**Calmodulin-dependent protein kinase IV mediated antagonism of BMP signaling regulates lineage and survival of hematopoietic progenitors**


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**SUMMARY**

In the current study, we show that bone morphogenetic proteins (BMPs) play a role in hematopoiesis that is independent of their function in specifying ventral mesodermal fate. When BMP activity is upregulated or inhibited in *Xenopus* embryos hematopoietic precursors are specified properly but few mature erythrocytes are generated. Distinct cellular defects underlie this loss of erythrocytes: inhibition of BMP activity induces erythroid precursors to undergo apoptotic cell death, whereas constitutive activation of BMPs causes an increase in commitment of hematopoietic progenitors to myeloid differentiation and a concomitant decrease in erythrocytes that is not due to enhanced apoptosis. These blood defects are observed even when BMP activity is misregulated solely in non-hematopoietic (ectodermal) cells, demonstrating that BMPs generate extrinsic signals that regulate hematopoiesis independent of mesodermal patterning. Further analysis revealed that endogenous calmodulin-dependent protein kinase IV (CaM KIV) is required to negatively modulate hematopoietic functions of BMPs downstream of receptor activation. Our data are consistent with a model in which CaM KIV inhibits BMP signals by recruiting limiting amounts of CREB binding protein (CBP) away from transcriptional complexes functioning downstream of BMPs.

Key words: BMPs, *Xenopus laevis*, Hematopoiesis, CaM KIV, CBP, CREB

**INTRODUCTION**

The term hematopoiesis describes the generation, proliferation and differentiation of pluripotent, self-renewing hematopoietic stem cells (HSCs) into one of the various mature lineages of the blood (reviewed by Evans, 1997; Davidson and Zon, 2000). The first wave of hematopoiesis is primitive hematopoiesis, which gives rise primarily to embryonic erythrocytes (red blood cells, RBCs). In amphibian embryos primitive hematopoiesis takes place in the ventral blood island (VBI, the mammalian equivalent of the extraembryonic yolk sac) (Mangia et al., 1970; Turpen et al., 1981; Turpen and Knudson, 1982; Kau and Turpen, 1983; Maeno et al., 1985a; Maeno et al., 1985b). The second wave of blood formation is definitive hematopoiesis, which yields adult erythrocytes as well as progenitors of the lymphoid (lymphocyte) and myeloid (granulocyte, monocyte and platelet) lineages. Definitive hematopoiesis initiates in a region near the aorta, gonads and mesonephros, also called the dorsal lateral plate in *Xenopus laevis* (Kau and Turpen, 1983; Maeno et al., 1985a; Maeno et al., 1985b; Weber et al., 1991). HSCs derived from this region will later migrate to seed other hematopoietic organs such as the liver and spleen (Chen and Turpen, 1995; Sanchez et al., 1996).

Recent fate mapping studies in *Xenopus* have shown that primitive blood is derived from blastomeres that reside on both the dorsal and ventral side of early cleavage stage embryos (Lane and Smith, 1997; Mills et al., 1999; Ciau-Uitz et al., 2000). While dorsal blastomeres give rise to erythrocytes in the anterior portion of the VBI, ventral blastomeres populate posterior portions of the VBI (Tracey et al., 1998; Ciau-Uitz et al., 2000). Tissue explant and transplant studies, however, suggest that primitive erythrocytes are derived from mesoderm that resides on the ventral, and not the dorsal side of gastrula through to tailbud stage embryos (Kau and Turpen, 1983; Maeno et al., 1985b; Weber et al., 1991; Maeno et al., 1992; Maeno et al., 1994a; Zhang and Evans, 1996; Turpen et al., 1997; Mead et al., 1998). Dorsal mesoderm, in fact, inhibits differentiation of ventral mesoderm to erythrocytes when the two tissues are co-cultured following isolation from gastrula (Maeno et al., 1994b) or neurula (Maeno et al., 1992) stage embryos. Thus, although blood descends from dorsal and ventral blastomeres of cleavage stage embryos, hematopoietic tissue is restricted to ventral regions of tailbud, and possibly gastrula, stage embryos.

Commitment of cells to the hematopoietic lineage is initiated by ventral patterning of the mesoderm during...
gastrulation, a process that requires BMPs (reviewed by Nieto, 1999). BMPs are members of the transforming growth factor-β superfamily and transmit their signals via transmembrane serine-threonine kinase receptors (reviewed by Christian and Nakayama, 1999; Massague and Wotton, 2000). These receptors propagate the signal by phosphorylating a BMP pathway-specific Smad (Smad1, 5 or 8) that then forms hetero-oligomers with a pathway-shared Smad, Smad4. This complex translocates into the nucleus to activate transcription of target genes. Although Smads can bind DNA weakly and may function as direct transcriptional co-activators in some contexts, they generally function in concert with unrelated sequence-specific DNA binding proteins, and require transcriptional co-activators, such as CREB binding protein (CBP) (reviewed by Massague and Wotton, 2000).

Evidence that BMPs are required for specification of ventral fate has come from studies in a variety of model systems (reviewed by Nieto, 1999). When endogenous BMP signaling is blocked by introduction of BMP inhibitors into ventral cells of *Xenopus* embryos, ventral tissues fail to differentiate and a secondary dorsal axis is formed. Conversely, when cells on the dorsal side of *Xenopus* embryos are made to misexpress BMP4, they form ventral mesoderm rather than notochord. Zebrafish embryos mutant for BMPs, or for the BMP antagonist chordin, show analogous dorsalized and ventralized phenotypes, respectively.

