qi et al., 2004].

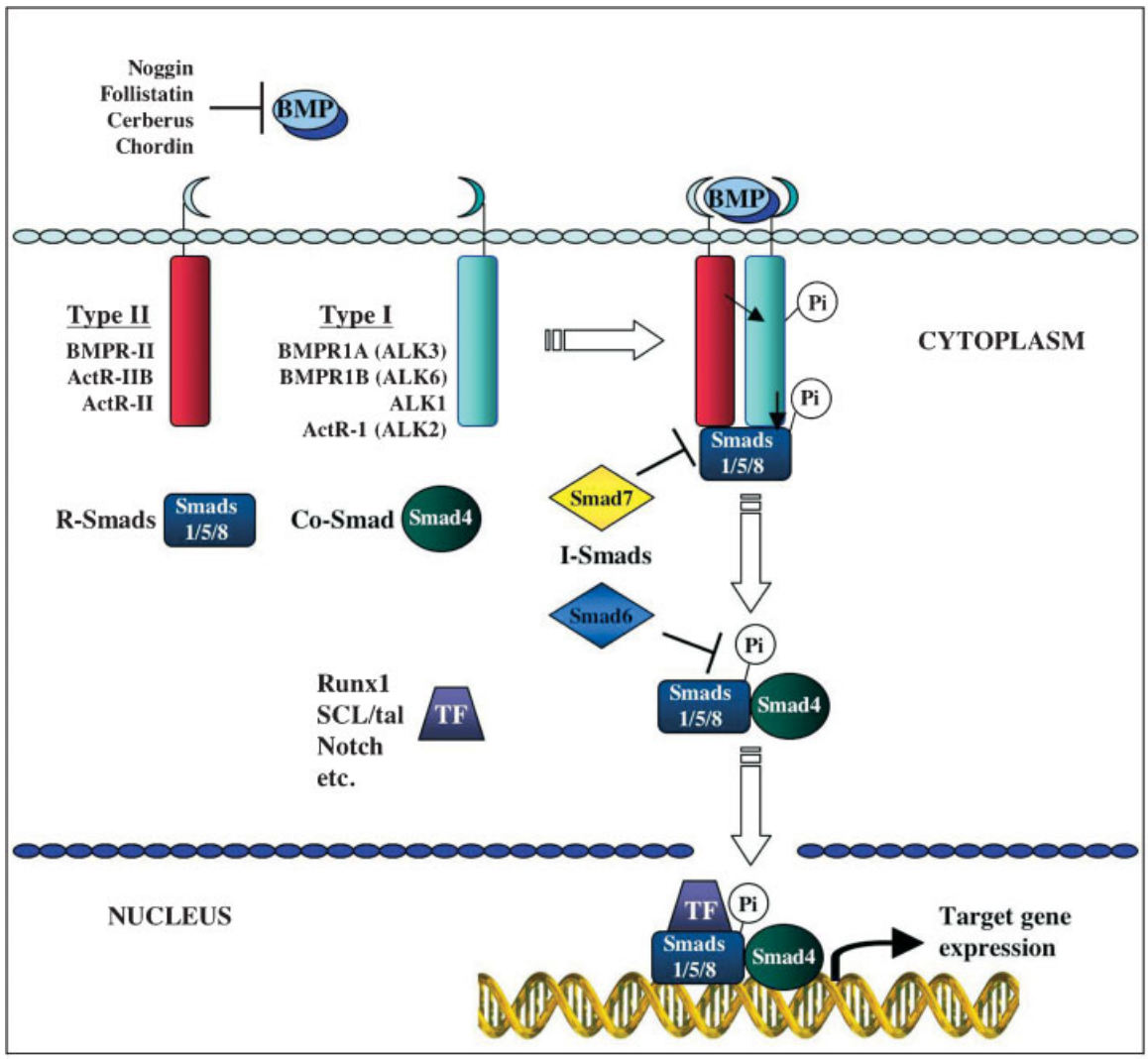

Fig. 1. The BMP signaling cascade. BMP 2, 4, and 7 ligands bind to the Type II receptors. Upon binding, Type I and II receptors heterodimerize, and Type II phosphorylates Type I. This leads to the phosphorylation and activation of one of the receptor (R-) Smads (1, 5 or 8). The R-Smads then interact with the co-Smad, Smad 4. This complex translocates to the nucleus, interacting with other transcription factors such as Runx1, SCL/tal or Notch to up- or downregulate transcription of target genes. The I-Smads (Smads 6 and 7) and other proteins antagonize the pathway at various points. Alternative (Smad-independent) pathways (e.g., p38 MAPK) are not shown.

