Crosstalk between BMP and CaM-KIV:

A novel signaling pathway in hematopoiesis

by

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ABSTRACT

Bone morphogenetic proteins (BMPs) have important signaling roles in embryonic development. During gastrulation they ventrally pattern the mesoderm and ectoderm, and later in development they function in the specification and/or differentiation of many tissues and organs including the heart and the blood. The exact nature of BMP's signaling role in the development of these two vital systems has not been identified, and it is this gap in knowledge this thesis attempts to fill.

To begin to examine potential roles for bone morphogenetic proteins (BMPs) in cardiogenesis, we used targeted misexpression of intracellular BMP inhibitors to selectively disrupt BMP signaling in a subset of embryonic cells, including those that give rise to the heart and dorsal anterior endoderm, in *Xenopus*. Early expression of markers of precardiac fate was not perturbed in BMP-deficient embryos. At later stages, however, most BMP-deficient embryos showed endodermal defects, a reduction in cardiac muscle-specific gene expression, a decrease in the number of cardiomyoctes and/or cardia bifida. Our results provide evidence that BMPs are not required for the initial induction of the cardiac field but are essential for patterning of the endoderm, for migration and/or fusion of the heart primordia and for cardiomyocyte differentiation.

To examine a potential non-cell autonomous role for BMPs in hematopoiesis, we experimentally misregulated the signaling pathway, by injecting intracellular inhibitors or a constitutively active receptor, specifically in cells that do not give rise to blood. Up or down-regulation of BMP resulted in anemia but due to distinct mechanisms:

hyperactivation of BMP caused a shift in cell fate towards the myeloid or white blood cell lineage, while inhibition of BMP caused an increase in erythrocyte-specific apoptosis. Interestingly, the same phenotypes were seen when the calcium/calmodulin-dependent protein kinase IV (CaM KIV) was blocked or hyperactivated, respectively. Further investigations revealed that CaM KIV is capable of antagonizing BMP signaling downstream of receptor activation, and that a balance between the two signaling pathways is required for proper blood development. Crosstalk between these two signaling pathways had not previously been reported. Based on our findings, we suggest a model for CaM KIV inhibition of BMP in which CaM KIV phosphorylation of CREB (or another CaM KIV substrate) allows CREB to bind to CBP preventing CBP from operating as a cofactor in Smad-induced transcription of genes that function during hematopoiesis.

INTRODUCTION

Xenopus laevis is a useful vertebrate system for studying early embryonic development for many reasons including its large, externally fertilized eggs that can be experimentally manipulated in a number of ways. In order to investigate the role of growth factors and signaling molecules in the development of specific organs and tissues, synthetic RNA encoding wild type or mutant versions of genes of interest can be injected directly into the developing embryo. The unfertilized egg has an animal-vegetal polarity, where the pigmented animal pole eventually differentiates into ectoderm, neural structures and skin, and the yolky vegetal hemisphere gives rise to gut and associated endodermal organs. The third primary germ layer, the mesoderm, is derived from interactions between the ectoderm and endoderm. By the 4-cell stage (2 hours of development), the dorsal hemisphere can be visually distinguished from the ventral by a difference in pigmentation. Therefore, very early in development the experimental RNA can be targeted to a specific region of the developing embryo that will give rise to the structure or tissue being investigated.

One family of signaling molecules that has been extensively studied using the *Xenopus* system is bone morphogenetic proteins (BMPs). BMPs are members of the TGF-β superfamily of signaling molecules and play multiple roles during embryonic development. TGF-β ligands bind to a heterocomplex of type I and type II transmembrane serine-threonine kinases to form a high affinity receptor. Once the ligand has bound, the type II receptor transphosphorylates the type I receptor which then propagates the signal by phosphorylating a signal-transducing Smad. Smads are a group

of structurally related proteins that have been shown to transduce signals downstream of BMP and other TGF-β family receptors (reviewed by Christian and Nakayama, 1999; Massague, 1998). Smad proteins share two conserved domains at the amino- and carboxy-terminae, called MH1 and MH2 (for Mad Homolgy domain) respectively, and a poorly conserved linker region. The MH2 domain is the effector domain whereas the MH1 domain is the regulatory domain that binds the MH2 domain and suppresses its function (Hata *et al.*, 1997). Smads 1, 5, and 8 are phophorylated by and transduce signals downstream of BMP receptors, whereas Smads 2 and 3 are phosphorylated by and transduce signals downstream of activin or TGF-β receptors. This phosphorylation relieves MH2 inhibition and allows the signal-transducing Smad to heterocomplex with Smad4, the common partner of all pathway-restricted Smads. This heterocomplex can then translocate to the nucleus and induce transcription of target (reviewed by Christian and Nakayama, 1999; Massague, 1998).

A class of inhibitory Smads have been identified that includes Smad6 and Smad7 (Imamura *et al.*, 1997; Nakayama *et al.*, 1998a). These Smads lack the C-terminal phosphorylation sequence and function to block BMP signaling downstream of ligand binding. Their mechanism of action remains controversial, but several models have been proposed. Both Smad6 and Smad7 can bind to the intracellular domain of type I receptors and thereby prevent phosphorylation-mediated activation of signal-transducing Smads (Hayashi *et al.*, 1997; Imamura *et al.*, 1997) (Figure 1B). In contrast, recent evidence shows Smad6 can bind directly to Smad1 at lower levels, thereby inhibiting interaction with Smad4 and subsequent gene transcription (Hata *et al.*, 1997) (Figure 1C).

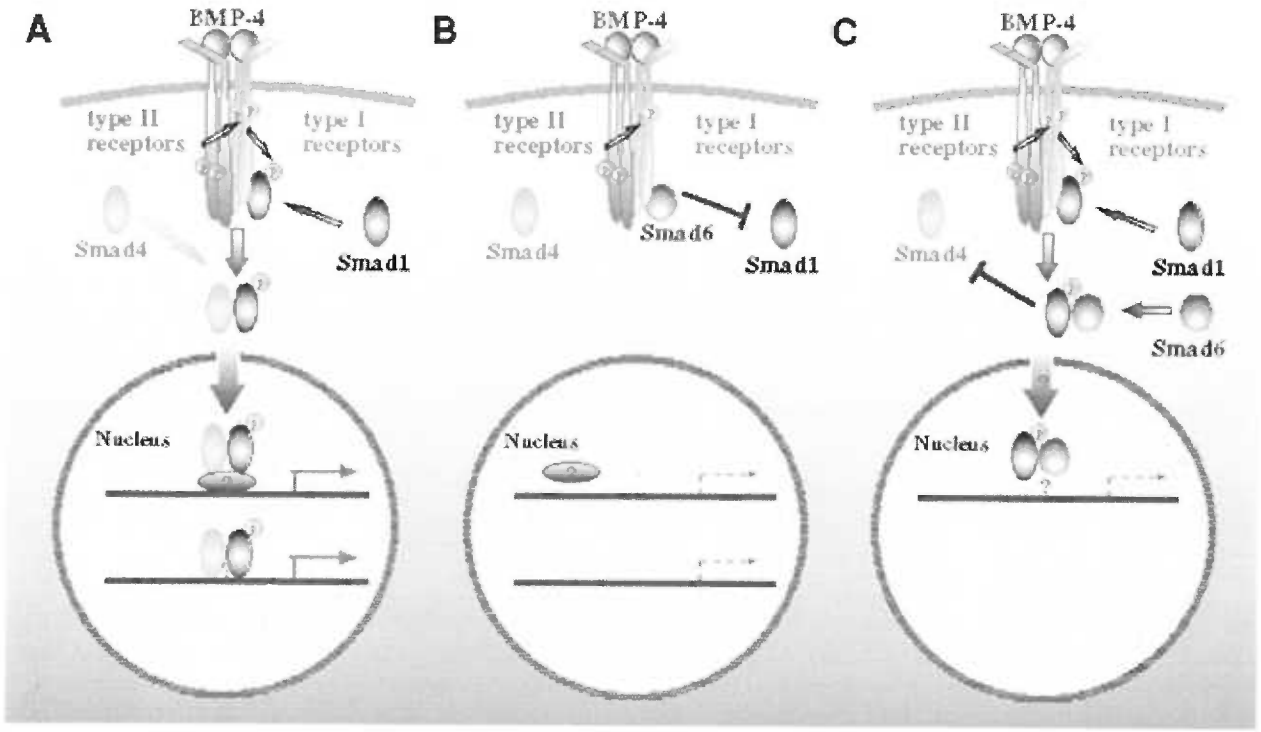

Figure 1. from Christian and Nakayama, 1999 A: Model of how Smad1 signals downstream of BMPs. **B:** Proposed model for Smad6 inhibition of BMP signal transduction in which Smad6 competes with Smad1 for association with the activated receptor complex. **C:** Alternative model for Smad6 inhibition of BMP signaling in which Smad6 competes with Smad4 for binding to phosphorylated Smad1.

BMPs were originally identified as cytokines that could induce ectopic bone formation (Wozney *et al.*, 1988), but have since been shown to be involved in both the ventral patterning of the mesoderm and the patterning and differentiation of many tissues and organs (Hogan, 1996). The role of BMPs in establishing the dorsal-ventral axis was most clearly shown in experiments in which BMP was overexpressed or experimentally inhibited in *Xenopus* embryos. Overexpression of BMP-4 leads to a ventralized phenotype that is characterized by a lack of dorsoanterior structures (Dale *et al.*, 1992). Conversely, when BMP signaling is blocked by a dominant-negative BMP receptor,

ventral mesoderm develops as dorsal instead (Graff *et al.*, 1994). Inhibition of BMP signals with antagonistic Smads also dorsalizes the embryo and a secondary dorsal axis may be formed (Nakayama *et al.*, 1998a) (Figure 2).

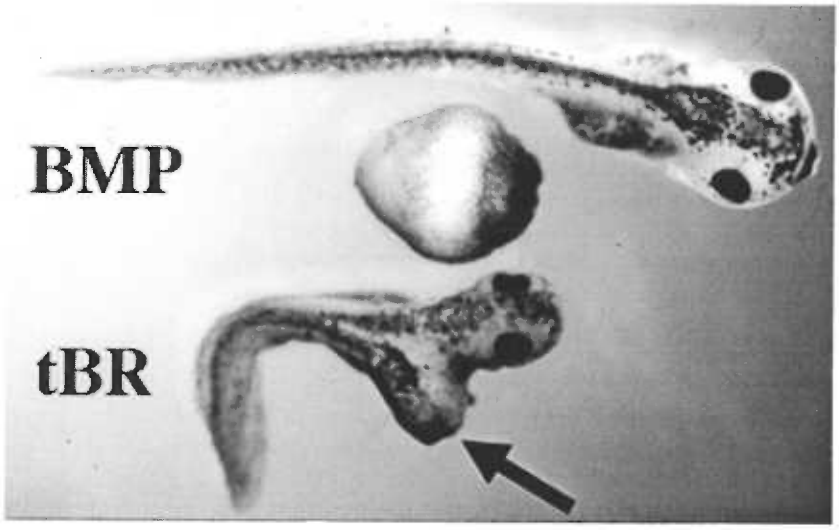

Figure 2. BMP signaling has a role in ventral patterning of the mesoderm. When BMP is overexpressed on the dorsal side of the embryo the embryo is ventralized, as marked by loss of dorsal structures (head, notochord, and muscle). Conversely, when endogenous BMP function is inhibited with a dominant-negative receptor, tBR, the ventral side of the embryo develops dorsal structures, as indicated by formation of a secondary dorsal axis.

Evidence supports an important role for BMPs in both heart and blood development. The vertebrate heart is derived from paired regions of dorsolateral mesoderm which forms the cardiac field on the dorsal side of the embryo (Figure 3). This field is a region competent to respond to inductive signals (Fishman and Chien, 1997). Explant experiments in *Xenopus* by Sater and Jacobson showed dorsalizing signals from the blastopore lip during gastrulation to be the earliest required step in establishment of heart mesoderm (Sater and Jacobson, 1990). Several studies have

concluded that the underlying anterior endoderm signals to the dorsalized mesoderm to induce cardiac differentiation (Nascone and Mercola, 1995; Schultheiss *et al.*, 1995; Tonegawa, 1996). Activin-A and FGF-2 have been suggested to be this signal as they are both expressed in the embryonic endoderm and can mimic the cardiogenic effects of the anterior endoderm (Sugi and Lough, 1995). Activin-A has also been shown to induce expression of the cardiac specific marker, XMHC-α, in *Xenopus* animal caps (Logan and Mohun, 1993). Studies in chick have implicated BMPs in inducing cells in lateral subdomains of the cardiac field to differentiate into heart (Schultheiss *et al.*, 1997). In these studies, ectopic BMP induced expression of Nkx2.5, one of the earliest markers of cardiac specification (Lints *et al.*, 1993).

Once the heart primordia have been established, they migrate anteriorly and ventrally during gastrulation to eventually fuse and form a single, ventral heart tube (Figure 3). The linear heart tube is the earliest functioning vertebrate heart. GATA transcription factors, 4, 5, and 6, are expressed in distinct but overlapping patterns that include the cardiac mesoderm prior to heart tube formation (Jiang and Evans, 1996). Depletion of all three of these genes in chick, using antisense oligomers, results in cardia bifida, a failure of the two cardiac primordia to fuse at the ventral midline, and in the most extreme cases two hearts form (Jiang *et al.*, 1998).

The heart tube then loops to the right converting the anterior-posterior patterning of the tube into a left-right asymmetry. Many genes have been shown to be asymmetrically expressed and suggested to play a role in left-right patterning. One example is the basic Helix-Loop-Helix factor, *eHAND*, also known as *Thing-1* (Hollenberg *et al.*, 1995; Srivastava *et al.*, 1995). *eHAND* is expressed in a left-dominant

pattern in the myocardium of the heart tube prior to looping, and this expression appears to be controlled by the homeodomain factor Nkx2.5 (Biben and Harvey, 1997). Nkx2.5 mutant mice fail to progress beyond the linear heart tube stage and lack left-sided *eHAND* expression (Biben and Harvey, 1997). Ectopic expression of BMPs in *Xenopus* animal cap explants strongly induces *eHAND* expression (Sparrow *et al.*, 1998). BMPs have also been proposed to have a direct role in heart tube looping. In Zebrafish, BMP-4 was shown to be predominantly on the left side of the heart tube, and the direction of looping appeared to be dependent on this expression pattern (Chen *et al.*, 1997).

Unlike higher vertebrates, *Xenopus* only have three chambers in the adult heart, but the single ventricle is designed so oxygenated and deoxygenated blood flow through it in fairly separate streams (Fishman and Olson, 1997). Trabeculae form in the inner spongy layer of the ventricle in response to signals from the endocardium, possibly neuregulin (Fishman and Chien, 1997).

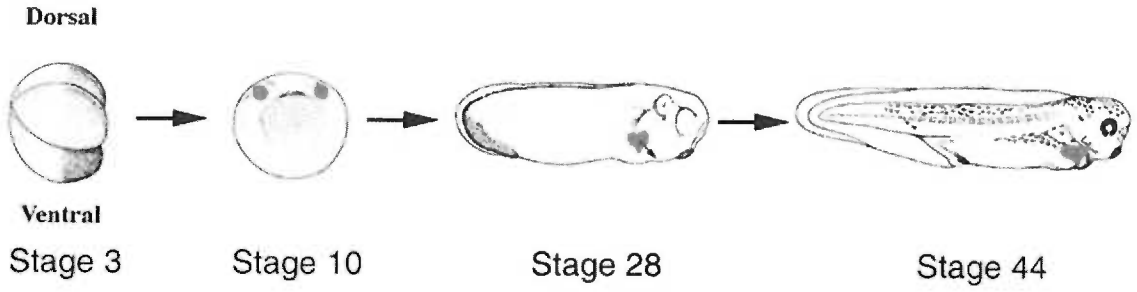

Figure 3. Summary of heart development in *Xenopus*. At stage 3 the dorsal-ventral axis becomes distinct. The cardiac primordia are specified from the dorso-lateral mesoderm by stage 10. The primordia have migrated ventrally and fused into a single heart tube by stage 28, and by stage 44 septation has occurred resulting in the fully developed 3-chambered heart.

BMPs have also been implicated in blood development. In contrast to the dorsal heart rudiments, primitive hematopoietic tissue has been shown to be derived from the ventral mesoderm by explant and axis disruption experiments (Kau and Turpen, 1983; Maeno *et al.*, 1985). Two recent fate map studies, however, have suggested that a percentage of blood may be derived from more dorsal blastomeres (Lane and Smith, 1999; Mills *et al.*, 1999).

In *Xenopus* embryos, primitive blood forms on the ventral side of the tailbud embryo in what is called the ventral blood island (VBI) (Nieuwkoop and Faber, 1967) (Figure 4). The VBI contains pluripotential hematopoietic stem cells (HSCs) which can give rise to all the cells found in the embryonic blood. The erythroid lineage (red blood cells) represents one line of decent from HSCs. Although the erythroid progenitor cells are restricted to forming only red blood cells, there are many generations of precursor cells. Most of these precursors express α-T globin mRNA. Visualizing *globin* expression by in situ hybridization reveals the characteristic V-shape of the VBI in tailbud stage embryos. Approximately 20 hours of development later the heart starts beating, the blood enters circulation, and the VBI is no longer a distinct structure (Kelley *et al.*, 1994).

