GATA-2 functions downstream of BMPs and CaM KIV in ectodermal cells during primitive hematopoiesis

by

Gokhan Dalgin

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ABSTRACT

In vertebrates, blood originates from the mesoderm. In *Xenopus*, generation of the hematopoietic system during development requires not only local signals from the mesoderm but also signals from the neighboring ectoderm. Ectodermal signals are modulated by both extra- and intracellular factors. However, it has not been shown exactly what these factors are. Experimental results summarized in this thesis will help to fill this gap in knowledge.

Previously we have shown that bone morphogenetic protein (BMP) function is required to generate the ectodermal signals that are required for differentiation of ventral mesoderm as blood. When BMP signals are blocked in ectodermal cells of *Xenopus* embryos, defects in primitive hematopoiesis are observed. Moreover, we have shown that calmodulin-dependent kinase IV (CamKIV) negatively regulates BMP mediated transcriptional responses in ectodermal cells during hematopoiesis.

In this thesis, I demonstrate that the transcription factor GATA-2 is also required in the ectoderm for normal primitive hematopoiesis. Blocking the expression of endogenous GATA-2, by injecting GATA-2 Morpholinos (GATA-2 MOs) into ectodermal cells of *Xenopus* embryos caused hematopoietic defects similar to those observed following misregulation of BMPs or CaM KIV in the same germ layer. Moreover, I show that BMPs, CaM KIV and GATA-2 act in a linear pathway during primitive hematopoiesis. My data suggest that expression of GATA-2 is regulated at transcriptional level by BMPs, and that GATA-2 is

posttranslationally modulated downstream of CamKIV. Based on our current findings, we propose a model in which BMPs induce the transcription of GATA-2 in the ectoderm while CaM KIV negatively regulates acetylation of GATA-2. The mechanism by which CaM KIV does so involves competition between GATA-2 and a CaM KIV substrate for a limited amount of CBP. These results may help us to understand the critical steps in primitive erythropoiesis early in development, and therefore will expand our knowledge of how specific blood lineages are generated.

CHAPTER 1

Introduction

Multicellular organisms begin with a fertilized egg (zygote), which gives rise to many different cell types that form the organism. The allocation of different cell-types into specific tissues and organs is governed by a mechanism called specification and differentiation. During the development of the embryo, a few types of conserved molecules, called morphogens, regulate these specification and differentiation steps. However, regulation of these steps is very complex, and the function of the morphogens is not well understood. For example, one family of morphogens, the bone morphogenetic proteins (BMPs), are known to pattern the mesoderm, but the molecular mechanism by which they do so, such as what genes are activated by BMPs and how they function, is not well known. In vertebrates, blood originates from mesoderm and various extra- and intracellular molecules direct the fate of the blood cells. Different signaling molecules and transcription factors are involved in the specification of blood cells, but it is still unknown how and when these molecules function. One of the best developmental systems to identify these unknown steps is *Xenopus laevis* embryos.

Advantages of using *Xenopus laevis* embryos in developmental studies.

Xenopus laevis embryos are an excellent model system to analyze early developmental processes. They have large eggs that can be fertilized *in vitro* and

develop externally. Animal and vegetal poles of the embryo have differences in pigmentation; moreover, at the 4-cell stage (2 hours of development), dorsal and ventral hemispheres can be distinguished. These differences are not only helpful for targeted injections of RNA or DNA to the future germlayers, but also make explantation experiments easier compared with other vertebrate embryos. Furthermore, RNAs encoding dominant-negative or constitutively active forms of the desired molecules can easily be injected into early-stage embryos to elucidate the function of the analyzed molecule. Recently, a different type of antisense technology, called morpholino oligonucleotides (MOs) has emerged, which can be used to block the translation of a desired mRNA (Heasman J, 2002). MOs are very useful for several reasons. First, they are designed for the specific RNA of choice. Second, since MOs are DNA analogs, they are not susceptible to enzymatic degradation; therefore, they are more stabile than other antisense molecules (Hudziak et al., 1996). Finally, they have fewer toxic effects compared to DNA antisense oligos.

Structure and function of TGF-β superfamily members.

By utilizing the advantages of *Xenopus* embryos, many molecules involved in early vertebrate development have been studied, including BMPs. BMPs are members of the transforming growth factor β (TGF-β) superfamily of signaling molecules and play multiple roles during embryonic development (Nakayama et al., 2000). BMP ligands bind to a heterocomplex of structurally

similar type I and type II transmembrane serine-threonine receptor kinases. Once the ligand binds to the receptor complex, the type II receptor trans-phosphorylates the type I receptor. Following the phosphorylation of the type I receptor, a BMP-receptor-specific signal transducer, Smad-1, -5, or 8, is phosphorylated. This phosphorylation allows these Smads to hetero-oligomerize with Smad—4. The Smad complex then translocates into the nucleus and, in conjunction with other nuclear cofactors, activates the transcription of target genes (Nakayama et al., 2000). This basic signal transduction pathway is illustrated in Fig. 1 and described in more detail below.

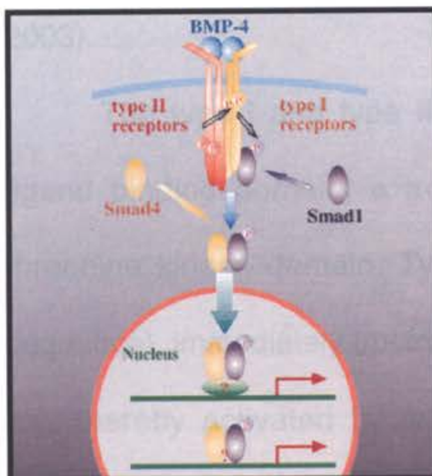

Figure1. The BMP signaling pathway, represented as the homodimeric BMP-4 ligand, binds to the receptor complex, which activates the downstream signaling molecules, Smad-1, -5 or-8. See text for details

TGF-β superfamily ligands and receptors.