R-Smad/Smad 4 complex also associates with co-activators or co-repressors [von Bubnoff and Cho, 2001; Canalis et al., 2003; de Caestecker, 2004] to control the expression of genes such as *AML1/Runx1/Cbfa2* and *Gata-2* [Liu et al., 2003] (Fig. 1). The Ski oncoprotein, which is highly expressed in HSCs, is a Smad-dependent co-repressor that blocks BMP signaling by disrupting R-Smad/Smad 4 complexes [Canalis et al., 2003]. The activity of R-Smads and Smad 4 can also be modulated by targeted ubiquitination or by cytosolic adapter proteins/chaperones which control access of Smads to TGFβ/BMP receptors or regulate their intracellular localization [von Bubnoff and Cho, 2001; de Caestecker, 2004].

It is now clear that BMPs can function as morphogens, molecules that elicit distinct developmental outcomes in a dose-dependent manner. The effective concentration of active BMP outside the cell is regulated, in part, by secreted antagonists such as noggin, chordin, follistatin, Twisted gastrulation (Tsg), and Cerberus [Fig. 1, and reviewed at length in Canalis et al., 2003]. Some of these proteins (e.g., noggin) are themselves induced by BMP signaling and appear to function as part of a negative feedback loop. Follistatin, initially

identified as an activin antagonist, can also bind BMP2/4. However, in contrast with noggin, follistatin expression is downregulated by BMPs. Tsg is unique in its potential to antagonize or promote BMP activity [Canalis et al., 2003].

BMPS AND THE INITIATION OF HEMATOPOIETIC DEVELOPMENT

What signals switch on the developmental program of blood formation in embryonic mesoderm? Early studies in *Xenopus* pointed to a role for BMP signaling in ventral patterning of mesoderm during gastrulation [von Bubnoff and Cho, 2001]. Targeted ablation of the mouse *Bmp4* [Winnier et al., 1995; Lawson et al., 1999], *Bmp2* [Zhang and Bradley, 1996], and *Bmpr1a*/*Alk3* [Mishina et al., 1995] genes led to similar conclusions. Because primitive hematopoietic cells arise from "ventral" (hematopoietic) mesoderm, it has been assumed that BMPs are essential for the earliest phase of hematopoiesis. Whether BMP4 functions solely as an inducer of hematopoietic mesoderm or plays an additional, independent role in hematopoiesis has been a matter of some dispute. Ectopic expression of BMP4 in cDNA-injected early stage frog embryos resulted in loss of muscle and notochord and enhanced production of blood cells (i.e. the embryos were "ventralized"). Similarly, injection of BMP4 mRNA into animal cap (ectodermal) explants led to activation of hematopoietic genes such as *Gata-2* and *SCL*/*tal* [reviewed by Walters et al., 2002]. BMPs induce hematopoietic differentiation of ES cells [see refs. cited by Baron, 2003; Chadwick et al., 2003] and can regulate self-renewal or differentiation of HSCs from human cord blood [Bhatia et al., 1999] (Fig. 2). Hematopoiesis was blocked when a dominant negative BMP2/4 receptor or noggin, a BMP antagonist, was injected into *Xenopus* animal caps [see additional citations in von Bubnoff and Cho, 2001]. These studies indicated that formation of ventral mesoderm is not a default state but requires an active signal [reviewed by von Bubnoff and Cho, 2001].