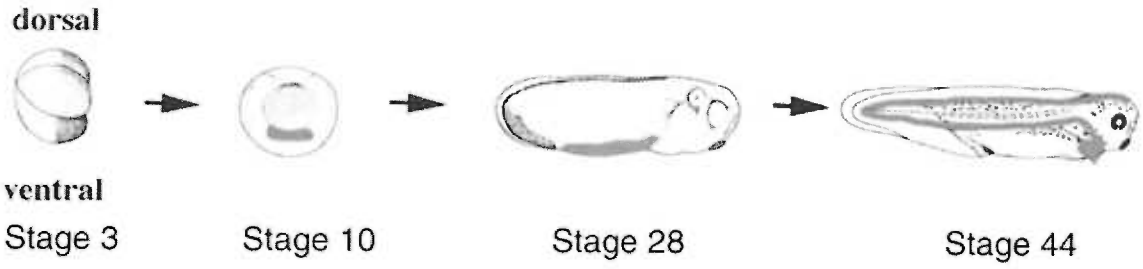

Figure 4. Summary of blood development in *Xenopus*. By stage 10 (gastrula) the ventral mesodermal cells are committed to the primitive blood lineage. The hematopoietic pogenitors in the ventral blood island (VBI) express globin by stage 28 (tailbud), and circulation begins at stage 44 (tadpole).

Many transcription factors play a role in hematopoiesis. Transcription factors belonging to the family of GATA-binding proteins are involved in differentiation of blood cells (Kelley *et al.*, 1994). Targeted gene disruption in mouse revealed an important role for both GATA-1 and GATA-2 in erythropoiesis (Pevny *et al.*, 1991; Tsai *et al.*, 1994). GATA-1 has been shown to function not only in erythroid differentiation but also in downregulating GATA-2 (Briegel *et al.*, 1996).

Another transcription factor involved in blood development is the basic helix-loop-helix transcription factor Stem Cell Leukemia (SCL) (Mead *et al.*, 1998). SCL cannot induce hematopoiesis in animal pole ectoderm, but can direct mesoderm to a hematopoietic fate. Therefore, SCL acts downstream of patterning to play a role in blood formation in mesodermal cells (Mead *et al.*, 1998). These experiments suggest that SCL is important for formation of HSCs, GATA-2 is required for HSC proliferation, and GATA-1 plays a role in differentiation. (reviewed by Huber and Zon, 1998).

Ventral patterning by BMP may initiate the hematopoietic program (Huber and Zon, 1998). BMPs have also been implicated in subsequent steps in hematopoiesis. For example, BMP-4 can induce expression of GATA-1, GATA-2, and SCL in animal pole explants (Maeno *et al.*, 1996; Zhang and Evans, 1996). One of the aims of this thesis is to more clearly define the role of BMPs in hematopoiesis.

BMPs are only one of a plethora of signaling pathways that function to direct the complicated process of embryonic development. Recently, the role of the calcium/calmodulin-dependent protein kinase IV (CaM KIV) during embryonic development was explored for the first time (Wayman et al., submitted). CaM KIV is a member of a recently characterized CaM kinase cascade (reviewed by Soderling, 1999). Binding of Ca^{2+} /CaM to CaM KIV serves two purposes, first it relieves autoinhibition. At low intracellular calcium, an autoinhibitory sequence interacts with the adjacent catalytic domain and maintains the kinase in an inactive state. Association of Ca^{2+} /CaM with the overlapping CaM binding domain removes the autoinhibitory segment from the catalytic core, thereby activating the kinase. Secondly, Ca^{2+} /CaM binding allows a critical threonine residue, located within the activation loop, to be phosphorylated by a CaM-dependent kinase kinase (CaM KK). Phosphorylation of this Thr196 by CaM KK results in a 10-20 fold activation of CaM KIV.

The substrate specificity of CaM KIV is not well defined, but it has been shown to phosphorylate and regulate several transcription factors including cAMP-responsive element-binding protein (CREB) (Enslen *et al.*, 1995; Matthews *et al.*, 1994), cAMP-responsive element-modulator τ (CREMτ), serum response factor (SRF), MEF2 (Blaeser

et al., 2000) and activating transcriptional factor-1 (ATF-1) (Sun, 1996). Consistent with a role in regulating transcription, CaM KIV and CaM KK show strong nuclear localization, but are also present in the cytosol (Sakagami, 1999).

In addition to responding to intracellular calcium, the CaM kinase cascade participates in crosstalk with other signaling cascades. For example, CaM KIV directly phosphorylates and inhibits the activity of type I adenylyl cyclase (Wayman, 1996) and nitric-oxide synthase (Hayashi, 1999), and it may indirectly activate mitogen-activated protein kinase (MAP) pathways (Enslen, 1996) (Figure 5).

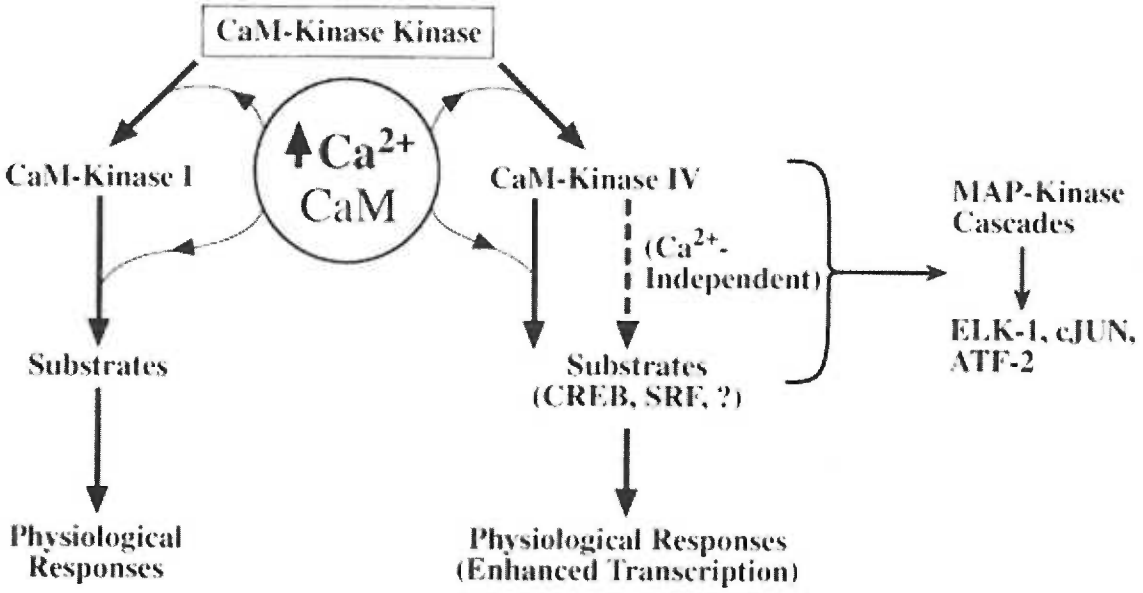

Figure 5. Model of the CaM-kinase cascade from Soderling, 1996. The cascade is triggered by elevation of intracellular Ca^{2+} which activates CaM. The Ca^{2+} /CaM complex activates CaM KK and binds to CaM KIV exposing the activation loop for phosphorylation by CaM KK. Phosphorylation of CaM KIV by CaM KK increases both its total activity and its activity in the absence of Ca^{2+} /CaM. Crosstalk with other signal transduction pathways is hypothesized.

BMPs can also interact with other signaling pathways. For example, it has been shown that the MAP kinase cascade can inhibit a specific branch of BMP signaling by phosphorylating the downstream transducer, Smad1 (Kretzschmar *et al.*, 1997). Recently, we collaborated with Gary Wayman and Tom Soderling to describe the role of CaM KIV in heart (Wayman et al., manuscript in preparation) and blood (Wayman et al., submitted) development. In this thesis I describe the role of BMP in heart and blood development, and in chapter 2, I present evidence that supports a model of BMP and CaM KIV crosstalk during hematopoiesis (Figure 8, chapter 2). Interaction between these two pathways has not been previously described and leads to a better understanding of BMP's role in vertebrate cardiovascular development.

Chapter 1

Bone morphogenetic protein function is required for terminal differentiation, but not for initial specification, of the heart.

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ABSTRACT

To examine potential roles for bone morphogenetic proteins (BMPs) in cardiogenesis, we used targeted misexpression of intracellular BMP inhibitors to selectively disrupt BMP signaling in a subset of embryonic cells in *Xenopus,* including those that give rise to the heart and dorsal anterior endoderm. Early expression of markers of precardiac fate was not perturbed in BMP-deficient embryos. At later stages, however, most BMP-deficient embryos showed endodermal defects, a reduction in cardiac muscle-specific gene expression, a decrease in the number of cardiomyoctes and/or cardia bifida. Our results provide evidence that BMPs are not required for the initial induction of the cardiac field but are essential for patterning of the endoderm, for migration and/or fusion of the heart primordia and for cardiomyocyte differentiation.

Key words: *Xenopus*, bone morphogenetic proteins, embryogenesis, cardiogenesis, cardia bifida, Nkx2.5, GATA-4, Smad6, endoderm, myocardium, endocardium.

INTRODUCTION

The vertebrate heart is derived from paired regions of dorsolateral mesoderm that form the cardiac field (reviewed by Fishman and Chien, 1997). These primordia become specified to the cardiac lineage in response to inductive signals from dorsal mesodermal and endodermal cells prior to the end of gastrulation (Jacobson and Sater, 1988; Nascone and Mercola, 1995; Sater and Jacobson, 1990; Schultheiss *et al.*, 1995). During neurula stages, heart progenitors migrate to the anterior ventral midline, fuse to form a single tube and begin to differentiate as cardiomyocytes. Following fusion, the heart tube loops to the right, converting its initial anterior-posterior pattern into a left-right asymmetry. Subsequently, morphogenetic cell movements, coupled with cell proliferation, subdivide the heart into a multi-chambered functional organ.

Several families of cardiac-enriched transcriptional regulators have been identified in recent years and these provide useful molecular markers to analyze specific aspects of heart development (reviewed by Fishman and Chien, 1997; Fishman and Olson, 1997). Nkx2.5, for example, is a NK-2 class homeodomain protein that is among the earliest markers of cardiac specification. *Drosophila* embryos mutant for the *Nkx* ortholog, *tinman* (*tin*), fail to form a heart (Azpiazu and Frasch, 1993; Bodmer, 1993) and the same is true of vertebrate embryos in which the function of two apparently redundant Nkx family members is simultaneously blocked (Fu *et al.*, 1998). Downstream of Nkx2.5, members of the MEF2 family of MADS-box transcription factors directly activate cardiac structural genes (reviewed by (Fishman and Olson, 1997) while the basic helix-loop-helix transcription factors, dHAND and eHand (also known as Thing1,

Hollenberg *et al.*, 1995) are required for morphogenesis of the heart and outflow tract (Srivastava *et al.*, 1995; Srivastava *et al.*, 1997). A fourth family of transcriptional regulators, the zinc finger binding proteins GATA-4, GATA-5 and GATA-6, are expressed very early in the cardiac program. These proteins are involved in the initial steps of cardiomyocyte differentiation, and in directing ventral migration and fusion of the cardiac primordia (reviewed by Charron and Nemer, 1999).

Cell-cell signaling molecules that are responsible for induction and patterning of the heart are less well studied, but a variety of embryological evidence suggests that BMPs are required for these processes. BMP-2 and -4, for example, can induce ectopic expression of the heart-specific genes, *Nkx2.5* and *GATA-4*, in non-precardiac mesoderm, and inhibition of BMP signaling blocks late expression of *Nkx2.5* and cardiac differentiation (Ladd *et al.*, 1998; Lough *et al.*, 1996; Schultheiss and Lassar, 1997). These studies have led to a model in which localized expression of BMPs in the endoderm selects which cells within the heart field will enter the cardiac lineage. Data obtained using an in vitro cell culture model for cardiac differentiation are consistent with the hypothesis that BMP function is required for induction of expression of cardiac-specific genes and for cardiomyoctye differentiation (Monzen *et al.*, 1999). BMPs have also been proposed to have a direct role in heart tube looping. In Zebrafish, BMP-4 is normally expressed predominantly on the left side of the heart tube, and appears to be in the pathway that specifies the direction of heart looping (Chen *et al.*, 1997).

Gene depletion studies in mice further support a role for BMPs in cardiac development but have not revealed a necessity for these factors in cardiac induction or myocyte differentiation. For example, mice lacking Smad6, an intracellular inhibitor of

BMP signaling, develop hyperplasia of the heart valves and defects in the aorticopulmonary septum (Galvin *et al.*, 2000). Mice deficient for BMP-2 (Zhang and Evans, 1996), or the downstream signal transducer, Smad5 (Chang *et al.*, 1999), display abnormal placement of the heart in the exocoelomic cavity, but induction and differentiation of the heart are not perturbed. Genetic analysis of BMP function is limited, however, by early lethality of mice lacking certain BMPs or their receptors (Mishina *et al.*, 1995; Winnier *et al.*, 1995), which precludes analyses of their later roles in organogenesis. Genetic analyses is also limited by functional redundancy among different BMP family members. Mice lacking either BMP-5 or BMP-7, for example, develop fairly normally whereas BMP-5; BMP-7 double mutants are embryonic lethal due to a variety of defects including delayed and abnormal development of the heart (Solloway and Robertson, 1999). In summary, although strong evidence suggests that BMPs function at multiple stages of cardiogenesis, including in the initial induction of the heart field, the exact nature of these roles has not been determined in vivo.

BMP family members initiate signaling by binding to a complex of structurally similar type I and type II transmembrane serine-threonine kinases (reviewed by Yamashita *et al.*, 1996). Multiple type I and type II receptors have been shown to be capable of binding BMPs. Following ligand binding, type II receptors transphosphorylate the type I receptors which then propagate the signal by phosphorylating one of the BMP pathway-specific signal transducing Smads, Smad1, Smad5 or Smad8 (reviewed by Christian and Nakayama, 1999; Massague, 1998; Piek *et al.*, 1999). The common Smad, Smad4, then heterodimerizes with phosphorylated signal-transducing Smads and this complex translocates into the nucleus and activates

transcription of target genes. A class of inhibitory Smads has also been identified that includes Smad6 and Smad7 (reviewed by Christian and Nakayama, 1999). These Smads lack the C-terminal phosphorylation sequence and function to block BMP signaling downstream of ligand binding.

In the current studies, we have taken advantage of the *Xenopus* system to selectively disrupt BMP signaling in a subset of embryonic cells, including those that give rise to the heart and to the anterior dorsal endoderm. This allowed us to bypass the early patterning defects and lethality that results from total loss of BMP function in order to analyze its role in cardiac development. Our results provide evidence that BMPs are not required for the initial induction of the cardiac field, but are essential for patterning of the endoderm, for migration and/or fusion of the heart primordia and for cardiomyocyte differentiation.

RESULTS

2.1 Dorsal inhibition of BMP signaling causes gut and heart defects.

To examine a possible role for BMPs in cardiac development, we injected RNA (300 pg) encoding the intracellular BMP antagonist, Smad 6 near the dorsal marginal zone (DMZ) of *Xenopus* embryos at the 4-cell stage. These embryos were then cultured until the tadpole stage 46. In all experiments, we also analyzed animals in which BMP signaling had been inhibited by injection of 150 pg RNA encoding a well characterized dominant mutant truncated BMP receptor (tBR),. Identical results were observed following injection of either Smad6 or tBR, thereby verifying that defects were specifically due to inhibition of the BMP pathway. Embryos in which BMP signaling was inhibited in dorsal cells appeared grossly normal at the tailbud stage, consistent with previously published results (Nakayama et al., 1998), but showed specific defects at the tadpole stage. In uninjected or mock-injected tadpoles, the gut showed a stereotypical coiling pattern (Fig 1A, white arrow). In contrast, almost all embryos in which BMP signaling was inhibited by injection of Smad 6 or tBR showed defects in gut coiling (Fig 1B) and, in some cases, the gut failed to coil and consisted of a linear tube (Fig 1C). In addition, the hearts of BMP-inhibited embryos appeared small and thin-walled (Fig 1E and F, black arrowhead) relative to control embryos (Fig 1D). Most of these embryos showed edema of the pericardial sac (Fig. 1F, black arrow) and of the overall body (Fig. 1C), possibly secondary to heart failure. In a subset of embryos, defects in head structures were also observed (Fig. 1C), consistent with the known role for BMPs in neural patterning (reviewed by Mehler *et al.*, 1997), but in many embryos heart and gut defects were observed independent of other axial defects (Fig. 1B). Subsequent analyses were restricted to embryos that did not show gross defects in axial patterning.

To further analyze the heart morphology of embryos in which BMP signaling was inhibited, histological sections of BMP-inhibited and control sibling tadpoles were examined. *Xenopus* tadpoles have a single ventricle which, in control embryos, has a thick, muscular wall (Fig. 2A, black arrow) that is highly trabeculated (white arrow). In embryos injected with either tBR or Smad6 RNA, the ventricular wall was thin and poorly trabeculated (Fig. 2B and C). In severely affected embryos, the heart consisted of a thin-walled tube that completely lacked trabeculation (Fig. 2C).

2.2 Inhibition of BMP signaling causes cardia bifida and a decrease in cardiac muscle-specific gene expression.

To begin to examine the underlying cause of the heart defects in embryos in which BMP signaling was inhibited, we analyzed expression of two markers of cardiomyocyte differentiation, troponin c (Tnc) and myosin light chain (MLC), by in situ hybridization. At tailbud stage 32, the heart primordia have migrated ventrally and, in 84% of control embryos, had fused at the ventral midline as indicated by the continuous band of Tnc (Fig. 3A) and MLC (Fig. 3D) staining throughout the heart tube. At the equivalent stage, TnC (Fig. 3B and C) and MLC (Fig. 3E and F) staining was greatly reduced in 85% of tBR-or Smad6-injected embryos . In addition, staining was observed in two discrete regions on either side of the midline in approximately 40% of embryos (Fig. 3B, C and E, white arrows) indicating that the two heart primordia had failed to fuse at the midline, a condition known as cardia bifida. We conclude that BMP signaling is required for cardiomyoctye differentiation and for proper migration and/or ventral fusion of the heart primordia.