The TGF-β superfamily of signaling molecules contains two subfamilies, the TGF-β/Activin/Nodal subfamily and BMP/GDF (growth and differentiation factor) subfamily (Shi and Massague, 2003). These subfamilies are characterized in terms of their sequence similarities and the receptors they utilize to activate

the distinct Smad family members. These two subfamilies regulate downstream target genes by binding to different classes of type I and type II receptors and activating different downstream Smad molecules. While the type I receptors for TGF-β/Activin/Nodal subfamily signaling molecules are ALK4, -5, or –7, the type II receptors are TβR-II and ActRIIB. By binding to these receptors, the TGF-β/Activin/Nodal subfamily activates Smad-2 or Smad-3 downstream signaling molecules. In contrast, the BMP/GDF subfamily of signaling molecules activate Smad-1, -5, or –8 by binding to BMPR-II or ActR-II/B (type II receptors) and ALK-2, -3, or –6 (type I receptors). In both cases, activated Smads form a heterocomplex with Smad-4 to translocate to the nucleus (Shi and Massague, 2003).

The type I and type II receptors consist of an N-terminal extracellular ligand binding domain, a transmembrane region, and a C-terminal serine-threonine kinase domain. Type I receptors contain a GS domain (SGSGSG sequence), immediately upstream of the kinase domain, which is phosphorylated and thereby activated by type II receptors (Shi and Massague, 2003). The deletion of the kinase domain or mutation of the GS domain of the type I receptor generates dominant negative or constitutively active forms of the receptor, respectively (Akiyama et al., 1997; Suzuki et al., 1994). These mutants are extremely useful in analyzing the developmental and cellular function of BMPs downstream of their receptors.

Studies in cell culture have shown that TGF-β superfamily signaling molecules activate type I and type II receptors by two different ligand-receptor interactions. First, TGF-β and Activin ligands have high affinity for type II receptors and low affinity for type I receptors. Once TGF-β or Activin ligand binds to the type II receptor, it allows the type I receptor to associate with the type II receptor-ligand complex, thereby activating subsequent signaling downstream of receptors (Attisano et al., 1993; Wrana et al., 1992). Second, in contrast to TGF-β and Activin, BMP ligands such as BMP-2 or BMP-4 have high affinity for the type I receptors and low affinity for type II receptors. Upon ligand binding to the type I receptor, the ligand-receptor complex aquires higher affinity for the type II receptor, thereby activating downstream signaling (Shi and Massague, 2003).

Downstream targets of activated BMP receptors.

In both types of ligand-receptor interactions, later signaling is due to activation of Smads. Smads are grouped into three subfamilies: receptor-regulated Smads (R-Smads; Smad-1,-2,-3,-5, and -8), a Co-mediator Smad (Co-Smad or Smad-4), and inhibitor Smads (I-Smads; Smad-6, and -7). Among the three classes of Smads, only R-Smads are phosphorylated and activated by type I receptor kinases. R-Smads and the Co-Smad contain a conserved N-terminal Mad Homology 1(MH1) domain and a C-terminal MH2 domain, which are separated by a proline-rich, non-conserved linker region. Most of the R-Smad and Smad-4 (Co-Smad) MH1 domains display sequence-specific DNA binding

activity. In contrast, the N-terminus of I-Smads has a weak sequence homology to the MH1 domain of R-Smads and does not bind to DNA (Shi and Massague, 2003). Smads bind to a consensus motif called the Smad-binding element (SBE) on target DNA, which has the sequence GCTC. Smad targets contain one or more of these SBEs; however, some target genes lack this motif. Smads have also been shown to interact with the sequence GCCGnCGC, called the GC-rich element. Although Smads bind to DNA with low specifity, it has been shown that they rely on various DNA-binding partners to regulate the transcription of target genes. The list of DNA-binding partners is growing; examples include forkhead family members FoxH1 or FoxHO, and other DNA-binding partners such as E2F4, and ATF3 (Massague et al., 2005).

The MH2 domain of Smads is crucial for receptor interaction, formation of Smad complexes, and for interacting with the nuclear pore complex for translocation from cytoplasm to nucleus (Shi and Massague, 2003). Furthermore, a conserved C-terminal SSXS motif of the MH2 domain on R-Smads is phosphorylated by type I receptors. This phosphorylation activates the R-Smads and leads to hetero-oligomerization with the Co-Smad (Smad-4), inducing the nuclear transport of this Smad complex (Attisano L et al., 2001; Christian and Nakayama, 1999). Finally, I-Smads (Smad-6 and Smad-7) are the negative regulators of TGF-β superfamily signaling molecules. They block the interaction of R-Smads with either type I receptor or Co-Smad, targeting the receptors for degradation (Shi and Massague, 2003).

BMPs' role in vertebrate development.

BMPs were originally isolated by their ability to induce bone formation (Wozney et al., 1988). BMPs are required for both vertebrate and invertebrate early development, and active BMP signaling is required for the specification of ventral fate (Hogan, 1996). It has been shown in *Xenopus* embryos that BMPs function at gastrulation to promote ventral fate and to block dorsalizing signals (Jones et al., 1996). Later in development, BMPs are expressed in many tissues and organs, suggesting multiple roles for BMPs.

BMPs are critical for mesoderm patterning. In *Xenopus*, when BMP signals are blocked by a dominant negative, truncated BMP receptor (tBR), with antisense BMP4, or with dominant negative-forms of BMP4 ligand (Graff, 1997), ventral mesoderm is converted to a dorsal fate. Conversely, overexpression of BMPs, either by the overexpression of the BMP ligands or by the constitutively active type I receptor, leads to loss of dorsoanterior structures, and the embryo is ventralized {Jones, 1992 #431;Dale L, 1992 #159

BMP-2, -4 and –7 are among the best studied members of the BMP family and mutational analyses in mice reveal multiple roles for these molecules in development. For example, homozygous mutants of BMP-7 have small kidneys and die shortly after birth from renal failure. They also have defects in eye and limb development. Moreover, homozygous BMP-2 mutant mice die at about embryonic day 9.5 (E9.5) with defects in amnion and heart development. Finally,

homozygous mutants of BMP-4 die at around gastrulation (E6.5) due to an inability to form embryonic mesoderm. However, some mutant BMP-4 embryos do develop further (possibly due to compensation by other BMPs), but they have defects in yolk sac hematopoiesis and severely truncated posterior development. These data, combined with *in vitro* studies, suggest a role for BMPs in hematopoiesis.