In *Xenopus* ectodermal explant cultures, the mesoderm inducers activin or bFGF were required in combination with BMP4 to achieve significant hematopoietic activation; BMP4 alone was not sufficient [von Bubnoff and Cho, 2001]. In mouse embryo ectodermal explant cultures in which hematopoietic and vascular development was activated by exogenous hedgehog protein, circumventing the requirement for visceral endoderm (VE) signals, *Bmp4* transcription was significantly increased [Dyer et al., 2001]. It was proposed that, in vivo, Indian hedgehog signaling from the VE may function in part through upregulation of *Bmp4* [Fig. 2 and Baron, 2001; Dyer et al., 2001]. Together, these studies suggest that hematopoietic fate is specified by BMP signaling during gastrulation. Other studies in the frog seem to indicate, however, that the hematopoietic activity of BMPs is more complex. BMPs and Ca^{2+}/calmodulin-dependent protein kinase IV (CaM KIV) may function in a common signaling pathway to control survival and lineage specification; CaM KIV inhibits the BMP pathway [Walters et al., 2002]. Constitutive activation of CaM KIV or ectopic expression of BMP inhibitors such as Smad 6 were shown to reduce survival of committed erythroid progenitors, while stimulation of the BMP pathway induced myeloid differentiation, apparently at the expense of erythroid differentiation [cited by von Bubnoff and Cho, 2001]. It is not yet clear whether the myeloid cells arose from primitive hematopoietic progenitors in the ventral blood island (a view favored by the authors) or independently, from a distinct population [Walters et al., 2002]. Moreover, the particular BMP(s) affected in these experiments are unknown: BMP2/4/7 are all expressed in gastrula stage frog embryos. In studies performed in vitro using recombinant BMP proteins, each BMP elicited distinct outcomes (proliferation/survival/differentiation) in a dose-dependent manner. High concentrations of BMP2 and BMP7 both inhibited proliferation of cord blood HSC, while high doses of BMP4 increased HSC expansion in culture [Fig. 2 and Bhatia et al., 1999].

LESSONS LEARNED FROM MOUSE MUTATIONS IN THE BMP PATHWAY

Targeted disruption of the mouse *Bmp4* [Winnier et al., 1995], *Bmp2* [Zhang and Bradley, 1996], *Bmpr*/*Alk3* [Mishina et al., 1995], or *Ecsit* [Xiao et al., 2003] genes resulted in severe mesoderm deficiency and early embryonic lethality. On certain genetic backgrounds, embryonic development of *Bmp4* null mutant embryos could progress to later stages