Because BMPs are required for ventral patterning of mesoderm, from which blood cells are derived, they are assumed to be essential for erythropoiesis. Consistent with this, ectopically expressed BMP4 induces expression of hematopoietic-specific genes in *Xenopus* ectodermal explants and expands the expression domain of erythroid-specific genes in whole embryos (Maeno et al., 1994b; Hemmati-Brivanlou and Thomsen, 1995; Maeno et al., 1996; Zhang and Evans, 1996; Huber et al., 1998; Miyamaga et al., 1999; Xu et al., 1999a; Deconinck et al., 2000). Furthermore, BMPs induce hematopoietic activity in embryoid bodies from mouse embryonic stem cells (Johansson and Wiles, 1995) and can regulate proliferation and differentiation of primitive HSCs in culture (Bhatta et al., 1999). Conversely, inhibition of BMP signaling in *Xenopus* embryos represses blood development (Maeno et al., 1994a; Zhang and Evans, 1996; Kumano et al., 1999), mice lacking the BMP signal transducer, Smad5, have disrupted vasculature and are anemic (Chang et al., 1999; Yang et al., 1999) and zebrafish embryos mutant for BMP2b (Mullins et al., 1996; Kishimoto et al., 1997; Nguyen et al., 1998) or BMP7 (Dick et al., 2000; Schmid et al., 2000) show a dorsalized phenotype accompanied by a decrease in RBCs. These findings have been interpreted to mean that high levels of BMP signaling specify hematopoietic fate during gastrulation (reviewed by Davidson and Zon, 2000).

Recent studies suggest that the role of BMPs in hematopoiesis may be more complex than previously thought. Introduction of intracellular inhibitors of BMP signal transduction into a subset of prospective blood progenitors on the ventral side of *Xenopus* embryos, for example, can induce dorsal fate, yet does not inhibit expression of globin (Kumano et al., 1999). This result raises the possibility that BMP signals may not need to be transduced within ventral mesodermal cells in order for erythrocytes to differentiate. Furthermore, although ectopic expression of two downstream effectors of BMP signaling, Xvent-1 and Xvent-2, causes an expansion of ventral structures, this is accompanied by a decrease in globin expression (Kumano et al., 1999; Xu et al., 1999a).

In the current studies we demonstrate that BMPs play a non cell-autonomous role in hematopoiesis that is independent of their function in ventral patterning of the mesoderm. Specifically, we find that intracellular BMP signals must be transduced in non-hematopoietic (ectodermal) cells to promote the survival of maturing erythrocytes, but that excess BMP signaling in these same cells enhances myeloid differentiation and represses erythropoiesis.

The hematopoietic defects that we observe following misregulation of BMP signaling phenocopy the effects of misregulating endogenous calmodulin-dependent protein kinase IV (CaM KIV) activity (Wayman et al., 2000), raising the possibility that these signaling molecules act in a common pathway. CaM KIV is a serine/threonine protein kinase that is activated by the ubiquitous calcium binding protein, calmodulin (reviewed by Soderling, 1999). The substrate specificity of this kinase is not well defined, but CaM KIV protein is predominantly nuclear and can phosphorylate and regulate a number of transcription factors, including cAMP-response element-binding protein (CREB). Once phosphorylated, CREB binds to the transcriptional co-activator CBP and this binding is sufficient to activate transcription of cAMP response element (CRE)-containing genes (Cardinaux et al., 2000).

We have found that BMPs and CaM KIV function in a common signaling pathway to regulate erythropoiesis. Our results suggest that the balance between endogenous BMP and CaM KIV signaling cascades in non-hematopoietic cells regulates expression of a cell-cell signaling molecule(s) that controls survival and lineage choice of HSCs. Finally, our data support a molecular model in which endogenous CaM KIV inhibits the BMP pathway by activating a substrate, possibly CREB, which binds CBP and competes for components of transcriptional complexes downstream of BMP receptor activation.

**MATERIALS AND METHODS**

**Embryo culture and manipulation**

*Xenopus* eggs were obtained, embryos injected with synthetic mRNAs and cultured as described previously (Moon and Christian, 1989). Embryonic stages are according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Capped synthetic RNA was generated by in vitro transcription of linearized template cDNAs using a Megascript kit (Ambion).

**β-gal staining, in situ hybridization and northern blot analysis of gene expression**

Embryos were stained for β-gal activity using Red-gal as a substrate, and processed for in situ hybridization as described previously (Nakayama et al., 1998). RNA was isolated and northern blots hybridized with antisense riboprobes as described previously (Christian and Moon, 1993). Following hybridization with globin and SCL riboprobes, filters were stripped and reprobed for expression of EF-1α as a loading control. Bands were visualized with a phosphorimager and quantified using the Macintosh IP lab get program.

**Collection and analysis of peripheral blood samples**

Tails were severed from tadpole stage embryos and larval hematopoietic cells were collected into amphibian PBS containing...
Bone morphogenetic proteins in hematopoiesis

0.5% BSA and 10 IU/ml of heparin. Cells were concentrated onto slides using a cytospin centrifuge. For general morphological examination, blood cells were stained with a Hema 3 stain set (Biochemical Sciences Inc.) and examined by light microscopy. Apoptotic cells in cytospin preparations of peripheral blood were detected by the TUNEL assay using a fluorescein apoptosis detection kit (Promega). Nuclei of cells were counter stained with propidium iodide to determine total numbers of cells.