Hematopoiesis in vertebrate embryos.

In vertebrates, hematopoiesis happens in two different steps. The first step is called primitive hematopoiesis, and it gives rise predominantly to nucleated primitive erythrocytes and some myeloid cells. The second step is called definitive hematopoiesis, and it gives rise to all different types of blood cells. Primitive and definitive hematopoiesis originate from two different anatomical sites in the vertebrate embryo. In frogs, primitive and definitive hematopoiesis occurs intraembryonically in the ventral blood island (VBI) and in the dorsal lateral plate (DLP), respectively. In mammals, primitive hematopoiesis takes place in the extraembryonic yolk sac, while definitive hematopoiesis occurs intraembryonically, initially in the aorta-gonad-mesonephros (AGM) region and subsequently in the placenta, liver and then bone marrow.

Hematopoiesis in mice.

Similar to all other vertebrates, hematopoietic cells in mice originate from the mesoderm. During gastrulation, the mouse embryo establishes mesoderm.

Before gastrulation, a part of the embryo called the epiblast (embryo proper) is in contact with the extraembryonic ectoderm (ExE) at the proximal site, and the rest of the embryo is surrounded by visceral endoderm (VE). The first sign of gastrulation is the formation of the primitive streak at the posterior epiblast-extraembryonic border region. During gastrulation, epiblast cells migrate through the primitive streak, where they form the mesodermal cells, triggered by signals coming from the surrounding Exe and VE. Signals from these two regions may also regulate the specification or differentiation of hematopoietic and endothelial lineage (haemangioblast) reviewed by {Baron, 2005 #469}.

Detailed cell tracing analysis suggested that derivatives of the lateral plate mesoderm (such as AGM), which will become definitive hematopoietic progenitors, exit the primitive streak anteriorly (Baron, 2005). In contrast, the first mesodermal cells emerging from the primitive streak move proximally towards the ExE region and differentiate into the blood islands of the yolk sac (McGrath and Palis, 2005). Differentiation of primitive hematopoietic and endothelial cells are observed in the yolk sac mesoderm by about E7.5 (Baron, 2005). Interestingly, recent studies suggest that commitment of mesodermal progenitors to these lineages happens in the posterior primitive streak even before they migrate to the ExE (Huber et al., 2004).

In mice, primitive erythrocytes that are generated by the yolk sac blood islands are nucleated; in contrast, definitive erythrocytes are enucleated. However, recently, antibodies generated against mouse βH1-globin (embryonic

globin) allowed identification of circulating enucleated primitive red blood cells in the embryo and showed that the majority of primitive erythroblasts lose their nuclei between E12.5 and E16.5 (Kingsley et al., 2004).

In vitro, primitive and definitive erythrocytes come from different colonies. When E7.25 mouse yolk sac cells are cultured in vitro, they form erythroid progenitors, called EryP-CFC, which give rise to large primitive erythroid cells expressing embryonic and adult globin (Palis et al., 1999; Wong et al., 1986). In contrast, burst-forming unit-erythroid (BFU-E) and colony-forming unit-erythroid (CFU-E) progenitors give rise to small, definitive red blood cells expressing only adult globins (Palis et al., 1999; Wong et al., 1986). About E8.25 these progenitors are restricted to the yolk sac and by E9.5 and E10 they are found in the circulation. However, these erythroid progenitors are also found extensively in the fetal liver, suggesting that the fetal liver is the site for definitive erythroid maturation in the fetus. Both hematopoietic stem cells (HSC) that emerge from the AGM and the yolk-sac-derived erythroid progenitors seed the fetal liver and help the fetus to generate definitive erythrocytes to support the growing midgestation fetus. Later in development and in adult life, the HSCs from the fetal liver will populate both the thymus and the bone marrow that generates the blood cells of adult mice (McGrath and Palis, 2005).

Hematopoiesis in Zebrafish.

Zebrafish hematopoiesis is similar to other vertebrates , and happens in two steps. However, zebrafish primitive hematopoiesis happens in two different

anatomical sites in the embryo, which are both derived from lateral plate mesoderm: the intermediate cell mass (ICM) and anterior lateral plate mesoderm (ALPM) (de Jong and Zon, 2005). As noted earlier, in contrast to mice, these primitive hematopoietic sites are intraembryonic. While the ICM is located between the notochord and the endoderm of the trunk, the ALPM is located under the head (Galloway and Zon, 2003). While erythrocytes develop only in the ICM, the ALPM gives rise to macrophages and other myeloid precursors. Some myeloid cells also come from the posterior ICM (Berman et al., 2005).

Blood circulation in zebrafish embryos starts about 24 hours postfertilization (hpf). At this stage of development the circulating blood is entirely generated by the first wave of hematopoiesis (that is primitive hematopoiesis). Prior to circulation, around 12 hpf, precursors of the circulating blood cells express hematopoietic specific markers such as *Scl, GATA-2 and Lmo2* in the two stripes of the lateral plate mesoderm (Detrich et al., 1995; Gering et al., 1998). However, at the same time the early erythroid marker *GATA-1* is expressed only in the ICM (Detrich et al., 1995) and an early myeloid marker PU.1 is expressed in the ALPM (Bennett et al., 2001; Lieschke et al., 2002). These data confirm the separation of two anatomical sites for the generation of primitive erythroid and myeloid hematopoiesis.