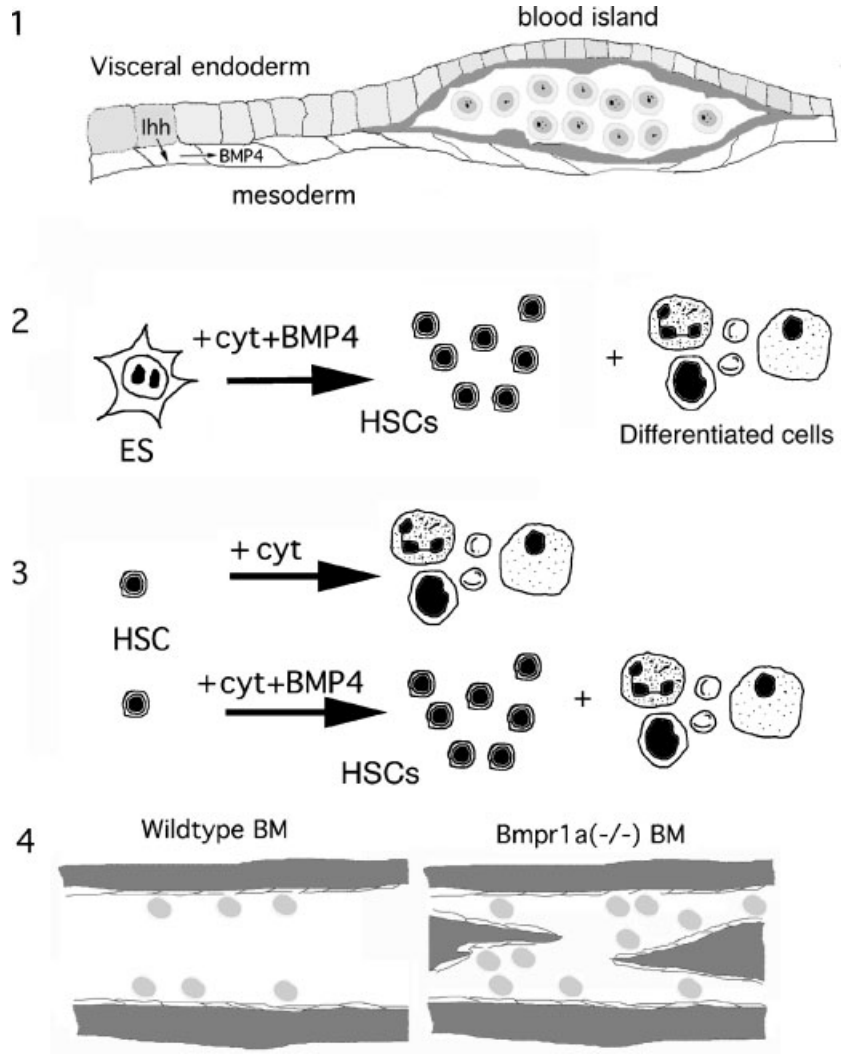

Fig. 2. Four settings in which BMP4 can influence hematopoietic development. (1) Induction of early hematopoietic cells from mesoderm in response to cues from the visceral endoderm (VE) [Baron, 2001; Dyer et al., 2001]. (2) BMP4 enhances the production of progenitors and differentiated hematopoietic cells from mouse, rhesus monkey, or human ES cells [Baron, 2003; Chadwick et al., 2003]. cyt, cytokine cocktail. (3) BMP4 promotes self-renewal of HSCs from human cord blood [Bhatia et al., 1999]. (4) The adult bone marrow microenvironment: in the absence of BMP signaling via BMPR-1a, osteoblast apoptosis is reduced and trabecular bone mass is increased. The HSC microenvironment is thereby extended and the numbers of HSCs increased [Zhang et al., 2003].

[Winnier et al., 1995; Lawson et al., 1999]. The YSs of these embryos were abnormal, with decreased extraembryonic mesoderm (ExM) and few, if any, primitive erythroid and endothelial cells [Winnier et al., 1995]. Prior to its expression in the epiblast and, later, in the ExM, *Bmp4* is expressed in the extraembryonic ectoderm (ExE). Tetraploid chimera analyses have established that Bmp4 produced by the ExE is required for establishment of primordial germ cell (PGC) and allantois lineages [Lawson et al., 1999], while ExM-derived Bmp4 is essential for normal localization of PGCs and development of the allantois [Fujiwara et al., 2001].

The early embryonic lethality exhibited by *Bmp4* and other mutants has precluded a detailed analysis of the role of the BMP pathway in hematopoietic development. Analysis of conditional null mutant mice in which gene expression can be selectively ablated in specific lineages (depending on the availability of appropriate Cre-expressing mouse lines) may facilitate progress in this area.

Smad proteins may have both positive and negative biological effects. For example, Smad 5 is required for survival of mesenchyme and for normal vascular development in the embryo but—perhaps somewhat surprisingly, given its

function in the BMP pathway—it has also been implicated in the negative regulation of primitive hematopoietic progenitor cells during embryonic hematopoiesis [Liu et al., 2003]. *Smad5* null mutant ES-cell derived embryoid bodies (EBs) show decreased sensitivity to TGF-β1 [Liu et al., 2003], a molecule known to inhibit proliferation of hematopoietic progenitors. Interestingly, ectopic vasculogenesis and hematopoiesis is observed in the amnions of *Smad5* deficient embryos [Chang et al., 1999]. While the inappropriate formation of hemato-vascular cell types has been interpreted as "mislocation" of allantois tissue in the mutants [Chang et al., 1999], it could also reflect the absence of an inhibitory activity of Smad 5 in the amnion.