2.3. Embryos in which BMP signaling is inhibited have fewer cardiomyocytes.

The decrease in Tnc and MLC message in embryos in which BMP signaling was inhibited could be due to a decrease in the number of myocardial cells, a decrease in the

size of myocardial cells, or failure of prospective myocardial cells to differentiate as cardiomyocytes. To begin to distinguish between these possibilities, embryos were stained for Tnc at tailbud stage 32 and transverse sections through the heart tube were co-stained with SYTOX green to visualize nuclei of individual cells. Embryos in which Tnc staining was barely detectable were excluded from this analysis since it was not possible to accurately judge relative anterior-posterior position within the heart tube. Thus, the examples shown here are representative of less severely affected embryos. Examination of sections through comparable regions of the heart tube in BMP-inhibited (Fig. 4C-F) and control (Fig. 4A and B) embryos revealed that the decrease in TnC staining in BMP-inhibited embryos was accompanied by a decrease in myocardial cell number. For example, approximately sixty per cent as many myocardial cells were present in the BMP-deficient embryo shown in Figure 4D as in the control sibling (Fig. 4B). In addition, the endocardium appeared to be poorly developed (Figure 4C and E, white arrowheads) and the heart tubes had failed to fuse in a subset of BMP-deficient embryos (Fig 4E and F). These results demonstrate that BMP signaling is essential for differentiation of the proper number of cardiomyoctes, and for normal migration and/or fusion of the cardiac primordia.

2.4. Inhibition of BMP signaling does not perturb initial specification of the heart field.

To investigate the possibility that the decrease in the number of differentiated cardiomyocytes in BMP-deficient embryos resulted from a decrease in the number of cells initially specified to the cardiac fate, we analyzed expression of the homeobox transcription factor, Nkx2.5. Nkx2.5 is the earliest known marker of the cardiac lineage and previous studies in chick and fly suggest that BMP signaling is both necessary and sufficient for expression of Nkx2.5 in precardiac mesoderm (Frasch, 1995; Schultheiss and Lassar, 1997). Surprisingly, no significant difference was observed in the level of

expression of Nkx2.5 in BMP-deficient neurula stage embryos relative to controls as analyzed by in situ hybridization (data not shown) and reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 5, stage 19). In contrast, when gene expression was analyzed in older (tailbud, stage 30) sibling embryos from the same experiment, expression of Nkx2.5 was significantly decreased and expression of Tnc was almost abolished in tBR-injected embryos (Fig. 5). These results demonstrate that the decrease in the number of differentiated cardiomyocytes in BMP-deficient embryos is not due to a decrease in the number of cells that are initially specified to the cardiac lineage.

To further investigate a possible role for BMP signaling in early transcriptional regulation of cardiac fate, we analyzed expression of GATA-4 in embryos in which BMP signaling was inhibited. GATA-4, like Nkx2.5, is expressed in precardiac mesoderm and BMPs can induce ectopic expression of this gene in non-cardiac mesoderm (Schultheiss *et al.*, 1997). Furthermore, GATA-4 has been shown to regulate the expression of several cardiac muscle-specific genes, such as MHC and Tnc (Ip *et al.*, 1994), and mice carrying a homozygous null mutation at the GATA-4 allele show cardia bifida and defects in endoderm development similar to those observed in BMP-deficient *Xenopus* embryos (Kuo *et al.*, 1997; Molkentin *et al.*, 1997). Inhibition of BMP signaling did not cause a significant decrease in the level of GATA-4 transcripts in neurula (st. 19) or tailbud (st. 30) stage embryos relative to controls (Fig. 5). Thus, although GATA-4 is a logical candidate for a gene whose expression or function might be perturbed in BMP-deficient embryos, our results demonstrate that BMP signaling is not required for expression of GATA-4.

DISCUSSION

3.1. BMP signaling is not essential for induction of the cardiac field

Embryological studies have shown that anterior endoderm is the source of instructive signals that induce the heart to form from overlying mesoderm, and BMPs have been implicated in this process. In chick, for example, exogenously supplied BMP-2 induces ectopic expression of the early cardiac markers, Nkx2.5 and GATA-4, but only within anterior mesodermal cells located close to the heart forming field (Ladd *et al.*, 1998; Schultheiss and Lassar, 1997). More distally located posterior mesoderm can be made competent to respond to BMP-4, however, by pretreatment with FGFs (Ladd *et al.*, 1998; Lough *et al.*, 1996). Conversely, application of the BMP-inhibitor, noggin, blocks late expression of Nkx2.5 and cardiac differentiation (Ladd *et al.*, 1998; Schultheiss and Lassar, 1997). Collectively, these data have been interpreted to support a model in which an initial signal, possibly provided by FGFs, sets up a cardiogenic field in the anterior mesoderm that is competent to respond to BMPs. Subsequently, BMPs (most likely BMP-2 or -4) supplied by the underlying endoderm specify which cells within this field will enter the cardiac myocyte lineage (Schultheiss and Lassar, 1997) reviewed by (Mohun and Sparrow, 1997).

Genetic studies in *Drosophila* also support a role for BMPs in initiation of the cardiac program. Specifically, flies mutant for the BMP-2/4 ortholog, *decapentaplegic* (*dpp*), fail to maintain expression of the Nkx2.5 ortholog, *tinman* (*tin*) in cardiac mesoderm (Frasch, 1995) and the heart does not form (Azpiazu and Frasch, 1993;

Bodmer, 1993). Conversely, misexpressed Dpp can activate expression of *tin* in ectopic sites (Frasch, 1995).

Our results are consistent with previous studies showing an essential role for BMPs in cardiogenesis but they do not support a model in which BMP signaling is required for the initial establishment of the cardiac field. Specifically, embryos in which BMP signaling is selectively blocked in dorsal cells express early markers of cardiac specification, such as *Nkx2.5 and GATA-4*, although expression of Nkx2.5 is not maintained at later stages. This is similar to what is observed in *Drosophila* in that *tin* is initially broadly expressed in a Dpp-independent manner. Maintenance of expression of *tin* in the cardiac mesoderm, however, is strictly dependent on Dpp (Frasch, 1995). Previously published data have shown that expression of Nkx2.5 is absent in explants of precardiac mesoderm cultured in the presence of BMP antagonists (Schultheiss and Lassar, 1997). Our results do not contradict this data since expression of Nkx2.5 was not analyzed until after the onset of terminal differentiation in the previous studies, and we also observe a decrease in Nkx2.5 expression in BMP-deficient embryos at this stage. Taken together, our results suggest a role for BMPs in maintaining but not initiating Nkx2.5 expression.

3.2. Inhibition of BMP signaling causes gut and heart defects similar to those observed following loss of function of GATA factors.

Our results demonstrate that, although BMPs are not required for initial establishment of the cardiac field, they are essential for patterning the endoderm and for cardiomyocyte differentiation. When analyzed shortly after the onset of terminal

differentiation, expression of cardiac muscle-specific genes was repressed and fewer myocardial cells were present in BMP-inhibited embryos than in controls. Further analyses will be required to determine whether the decrease in cell number is due to a defect in proliferation or an increase in cell death.

In addition to inhibition of cardiomyocyte differentiation, embryos in which BMP signaling was blocked showed defects in differentiation and patterning of the gut, and cardia bifida. Cardia bifida persisted at least until the late tailbud stages, but formation of two complete hearts at the tadpole stage was rare. In contrast, chick embryos with experimentally induced cardia bifida develop paired functional hearts (Gannon and Bader, 1995). Thus, it is possible that inhibition of BMP signaling delays, rather than prevents, fusion of the heart tubes, or that compensatory mechanisms within the *Xenopus* embryo prevent the development of two functional hearts at the tadpole stage.

The nature of the molecular defect(s) responsible for the gut and heart abnormalities in BMP-deficient embryos is unknown, but the similarities between these defects and those observed following loss of function of one or more GATA factors raises the possibility that BMPs may be required for GATA function. Genetic studies have implicated GATA-4, GATA-5 and GATA-6 as important regulators of gut endoderm development. Each of these transcription factors is expressed in endoderm and gut-derived organs in a variety of organisms, and can activate expression of endoderm specific genes (Bossard and Zaret, 1998; Gao *et al.*, 1998). Furthermore, gene inactivation studies have shown that GATA family members are critical for endoderm specification, patterning or differentiation in mice (Kuo *et al.*, 1997; Molkentin *et al.*, 1994; Soudais *et al.*, 1995), zebrafish (Reiter *et al.*, 1999), flies (Rehorn *et al.*, 1996) and

worms (Fukushige *et al.*, 1998; Zhu *et al.*, 1998; Zhu *et al.*, 1997). These same GATA factors play critical roles in heart development (reviewed by (Charron and Nemer, 1999). Mice deficient for GATA-4 develop cardia bifida (Kuo *et al.*, 1997; Molkentin *et al.*, 1997) similar to that observed following inhibition of BMP signaling, and this can be rescued by restoring expression of GATA-4 within endodermal cells (Narita *et al.*, 1997). Cardia bifida is also observed in chick embryos following partial depletion of GATA-4, -5 and -6 (Jiang and Evans, 1996), and this again appears to be due to defects in the endoderm (Ghatpande *et al.*, 2000). Zebrafish embryos mutant for GATA-5 also display cardia bifida, but in addition have decreased numbers of myocardial precursors, defects in ventricular differentiation and reduced expression of cardiac-specific genes such as Nkx2.5 (Reiter *et al.*, 1999). Consistent with this latter finding, strong evidence exists that GATA-4, -5 and/or -6 can regulate transcription of a variety of cardiac-specific transcription factors, such as Nkx2.5 (Lien *et al.*, 1999; Searcy *et al.*, 1998) as well as genes encoding structural components of the myocardium (Ip *et al.*, 1994; Jiang and Evans, 1996; Molkentin *et al.*, 1994); reviewed by (Evans, 1997b). Collectively, the similar phenotypes that are observed following disruption of either BMP or GATA function, including gut defects, cardia bifida, defective differentiation of the ventricle and diminished expression of cardiac-specific genes, including Nkx2.5, raise the possibility that BMP signaling is necessary for proper function of one or more GATA factors.

3.3. Cardiac defects may be an indirect consequence of loss of BMP function in endodermal cells.

The observation that BMP-deficient embryos show severe defects in endodermal patterning, together with previous evidence that endodermally derived signals are essential for myocardial and endocardial differentiation and for proper morphogenesis of the heart (reviewed by (Fishman and Chien, 1997), make it tempting to speculate that the primary defect in these embryos may be of endodermal origin. As discussed above, studies of GATA-deficient animals demonstrate that embryonic endoderm plays a critical role in directing proper migration and/or fusion of the cardiac primordia into a single tube and thus it is feasible that the cardia bifida observed in the absence of BMP signals develops secondary to defects in the endoderm. Likewise, endoderm appears to play a role in directing endocardial development (Sugi and Markwald, 1996) and BMP-deficient animals show morphologically apparent defects in the endocardial layer of the heart. Signals from the endocardium to the myocardium are also important for the generation of ventricular trabeculae (reviewed by Fishman and Olson, 1997) and endocardial defects may underlie the lack of trabeculation in the ventricles of BMP-deficient animals. Further studies involving targeted inhibition of BMP signaling in specific germ layers will be necessary to clarify this issue.

MATERIALS AND METHODS

4.1. Embryo culture and manipulation

Xenopus eggs were collected and fertilized, embryos were injected with synthetic mRNAs and then cultured as previously described (Moon and Christian, 1989). Embryonic stages are according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Capped synthetic RNA encoding Smad 6 or tBR (Nakayama *et al.*, 1998a) was generated by in vitro transcription of linearized template cDNAs using a MegaScript kit (Ambion).

4.2. In situ hybridization and histological analysis

Embryos were processed for in situ hybridization as previously described (Nakayama *et al.*, 1998a). Following in situ hybridization, some embryos were dehydrated in methanol, embedded in paraffin and sections 10-12 μM thick were cut and counterstained with eosin (Christian and Moon, 1993). SYTOX green nuclear stain (Molecular Probes) was used at a 1:500 dilution in TE buffer for 15 minutes on sections that were cleared with Xylene and then gradually rehydrated.

4.3. RT-PCR analysis of gene expression

RNA was isolated and RT-PCR analysis was performed as previously described (Cui *et al.*, 1996). PCR conditions and cycle number was determined for each primer pair so that PCR products were examined during the exponential phase of amplification. GATA-4 (Jiang and Evans, 1996), and EF1α (Tsuneizumi *et al.*, 1997) primers have been reported, other primers are as follows: Nkx2.5 forward, 5'-TTGTAGGAGTGCAGCCCATT-3';

reverse, 5'-TTTCCCAGTACCGTCAGAGT-3'; Tnc forward, 5' GCTTGTCCCGATCT GAAC ; reverse, 5' GAGATTGGCCCGTAGATC. TnC, Nkx2.5, and EF1α were amplified for 25, 23 and 18 cycles respectively, under the following conditions: 95°C for 5 minutes prior to cycling at 94°C for 30 seconds; 59°C for 30 seconds; and 72°C for 45 seconds. GATA-4 was amplified for 32 cycles at an annealing temperature of 61°C.

Acknowledgements

We thank Paul Krieg and Tim Mohun for the Tnc and MLC plasmids, respectively, and members of the Christian laboratory for comments on the manuscript. This work was supported in part by grants from the NIH (HD01167) and the American Heart Association (9750143N) to J.L.C. M.J.W. is the recipient of a Tartar Trust Fellowship.

FIGURE LEGENDS

Figure 1. Disruption of BMP signaling in dorsal cells causes gut and heart defects. Low magnification ventral views (A-C) and high magnification lateral views (D-F) of control stage 46 tadpoles (A, D) or representative sibling embryos in which BMP signaling was inhibited in dorsal cells by injection of RNA encoding tBR or Smad6 (B-C; E-F). White arrows denote the gut, black arrows denote the heart which is small and thin walled in BMP-deficient embryos. Arrowhead in F indicates the edematous pericardial sac surrounding the heart of a BMP-deficient embryo.

Figure 2. Histological analysis of heart morphology in BMP-deficient embryos. Transverse sections through the hearts of control tadpoles (A) or sibling tadpoles in which BMP signaling was inhibited within dorsal cells (B-C). Black arrows indicate the ventricular wall, white arrows point to trabeculae.

Figure 3. Inhibition of BMP signaling causes cardia bifida and a decrease in cardiac muscle-specific gene expression. Ventral views of tailbud stage 32 control embryos (A, D) or sibling embryos in which BMP signaling was inhibited in dorsal cells by injection of RNA encoding tBR or Smad6 (B-C, E-F). White arrows indicate region(s) of expression of TnC (A-C) or MLC (D-F) visualized by whole mount in situ hybridization.

Figure 4. Dorsal inhibition of BMP signaling causes a decrease in the number of cells committed to differentiate as cardiomyocytes. Transverse sections through the heart

tubes of TnC stained control tailbud stage 32 embryos (A, B) or sibling embryos in which BMP signaling was inhibited in dorsal cells by injection of RNA encoding tBR or Smad6 (C-F). A, C and E are bright field views to show Tnc staining of the myocardium while B, D and F are corresponding fluorescent views of the same sections that were counterstained with SYTOX green to label nuclei of individual cells. White arrows indicate the pericardium, shorter white arrows the myocardium and white arrowheads the endocardium of the heart tube.

Figure 5. Downregulation of BMP signaling does not perturb the initial specification of cardiac fate but does lead to a decrease in myocardiocyte differentiation. RT-PCR analysis of Nkx2.5, GATA-4 and Tnc expression in neurula stage 19 and/or tailbud stage 32 control embryos relative to sibling, BMP-deficient embryos. Negative controls in which reverse transcriptase was omitted (RT-) were prepared in parallel for each reaction using equivalent amounts of control RNA. Amplified bands were visualized with a Molecular Dynamics phosphorimager and the Macintosh IP lab gel program was used to quantitate relative levels of expression of each gene (expressed as % of control), normalized relative to levels of EF1α.