RESULTS

Misregulation of BMP signaling blocks expression of globin

To examine the role of BMPs in hematopoiesis, we analyzed blood development in embryos in which BMP signaling was either inhibited or upregulated in a subset of embryonic cells. RNA encoding a dominant negative truncated BMP receptor (tBR; 200 pg) (Graff et al., 1994) or a constitutively active form of a type I BMP receptor (caALK3, 600 pg) (Shibuya et al., 1998) was injected into two dorsal or ventral blastomeres of four-cell embryos. Expression of the erythrocyte marker, globin, was examined in whole tailbud stage embryos. Injection of this dose of RNA did not perturb dorsal-ventral patterning in the majority of embryos, thus allowing us to examine the potential roles for BMPs in blood development independent of mesodermal patterning.

Globin was expressed throughout the VBI in embryos injected with a control RNA, β-galactosidase (β-gal), as analyzed by whole-mount in situ hybridization (Fig. 1A). By contrast, expression of globin was dramatically reduced in embryos in which BMP signaling was blocked in either ventral or dorsal cells by misexpression of tBR (Fig. 1A; Table 1). Unexpectedly, constitutive activation of the BMP pathway, by injection of RNA encoding caALK3 into either ventral or dorsal cells, also led to a loss of expression of globin (Fig. 1A; Table 1). Northern blot analysis confirmed that inhibition or activation of BMP signaling in either dorsal or ventral cells of Xenopus embryos led to a significant decrease in globin transcripts (Fig. 1B, left panel).

Our finding that activation of the BMP pathway inhibits expression of globin was unexpected because others have shown that injection of BMP4 RNA into dorsal cells of embryos causes overcommitment of mesoderm to a ventral fate, leading to an expansion of the globin expression domain (Maeno et al., 1994b; Hemmati-Brivanlou and Thomsen, 1995; Maeno et al., 1996). This discrepancy might be explained if caALK3 is activating a pathway distinct from that activated by BMP4 ligand, or if BMP4 signaling plays a role in hematopoiesis that is independent of its role in ventral patterning. To test these possibilities, we injected RNA encoding BMP4 (500 pg) near the dorsal or ventral midline of four-cell embryos and analyzed expression of globin at tailbud stage 32. When BMP4 RNA was injected into ventral cells, dorsoventral patterning was not perturbed but expression of globin was nearly ablated in all embryos as determined by in situ hybridization (Fig. 1A; Table 1) and northern blot analysis (Fig. 1B, right panel). When BMP4 was injected into dorsal cells, embryos displayed ventralized phenotypes ranging from complete loss of all dorsal and anterior structures [dorsoanterior index (DAI) of 0 according to the scale of Kao and Elinson, 1988] to completely normal (DAI of 5). Embryos that appeared normal or had only a minimal loss of dorsal and

Fig. 1. Misregulation of BMP signaling in Xenopus embryos inhibits erythropoiesis. RNAs encoding β-gal, tBR, caALK3 or BMP4 were injected near the ventral (VMZ) or dorsal marginal zone (DMZ) of 4-cell embryos. (A) Tailbud stage 32 embryos were stained for globin RNA (purple) by in situ hybridization. BMP-injected embryos were subdivided into those with normal dorsal-ventral pattern (DAI 5) and those with a ventralized phenotype (DAI 0). Ventral views of embryos are shown except for the BMP-injected, DAI 0 embryo for which ventral and lateral views are provided. (B) Northern blot analysis of globin expression. Levels of globin transcripts, normalized relative to levels of EF-1α transcripts, are expressed as percentage of control, below each lane.
anterior structures (DAI 2-5) showed a strong reduction in expression of globin (Fig. 1A,B). By contrast, at least some embryos in which the majority of mesoderm was committed to a ventral fate (DAI 0-1) showed an expansion in the globin expression domain (Fig. 1A), consistent with what has been previously reported. These embryos also showed an increase in expression of globin relative to the ubiquitously expressed gene, EF-1α (Fig. 1B, right panel). This increase in globin transcripts is inconsistent with the decrease in globin expression in BMP overexpressing embryos with normal dorsoventral pattern. This may, however, be due to an increase in the fraction of cells that express globin in ventralized embryos which would mask a secondary inhibition of globin transcription in individual cells. Taken together, these findings suggest that BMPs play a role in erythropoiesis that is independent of their function in specifying ventral mesodermal fate. Our results raise two further questions: why is expression of globin repressed following either activation or inhibition of BMPs, and why is expression of globin almost completely repressed following misregulation of BMPs in only a subset of embryonic cells?

BMP signaling regulates hematopoiesis non cell-autonomously

Anterior and posterior portions of the VBI are derived from dorsal and ventral regions of the embryo, respectively, which might explain why misregulation of BMP signaling in either dorsal or ventral cells led to a loss of globin transcripts. We did not, however, observe a correlation between the site of injection of mutant BMP receptor RNAs (DMZ versus VMZ) and the region of the VBI (anterior versus posterior) in which globin expression was absent. Rather, staining was completely lost or greatly diminished throughout the entire extent of the VBI in most experimental embryos (Fig. 1A; Table 1). This finding, together with our data showing that inhibition or activation of BMP signaling in a subset of cells almost completely represses globin expression suggests that proper regulation of BMP signaling may be required to generate a non cell-autonomous secondary signal that regulates erythropoiesis.