As in other vertebrates, the second step of hematopoiesis, definitive hematopoiesis, happens in a different anatomical location. The first site of definitive hematopoiesis in zebrafish is the ventral wall of the dorsal aorta (the

equivalent of AGM in mammals) (de Jong and Zon, 2005). By 4 to 5 hpf, blood forming regions relocate to the kidney marrow, and this site remains the life long source of HSCs in zebrafish (Galloway and Zon, 2003).

Hematopoiesis in *Xenopus* and the role of BMPs.

As noted above, in *Xenopus*, the site for primitive hematopoiesis is called the ventral blood island (VBI), which is analogous to the yolk sac of mammals (Fig. 2).

Previous studies have suggested that the VBI is formed in response to high levels of BMPs. Since BMPs confer ventral pattern on the mesoderm (Dale L et al., 1992; Jones et al., 1992; Koster et al., 1991), and most precursors of the VBI come from the ventral mesoderm (VM) (Ciau-Uitz et al., 2000; Lane and Sheets, 2002; Lane and Smith, 1999), BMPs are known to be crucial for blood formation in vertebrates.

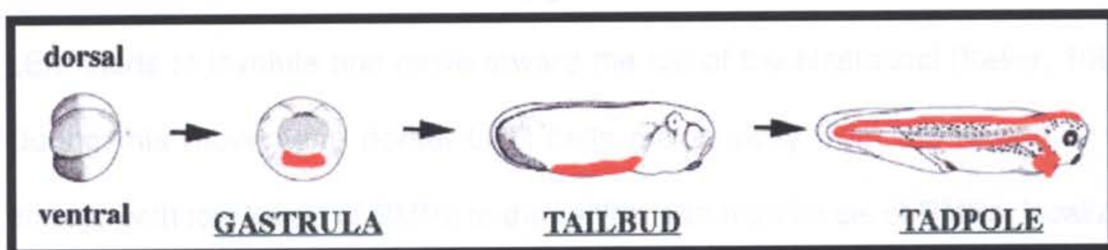

Figure 2. Summary of blood development in *Xenopus*. At the gastrula stage ventral mesodermal cells (Red) commit to give rise to primitive blood cells. At the tailbud stage, these cells populate a region called the VBI (Red) and start to differentiate, as indicated by expression of globin. At the tadpole stage, blood starts to circulate.

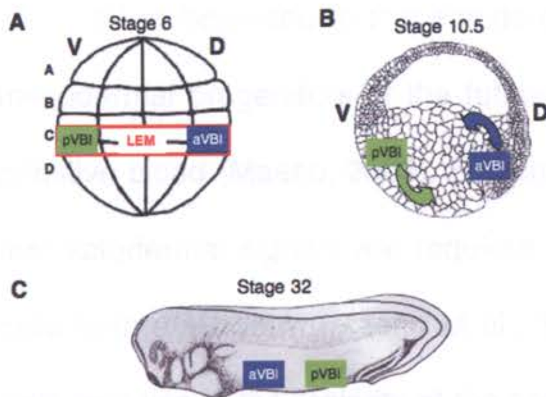

Figure 3. (A) At stage 6 (32 cell) the lower portion of the C tier and the upper portion of the D tier of cells is called leading edge mesoderm (LEM) and it is fated to contribute to the ventral blood island. (B) At gastrula stage dorsal mesoderm involutes and moves toward the blastocoel (blue arrow) and later ends up at the roof of the archenteron where it is in close contact with ectodermal cells and away from BMP inhibitors. (C) During gastrulation cells that come from the dorsal LEM end up in the anterior ventral blood island (aVBI), whereas, cells derived from the ventral LEM forms the posterior ventral blood island (pVBI). Adapted from (Lane and Smith, 1999).

However, elegant lineage tracing experiments have shown that blood also comes from the dorsal mesoderm, where there are low BMP levels (Lane and Sheets, 2002; Lane and Smith, 1999). Specifically, the entire lower portion of the marginal zone of the 64-cell embryo, which is called the leading edge mesoderm (LEM), contributes to the VBI. During gastrulation, the dorsal-most portion of the LEM starts to involute and move toward the top of the blastocoel (Keller, 1991). During this movement, dorsal LEM cells move away from the region of the embryo with low levels of BMPs to the region with high levels of BMPs, localizing at the site of the anterior VBI, close to the ventral midline and underneath the ectodermal cells (Fig. 3). While the anterior VBI is formed by these dorsal LEM cells, the posterior blood island is formed by the ventral LEM cells (Lane and Smith, 1999; Walmsley et al., 2002).

It has been shown that ectodermal cells that are in close contact with the mesodermal progenitors of the future VBI are crucial for the differentiation of primitive blood (Maeno, 2003). Evidence from explant assays in *Xenopus* show that ectodermal signals are required for the proper differentiation of erythroid cells from mesoderm (Maeno et al., 1994b). Specifically, as noted earlier, VM cells give rise to the majority of the posterior VBI. When stage-10 VM is isolated from the embryo and cultured alone until tailbud stage, it fails to express the erythrocyte marker globin. However, globin expression is detected when stage-10 VM is co-cultured with the same-stage animal cap (prospective ectoderm) (Fig. 4). Interestingly, when stage 10 VM is co-cultured with stage 7 animal cap, the explants fail to express globin. Conversely, when the stage 7 animal cap was taken from an embryo that was previously injected with either BMP-4 or GATA-2 RNA and then co-cultured with stage 10 VM, globin expression is restored (Maeno et al., 1996) (illustrated in Fig. 4). Moreover, when VM alone is taken from stage 16 embryos (neurula stage), it is able to differentiate as erythrocytes in culture (Maeno M, 1992).

These data suggest that stage 10 VM cells include precursors that will give rise to primitive erythrocytes; however, around gastrulation and before neurulation they require ectodermal signals to differentiate as red blood cells. Consistent with these data, in our lab, we have shown that BMP function is required non-cell autonomously, in the ectoderm, for the differentiation of primitive blood cells (Walters et al., 2002) (explained in detail below).