Figure 1

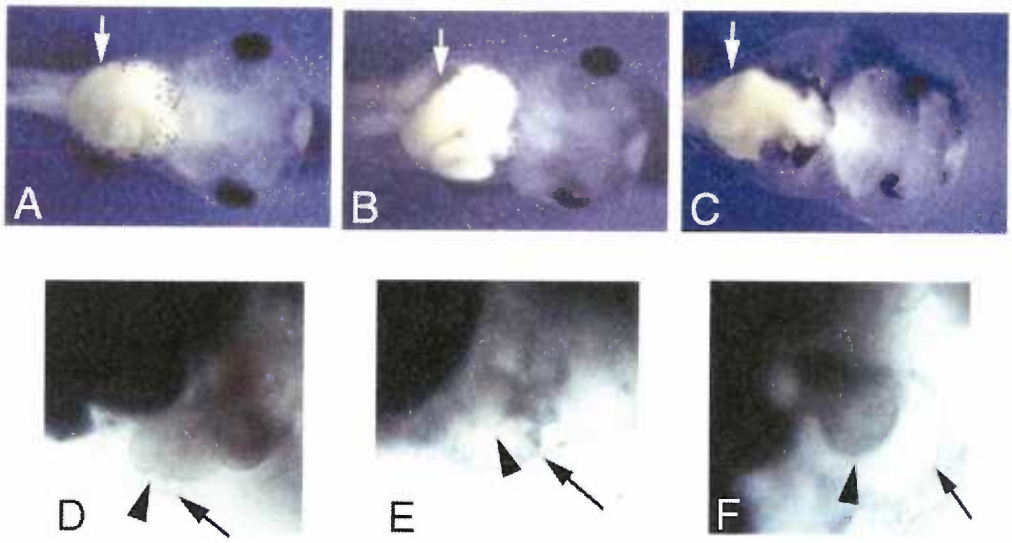


Figure 2

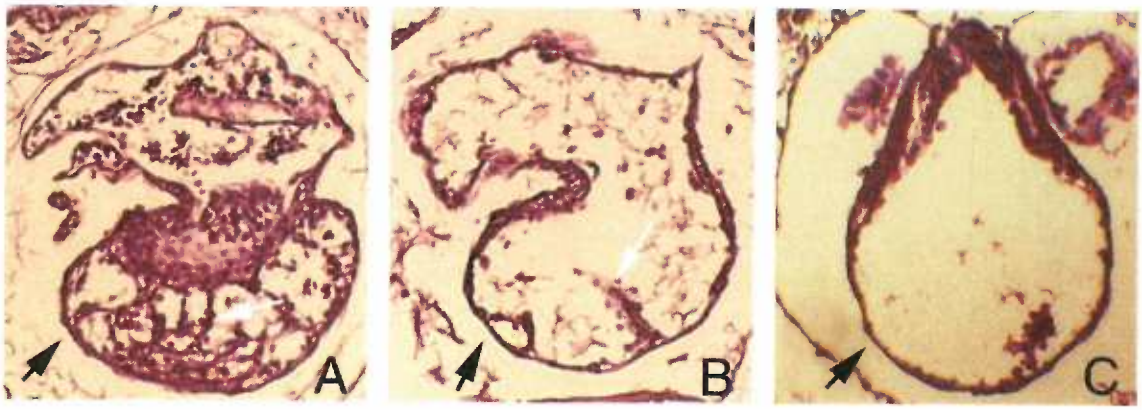


Figure 3

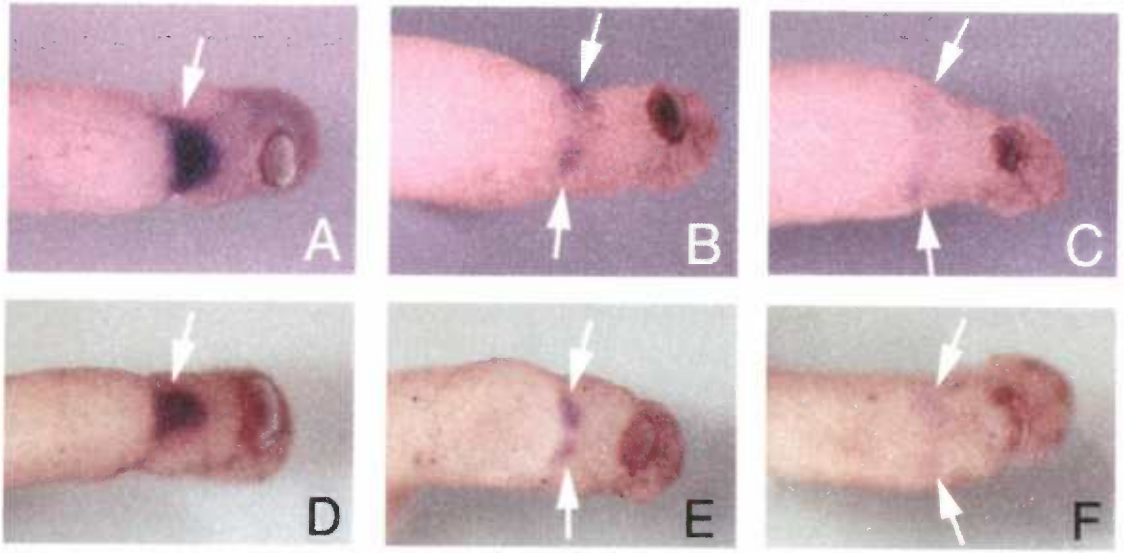


Figure 4

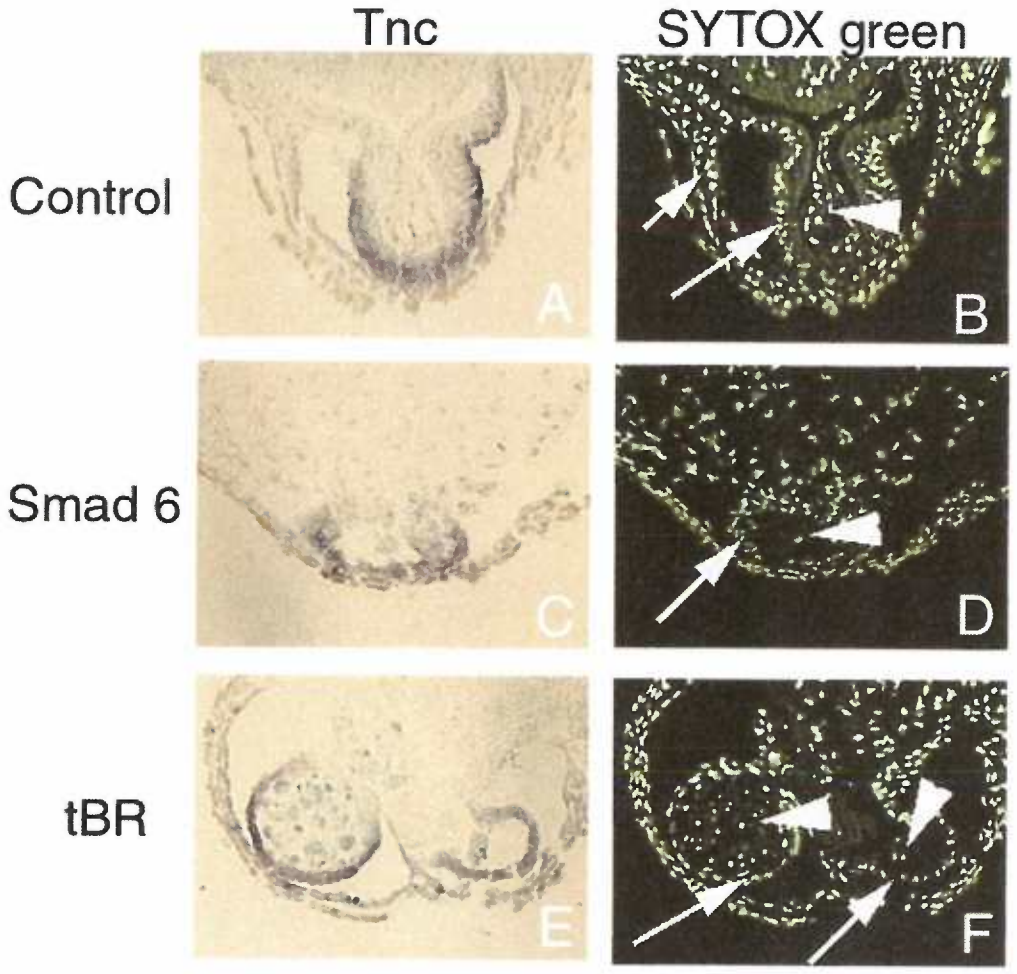
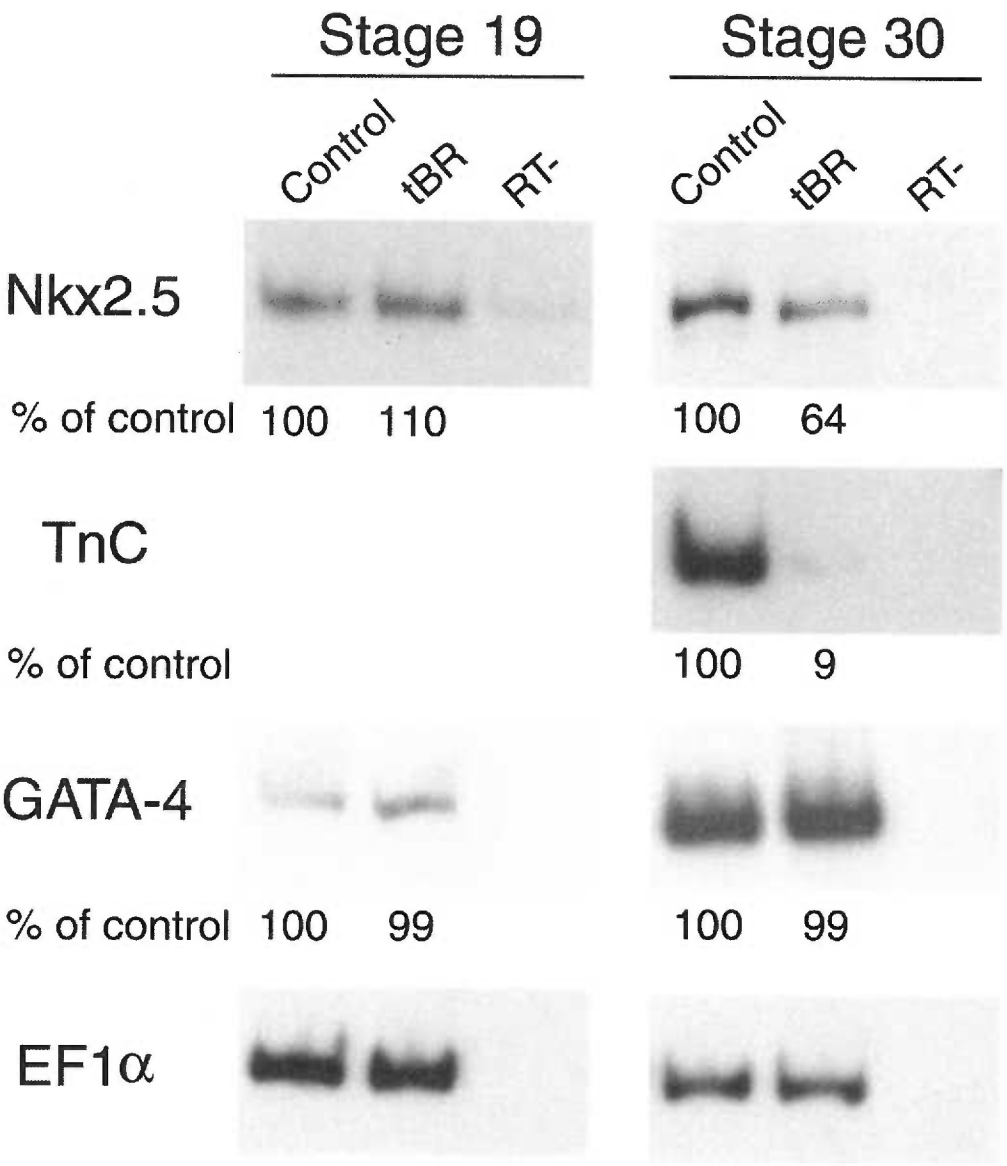


Figure 5


Stage 19
Stage 30
Control
tBR
RT-
Control
tBR
RT-
Nkx2.5
% of control 100 110 100 64
TnC
% of control 100 9
GATA-4
% of control 100 99 100 99
EF1α

Chapter 2

Crosstalk between BMP and CaM KIV: A novel signaling pathway in hematopoiesis

Melinda J. Walters, Gary A. Wayman, Richard H. Goodman, Thomas R. Soderling and Jan L. Christian

ABSTRACT

To investigate a potential non-cell autonomous role for bone morphogenetic proteins (BMPs) in hematopoiesis, we experimentally misregulated the signaling pathway in *Xenopus* embryos, by injecting intracellular inhibitors or a constitutively active receptor, specifically in cells that do not give rise to blood. Up or down-regulation of BMP resulted in anemia but due to distinct mechanisms: hyperactivation of BMP led to a shift in cell fate resulting in increased white blood cells at the expense of red blood cells, while inhibition of BMP caused an increase in red blood cell-specific apoptosis. The same phenotypes were seen when CaM KIV was blocked or hyperactivated, respectively. We found that CaM KIV is capable of antagonizing BMP signaling downstream of receptor activation, and that a balance between the two signaling pathways is required for proper blood development. Based on our findings, we propose a model for CaM KIV antagonism of BMP in which CaM KIV phosphorylation of CREB (or another CaM KIV substrate) allows CREB to bind to CBP preventing CBP from operating as a cofactor in Smad-induced transcription of hematopoietic genes.

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 (Evans, 1997a). The first wave of hematopoiesis is primitive hematopoiesis, which gives rise primarily to erythrocytes (red blood cells). In *Xenopus*, primitive hematopoietic tissue has been shown to be derived from the ventral mesoderm of gastrula stage embryos by explant and axis disruption experiments (Kau and Turpen, 1983; Maeno *et al.*, 1985). Hematopoietic precursors begin to differentiate in the ventral blood island (VBI) (the equivalent of the mammalian yolk sac) by the tailbud stage. The second wave of hematopoiesis produces definitive hematopoietic cells, which are derived from both the VBI and the mesoderm of the dorsal lateral plate (DLP). This wave yields adult erythroid cells as well as progenitors of the lymphoid (lymphocyte) and myeloid (granulocyte, monocyte and platelet) lineages (Kau and Turpen, 1983; Maeno *et al.*, 1985; Turpen *et al.*, 1997; Weber *et al.*, 1991). HSCs derived from the DLP will later migrate to seed other hematopoietic organs such as the liver and spleen (Chen, 1995).

The hematopoietic program is initiated by ventral patterning of the mesoderm, a process that requires bone morphogenetic proteins (BMPs) (Huber and Zon, 1998). BMP-4 can induce expression of the hematopoietic-specific transcription factors GATA-1, GATA-2, and SCL in animal pole explants from *Xenopus* (Maeno *et al.*, 1996; Zhang and Evans, 1996) and induces hematopoietic activity in embryoid bodies from mouse embryonic stem cells (Johansson and Wiles, 1995). BMPs may subsequently regulate the

proliferation and differentiation of primitive hematopoietic cells (Bhatia *et al.*, 1999). In addition, mice lacking the BMP signal transducer, Smad5, have disrupted vasculature and anemia (Chang *et al.*, 1999; Yang *et al.*, 1999). Clearly, BMP signaling is important in hematopoiesis, but the exact nature of its role has not been determined.

BMPs are members of the TGF-β superfamily of signaling molecules. BMP family members initiate signaling by binding to a heterocomplex of type I and type II transmembrane serine-threonine kinases to form a high affinity receptor (Yamashita *et al.*, 1996). Following ligand binding, type II receptors transphosphorylate the type I receptors which then propagate the signal by phosphorylating one of the BMP pathway-specific signal-transducing Smads, Smad1, Smad5, or Smad8 (reviewed by Christian and Nakayama, 1999; Massague, 1998; Piek *et al.*, 1999). The common Smad, Smad4, then heterodimerizes with phosphorylated signal-transducing Smads and this complex translocates into the nucleus and activates transcription of target genes. A class of inhibitory Smads has also been identified that includes Smad6 and Smad7 (reviewed by Christian and Nakayama, 1999). These Smads lack the C-terminal phosphorylation sequence and function to block BMP signaling downstream of ligand binding.

Several identified extrinsic factors are known to be involved in embryonic hematopoiesis, including BMPs, which are involved in specification of ventral fate within the mesoderm and regulation of hematopoietic transcription factor expression. Unidentified extrinsic signals provided by endodermal (Belaoussoff *et al.*, 1998; Yoder *et al.*, 1994) and endothelial cells (Fennie *et al.*, 1995; Ohneda *et al.*, 1998; Yoder *et al.*, 1994) can also influence the proliferation, survival and/or lineage fate of HSCs. Recently, we provided evidence that calcium/calmodulin-dependent protein kinase IV,

CaM KIV, has a critical role in the generation of extrinsic signals that regulate several different steps in erythroid development (Wayman et al., submitted).

CaM KIV is a member of a family of serine/threonine protein kinases dependent on the Ca^{2+} binding protein, calmodulin, that are activated as a part of a recently characterized CaM kinase cascade (Soderling, 1996). These kinases share certain structural motifs such as a conserved catalytic domain positioned adjacent to overlapping autoinhibitory and CaM binding domains. When Ca^{2+} and calmodulin (CaM) bind to CaM KIV it relieves autoinhibition and allows a critical threonine residue, located within the activation loop, to be phosphorylated by a CaM-dependent kinase kinase (CaM KK). Phosphorylation of this Thr196 by CaM KK results in a 10-20 fold activation of CaM KIV.

The substrate specificity of CaM KIV is not well defined, but it has been shown to phosphorylate and regulate a number of transcription factors including activating transcriptional factor-1 (ATF-1), serum response factor (SRF), MEF2, cAMP-responsive element-modulator τ (CREMτ), and cAMP-responsive element-binding protein (CREB) (Blaeser *et al.*, 2000; Enslen *et al.*, 1995; Matthews *et al.*, 1994; Sun, 1996). Consistent with a role in regulating transcription, CaM KIV and CaM KK show strong nuclear localization, but are also present in the cytosol (Sakagami, 1999).

CREB is essential for many cellular processes, including proliferation and differentiation, and is activated by a diverse array of stimuli. Phosphorylation of CREB at a specific residue, serine 133, in the kinase inducible domain (KID) is required for CREB-induced gene transcription (reviewed by Shaywitz and Greenberg, 1999). CaM KIV is one of the protein kinases that is responsible for phosphorylation at this site.