To test the hypothesis that BMP function is required in non-hematopoietic cells for normal blood development, we targeted injections of RNA encoding tBR or caALK3 to dorsal midline blastomeres at the extreme animal pole of 32-cell embryos (A1 blastomeres according to the nomenclature of Nakamura and M. J. Walters and others

Table 1. Misregulation of BMP signaling in a subset of embryonic cells inhibits expression of globin throughout the VBI

<table>
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<th>RNA</th>
<th>Injection site</th>
<th>% Embryos expressing globin in the VBI</th>
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<td>Decreased or absent</td>
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<td>Through</td>
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<tr>
<td>tBR</td>
<td>DMZ</td>
<td>89</td>
</tr>
<tr>
<td>tBR</td>
<td>VMZ</td>
<td>69</td>
</tr>
<tr>
<td>caALK3</td>
<td>DMZ</td>
<td>61</td>
</tr>
<tr>
<td>caALK3</td>
<td>VMZ</td>
<td>70</td>
</tr>
<tr>
<td>BMP4</td>
<td>DMZ</td>
<td>74</td>
</tr>
<tr>
<td>BMP4</td>
<td>VMZ</td>
<td>100</td>
</tr>
<tr>
<td>β-gal</td>
<td>DMZ</td>
<td>4</td>
</tr>
<tr>
<td>β-gal</td>
<td>VMZ</td>
<td>9</td>
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<tr>
<td>none</td>
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RNAs were injected into two blastomeres near the dorsal (DMZ) or ventral marginal zone (VMZ) of four-cell embryos. Expression of globin was analyzed by in situ hybridization at stage 32. Embryos were scored for decreased or absent staining predominantly in the anterior or posterior portions of the VBI or throughout the entire VBI.

Fig. 2. BMP signaling in ectodermal cells regulates erythropoiesis. (Top) Two dorsal animal pole (A1) blastomeres of 32-cell embryos were injected with β-gal RNA alone or together with dominant negative (tBR) or constitutively active (caALK3) BMP receptor RNAs as illustrated. (A) Tailbud stage 32 embryos were stained for β-gal activity (red stain, white arrows) and then for globin RNA (purple stain, black arrows) by in situ hybridization. (B) Control embryos (β-gal), as well as embryos in which BMP signaling was misregulated in A4 or A1 blastomeres, were analyzed for expression of globin by northern blot hybridization. Levels of globin transcripts, normalized relative to levels of EF-1α transcripts, are expressed as a percentage of control, below each lane.
Fig. 3. Misregulation of BMP signaling does not perturb specification of hematopoietic fate. Embryos were injected with RNA encoding caALK3 (near the VMZ) or tBR or Smad6 (near the DMZ) at the 4-cell stage. Expression of SCL was analyzed at neurula (St. 18) and tailbud (St. 32) stages by northern blot hybridization. The blot was stripped and reprobed for globin, and then EF-1α transcripts. The difference in SCL levels in tailbud stage embryos injected with Smad6 as opposed to tBR was not reproducible.

**Table 2. Misregulation of BMP signaling in non-hematopoietic cells inhibits globin expression**

<table>
<thead>
<tr>
<th>RNA site</th>
<th>% Embryos expressing globin</th>
<th>n</th>
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<tbody>
<tr>
<td>A1</td>
<td>+/–</td>
<td>72</td>
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<tr>
<td>A4</td>
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<td>A1</td>
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<td>A4</td>
<td>++</td>
<td>80</td>
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<tr>
<td>A1</td>
<td>+/–</td>
<td>64</td>
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RNA encoding β-galactosidase (β-gal) was injected alone or together with RNA encoding tBR or caALK3 into both A1 or A4 blastomeres of 32-cell embryos as illustrated above Fig. 2. Expression of globin was analyzed by in situ hybridization at stage 32 and was scored as follows: +/-, absent or barely visible staining; ++, slight decrease in staining; ++++, strong staining. n, sample size.

Kishiyama, 1971). These blastomeres give rise to anterior ectodermal derivatives and do not contribute to erythroid cells in the VBI (Mills et al., 1999). RNA encoding β-gal (100 pg) was co-injected as a lineage tracer as illustrated at the top of Fig. 2. Half of the injected embryos in each group were stained for β-gal activity at the tailbud stage, to verify the accuracy of injections, followed by in situ hybridization to detect globin transcripts. RNA was extracted from the remaining embryos for analysis of globin by northern blot hybridization. β-gal activity was restricted to anterior regions of injected embryos as indicated by red staining (Fig. 2A, white arrows). Inhibition or constitutive activation of BMP signaling in A1 cells caused a severe decrease in globin expression in many embryos as demonstrated by in situ hybridization (Fig. 2A; Table 2) and northern blot analysis (Fig. 2B). Inhibition or activation of BMP signals in neighboring A4 blastomeres, which contribute to only a minority of cells in the VBI, also led to a substantial reduction in expression of globin (Fig. 2B; Table 2). These results demonstrate that proper regulation of BMP signaling is required in non-hematopoietic (ectodermal) cells, at some time prior to the tailbud stage, to enable ventral mesoderm to differentiate as RBCs.