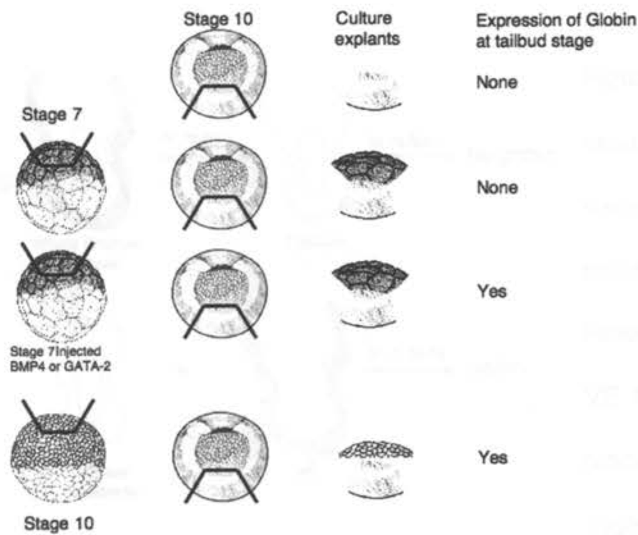

Figure 4. Schematic representation of the explant assay. At stage-10 ventral mesoderm can only form blood if it is co-cultured with ectoderm. While stage-10 ectoderm can induce blood formation in this explant assay, stage-7 ectoderm requires BMPs or GATA-2. Adapted from (Maeno et al., 1994b).

Similar to what has been identified in *Xenopus*, interaction of non-hematopoietic tissue with hematopoietic mesoderm is required primitive hematopoiesis (Belaoussoff et al., 1998). Specifically, during gastrulation in mice, only the posterior mesodermal cells that are in close contact with the visceral-endoderm (VE) will generate the hematopoietic cells of the yolk sac (Baron, 2005). When pre- or early gastrula stage epiblasts are stripped of their VE, they are unable to activate the transcription of erythroid markers such as *GATA-1* and *globin*; therefore, erythropoiesis is defective (Belaoussoff et al., 1998). However, blood formation is restored in the explants when VE from donor embryos is co-cultured with the naked epiblast (Belaoussoff et al., 1998) (Fig.5).

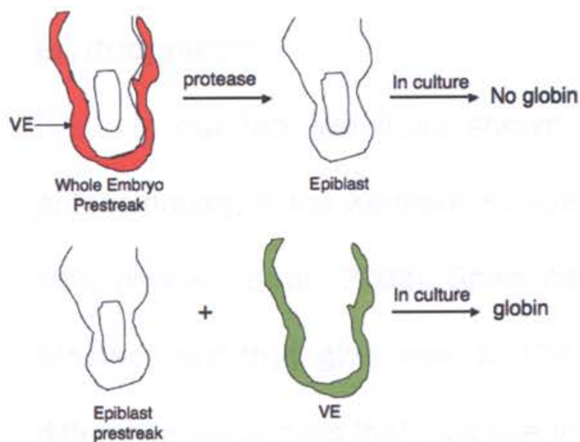

Figure 5. Schematic representation of explant assays in mice embryos. Epiblast stripped of its visceral endoderm (VE,red) cannot form blood cells as defined by expression of globin. However, if the same epiblast is co-cultured with VE from another embryo (green) it can form blood cell. Adapted from (Belaoussoff et al., 1998).

As noted earlier, in *Xenopus*, the non-cell autonomous signals from ectoderm are required before neurulation; similar to this requirement, mouse E6.75 mesodermal explants can differentiate as erythrocytes in culture suggesting that signals from the VE have been transmitted before this stage (Belaoussoff et al., 1998).

Interestingly, a signaling molecule *Indian hedgehog* (*Ihh*) is expressed in the VE (Farrington et al., 1997) and recombinant Ihh protein can restore the loss of VE in the explant assays (Dyer et al., 2001). These data suggest that *Ihh* might have inducing activity in the VE for the formation of erythropoietic precursors in the neighboring mesoderm. However, embryos deficient for *Ihh* show vascular defects but primitive erythroid cells looke normal, (Baron, 2005) suggesting that *Ihh* is not an essential factor in the VE for erythropoiesis and that there are other molecules also involved in this process.

The roles of ectodermal BMPs and CaM KIV in *Xenopus* primitive erythropoiesis.

In our lab, we have shown that BMP function is required non-cell autonomously, in the *Xenopus* ectoderm, for the differentiation of primitive blood cells (Walters et al., 2002). Specifically, when BMPs are downregulated in the blastomeres that give rise to the ectoderm, this causes defects in the differentiation of cells that give rise to primitive blood. We have also shown that calcium/calmodulin-dependent protein kinase IV (CaM KIV) antagonizes the BMP pathway in the ectoderm.

CaM KIV is predominantly a nuclear enzyme, which is dependent on the Ca^{2+}-binding protein calmodulin (Soderling, 1999). The second messenger Ca^{2+} plays its role by utilizing Ca^{2+}-binding proteins. One of the well-known intracellular Ca^{2+}-binding proteins is calmodulin (CaM) (Krebs, 1998). Calmodulin binds and activates many different enzymes, including protein kinases, protein phosphatases, adenylyl cyclases etc, but two of the best known are CaM-kinases and calcineurin (Soderling, 1999). To date, all CaM-dependent kinases identified are serine/threonine kinases which recognize the consensus sequence R-X-X-S/T (Anderson and Kane, 1998).

CaM kinases have a conserved catalytic domain adjacent to a regulatory region that consist of an autoinhibitory domain (AID) and the CaM-binding domain (CBD). In addition, CaM KIV has a unique serine-rich, amino-terminal

domain that might play a role in enzyme activation (Anderson and Kane, 1998). Analysis of CaM KIV from brain suggested activation of the enzyme is Ca^{2+}/CaM dependent, and this interaction triggers auto-phosphorylation of Thr196 (McDonald et al., 1993). Interestingly, comparisons of tissue-purified CaM KIV and CaM KIV expressed in cell culture show slower activation of CaM KIV isolated from primary tissues than that expressed in cultured cells. However, activation in tissue culture is improved when the cells are treated with brain extract (Krebs, 1998). This led to the discovery of CaM-dependent kinase kinases (Cam KK), and subsequent studies showed that CaM KK phosphorylates Thr196, which increases the activation of Cam KIV by 10-20 fold (Soderling, 1999).