Once CREB is phosphorylated, it associates with CREB-binding protein (CBP), and this interaction appears to be important for CREB activity. Dimers of phosphorylated CREB may bind to DNA at cAMP-responsive elements (CREs) and then recruit the coactivator, CBP, which is suggested to function to link CREB to the basal transcription machinery (reviewed by Shaywitz and Greenberg, 1999).

CBP has been implicated in several aspects of embryonic development including hematopoiesis. Mice lacking CBP have severe anemia that is suggested to be due to defects in the vascular endothelial cells that comprise the hematopoietic microenvironment (Oike *et al.*, 1999). CBP has also been shown to be a co-activator of many transcription factors other than CREB including the erythroid-specific GATA factor GATA-1 (Blobel *et al.*, 1998; Takahashi *et al.*, 2000). Interaction of CBP with Smad2 and Smad4, components of the TGF-β signaling pathway, has been shown to be required for endothelial cell development (Topper *et al.*, 1998). CBP also acts as a cofactor for BMP-specific Smad-induced transcription. In *Drosophila*, CBP loss-of-function mutants have defects identical to those seen in decapentaplegic (the BMP orthologue) mutants (Waltzer and Bienz, 1999). In *Xenopus* animal cap assays, CBP function was shown to be required for BMP signaling (Kato *et al.*, 1999). CBP was also demonstrated to link Smad1 to the transcription factor STAT3 leading to synergistic activation of target genes (Nakashima *et al.*, 1999).

In this study, we demonstrate a non-cell autonomous role for BMP signaling in hematopoiesis by experimentally misregulating the signaling pathway specifically in cells that do not give rise to blood. Up or down-regulation of BMP resulted in anemia but due to distinct mechanisms: hyperactivation of BMP caused a shift in cell fate towards the

myeloid or white blood cell lineage, while inhibition of BMP caused an increase in erythrocyte-specific apoptosis. The same phenotypes were seen when CaM KIV was blocked or hyperactivated, respectively. We found that CaM KIV is capable of antagonizing BMP signaling downstream of receptor activation, and that a balance between the two signaling pathways is required for proper blood development. Our results suggest that CaM KIV phosphorylation of CREB (or another CaM KIV substrate) allows CREB to bind to CBP preventing CBP from operating as a cofactor in Smad-induced transcription of hematopoietic genes.

MATERIALS AND METHODS

Embryo culture and manipulation

Xenopus eggs were obtained, embryos injected with synthetic mRNAs and cultured as described (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).

ß-galactosidase staining and in situ hybridization

Embryos were stained for ß-galactosidase activity using Red-gal as a substrate, and processed for in situ hybridization as described (Nakayama *et al.*, 1998b).

Northern blot and RT-PCR analysis of gene expression

RNA was isolated and reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed as described (Cui *et al.*, 1996) using the following PCR conditions: 95° C for 5 minutes, followed by 30 cycles at 94° C for 30 seconds; 55° C for 30 seconds; and 72° C for 30 seconds. Cycle number was determined empirically for each primer pair so that PCR products were examined during the exponential phase of amplification. EF1α (Tsuneizumi *et al.*, 1997) and GATA-1 (Zon *et al.*, 1991) primers have been published. Northern blot hybridization was performed as described (Christian and Moon, 1993).

Collection and analysis of peripheral blood samples

Tails were severed from tadpole stage embryos and larval hematopoietic cells were collected into amphibian PBS containing 0.5 % BSA and 10 IU/ml of heparin. Cells were concentrated onto slides using a cytocentrifuge. 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 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

Inhibition of BMP signaling causes defects in blood development.

In our previous investigation into the role of BMPs in embryonic development, we discovered that injection of RNA encoding the intracellular BMP inhibitors Smad 6 or tBR into cells on the dorsal side of *Xenopus* embryos caused defects in gut coiling and cardiac development (Walters et al, submitted). In addition, embryos in which BMP signaling was inhibited dorsally appeared to have pale hearts and few circulating red blood cells (RBCs) (data not shown). Blockade of BMP signaling in ventral blastomeres causes these cells to adopt a more dorsal fate (Graff, 1997) and inhibits blood development (Lane and Smith, 1999). In contrast, dorsal disruption of BMP signaling does not change mesodermal cell fate and is reported not to inhibit blood development (Kumano *et al.*, 1999). To examine potential roles for BMP signaling in blood development, 400 pg of RNA encoding tBR or Smad6 was injected into two dorsal or ventral blastomeres of four-cell embryos and expression of the erythrocyte marker, α-T globin, was examined at the tailbud stage. In control embryos, globin was expressed throughout the ventral blood island (VBI) as analyzed by whole-mount in situ hybridization (Fig. 1A). Globin expression was dramatically reduced throughout the VBI when BMP signaling was blocked on either the ventral or dorsal side by misexpression of Smad 6 or tBR (Fig. 1A). Northern blot analysis confirmed that inhibition of BMP signaling in either dorsal or ventral cells of *Xenopus* embryos led to a significant decrease in globin transcripts (Fig. 1B).

BMP signaling regulates hematopoiesis non-cell autonomously.

Recent fate mapping studies have shown that both dorsal and ventral blastomeres of *Xenopus* embryos contribute to the VBI (Lane and Smith, 1999; Mills *et al.*, 1999; Tracey *et al.*, 1998), and this might explain why blocking BMP signaling in dorsal cells disrupts globin expression in a ventral region. However, our findings that inhibition of BMP signaling in dorsal blastomeres alone almost completely represses globin expression in some embryos suggests a possible non-cell autonomous role for BMP signaling in blood development. To test this hypothesis, we targeted injections of RNA encoding Smad 6 or tBR (400 pg) to dorsal midline blastomeres at the extreme animal pole of 32-cell stage embryos (A1 blastomeres by the nomenclature of (Nakamura and Yamada, 1971), D1.1.1 by the nomenclature of (Mills *et al.*, 1999). These blastomeres give rise mostly to brain and do not contribute to erythroid cells in the VBI (Mills *et al.*, 1999). RNA encoding ß-galactosidase (100 pg) was also injected into these blastomeres as a lineage tracer, either alone or with the BMP inhibitors, as illustrated above Fig.ure 2. Half of the injected embryos in each group were stained for ß-galactosidase 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 expression by Northern blot hybridization. ß-galactosidase activity was seen in the most anterior structures of the injected embryos as indicated by red staining (Fig. 2A, white arrows). Injections of tBR caused a severe decrease in globin expression in many embryos as determined by in situ hybridization (Fig. 2A) and Northern blot analysis (Fig. 2B). Surprisingly, when BMP signaling was upregulated in A1 blastomeres, by injection of RNA (600 pg) encoding a constitutively active form of a type I BMP receptor (CA-

ALK3), which phosphorylates downstream Smads independent of ligand binding (Akiyama *et al.*, 1997; Hoodless *et al.*, 1996), a significant reduction in globin message was again observed (Fig. 2A and B). These results demonstrate that proper regulation of BMP signaling is essential for expression of globin in the VBI, and that BMP function is required non-cell autonomously, in neighboring tissues, to allow for normal RBC development.

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 was misregulated. To investigate this possibility, we analyzed expression of GATA-1, which is an early marker of blood development (Kelley *et al.*, 1994). Since BMP function is necessary and sufficient for cells to adopt a ventral fate (reviewed by (Harland, 1994), and since BMPs can function non-cell autonomously to regulate RBC development (Fig. 2), we targeted RNA encoding tBR (400 pg) to the dorsal side of embryos and RNA encoding CA-ALK3 (600 pg) to the ventral side of 4-cell embryos in this and all subsequent experiments. In this way we were able to misregulate BMP signaling, and thus interfere with RBC development, without altering early dorsoventral patterning. Neither inhibition of BMP signaling in dorsal cells, nor hyperactivation of BMP signaling in ventral cells caused a decrease in GATA-1 expression at neurula stage 19 (Fig. 3). At tailbud stage 30, however, GATA-1 expression was reduced in sibling embryos in which BMP signaling was perturbed (Fig. 3). Expression of globin was also repressed in sibling tailbud stage embryos from this same experiment, as analyzed by whole mount in situ

hybridization (data not shown). We conclude that misregulation of BMP signaling does not disrupt the initial specification of hematopoietic fate.

Activation and inhibition of BMP signaling cause distinct hematopoietic defects.

To further investigate how either activation or inhibition of the BMP signaling pathway could result in repression of globin expression, we looked for phenotypic differences in RBCs collected from sibling stage 43 tadpoles in which BMP signaling was either upregulated or downregulated. 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 easily 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, arrow) while WBCs and are larger, irregularly shaped and have lightly stained cytoplasm (Fig. 4A, arrowhead). Blood from mock-injected or uninjected control embryos had an average of 75 blood cells per visual field (see methods), about 10% of which were WBCs (Fig. 4A and B). Tadpoles in which BMP signaling was enhanced by injection of CA-ALK3 showed an increase in the number of WBCs and a concomitant decrease in the number of RBCs (Fig. 4A and B) such that the white to red cell ratio was approximately 7-fold higher than in controls (Fig. 4C). These results suggest that BMPs can enhance commitment of HSCs to the myeloid (WBC), as opposed to erythroid (RBC) fate.

Embryos in which BMP signaling was disrupted 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 were irregularly shaped and more lightly stained than those from sibling controls (Fig. 4A). To determine whether the abnormal morphology and decreased number of RBCs was due to an increase in programmed cell death, we used the TUNEL assay to quantitate the number of apoptotic cells in blood samples from BMP deficient embryos relative to controls. As shown in Figure 4D and E, inhibition of BMP signaling following misexpression of either Smad 6 or tBR caused a significant increase in the fraction of blood cells that were apoptotic. We cannot definitively identify the apoptotic cells as RBCs since the TUNEL assay is not compatible with counterstains. The observation that inhibition of BMP signaling resulted in a significant decrease in RBCs, but not WBCs, however, suggests that the apoptotic cells are predominantly or solely RBCs. We conclude that the decrease in globin expression observed when the BMP pathway is either blocked or hyperactivated is caused by two distinct mechanisms: upregulation of BMP signaling causes commitment of hematopoietic precursors to myeloid differentiation at the expense of erythroid differentiation, whereas inhibition of BMP signaling leads to increased apoptosis of erythroid precursors.

Misregulation of BMP and CaM KIV causes reciprocal defects in heart and blood development.

Misregulation of CaM KIV activity in *Xenopus* embryos causes defects in the gut, heart and blood that are indistinguishable from those observed following misregulation of

BMP activity, suggesting that there may be crosstalk between these two pathways. Specifically, inhibition of BMP signaling (Walters et al., submitted) or upregulation of CaM KIV activity (Wayman, manuscript in preparation) in dorsal cells disrupts normal coiling of the gut and causes defects in heart development, including cardia bifida, decreased expression of cardiac muscle-specific genes and formation of a thin, non-trabeculated ventricle. Furthermore, inhibition of CaM KIV leads to an increase in WBCs at the expense of RBCs, while constitutive activation of CaM KIV causes an increase in apoptosis, and consequent decrease in the number of differentiated RBCs (Wayman et al., submitted). These blood defects are identical to those observed following upregulation or inhibition of BMP signaling, respectively (Fig. 4, this manuscript). Finally, both BMPs and CaM KIV function non-cell autonomously to regulate blood development (Fig. 2, this manuscript and Wayman et al., submitted). Importantly, whereas inhibition and constitutive activation of BMP signaling leads to duplication or loss of dorsal structures, respectively (Maeno *et al.*, 1994; Suzuki *et al.*, 1994), misregulation of CaM KIV activity does not perturb dorsoventral patterning (Wayman et al., submitted). Thus, any crosstalk between BMP and CaM KIV signaling cascades must be restricted to specific developmental contexts such as hematopoiesis and cardiac development.

The observation that activation of CaM KIV phenocopies the effect of blocking BMP signaling during hematopoiesis, and vice versa, suggests that these molecules operate in the same or parallel signaling cascades and that activation of one pathway can negatively modulate the other. To begin to ask whether one of these signaling cascades is dominant and/or functions downstream of the other, we simultaneously activated both

pathways by co-injecting RNA encoding CA-ALK3 (600 pg) together with constitutively active forms of CaM KIV (CaM KIVc) and its upstream kinase, CaM KK (CaM KKc) (100 pg each), near the dorsal midline 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 CaM KIVc (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 and B). This supports the hypothesis that CaM KIV functions to block transduction of BMP signals downstream of receptor activation. Conversely, whereas inhibition of CaM KIV, achieved by injecting RNA (1 ng) encoding a dominant negative form of CaM KIV (Dn CaM KIV, (Ahn *et al.*, 1999; Gringhuis *et al.*, 1997), led to an increase in WBCs at the expense of RBCs, inhibition of the BMP pathway alone, or simultaneous inhibition of the BMP and CaM KIV pathways led to a decrease in RBCs without affecting the number of WBCs (Fig. 5C and D). Thus, inhibition of endogenous CaM KIV can not cause an overcommitment of HSCs to myeloid differentiation in the absence of endogenous BMP signals. Collectively, these results are consistent with the hypothesis that activation of CaM KIV is required to negatively modulate BMP signaling in order for HSCs to commit to an erythroid pathway of differentiation. Furthermore, an active BMP signaling pathway is required for survival of RBCs, and CaM KIV can block this pathway downstream of receptor activation.

Constitutively active CREB phenocopies active CaM KIV or dominant negative BMP.

CaM KIV could potentially inhibit BMP signals at the transcriptional level, through activation of the transcription factor CREB, and recruitment of the transcriptional co-activator CBP. CaM KIV is predominantly nuclear and CREB is one of its best-characterized substrates (Soderling, 1999). Once phosphorylated, CREB binds to CBP and this binding is sufficient to activate transcription of CREB-responsive genes (Cardinaux *et al.*, 2000; Shaywitz, 1999). Since analysis of CBP-deficient mice has demonstrated a requirement for CBP in hematopoiesis (Oike *et al.*, 1999), and since interaction of Smads with CBP is required for transcription of many TGF-ß and BMP target genes (Feng *et al.*, 1998; Janknecht *et al.*, 1998; Nakashima *et al.*, 1999; Nishihara *et al.*, 1998; Topper *et al.*, 1998), it seemed feasible that CaM KIV might inhibit BMP signaling by CREB-mediated sequestration of CBP.

To begin to investigate the hypothesis that activation of CREB can inhibit BMP function during hematopoiesis, we injected RNA encoding a constitutively active form of CREB ($CREB_{DIEDML}$) into *Xenopus* embryos and asked whether this generated the same blood phenotype as does blockade of BMP signaling. $CREB_{DIEDML}$ carries a six amino acid substitution in the activation domain that enables it to bind CBP and activate transcription of cAMP response element (CRE)-containing reporter genes in a phosphorylation-independent manner (Cardinaux *et al.*, 2000). Injection of 1 ng of RNA encoding $CREB_{DIEDML}$ near the VMZ of four-cell embryos greatly decreased expression of globin in 70% of injected embryos (n=42) as analyzed by in situ hybridization (Fig. 6A). The loss of globin expression is caused by a specific decrease in the number of RBCs, without a concomitant increase in WBCs (Fig. 6B). This phenotype is identical to that observed following inhibition of BMP signaling, or constitutive activation of CaM

KIV (Fig. 5), 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 assayed the activity of a constitutively active form of CREB in which the DNA binding domain has been replaced with the GAL4 DNA binding domain (GAL4-$CREB_{DIEDML}$). Injection of 1 ng of RNA encoding GAL4-$CREB_{DIEDML}$ near the VMZ of four-cell embryos inhibited globin synthesis (Fig. 6A) and caused a specific reduction in RBC number (Fig. 6B). In 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 hematopoiesis.

The observation that GAL4-$CREB_{DIEDML}$ 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 upregulation of BMP signaling or inhibition of CaM KIV activity. To test this, we upregulated BMP signaling in ventral cells either directly, by injecting RNA encoding CA-ALK3 (600 pg), or indirectly by injecting RNA encoding DnCaM KIV (1 ng), in the presence or absence of RNA encoding GAL4-$CREB_{DIEDML}$ (1 ng). Misexpression of DnCaM KIV (Fig. 7A and B) or CA-ALK3 (Fig. 7C and D) alone led to a decrease in RBCs that was accompanied by an increase in WBCs, such that the WBC:RBC ratio was increased 7-fold. Co-expression of GAL4-$CREB_{DIEDML}$ 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-CREB$_{DIEDML}$ 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. To directly test this hypothesis we asked whether addition of excess CBP could phenocopy inhibition of endogenous CaM KIV and/or block hematopoietic defects caused by constitutive activation of CaM KIV. Injection of RNA encoding CBP (3 ng) near the animal pole of ventral blastomeres of four cell embryos did not cause gross morphological defects despite the fact that wild type CBP protein was present at levels approximately 10-fold greater than endogenous at least until stage 14 (data not shown). Analysis of peripheral blood smears from CBP-injected embryos revealed a decreased number of RBCs and a concomitant increase in WBC number, leading to a 3-fold elevation in the WBC:RBC ratio (Fig. 7E and F). Furthermore, whereas aberrant activation of CaM KIV causes a specific decrease in RBCs without an increase in WBCs, activation of CaM KIV in the presence of excess CBP causes an increase in WBCs at the expense of RBCs (Fig. 7E and F). These results support the hypothesis that CBP is present in limiting amounts, and that endogenous CaM KIV activates a substrate that sequesters CBP away from transcriptional components of the BMP signaling cascade that are essential for hematopoiesis, but not for other BMP-regulated developmental processes such as dorsal-ventral patterning.