BMPs AS EFFECTOR MOLECULES IN LATER STAGES OF HEMATOPOIETIC DEVELOPMENT

As noted earlier, the AGM is an important region of hematopoietic development in the embryo proper. Clusters of CD34+ and CD45+ cells have been observed in very close apposition to the endothelial lining of the ventral wall of the dorsal aorta in human, mouse, and chick embryos [reviewed by Speck et al., 2002]. The Runx1 transcription factor is expressed in and is required for formation of intra-aortic clusters, suggesting that these are the HSCs of the AGM [Speck et al., 2002]. *Runx1* is expressed not only in these cells but also in similar clusters that appear to bud out from the endothelium of the vitelline and umbilical arteries [Speck et al., 2002]. Interestingly, the "budding" process was greatly dampened in embryos haploinsufficient for *Runx1* and mesenchymal HSCs were detected in increased numbers than in wild type [N. Speck, personal communication, and North et al., 2002]. Mesenchymal "angio-hematopoietic" stem cells are seen in the AGM region of human embryos (B. Péault, personal communication). Polarized expression of BMP4 protein has been observed in the human AGM, suggesting a direct role for this signaling molecule in hematopoietic specification of mesoderm in vivo [Speck et al., 2002]. It is tempting to speculate that BMP4 signaling may induce expression of *Runx1* in the AGM mesenchyme.

BMP signaling has been implicated in the maintenance of the niche (microenvironment) controlling HSC number. BMP receptor 1A (BMP-R1A) is expressed by osteoblasts present in the bone marrow, the microenvironment in which adult HSCs reside. When BMP-R1A expression was conditionally ablated in osteoblasts, the frequency of this cell type increased, presumably because of decreased apoptotic signaling via this receptor [Zhang et al., 2003]. Osteoblasts increased dramatically in number and associated via tight junctions, with a corresponding increase in the number of HSCs [Zhang et al., 2003]. Regulation of the HSC niche by osteoblasts, possibly by Notch activation, was demonstrated in an independent study [Calvi et al., 2003]. Thus, the interface between osteoblasts and HSCs is a crucial component of the stem cell niche in bone marrow (Fig. 2).

The TGFβ/BMP accessory receptor endoglin [de Caestecker, 2004] is differentially expressed in long-term repopulating hematopoietic stem cells (LTR-HSC) and has been used as a marker for purification of these cells from adult mouse bone marrow [Chen et al., 2003].

CROSS-TALK BETWEEN THE BMP AND OTHER SIGNALING PATHWAYS

The BMP and TGFβ pathways can function antagonistically, for example through competition for limiting amounts of the common Smad 4. The BMP cascade may also cooperate with or antagonize other signaling systems. Ecsit, an adaptor in the Toll receptor pathway, associates with the Smad 1/Smad 4 complex, inhibiting BMP target gene transcription [Xiao et al., 2003]. Alternative (Smad independent) signaling pathways may be regulated by BMP signaling. BMP2 can activate PI3 kinase, Akt kinase, or p38 MAPK in a number of cell types [von Bubnoff and Cho, 2001; de Caestecker, 2004]. The MAP kinase pathway, which mediates the effects of certain receptor tyrosine kinsases (RTK), can regulate Smad activity through differential phosphorylation and, therefore, nuclear localization [de Caestecker, 2004]. BMP4 synergizes with vascular endothelial growth factor (VEGF), whose activity is mediated by the RTK Flk1/VEGFR2, to enhance the production of erythroid, myeloid, and lymphoid cells in ES cell-derived EBs [Fig. 2 and Nakayama et al., 2000]. In *Drosophila*, BMP and RTK signaling synergize to regulate expression of key transcription factors directly, via Smad and cAMP response elements [von Bubnoff and Cho, 2001], and it is possible that similar interactions at the level of the promoter

may also occur in vertebrates. Cross-talk between BMP signaling and Ca^{2+}/calmodulin signaling was mentioned earlier and is discussed in more detail in another review [von Bubnoff and Cho, 2001]. Activation of the BMP type II receptor by BMP4 interferes with its interaction with the cytoskeletal regulator LIM kinase 1 (LIMK1), releasing LIMK1 to function in reorganization of the actin cytoskeleton [Foletta et al., 2003]. Such observations suggest mechanisms by which BMPs may regulate processes such as cell differentiation and migration [de Caestecker, 2004].