Despite the identification of a consensus sequence on target substrates, the substrate specifity of CaM KIV is not clear. Nonetheless, CaM KIV is a major mediator of Ca^{2+} induced gene expression and it has been shown that it phosphorylates and regulates transcription factors such as activating transcription factor-1 (ATF-1), serum response factor (SRF), cAMP-responsive element-modulator τ (CREMτ), and cAMP-responsive element-binding protein (CREB) (Krebs, 1998).

CREB was first identified as a cAMP-dependent transcription factor that is activated by protein kinase A (PKA) (Gonzalez and Montminy, 1989). However, it has been shown that it can be also activated by Ca^{2+}/CaM-dependent kinases, and it can activate expression of genes containing a cAMP response element

(CRE) in their promoters. Activation of CREB requires the phosphorylation of Ser133 which is located in a region of CREB called the kinase-inducible-domain (KID). Subsequently, analysis of a human thyroid cDNA expression library identified a protein that specifically interacts with phosphorylated Ser133, called CREB-binding protein (CBP) (Chrivia et al., 1993). A region called the KID interaction (KIX) domain in the N-terminus of CBP binds to phosphorylated-CREB, and this interaction is important for CREB activity. Furthermore, one of the most important functions of CBP is that it has histone acetyltransferase (HAT) activity. Acetylation of histones leads to a more open configuration of chromatin. As a result, transcription factors that are important for the regulation of genes can access DNA. Therefore, CREB binds to CRE on target promoters and, when phosphorylated on Ser-133 can recruit CBP. At this point, the complex can modulate transcription of target genes. Recently, it has been shown that CBP not only acetylates histone proteins but also acetylates transcription factors such as GATA-1 and GATA-2, and other transcription factors (Blobel, 2000).

In our lab we provided evidence that either downregulation of BMPs or upregulation of Cam KIV in the *Xenopus* ectoderm caused a decrease in the number of circulating blood cells and this is due to the increase in red blood cell apoptosis. Moreover, we have shown that hematopoietic defects are caused by CaM KIV-mediated antagonism of BMPs and this is due to competition between the CaM KIV and BMP signaling pathways for the limited amount of CBP in the nucleus (Walters et al., 2002).

Interestingly, while constitutively active CREB (caCREB; a CREB molecule that can interact with CBP and thus activate transcription of CRE-containing reporter genes in the absence of phoshorylation, (Cardinaux et al., 2000) can phenocopy this defect, a mutant form of caCREB that cannot bind to the CRE (because it is DNA binding domain is replaced with GAL-4 DNA binding domain) also confers the same defects. In contrast, a form of CREB that is unable to bind CBP in the absence of phosphorylation has no effect on blood cells. Taken together, these data suggest that activation of CREB target genes is not involved in the CaM KIV-mediated suppression of BMPs and therefore not

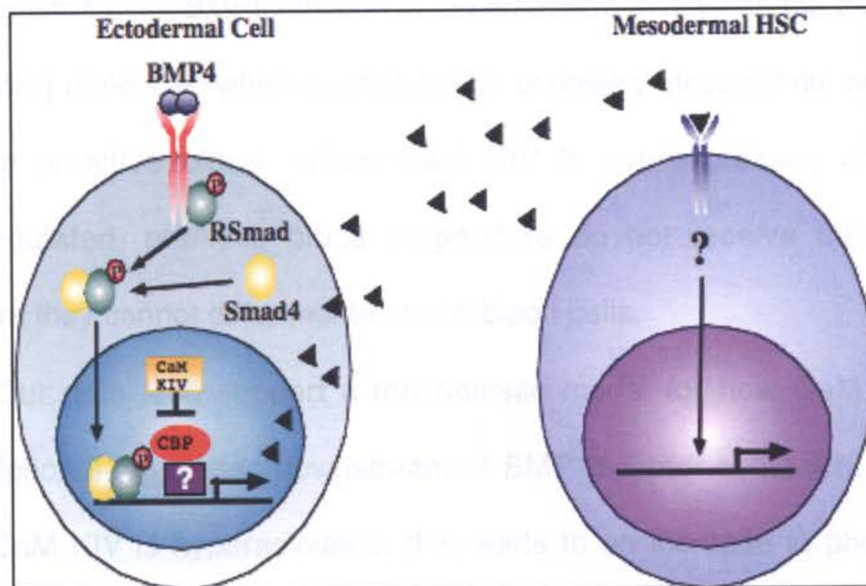

Figure 6. Model explaining the activation of the ectodermal signal that is required for the mesoderm to differentiate as primitive blood cells. BMPs (illustrated as BMP4) activate the transcription of a signaling molecule via Smads and CaM KIV antagonizes this transcriptional response. Therefore, ectodermal signals that are required for the mesoderm to differentiate as blood are tightly regulated by these two signaling pathways.

unable to bind CBP in the absence of phosphorylation has no effect on blood cells. Taken together, these data suggest that activation of CREB target genes is not involved in the CaM KIV-mediated suppression of BMPs and therefore not involved in hematopoiesis. Instead, we proposed that CaM KIV antagonizes BMP signaling by activating a substrate (CREB) that competes with the BMP signaling pathway for the limited amount of CBP. These data, along with the embryological evidence (as explained next) led us to develop a model to explain the role of ectodermal BMPs and CaM KIV during regulation of hematopoiesis (Fig. 6).

During gastrulation, BMPs function in the ectoderm to activate the transcription of a signaling molecule, which signals to the overlying mesodermal cells that give rise the primitive blood. When CaM KIV is overexpressed or BMPs are downregulated, primitive blood progenitors do not receive this signal and therefore they cannot differentiate as red blood cells.