DISCUSSION

In this study, we have provided evidence that BMP signaling is required during hematopoiesis to promote the survival of maturing erythrocytes. In addition, the excess WBCs produced at the expense of RBCs when BMP signaling is hyperactivated suggests that BMP signaling must be downregulated to initiate erythroid, as opposed to myeloid, differentiation during primitive hematopoiesis. Our findings support a hypothesis in which BMP signaling is regulated during hematopoiesis by CaM KIV, and CaM KIV exerts its inhibitory effect by sequestering CBP away from transcriptional components of the BMP pathway by activation of a substrate, possibly CREB.

Regulation of BMP signaling is important for both lineage specification and erythrocyte maturation.

In *Xenopus*, the first wave of hematopoiesis during embryogenesis is primitive hematopoiesis, which occurs in the VBI and produces primarily erythrocytes. The pressures to produce RBCs over WBCs probably include the small size of the embryo at this stage, the rapid growth rate, and the high oxygen demands. Transplantation experiments have revealed the pluripotential nature of HSCs and the role of their environment in lineage determination (Turpen *et al.*, 1997). The specific factors in the environment that are required to restrict the differentiation of primitive HSCs to an erythroid fate have not yet been identified. We recently provided evidence that CaM KIV is required to generate these signals (Wayman et al., submitted) based on the overproduction of WBCs at the expense of RBCs when CaM KIV activity was blocked in

Xenopus embryos (Wayman et al., submitted and current study). Our current results suggest that endogenous CaM KIV functions to antagonize BMP signaling in order to promote the differentiation of RBCs over WBCs. If CaM KIV is inhibited or BMP hyperactivated, excess WBCs are produced. When both signaling pathways are hyperactivated, the number of WBCs is equivalent to controls, but RBC number is still reduced as seen when CaM KIV alone is hyperactivated or when BMP is inhibited directly. The BMP-deficient phenotype observed when both pathways are blocked can be explained by the hypothesis that CaM KIV functions downstream of receptor activation to antagonize BMP signaling.

BMP signaling has a role in the extrinsic regulation of hematopoiesis.

Many factors have been shown to intrinsically regulate all steps in hematopoiesis, from proliferation and differentiation to maturation. The basic helix-loop-helix gene, SCL (stem cell leukemia, (Mead *et al.*, 1998), specifies HSCs from mesoderm after dorsal-ventral patterning. In addition, transcription factors belonging to the family of GATA-binding proteins are involved in the differentiation of blood cells (Kelley *et al.*, 1994). GATA-2 is important for the maintenance and proliferation of pluripotent progenitor cells, and mice deficient for GATA-2 die with severe anemia (Tsai *et al.*, 1994). Expression of GATA-2 must be downregulated for differentiation of progenitors, however, and enforced expression blocks normal hematopoiesis (Persons *et al.*, 1999). GATA-1 expression increases as GATA-2 decreases (reviewed by Orkin, 1992) and has been shown to be required for the terminal differentiation of erythrocytes.

Extrinsic factors produced by the endodermal and endothelial microenvironment are also required for hematopoiesis. Explant experiments in *Xenopus* have demonstrated that in vivo interactions between the ventral mesoderm and adjacent tissues are required to establish erythroid precursors (Maeno *et al.*, 1985). Other studies have shown that endothelial cells mediate the expansion of erythroid and myeloid precursor cells (Fennie *et al.*, 1995; Ohneda *et al.*, 1998). Although there is significant evidence that signals from the microenvironment are required for normal blood development, the exact nature of these extrinsic signals remains unclear. In zebrafish, a protein encoded by the *cloche* gene has been shown to function non-cell autonomously to promote the differentiation of erythrocytes (Parker and Stainier, 1999). Our recent studies demonstrate a role for CaM KIV in the generation of extrinsic signals that regulate several different steps in erythroid development (Wayman et al., submitted).

A model for BMP function in hematopoiesis was recently proposed in which BMP is secreted from ectodermal cells and is required to signal directly to mesodermal cells that will become blood (Kumano *et al.*, 1999). Injection of the intracellular BMP inhibitors into the ventral animal pole cells (A4) at the 32-cell stage resulted in a decrease in globin expression (Kumano *et al.*, 1999). They conclude that the VBI is induced by direct contact with ectodermal cells during late gastrulation. In this study, we observed defects in hematopoiesis when we misregulated BMP signaling in the dorsal animal pole cells (A1) of 32-cell stage embryos. These blastomeres do not give rise to erythrocytes (Mills *et al.*, 1999), nor do they ever come into contact with hematopoietic cells directly. We interpret these results as indication that BMP signaling is acting non-cell autonomously in hematopoiesis. All the blastomeres at this stage give rise to vascular

endothelial cells (Mills *et al.*, 1999), therefore, it is conceivable that lack of functional BMP signaling in the surrounding endothelial cells alters the expression or function of some as yet unknown factor(s) required for hematopoiesis.

The constitutively active receptor used in these experiments, CA-ALK3, was identified as a type I receptor for BMP-2, -4, and –7, and both the dominant negative receptor, tBR, and the antagonistic Smad, Smad6, block signaling by BMP-2 and –4. Therefore, it is likely that the phenotypes we observed were due to misregulation of BMP-2 or -4. The expression pattern of BMP-4 is consistent with this interpretation as well. Initially, BMP-4 is distributed evenly across the dorsal-ventral axis, by late gastrula it is restricted ventrally, and by the tailbud to tadpole stages it is increasingly restricted to specific regions including the heart, the eye, and the dorsal-most regions of the brain. Consistent with our hypothesis of non-cell autonomous BMP function in blood development, the progeny of the dorsal animal pole cells (A1) that were targeted in our experiment include the dorsal-most regions of the brain. In addition, CaM KIV is ubiquitously expressed (Wayman et al., submitted) and therefore present in the progeny of the A1 blastomeres as well.

CaM KIV modulates BMP signaling via CREB activation and CBP binding.

CaM KIV is one of several protein kinases capable of phosphorylating the transcriptional coactivator CREB. Phosphorylation of CREB enables it to bind to CBP, and CBP has been shown to be important for signaling by some Smad proteins. CaM KIV can also directly phosphorylate CBP (Chawla *et al.*, 1998), but the BMP-deficient phenotype observed when constitutively active-CREB is injected suggests it is CaM

KIV's phosphorylation of CREB, or another substrate capable of sequestering CBP, and not direct activation of CBP that is responsible for downregulating BMPs.

The phosphorylation-independent mutant version of CREB ($CREB_{DIEDML}$) phenocopied constitutively active CaM KIV causing a decrease in RBCs without affecting WBC number. A constitutively active CREB that also lacked a DNA binding domain (GAL4-$CREB_{DIEDML}$) also altered hematopoiesis in a BMP-deficient or hyperactive CaM KIV manner, while a wild type CREB only lacking its DNA-binding domain (GAL4-CREB) had no effect on blood development. This suggests that activated CREB, by CaM KIV phosphorylation or constitutively active mutations, inhibits BMP by binding to CBP and not by transcriptional activation of an antagonistic factor. Injection of wild type CBP further supports this conclusion as it induces an overactive BMP phenotype, with excess WBCs at the expense of RBCs, even in the presence of the constitutively active CaM KIV.

CBP is a required component common to coactivator complexes of diverse transcription factors, including the hematopoiesis-specific transcription factor, GATA-1 (reviewed by Blobel, 2000). Mice deficient for CBP have severe hematopoietic defects, which may be due to a defect in the endothelial microenvironment suggesting a non-cell autonomous role for CBP in hematopoiesis (Oike *et al.*, 1999). Normal levels of expression of CBP are specifically required, as demonstrated by the defects in hematopoiesis seen in mice in which a single CBP allele had been inactivated (Kung *et al.*, 2000). CBP appears to be in limiting amounts within the cell, which allows competition for CBP to be a method of gene regulation (Janknecht and Hunter, 1996; Kamei *et al.*, 1996).

Our findings suggest a model in which competition for CBP is the means of CaM KIV inhibition of BMP signaling. An active BMP signal results in the translocation of a phosphorylated Smad complex into the nucleus. In the absence of CaM KIV signaling, this complex can initiate transcription of genes required for WBC fate or RBC survival. In our model, CBP links the active Smad complex to transcription factor(s) whose function is required for normal erythropoiesis (Figure 8A). When CaM KIV is activated by its upstream kinase, it phosphorylates downstream substrates including CREB. Once phosphorylated, CREB can bind to CBP. In our model, CREB binding to CBP specifically precludes the linking of Smads to the hematopoiesis-specific transcription factor(s) and results in higher levels of RBC apoptosis (Figure 8B).

It is also possible that following activation by CaM KIV, CREB binds to the limiting amount of CBP and displaces Smad proteins. However, hyperactivation of CaM KIV does not duplicate all the phenotypes observed when BMP signaling is blocked. For example, BMP signaling inhibition on the ventral side of *Xenopus* embryos has been shown to dorsalize the embryo as indicated by the formation of a secondary dorsal axis (Graff *et al.*, 1994; Nakayama *et al.*, 1998b), but ventral hyperactivation of CaM KIV signaling does not duplicate this phenotype. If CaM KIV blocked all BMP signaling we would expect to see these dramatic changes in the dorsal-ventral axis. Therefore, we favor a model in which CBP displaces another component of the transcriptional complex that functions only in the context of hematopoietic and cardiac development. Binding of certain transcription factors to CBP, such as those involved in inducing ventralizing genes, may not be affected by the binding of the CaM KIV substrate to CBP.

In conclusion, data we have presented here suggest novel crosstalk between BMP and the CaM KIV cascade during hematopoiesis. CaM KIV appears to antagonize BMP signaling, and our evidence supports the hypothesis that it is doing so by activating a CBP-binding substrate and preventing CBP from acting as a coactivator of BMP-specific hematopoietic target genes.

FIGURE LEGENDS

Figure 1. Inhibition of BMP signaling in *Xenopus* embryos inhibits erythropoiesis. (A) RNAs encoding the dominant negative BMP receptor (tBR) or the inhibitory Smad (Smad 6) were injected near the ventral (VMZ) or dorsal marginal zone (DMZ) of 4-cell embryos. Ventral views of tailbud stage (st. 28) embryos that have been stained for globin RNA (purple) by in situ hybridization are shown. (B) Northern blot analysis of globin expression in tailbud (st. 28) stage embryos that have been injected at the 4-cell stage with RNAs encoding tBR or Smad 6 as indicated above each lane. Ethidium bromide staining of RNA gel prior to transfer is shown to demonstrate that equivalent amounts of RNA are present in each lane.

Figure 2. BMP signaling plays a non-cell autonomous role in regulating hematopoiesis. (A) Two dorsal midline animal pole blastomeres of 32-cell embryos (called A1) were injected with β-galactosidase (β-gal) RNA alone or together with dominant negative (tBR) or constitutively active (CA-ALK3) BMP receptor RNAs as illustrated above the figure. Embryos were cultured to the tailbud stage (st. 28) and stained for β-galactosidase (red stain, white arrows) activity and then for globin RNA (purple stain, black arrows) by in situ hybridization. A lateral (top row) and ventral (bottom row) view of each embryo is shown. (B) Northern blot analysis of globin expression in tailbud (st. 28) stage embryos in which BMP signaling was misregulated in dorsal midline, animal pole blastomeres (A1) of 32-cell embryos. Ethidium bromide staining of RNA gel prior to transfer is shown as a loading control.

Figure 3. Misregulation of BMP signaling does not perturb specification of hematopoietic fate. RT-PCR analysis of GATA-1 expression in neurula (st. 19) and tailbud (st. 30) stage embryos that had been injected near the VMZ (CA-ALK3) or DMZ (tBR) at the four cell stage as indicated above each lane. Analysis of EF1α expression was used as a control for equal loading. Bands were visualized with a phosphorimager and quantified using the MacIntosh IP lab get program. Relative levels of GATA-1 transcript are expressed as per cent control below each lane.

Figure 4. Activation and inhibition of BMP causes distinct defects in hematopoiesis. (A) Wright-giemsa stained cytospin preparations of blood collected from control tadpoles or from tadpoles in which BMP signaling had been misregulated. Arrows indicate RBCs; arrowheads indicate WBCs. (B) The mean number (± SEM) of WBCs (open bars), RBCs (red bars) and total blood cells (shaded bars) present in three random fields of cytospin blood preparations from control or experimental embryos is shown. (C) Ratio of WBCs to RBCs, calculated from the data shown in (B). (D) Apoptotic cells in cytospin preparations of blood collected from control and experimental animals detected using a TUNEL assay. Nuclei of cells were stained with propidium iodide. Arrows indicate TUNEL-positive cells. (E) Per cent of blood cells that are apoptotic in control and experimental animals as determined by counting total number of cells and number of TUNEL-positive cells present in three random fields of cytospin blood preparations from at least 24 embryos. Data presented is the mean ± SEM.

Figure 5. CaM KIV antagonizes BMP signaling. (A and C) The mean number (± SEM) of WBCs (open bars), RBCs (red bars) and total blood cells (shaded bars) present in three random fields of cytospin blood preparations from control embryos or embryos injected with RNA encoding mutant forms of BMP and/or CaM KIV as indicated on the X-axes. (B and D) The average ratio of WBCs to RBCs (± SEM), calculated from the data shown in (A and C).

Figure 6. Constitutively active CREB phenocopies active CaM KIV and dominant negative BMP. (A) Ventral view of tailbud (st. 28) stage embryos in which globin expression has been analyzed by whole mount in situ hybridization. Embryos were injected near the VMZ at the 4-cell stage with RNAs encoding β-galactosidase (control), constitutively active CREB ($CREB_{DIEDML}$), constitutively active CREB without a DNA binding domain (GAL4-$CREB_{DIEDML}$), or wild type CREB without a DNA binding domain (GAL4-CREB) as indicated above each photo. (B) The mean number (± SEM) of WBCs (open bars), RBCs (red bars) and total blood cells (shaded bars) present in three random fields of cytospin blood preparations from control or experimental embryos is shown.

Figure 7. (A, C and E) The mean number (± SEM) of WBCs (open bars), RBCs (red bars) and total blood cells (shaded bars) present in three random fields of cytospin blood preparations from embryos injected with RNA encoding β-galactosidase (control), mutant forms of CaM KIV, BMP, and CREB, or wild type CBP, either alone or in

combination, as indicated on the X-axes. (B, D and F) The average ratio of WBCs to RBCs (± SEM), calculated from the data shown in (A, C and E).

Figure 8. A model of CaM KIV's inhibition of BMP signaling during hematopoiesis (details in text). (A) Nuclear component of an active BMP signal in which the Smad complex is linked to a hematopoietic-specific transcription factor by the coactivator CBP. (B) CaM KIV inhibition of the interaction between the Smad complex and transcriptional components by activating a downstream substrate such as CREB.

Chapter 3

THESIS SUMMARY

The role of BMPs in cardiac development

In the first chapter of this thesis, I have described findings that aid in our understanding of the part BMP signaling plays in the formation of the vertebrate heart. I disrupted BMP signaling in *Xenopus* embryos by injecting synthetic RNA encoding well-characterized intracellular BMP inhibitors into specific blastomeres that give rise to heart and anterior endoderm. This disruption caused defects in endoderm patterning, failure of the two heart primordia to migrate and/or fuse at the ventral midline, and defects in cardiomyocyte differentiation. Interestingly, the initial specification of the heart field, as assayed by Nkx2.5 and GATA-4 expression, was unaffected. Therefore, BMP is required for the terminal differentiation, but not the initial induction, of the heart field.

BMP signaling in blood development

The results of my investigations into the role of BMP in hematopoiesis are described in chapter two. As in heart development, the initial specification of this cell type is not dependent on BMP signaling, as determined by analysis of early hematopoietic markers. Specific defects were observed following specification, however. Blood cell development was found to require an intact signaling pathway in the hematopoietic environment by targeting inhibitors to specific blastomeres at the 32-cell stage that do not give rise to blood. Surprisingly, hyperactivation of the pathway, by injecting synthetic RNA encoding a constitutively active BMP receptor, also disrupted

blood cell development non-cell autonomously. Further investigation revealed the decrease in red blood cells (RBCs) caused by up- or down-regulation of BMP signaling was due to distinct mechanisms. We determined that hyperactivation of BMP signaling caused a change in cell fate resulting in increased white blood cells (WBCs) at the expense of RBCs. Inhibition of BMP signaling also decreased RBCs, but did so by increasing the frequency of apoptosis in these cells. Therefore, a balanced BMP signal is required non-cell autonomously for normal hematopoiesis (Figure 1).