**Misregulation of BMP signaling does not disrupt specification of hematopoietic fate**

A decrease in the initial specification of hematopoietic stem cells (HSCs) could account for the decrease in globin expression observed when BMP signaling is misregulated. To investigate this possibility, we analyzed expression of stem cell leukemia (SCL), a transcription factor that is expressed in hematopoietic mesoderm early in embryogenesis and is proposed to specify hematopoietic fate (Mead et al., 1998). In this and all subsequent experiments, we targeted RNA encoding tBR (400 pg), or the intracellular BMP inhibitor, Smad6 (Nakayama et al., 1998) (200 pg) to the dorsal side and RNA encoding caALK3 (600 pg) to the ventral side of 4-cell embryos. This allowed us to interfere with BMP signals that are required for erythropoiesis without altering BMP function in early dorsoventral patterning. Neither inhibition of BMP signaling in dorsal cells, nor hyperactivation of BMP signaling in ventral cells caused a significant decrease in expression of SCL, normalized relative to EF-1α, in neurula stage 18 embryos (Fig. 3). At tailbud stage 32, however, embryos in which BMP signaling was perturbed showed a 30-70% decrease in expression of globin and SCL relative to un.injected siblings (Fig. 3). Identical results were obtained when we examined expression of GATA-1, another early marker of blood development (data not shown) (Kelley et al., 1994). We conclude that the loss of globin transcripts in embryos in which BMP signaling is misregulated is not due to a failure to specify hematopoietic fate within the ventral mesoderm.

**Activation and inhibition of BMP signaling cause distinct hematopoietic defects**

To investigate why activation and inhibition of BMP signaling both repress expression of globin, we looked for phenotypic differences in RBCs collected from sibling stage 43 tadpoles in which BMP signaling was perturbed. At this stage of development, all of the circulating blood is derived from the VBI as a result of primitive hematopoiesis (Turpen et al., 1997). Peripheral blood was collected onto slides by cytocentrifugation and stained with a Wright-Giemsa differential stain. White blood cells (WBCs) are predominantly of the monocyte/macrophage lineage at this stage of development (Ohinata et al., 1990) and can be distinguished from RBCs by morphology and staining characteristics (Hadji-Azimi et al., 1987). RBCs are small and round with a large nucleus and darkly stained cytoplasm (Fig. 4A, black arrows) while WBCs are larger, irregularly shaped and have lightly stained cytoplasm (Fig. 4A, white arrows). Blood from mock-injected or uninjected control embryos had an average of 75 blood cells per visual field, about 10% of which were WBCs (Fig. 4A,B). Tadpoles in which BMP signaling was upregulated showed an increase in the number of WBCs and a concomitant decrease in the number of RBCs (Fig. 4A,B) such that the white to red cell ratio was seven-fold higher than in controls (Fig. 4C). One simple interpretation of these results is that high levels of BMP signaling enhance commitment of HSCs to the myeloid (WBC) pathway of differentiation at the expense of erythrocytes. Alternatively, BMP may have independent positive and negative effects on committed myeloid and erythroid progenitors, respectively.

Embryos in which BMP signaling was inhibited showed a specific decrease in the number of RBCs whereas the number of
WBCs was not significantly perturbed (Fig. 4A,B). In addition, RBCs from BMP-deficient embryos stained more lightly with both Wright-Giemsa (Fig. 4A), and with the heme-specific stain, o-dianisidine (data not shown). To test the possibility that the decreased number of RBCs was due to an increase in programmed cell death, we used the TUNEL assay to quantitate apoptotic blood cells. As shown in Fig. 4D,E, inhibition of BMP signaling following misexpression of either Smad6 or tBR caused a significant increase in the fraction of blood cells that were apoptotic. The observation that inhibition of BMP signaling resulted in a significant decrease in RBCs, but not WBCs suggests that the apoptotic cells are predominantly or solely RBCs. By contrast, constitutive activation of the BMP pathway led to a decrease in the number of RBCs (Fig. 4B) but the fraction of apoptotic cells was unchanged (Fig. 4E). We conclude that the decrease in globin expression observed when the BMP pathway is blocked or hyperactivated is caused by two distinct mechanisms: upregulation of BMP signaling causes an increase in myeloid differentiation and a loss of erythrocytes, whereas inhibition of BMP signaling leads to increased apoptosis of erythroid precursors.

**CaM KIV negatively modulates BMP signaling in the context of hematopoiesis**

Misregulation of embryonic BMP activity causes blood defects that are indistinguishable from those observed following misregulation of CaM KIV activity (Wayman et al., 2000), suggesting that there may be crosstalk between these pathways. Specifically, inhibition of CaM KIV or upregulation of BMP signaling leads to an increase in WBCs and a decrease in RBCs, while constitutive activation of CaM KIV or inhibition of the BMP pathway causes increased apoptosis of RBC progenitors. In addition, both BMPs (Fig. 2) and CaM KIV (Wayman et al., 2000) function non cell-autonomously to regulate hematopoiesis. Importantly, whereas inhibition and constitutive activation of BMP signaling leads to duplication or loss of dorsal structures, respectively, misregulation of CaM KIV activity does not perturb dorsoventral patterning (Wayman...
et al., 2000). Thus, any crosstalk between BMP and CaM KIV must be restricted to specific developmental contexts such as hematopoiesis.