Our data also support a mechanistic model for how CaM KIV inhibits transcriptional responses downstream of BMP receptor activation. Specifically, when CaM KIV is hyperactivated, this leads to an increase in phosphorylated CREB which then competes with the transcription factors that are activated downstream of BMPs (the Smads) for binding to CBP. It is unlikely that phosphorlyated CREB competes directly with Smads for binding to CBP, given that upregulation of CaM KIV did not cause defects such as dorsalization, which are associated with the loss of interaction between Smad and CBP (Kato et al.,

1999). However, it is likely that activated CREB blocks the interaction of CBP with a hematopoietic-specific transcription factor, which functions together with Smads. As illustrated in figure 7 there are two possibilities for how this transcription factor is involved in CaM KIV mediated antagonism of BMP signaling to regulate primitive hematopoiesis. First, it is possible that this hematopoietic transcription factor is a transcriptional target of BMPs and it competes for the CBP on its target genes promoter with CREB (Fig. 7a). Second, it is possible that it functions together with Smads on a common promoter(s) to activate common target gene(s) and CREB can compete with this transcription factor, but not with Smads, for binding to CBP (Fig. 7b).

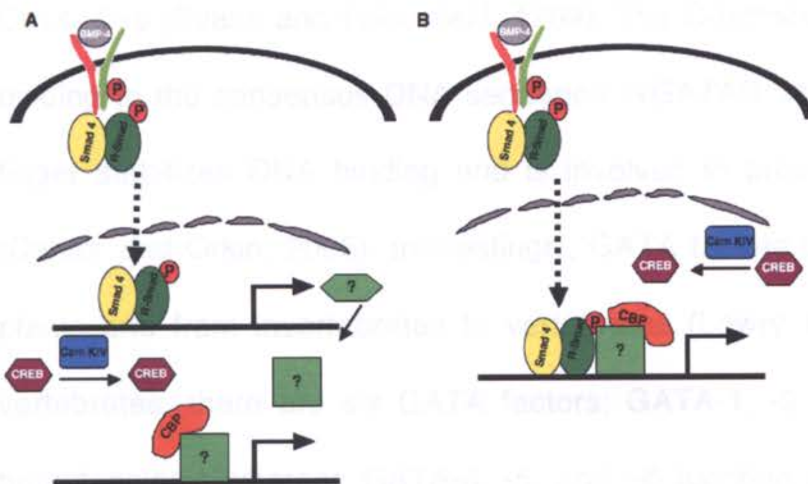

Figure 7. Model explaining the two different possibilities for how a transcription factor functions downstream of BMP receptor activation and is involved in CaM KIV mediated antagonism of BMP signaling during primitive hematopoiesis. (A) A BMP ligand (such as BMP4) binds to its receptor and Smads activate the synthesis of a transcription factor (green). The unidentified transcription factor then competes with CREB (or another CaM KIV substrate) for the CBP on its target gene promoter. (B) Alternatively, it can compete with CBP on a target gene promoter that functions together with Smads.

Transcription factors that are involved in hematopoiesis.

In *Xenopus* embryos, BMPs are involved in the activation of a number of hematopoietic-specific transcription factors, such as *GATA-1, GATA–2, SCL, LMO-2,* and *Xfli-1* (Sadlon et al., 2004). Among these, *GATA-2* is a direct target that is transcriptionally activated downstream of BMPsignaling (Friedle and Knochel, 2002).

The GATA family of transcription factors.

GATA transcription factors have two well-conserved zinc finger motifs, referred to as the amino (N)- and carboxy (C)-terminal zinc finger domains. These domains contain a consensus amino acid sequence of $CysX_2$ $CysX_{17}$ $CysX_2$ Cys (Evans and Felsenfeld, 1989). The C-terminal zinc fingers facilitate binding to the consensus DNA sequence WGATAR while the N-terminal zinc finger stabilizes DNA binding and is involved in protein-protein interactions (Cantor and Orkin, 2005). Interestingly, GATA factors are found from fungi to plants and from invertebrates to vertebrates (Lowry and Atchley, 2000). In vertebrates, there are six GATA factors; GATA-1, -2, and –3 have roles in hematopoiesis whereas GATA-4, -5, and –6 function in the formation of the heart, gut, liver, and gonads (Bresnick et al., 2005; Patient and McGhee, 2002). Both vertebrate and invertebrate GATA factors participate in protein complexes that either activate or repress transcription by binding promoter or enhancer regions of the target genes (Burch, 2005).

Distinct domains of GATA proteins have different functions during hematopoiesis. Mutational analysis in tissue culture cells has shown that the first 80 amino acids in the N-terminus of GATA-1 (NT) have strong trans-activation activity (Martin and Orkin, 1990). However, this N-terminal region is dispensable for erythroid and megakaryocytic cell differentiation (Blobel et al., 1995; Visvader et al., 1995; Weiss et al., 1997). In contrast, rescue analysis in GATA-1 mutant mice has shown that the C-terminal zinc finger is indispensable for both primitive and definitive hematopoiesis, while the N-terminal zinc finger and NT domains are both indispensable for definitive erythropoiesis (Shimizu et al., 2001).

The GATAs that are involved in hematopoiesis have unique mutant phenotypes. In mice, *GATA-1* is expressed in erythroid cells, mast cells, and megakaryocytes, and analysis of mutant mice shows that it is essential for erythropoiesis (Pevny et al., 1995; Pevny et al., 1991). Specifically, *GATA-1* null embryonic stem cells fail to contribute to adult red blood cells in chimeric mice, although they can contribute to all non-hematopoietic tissues and white blood cell fractions.