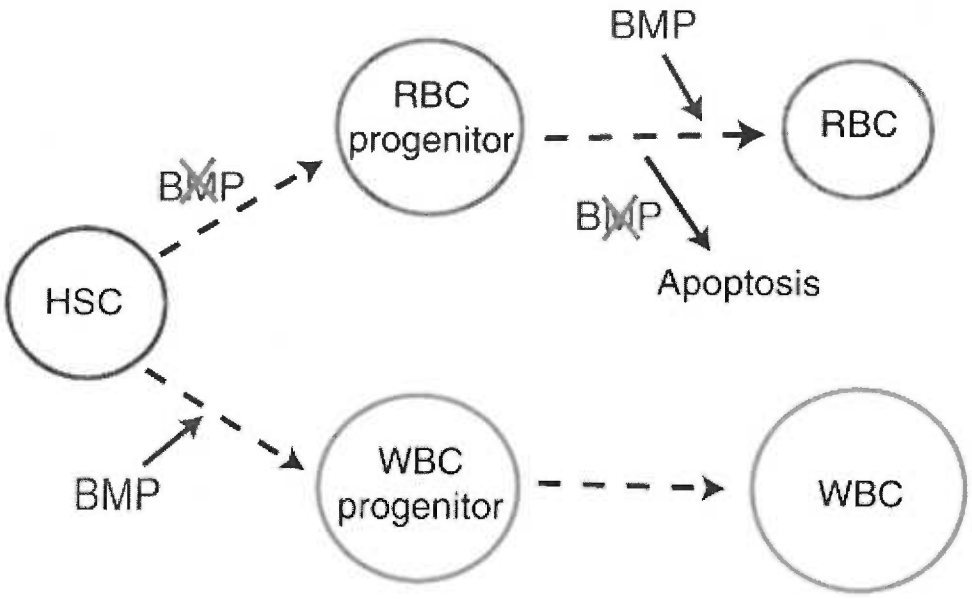


Figure 1. Model of BMP signaling role in hematopoiesis. As indicated in this simplistic drawing, BMP signaling is required non-cell autonomously for WBC lineage determination. In addition, BMP must be downregulated in the hematopoietic microenvironment for RBC production, and subsequently upregulated for RBC maturation.

Crosstalk between BMP and CaM KIV during hematopoiesis

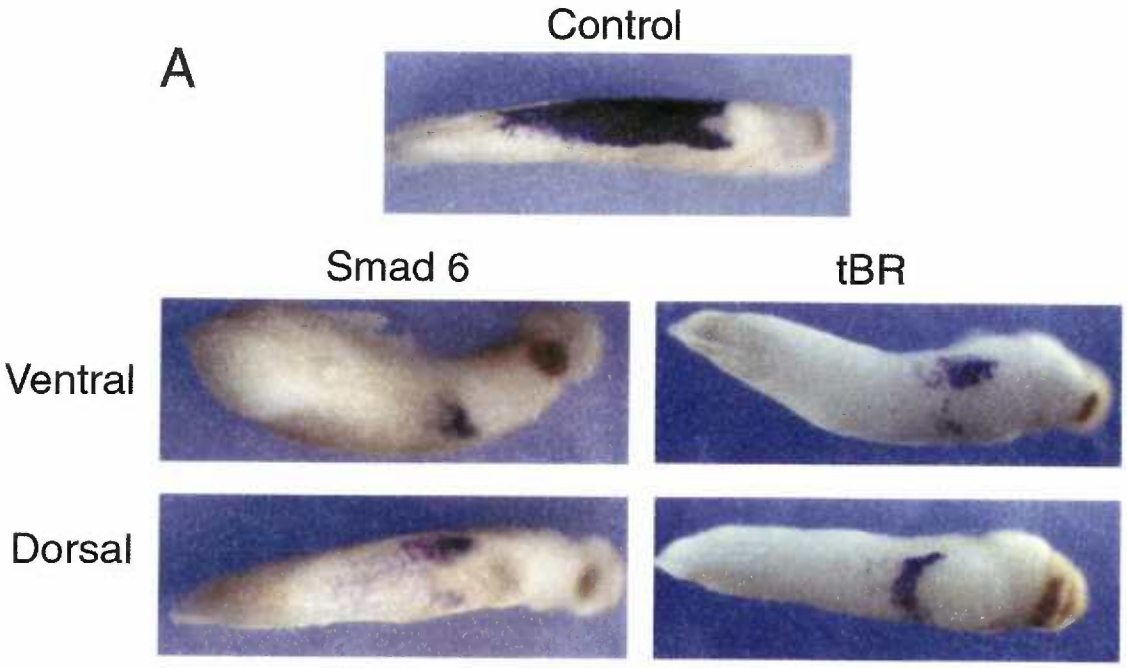
I participated in the recent studies by Gary Wayman from Tom Soderling's lab in which the role of the CaM KIV signaling cascade in vertebrate hematopoiesis was investigated (Wayman et al., submitted). These studies, in conjunction with those described in chapter 2 of this thesis, revealed reciprocal roles for CaM KIV and BMP in blood development, where BMP hyperactivation and CaM KIV inhibition both cause an increase in WBC number, and BMP inhibition and CaM KIV hyperactivation both increase RBC apoptosis. By misregulating both signaling pathways simultaneously, we determined that CaM KIV functions downstream of BMP receptor activation and antagonizes transduction of BMP signals. We hypothesized that CaM KIV inhibits BMP by activating a substrate, such as CREB, that can then bind to CBP and prevent CBP from acting as a coactivator for BMP-induced transcription of hematopoiesis-specific genes. This hypothesis was supported by injections of a phosphorylation-independent constitutively active CREB mutant that caused the same blood phenotype observed when CaM KIV was upregulated or BMP inhibited directly, even if BMP was hyperactivated or CaM KIV was blocked. In addition, injection of wild-type CBP caused an active BMP phenotype, even in the presence of constitutively active CaM KIV. Therefore, we propose a model describing the interaction of BMP and CaM KIV signaling at the level of the coactivator, CBP, in order to achieve the balance of signals required for lineage determination and hematopoietic cell survival (Figure 8, chapter 2).

Concluding remarks

In this thesis, I have further defined the role BMP signaling plays in vertebrate heart and blood development. I have also, in collaboration with Gary Wayman, identified novel crosstalk between BMP and CaM KIV in hematopoiesis. We have provided evidence supporting a model in which CaM KIV antagonizes BMP signaling in blood development by interfering with CBP's role in activating transcription of hematopoiesis-specific BMP target genes.

The future of this project is promising, with many interesting unanswered questions. One aspect we have yet to investigate is the timing during development that these signals are required. In the experiments I have described in this thesis, RNA was injected at the 4-, 8-, or 32-cell stage and therefore is present throughout development. In an effort to circumvent this limitation, I have constructed a hormone-inducible Smad6 with the hormone-binding domain of the glucocorticoid receptor. Addition of this hormone-binding domain to the C-terminus of other proteins has been shown to create a protein that is completely, reversibly, non-functional (Kolm and Sive, 1995). When the hormone binds, the change in conformation results in a fully functional protein as long as the hormone is present. With this construct, BMP signaling could be inhibited at any stage, and then inhibition relieved by removal of the hormone. Once the stage of development in which BMP function is required for hematopoiesis has been identified, expression cloning or arrays can be used to determine what genes are being regulated by BMP that in turn regulate hematopoiesis. Preliminary experiments with this construct were encouraging, and presumably further experiments will be carried out by future members of the Christian laboratory.

Figure 1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Control
Smad 6
tBR
Ventral
Dorsal

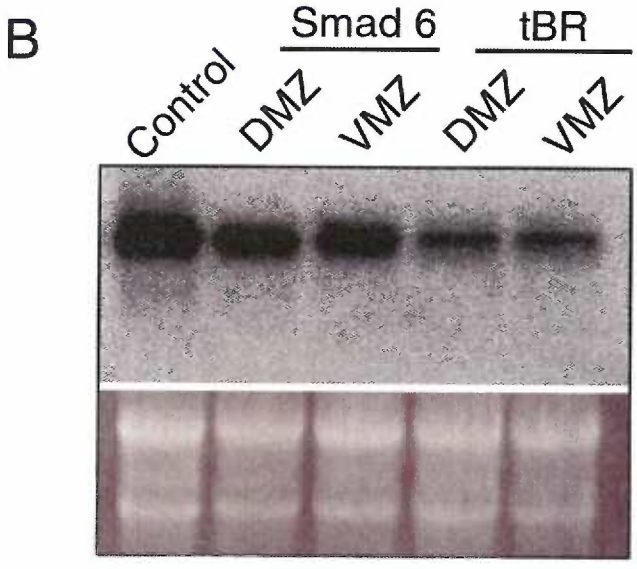
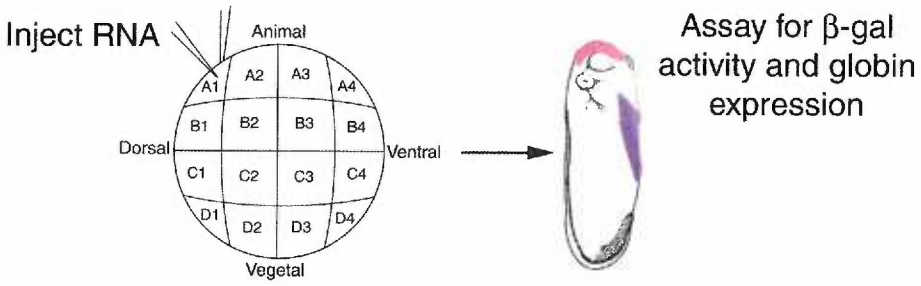
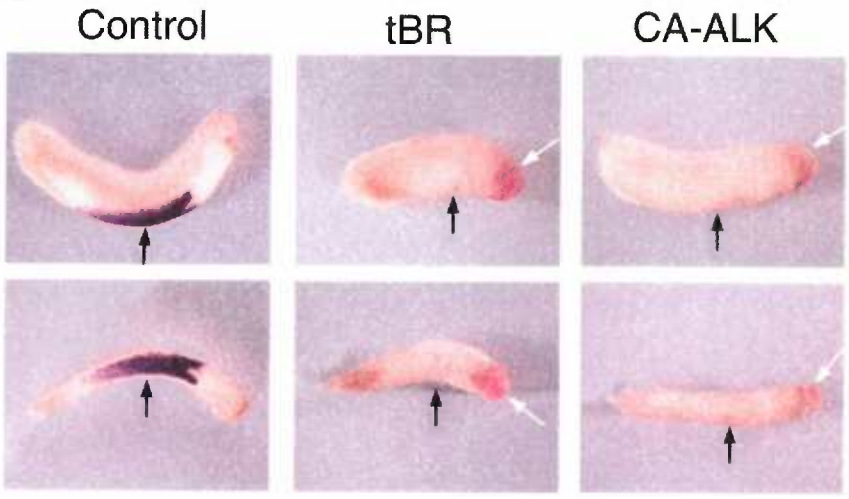

B
Smad 6
tBR
Control
DMZ
VMZ
DMZ
VMZ

Figure 2


Inject RNA
Animal
A1
A2
A3
A4
B1
B2
B3
B4
Dorsal
Ventral
C1
C2
C3
C4
D1
D2
D3
D4
Vegetal
Assay for β-gal activity and globin expression

A


Control
tBR
CA-ALK

B

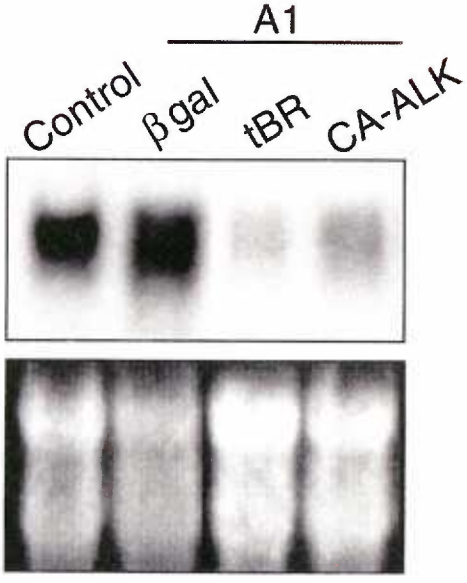

A1
Control
βgal
tBR
CA-ALK

Figure 3

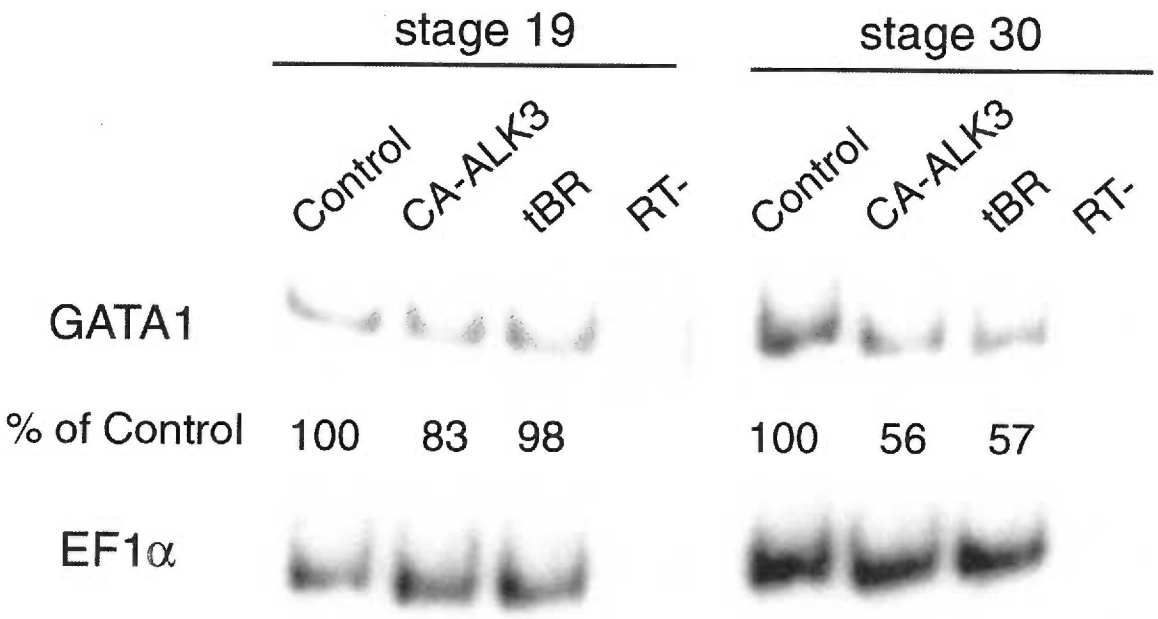

stage 19
stage 30
Control
CA-ALK3
tBR
RT-
Control
CA-ALK3
tBR
RT-
GATA1
% of Control
100
83
98
100
56
57
EF1α

Figure 4

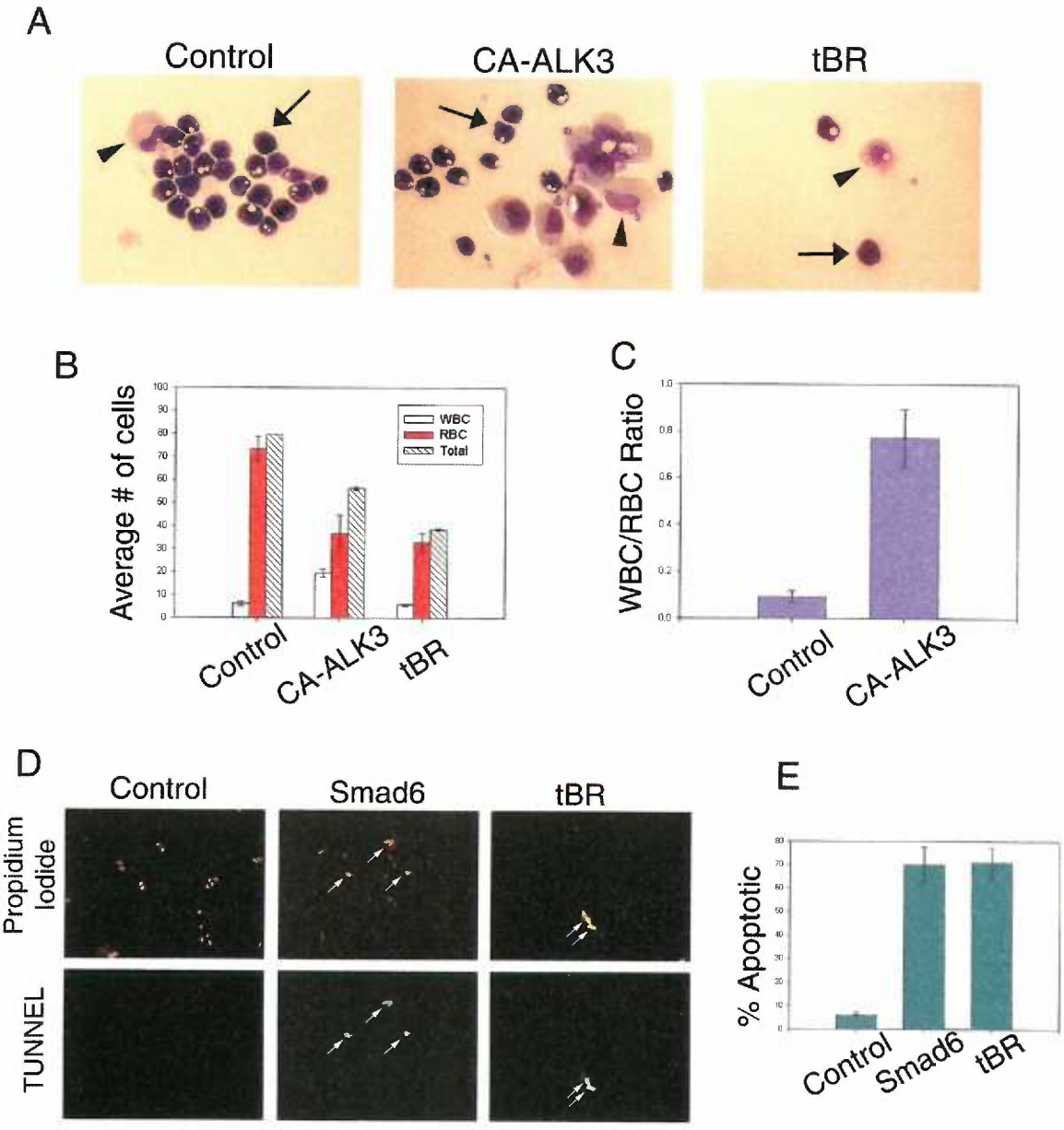

A
Control
CA-ALK3
tBR
B
Average # of cells
WBC
RBC
Total
Control
CA-ALK3
tBR
C
WBC/RBC Ratio
Control
CA-ALK3
D
Control
Smad6
tBR
Propidium Iodide
TUNNEL
E
% Apoptotic
Control
Smad6
tBR