The observation that activation of CaM KIV phenocopies hematopoietic defects caused by blockade of BMP signaling, and vice versa, suggests that activation of one pathway inhibits the other. To begin to determine whether one of these signals is dominant, or functions downstream of the other, we analyzed hematopoiesis in embryos in which both pathways were constitutively activated. RNA encoding caALK3 was co-injected with constitutively active forms of CaM KIV (caKIV) and its upstream kinase, CaM KK (caKK) (100 pg each), near the VMZ of 4-cell embryos. Co-expression of these two constitutively active kinases generates eight- to ten-fold greater calcium-independent CaM KIV activity than does expression of caKIV alone (Enslen et al., 1996). Whereas activation of the BMP pathway alone led to an increase in WBCs at the expense of RBCs, activation of the CaM KIV pathway alone, or co-activation of the CaM KIV and BMP pathways led to a decrease in RBCs without affecting the number of WBCs (Fig. 5A,B). This result supports the hypothesis that CaM KIV antagonizes BMP function downstream of receptor activation.

To further test the hypothesis that CaM KIV can function as an intracellular antagonist of BMP, we analyzed the phenotype of embryos in which both pathways were simultaneously blocked. Inhibition of endogenous CaM KIV, achieved by injecting RNA (1 ng) encoding a well characterized dominant negative form of CaM KIV (DnKIV) (Gringhuis et al., 1997; Ahn et al., 1999), led to an increase in WBCs at the expense of RBCs. By contrast, inhibition of the BMP pathway, or simultaneous inhibition of the BMP and CaM KIV pathways led to a decrease in RBCs without affecting the number of WBCs (Fig. 5C,D). Thus, inhibition of endogenous CaM KIV can only induce overcommitment of HSCs to myeloid differentiation when endogenous BMP signals are active. Collectively, these results are consistent with a model in which endogenous CaM KIV is required to negatively modulate BMP signaling downstream of receptor activation in order to prevent HSCs from overcommitting to a myeloid pathway of differentiation at the expense of erythrocytes.

**CaM KIV inhibits the BMP pathway via competition for CBP**

Transcriptional regulation through both the BMP and CaM KIV pathways involves the co-activator CBP. Specifically, CBP is an essential cofactor together with Smads for transcription of BMP and other TGFβ family target genes (reviewed by Massague and Wotton, 2000) while the best characterized transcriptional target of CaM KIV is CREB, the prototypical CBP-binding protein. Previous studies have provided evidence that CBP levels may be limiting in cells, such that activation of one CBP-utilizing pathway can inhibit transcriptional responses downstream of another (reviewed by Shaywitz and Greenberg, 1999). Thus, CaM KIV could potentially inhibit BMP signals through activation of a substrate such as CREB that recruits CBP away from transcriptional complexes functioning downstream of BMPs.

To test whether activation of CREB can phenocopy loss of BMP function during hematopoiesis, we injected RNA encoding a constitutively active form of CREB (caCREB) into *Xenopus* embryos and asked whether this caused a loss of RBCs. caCREB carries a six amino acid substitution in the activation domain that enables it to bind CBP and activate transcription of CRE-containing reporter genes in a phosphorylation-independent manner (Cardinaux et al., 2000). Injection of 1 ng of RNA encoding caCREB near the VMZ of four-cell embryos did not cause gross morphological abnormalities at the tailbud stage but led to greatly decreased expression of *globin* in 70% of injected embryos (n=42) (Fig. 6A). The loss of *globin* expression is caused by a specific decrease in the number of RBCs, without an increase in WBCs (Fig. 6B). This phenotype is similar to that observed following inhibition of BMP signaling, or constitutive activation of CaM KIV, consistent with the hypothesis that CaM KIV inhibits BMP signal transduction through activation of CREB.

To ask whether binding of CREB to CREs on target genes is necessary for inhibition of erythropoiesis, we asked whether a constitutively active form of CREB in which the DNA binding domain has been replaced with the GAL4 DNA binding domain (GAL4-caCREB) can inhibit erythropoiesis. Injection of 1 ng of RNA encoding GAL4-caCREB near the VMZ of four-cell embryos inhibited *globin* synthesis (Fig. 6A) and caused a specific reduction in RBC number (Fig. 6B). By contrast, injection of an equivalent amount of RNA encoding wild type GAL4-CREB, which is unable to bind CBP in the...
absence of phosphorylation, had no effect on globin synthesis (Fig. 6A) or RBC number (Fig. 6B). These data demonstrate that activation of CREB target genes is not required for inhibition of erythropoiesis.

The observation that GAL4-caCREB can phenocopy activation of CaM KIV and inhibition of BMP signaling during hematopoiesis is consistent with the hypothesis that CREB, or another CaM KIV substrate, acts downstream of CaM KIV to inhibit transduction of BMP signals. To test this, we upregulated BMP signaling in ventral cells either directly, by injecting RNA encoding caALK3, or indirectly by injecting RNA encoding DnKIV, in the presence or absence of RNA encoding GAL4-caCREB. Misexpression of DnKIV (Fig. 7A,B) or caALK3 (Fig. 7C,D) alone led to a decrease in RBCs that was accompanied by an increase in WBCs, such that the WBC/RBC ratio was increased sevenfold. Co-expression of GAL4-caCREB completely suppressed the increase in the WBC/RBC ratio and instead caused a specific loss of RBCs that is identical to that observed when GAL4-caCREB is expressed alone (Fig. 7A-D).