Functional, if not physical, interactions between the Wnt and BMP signaling pathways have been identified in a number of systems [von Bubnoff and Cho, 2001]. Interactions between the BMP and Wnt pathways may not necessarily occur intracellularly; indirect interactions might equally well result from regulated expression of, say, a *Bmp* gene by Wnt signaling, or the reverse. Studies in the frog have suggested that the noncanonical BMP-MAPK pathway may interact with Wnt/β-catenin signaling [von Bubnoff and Cho, 2001; de Caestecker, 2004]. Wnts [Reya et al., 2003], like BMPs [Bhatia et al., 1999], can regulate proliferation of mouse or human HSCs, respectively, and it is tempting to speculate that their pathways are interconnected in hematopoietic development. Recently, conditional ablation studies in mice have indicated that β-catenin, an intracellular mediator of Wnt signaling, is not essential for normal adult hematopoiesis or lymphopoiesis [Cobas et al., 2004]. Perhaps, then, the hematopoietic activity of Wnts is mediated by the noncanonical Wnt/Ca^{2+} pathway involving a calmodulin kinase (CaMK). Whether β-catenin is also dispensable for earlier stages of ontogeny is not known: the conventional knockout phenotype is early embryonic lethality, as null mutant embryos fail to form mesoderm.

Recent evidence has linked BMP signaling with the Notch pathway [e.g., see Itoh et al., 2004]. Notch signaling has been implicated in lineage decision making processes in a variety of cell types. Among the four vertebrate Notch proteins, Notch1 and Notch2 are expressed in hematopoietic stem and/or progenitor cells [reviewed in Kumano et al., 2003]. In *Notch*1- (but not *Notch*2-) deficient mouse embryos, definitive HSC activities from YS and P-Sp were found to be severely impaired, in vitro and in vivo [Kumano et al., 2003]. Among the genes whose expression was dramatically reduced in P-Sp organ cultures from *Notch*1 null mutant embryos, *Bmp4* is notable [Kumano et al., 2003]. *Bmp2* expression was not affected, suggesting that BMP4 is more critical for hematopoietic development [Kumano et al., 2003]. Addition of BMP4 protein to the *Notch1* deficient cultures failed to rescue the hematopoietic defect, however, indicating that both Notch and BMP signaling are required in this system [Kumano et al., 2003].

Several studies have provided evidence for cross-talk between the BMP-Smad and the JAK–STAT pathways. Leukemia inhibitory factor (LIF) acts through the gp130 receptor and STAT3, and in neural cells can synergize with BMP2 by means of a complex containing Smad 1, STAT3, and p300. The BMP and gp130 receptor signaling pathways appear to cooperate functionally in patterning of ventral mesoderm in the frog [von Bubnoff and Cho, 2001]. Recently, two reports have demonstrated that BMP4 cooperates with LIF to maintain self-renewal of mouse ES cells [Ying et al., 2003; Qi et al., 2004] by upregulating the inhibitor of differentiation (Id) helix–loop–helix proteins [Ying et al., 2003] and/or by inhibiting the p38 MAPK pathway [Qi et al., 2004]. The function of gp130-STAT3 in hematopoiesis is well established. Whether the BMP and JAK-STAT signaling might also act in concert to maintain HSC self-renewal is unknown. However, LIF does not appear to play a role in regulation of the pluripotential state of human ES cells. Recent work has demonstrated that inhibition of glycogen synthase kinase-3 (GSK-3) and subsequent activation of the canonical Wnt pathway controls self-renewal of both human and mouse ES cells [Sato et al., 2004].

It is worth noting that much of the evidence for cross-talk between the BMP and other signaling pathways rests on overexpression studies and, thus, nonphysiological conditions. Therefore, confirmation that these interactions are biologically significant is essential and should be possible through analysis of genetically manipulated animals or cell lines.

CONCLUDING REMARKS

Studies in vertebrate animals ranging from frogs to humans have established functions for BMPs (in particular, BMP4) in the initiation and maintenance of hematopoetic activity.

BMPs are involved in induction and patterning of mesoderm in the early embryo, in the formation of blood islands of the YS, and in regulating the stem cell niche in adult bone marrow. The diversity of cellular responses to BMP signaling may be broadened through cross-talk with TGFβ, Wnt, MAPK, Notch, Ca^{2+}/calmodulin, and JAK-STAT pathways. Interactions with additional signaling pathways may yet be discovered.

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