Figure 5

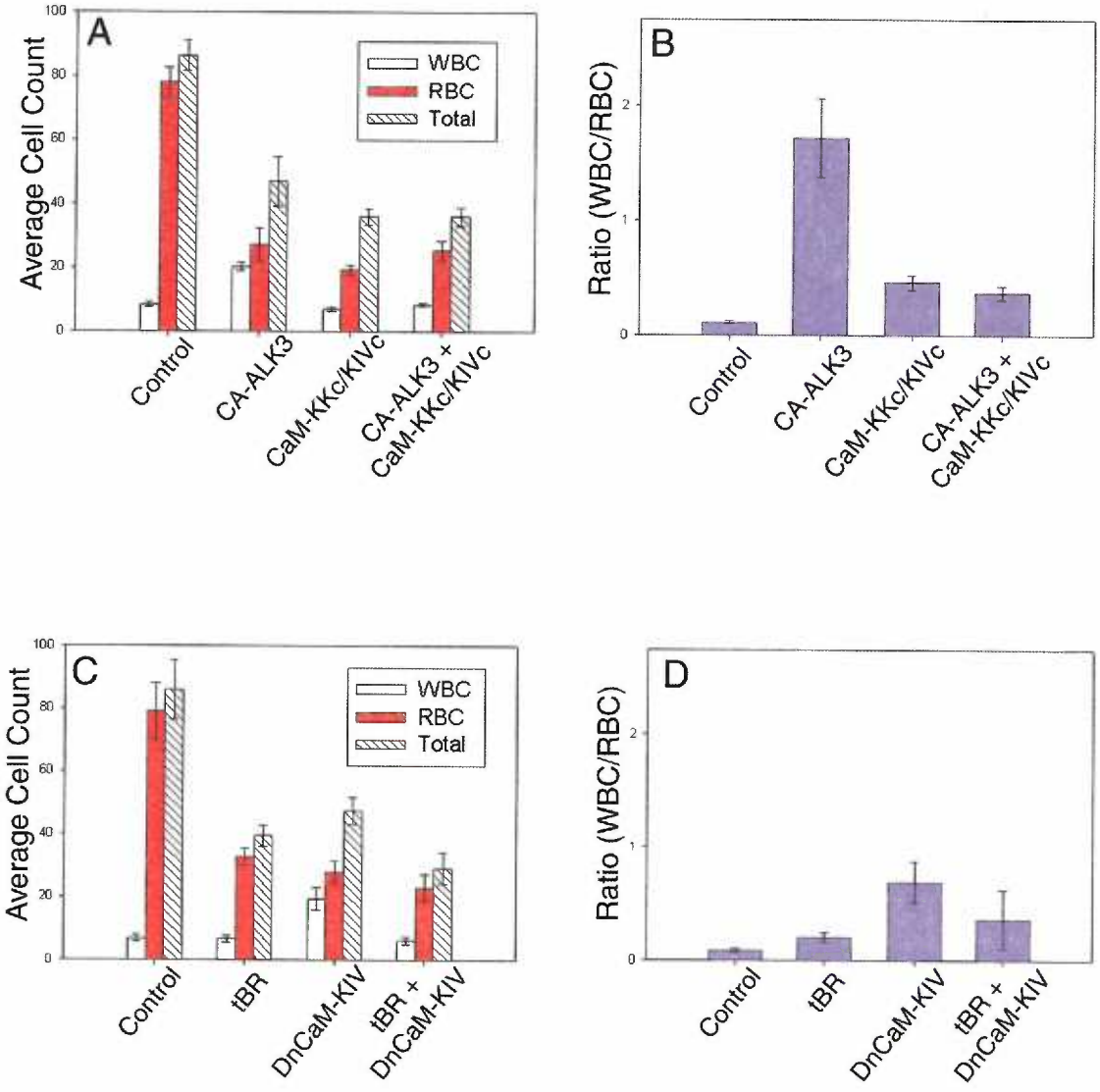

A
Average Cell Count
WBC
RBC
Total
Control
CA-ALK3
CaM-KKc/KIVc
CA-ALK3 + CaM-KKc/KIVc
B
Ratio (WBC/RBC)
Control
CA-ALK3
CaM-KKc/KIVc
CA-ALK3 + CaM-KKc/KIVc
C
Average Cell Count
WBC
RBC
Total
Control
tBR
DnCaM-KIV
tBR + DnCaM-KIV
D
Ratio (WBC/RBC)
Control
tBR
DnCaM-KIV
tBR + DnCaM-KIV

Figure 6

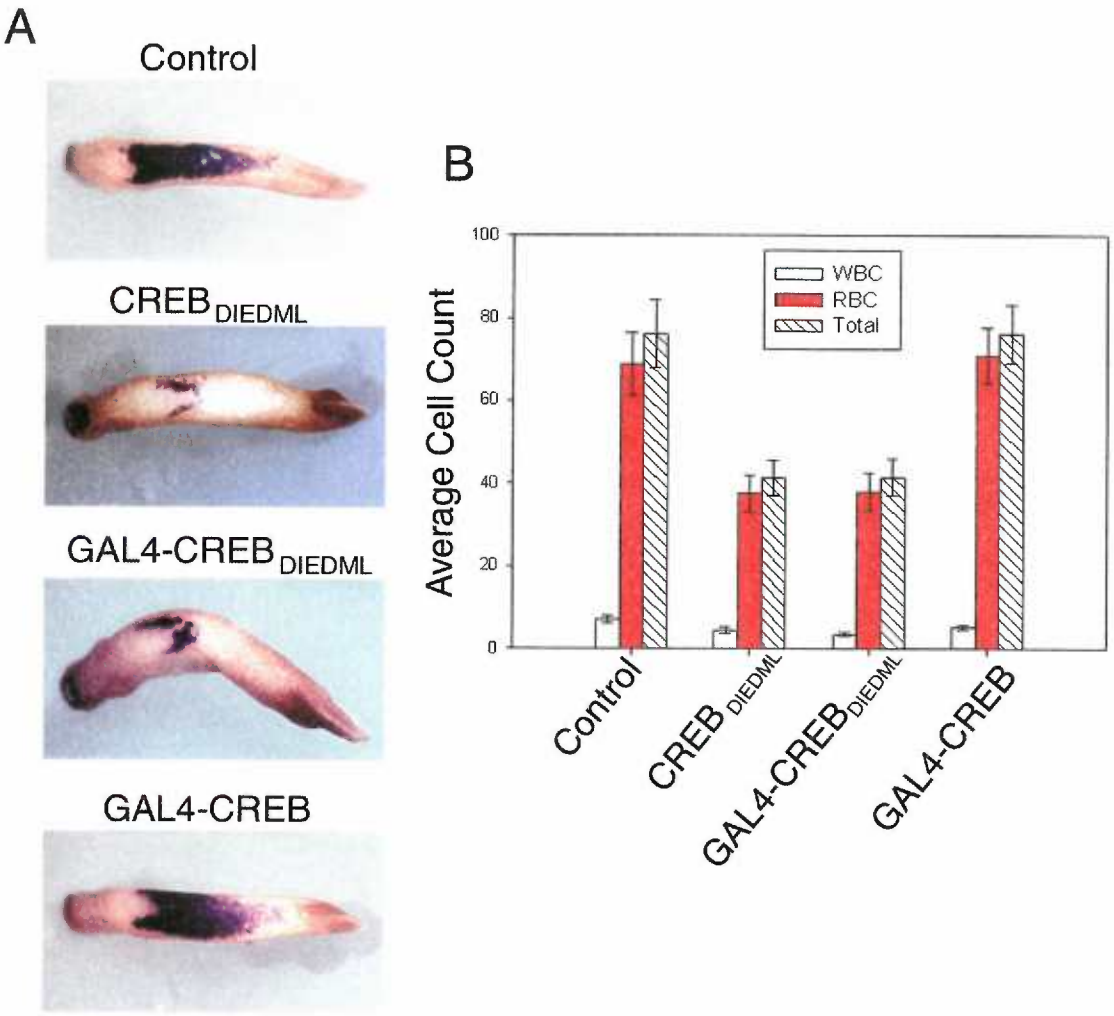

A
Control
CREB DIEDML
GAL4-CREB DIEDML
GAL4-CREB
B
Average Cell Count
100
80
60
40
20
0
WBC
RBC
Total
Control
CREB DIEDML
GAL4-CREB DIEDML
GAL4-CREB

Figure 7

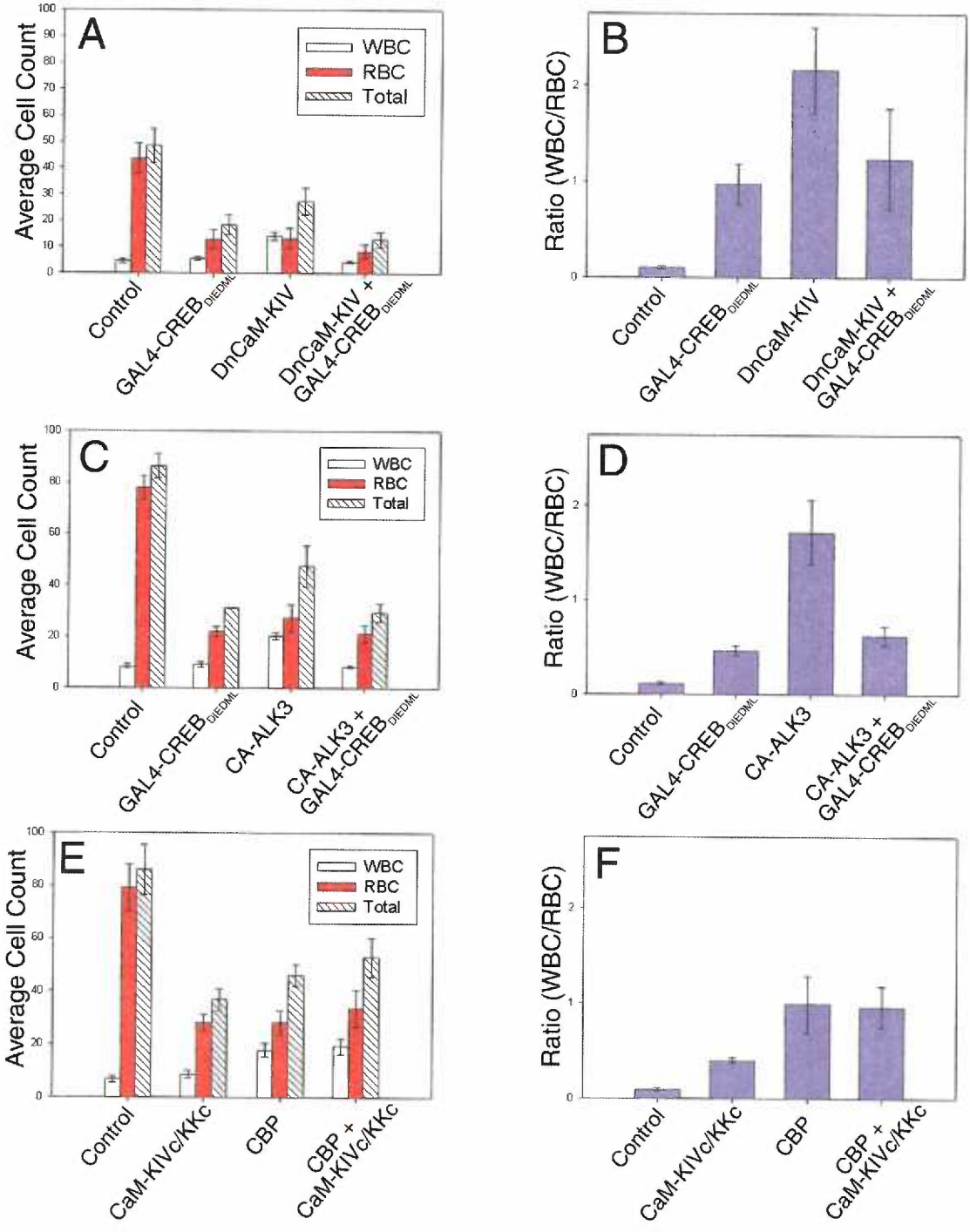
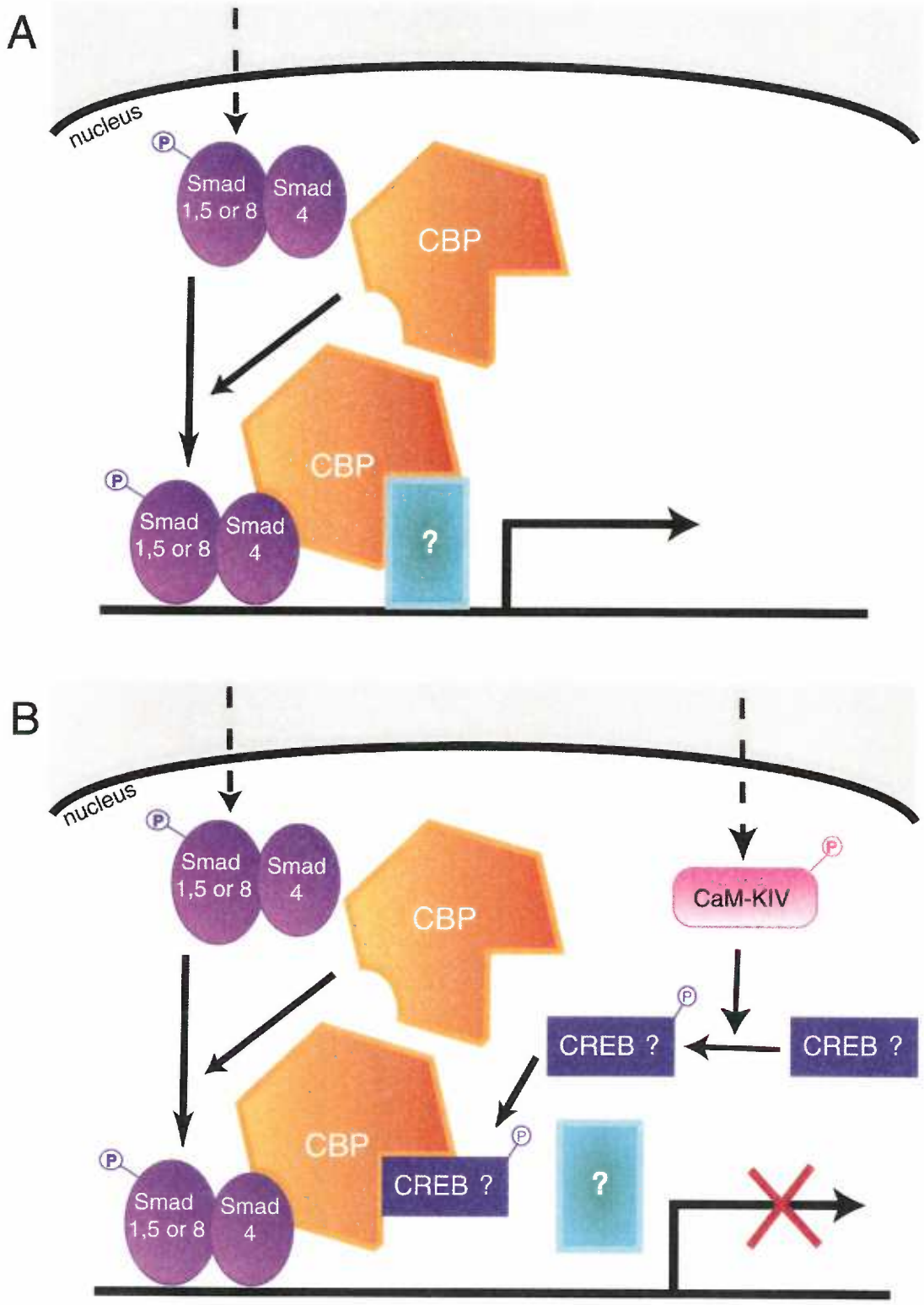

A
Average Cell Count
WBC
RBC
Total
Control
GAL4-CREB DIEDML
DnCaM-KIV
DnCaM-KIV + GAL4-CREB DIEDML
B
Ratio (WBC/RBC)
Control
GAL4-CREB DIEDML
DnCaM-KIV
DnCaM-KIV + GAL4-CREB DIEDML
C
Average Cell Count
WBC
RBC
Total
Control
GAL4-CREB DIEDML
CA-ALK3
CA-ALK3 + GAL4-CREB DIEDML
D
Ratio (WBC/RBC)
Control
GAL4-CREB DIEDML
CA-ALK3
CA-ALK3 + GAL4-CREB DIEDML
E
Average Cell Count
WBC
RBC
Total
Control
CaM-KIVc/KKc
CBP
CBP + CaM-KIVc/KKc
F
Ratio (WBC/RBC)
Control
CaM-KIVc/KKc
CBP
CBP + CaM-KIVc/KKc

Figure 8


A
nucleus
P
Smad 1,5 or 8
Smad 4
CBP
CBP
P
Smad 1,5 or 8
Smad 4
?
B
nucleus
P
Smad 1,5 or 8
Smad 4
CBP
P
CaM-KIV
P
CREB ?
CREB ?
CBP
P
CREB ?
?
P
Smad 1,5 or 8
Smad 4

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