All of the above data are consistent with the hypothesis that CaM KIV inhibits BMP signal transduction during hematopoiesis by activating a substrate that competes with BMP-regulated transcriptional complexes for access to limiting amounts of CBP. If this is true, then addition of excess CBP should phenocopy inhibition of endogenous CaM KIV and should block hematopoietic defects caused by constitutive activation of CaM KIV. When RNA encoding CBP (5 ng) was injected near the animal pole of ventral blastomeres of four-cell embryos, ectopic CBP protein was present at levels approximately ten-fold greater than endogenous protein at least
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DISCUSSION

In this study, we have identified a role for BMPs in hematopoiesis that is independent of their function in specification of ventral mesodermal fate. Specifically, we have shown that BMP signaling is required in non-hematopoietic cells to promote the survival of erythroid progenitors. In addition, our results suggest that excess BMP signaling can drive HSCs down the myeloid pathway of differentiation at the expense of erythrocytes. Finally, we show that endogenous CaM KIV functions to negatively regulate the BMP pathway during primitive hematopoiesis.

**BMP functions in non-hematopoietic tissues to regulate blood development**

Extrinsic signals produced by non-hematopoietic tissues are known to regulate blood development. Primitive murine blood progenitors isolated from the yolk sac, for example, are committed almost exclusively to erythroid differentiation but can be induced to adopt a myeloid fate when cultured in the presence of appropriate differentiation factors in vitro (Moore and Metcalf, 1970) or when transplanted to a definitive hematopoietic site in vivo (Huang and Auerbach, 1993). Similarly, primitive blood progenitors isolated from the ventral region of *Xenopus* embryos, which will normally differentiate as erythrocytes, instead form myeloid cells of the monocyte/macrophage lineage when transplanted into the dorsal lateral plate (Turpen and Smith, 1985). Factors produced by cell lines derived from the yolk sac (Yoder et al., 1994; Fennie et al., 1995) or other hematopoietic sites (Ohneda et al., 1998) can also direct the lineage commitment and maturation of HSCs. These studies demonstrate that primitive blood progenitors are pluripotent but that factors present in their environment direct them towards erythroid differentiation, whereas signals present in other hematopoietic sites can direct them to differentiate along the myeloid pathway.

Tissue recombination studies in *Xenopus* have shown that signals produced by dorsal and ventral ectodermal cells during gastrulation are required for ventral mesoderm to differentiate as erythrocytes (Maeno et al., 1992). These signals can be mimicked by overexpression of BMP4 in ectodermal cells (Maeno et al., 1994b). Conversely, ectodermal cells cannot stimulate erythropoiesis in ventral mesoderm made to express a dominant negative BMP receptor (Maeno et al., 1994a). These data support a model in which BMPs, secreted by ectodermal cells, signal to mesodermal cells, instructing them to form blood. A similar model was proposed based on the finding that inhibition of intracellular BMP signal transduction in the ventral ectoderm of whole embryos led to loss of expression of globin (Kumano et al., 1999). Because BMPs function in a positive feedback loop to regulate their own expression, the loss of globin was attributed to a loss of ectodermally derived BMP, which was proposed to be transmitted to prospective hematopoietic progenitors during gastrulation. In the current study, we show that misregulation of the intracellular BMP signaling cascade in either ventral or dorsal ectoderm inhibits erythropoiesis. Expression of BMP4 is normally extinguished in dorsal ectoderm during gastrulation, ruling out the possibility that our results are due to feedback inhibition of BMP expression in these cells. Our data instead suggest that ectodermal, rather than hematopoietic, cells must receive a BMP signal in order to generate a secondary, non cell-autonomous signal(s) that is/are essential for erythropoiesis. This signal is most likely transmitted during gastrulation since inhibition (Zhang and Evans, 1996) and upregulation (T. Nakayama and J. L. C., unpublished data) of BMP signaling after the late blastula stage, achieved by microinjection of plasmid expression constructs, is sufficient to disrupt erythropoiesis.

A simple model consistent with our data is that BMPs function in non-hematopoietic cells to regulate expression of a secondary signaling molecule(s) that is essential for the survival, and possibly proliferation, of erythroid progenitors. When BMP-mediated expression of this molecule is completely blocked, by constitutive activation of CaM KIV or ectopic expression of BMP inhibitors, HSCs commit to the erythroid pathway but progenitors are unable to survive. Conversely, when BMP-mediated transcription is upregulated, by expressing a constitutively active BMP receptor or by inhibiting endogenous CaM KIV, inappropriate high levels of the cell-cell signaling molecule(s) induce overcommitment of HSCs to myeloid differentiation. Consistent with this model, embryological studies have identified a signal generated in ectodermal cells during gastrulation that is required for normal erythropoiesis (Maeno et al., 1994b). Further studies have shown that a non cell-autonomous signal(s) functions shortly after this time (neurula stages 13-18) to regulate lineage commitment, proliferation and/or survival of HSCs (Turpen and Smith, 1985; Turpen et al., 1997).

The increase in WBC number in CA-ALK-injected embryos could potentially result from expansion of a population of progenitors that arise independent of the VBI, rather than from commitment of primitive HSCs to myeloid differentiation as proposed above. A unique population of macrophages has been identified that originate not from the blood islands, but from a region anterior to the heart (reviewed by Shepard and Zon, 2000). Although we cannot rule out the possibility that misregulation of BMPs induces proliferation of this distinct leukocyte population, this seems unlikely for several reasons. First, in situ hybridization analysis of an early myeloid marker (myeloperoxidase) in CA-ALK-injected tailbud stage embryos revealed that myeloperoxidase-positive cell number was unchanged outside of the VBI (data not shown). Second, non-VBI-derived macrophages migrate and populate the mesenchyme of many tissues, especially in the head region, but contribute few cells to the circulating blood in *Xenopus* (Ohinata et al., 1990). Thus, our data showing an increase in
circulating myeloid cells with a concomitant decrease in RBCs is more consistent with a lineage switch model than with two separate effects in two distinct blood compartments. Our results could also be explained, however, if BMP induces the expression of a gene product(s) that acts independently on erythroid and myeloid cells, for example by inhibiting proliferation or differentiation of committed erythroid progenitors, and promoting proliferation or differentiation of committed progenitors of the monocyte/macrophage pathway.

An intriguing possibility is that different levels of BMP signaling in ventral and dorsal ectodermal cells of gastrula stage embryos directs primitive and definitive hematopoiesis in the VBI and DLP, respectively. Specifically, it is feasible that CaM KIV differentially modulates BMP signaling such that specific BMP target genes are transcribed at slightly higher levels in dorsal than in ventral ectodermal cells. Dorsal ectodermal cells, which come to overlie the site of definitive hematopoiesis, would thus instruct HSCs to commit to the myeloid lineage whereas ventral ectodermal cells, which come to overlie the VBI would direct HSCs to differentiate as primitive erythrocytes. Consistent with this possibility, dorsal cells are reproducibly more sensitive to inhibition of BMP signaling, whereas ventral cells are more sensitive to upregulation of BMP signaling (Fig. 1B).

Many different members of the BMP family are expressed during embryogenesis but, based on the signaling specificity of the constitutively active and dominant mutant BMP receptors that were used in the current studies, BMP2, 4 and/or 7 are most likely required for hematopoiesis. Their genes are ubiquitously expressed during early gastrula stages (Hemmati-Brivanlou and Thomsen, 1995), as is CaM KIV (Wayman et al., 2000). Thus, the spatial and temporal patterns of expression of these signaling molecules are appropriate for their proposed roles in hematopoiesis.

**CaM KIV inhibits transcriptional responses downstream of BMPs**

Our data suggest a molecular mechanism for cross talk between the BMP and CaM KIV pathways. We hypothesize that CaM KIV inhibits BMP signaling by activating a CBP-binding substrate (possibly CREB), thereby preventing CBP from acting as a coactivator of BMP-specific target gene(s) that are required for hematopoiesis. In our model (Fig. 8, upper panel), BMP induces nuclear translocation of Smad heterooligomers that associate with CBP and with an unrelated, as of yet unidentified, DNA binding protein (purple box). Although this DNA binding protein is illustrated as being a direct component of the Smad/CBP complex, it is equally possible that Smads act synergistically with a trans-acting factor that binds to promoter regions of hematopoietic-specific genes and recruits CBP independent of direct interaction with Smads. When BMP and CaM KIV pathways are activated simultaneously, CaM KIV phosphorylates CREB which then binds to CBP and disrupts interactions between limiting amounts of CBP and the hematopoietic-specific DNA binding protein (lower panel). It is possible that phosphorylated CREB directly disrupts interactions between CBP and Smads. This is unlikely, however, since upregulation of CaM KIV signaling in early embryos selectively inhibits the ability of BMPs to regulate hematopoiesis but does not interfere with Smad/CBP-mediated specification of ventral fate (Kato et al., 1999).

CBP functions as a coactivator for diverse transcription factors, including the hematopoietic-specific transcription factor, GATA1, and genetic studies have demonstrated a requirement for CBP in hematopoiesis (reviewed by Blobel, 2000; Goodman and Smolik, 2000). Specifically, inactivation of a single CBP allele inhibits erythropoiesis (Kung et al., 2000), possibly in a non cell-autonomous manner (Oike et al., 1999), as does misregulation of CaM KIV or BMP signaling. Previous studies have suggested that CBP is present in limiting amounts in cells and that competition for binding to CBP can regulate gene transcription (Chakravarti et al., 1996; Kamei et al., 1996; Takahashi et al., 2000). Consistent with this, our data suggest that CaM KIV inhibits BMP signaling by CREB-mediated sequestration of limiting amounts of CBP. Furthermore, our studies extend the model of competition for CBP by utilizing an experimental paradigm in which endogenous CBP-interacting transcription factors are activated through signaling pathways rather than being overexpressed.

Our data do not exclude other possible mechanisms of CaM KIV-mediated repression of BMP signaling. It is possible, for example, that CaM KIV and BMPs independently induce expression of antagonistic target genes that act in parallel pathways. Our data showing that a form of CREB that binds...
CBP constitutively can phenocopy defects observed following constitutive activation of CaM KIV, and that activation of CREB target genes is not required for this activity, do not support this interpretation. It is also possible that CaM KIV phosphorylates and directly inhibits nuclear translocation or function of Smad1, 5 and/or 8. Consistent with this possibility, putative consensus CaM KIV motifs are present in BMP pathway-specific Smads and a distinct CaM kinase family member (CaM KII) has been shown to phosphorylate and inhibit nuclear translocation of Smad2 (Wicks et al., 2000). In preliminary experiments, however, we have not detected inhibition of Smad nuclear translocation in the presence of CaCaM KIV (G. A. W., unpublished data). Furthermore, if this model were correct, constitutive activation of CaM KIV should phenocopy complete loss of BMP function, including dorsalization of the embryo, and this is not observed. Finally, recent studies have shown that ectopically expressed calmodulin can either inhibit (Xu et al., 1999b) or enhance (Sherer and Graff, 2000) Smad1 activity in Xenopus embryos raising the possibility that the increase in Ca2+/calmodulin that

REFERENCES