GATA-2 is highly expressed in early hematopoietic progenitors, mast cells, and megakaryocytes; moreover, it is also expressed in neural and endothelial cells. *GATA-2 -/-* ES cells fail to develop all hematopoietic lineages and homozygous mutant *GATA-2* mice die at E10.5 due to failure of both primitive and definitive hematopoiesis (Tsai et al., 1994). Analysis of these mutant mice revealed that no *GATA-2* homozygous mutant embryos survived beyond E11.5

(before fetal liver stage). Although mutant embryos looked nearly normal, their yolk sac blood vessels were almost empty. Analysis of blood derived from the yolk sac of E13 chimeras (generated by injecting GATA-2 -/- ES cells into wild type blastocysts) showed that erythrocytes were predominantly nucleated. Since primitive, but not definitive erythrocytes remain nucleated, these data suggest that *GATA-2 -/-* chimeras have primitive hematopoietic potential. In contrast, analysis of hematopoietic colonies from the hematopoietic organs (such as bone marrow, spleen, thymus) of *GATA-2 -/-* chimeras at 1-3 months of age showed that *GATA-2 -/-* cells failed to contribute to the blood cells produced by these organs (Tsai et al., 1994). These data suggest that *GATA-2 -/-* cells are impaired with respect to their ability to contribute to hematopoiesis and *GATA-2* is essential for definitive hematopoiesis.

The primitive hematopoietic potential of *GATA-2* chimeras could be due to compensation by *GATA-1*. Evidence for this comes from a recent study showing that *GATA-1/GATA-2* double knockout mice exhibit almost no blood cells in the yolk sac and embryos die at midgestation from anemia (Fujiwara et al., 2004). This phenotype is more severe compared to either single knockout and suggests that *GATA-1* and *GATA-2* functionally overlap during primitive hematopoiesis. However, *in vitro* colony assays from the yolk sac of single and double knockouts suggested that loss of *GATA-2* causes more severe defects than does loss of *GATA-1* in definitive hematopoiesis.

Interestingly, while *GATA-1* null ES cells failed to produce primitive and definitive erythroid cells, they showed increased levels of *GATA-2* transcripts, suggesting that *GATA-1* suppresses the expression of *GATA-2* during erythroid development (Weiss et al., 1994). Consistent with this possibility, *GATA-1*-null G1E cells (immature erythroid cells derived from GATA-1-null embryonic stem cells) express endogenous *GATA-2* (Weiss et al., 1997), and induction of expression of GATA-1 in these cells, using a hormone inducible GATA-1 ER (Estrogen Receptor) construct, represses *GATA-2* transcription (Crispino et al., 1999; Grass et al., 2003). It has been suggested that GATA-1 binds to the GATA-2 promoter upstream of where GATA-2 normally binds and displaces GATA-2, thereby blocking auto-regulation of GATA-2 (Grass et al., 2003).

GATA-3 plays a slightly different role in hematopoiesis. It is highly expressed in T-lymphocytes and it is also expressed at lower level, in mast cells and in the developing brain. *GATA-3* mutants die at E11-12 due to internal bleeding and CNS deformation (Pandolfi et al., 1995).

GATA factors and human diseases.

Until now, no reports have shown inherited diseases resulting from a mutation in *GATA-2*. However, it has been shown that the *GATA-2* gene is located close to the breakpoint of 3q21 in myeloid leukemia. Some of the patients with 3q21 rearrangement displayed increased levels of GATA-2 transcripts. Since overexpression of *GATA-2* is seen in acute myeloid leukemia (AML)

irrespective of detectable 3q21 rearrangement, it is still possible that some other mechanism is involved in the misregulation of GATA-2 in these patients (Wieser et al., 2000).

One of the best characterized co-factors for GATA-1and GATA-2 is called "friend of GATA-1" (FOG-1). The critical residues for the GATA-1/FOG-1 interaction have been identified (Crispino et al., 1999), and the N-terminal zinc finger of GATA-1 is responsible for this interaction. Missense mutations in the N-terminal zinc finger of GATA1, such as substitution of valine 205 with methionine (V205M) disrupt the GATA-1/FOG interaction and result in abnormal hematopoiesis. For example, the V205M mutation in human *GATA-1* is responsible for dyserythropoietic anaemia and thrombocytopenia (Nichols et al., 2000). In addition, several mutations in the genes that are regulated by GATA-1 have been shown to be related to human hereditary disease (Ohneda and Yamamoto, 2002). Such diseases are delta-thalassemia, Bernard-Soulier syndrome, pyruvate kinase deficient anemia and congenital erythropoietic porphyria.

Mutations in the human GATA-3 gene have been shown to be responsible for HDR syndrome (hypoparathyroidism, sensorineural deafness, and renal dysplasia) (Muroya et al., 2001). Finally, a heterozygous G296S missense mutation of the GATA-4 gene is responsible for congenital heart defects (Garg et al., 2003).

Hematopoietic GATA transcription factors in *Xenopus*.

Xenopus homologs of hematopoietic GATA transcription factors have been isolated (Zhang and Evans, 1994; Zon LI et al., 1991). There are two *GATA-1* (*xGATA-1a* and *xGATA-1b*) genes in *Xenopus laevis*, which encode functional proteins that are 89% identical and show a similar binding specificity (Zhang and Evans, 1994). The xGATA-1 transcripts are detected at late gastrula, but the majority of the transcripts begin to accumulate at the neurula stage before the expression of *globin*, which is a differentiation marker for red blood cells. The transcripts are localized to the ventral region that gives rise to the VBI of the embryo. The *xGATA-1a* gene contributes to the majority of the *xGATA-1* expression during VBI formation. In contrast, the *xGATA-1b* gene contributes mostly to the GATA-1 transcripts in adult erythrocytes (Zhang and Evans, 1994). This observation suggests that these two GATA-1 genes are regulated differentially. Consistent with this, there are sequence differences in the proximal promoter regions, and the transcription start sites of the two genes are not conserved. These data suggest that cis-regulatory sequences are different between the two genes and therefore the microenvironment directs their transcription differently. As noted earlier, *GATA-1* is required for the differentiation of red blood cells (Weiss et al., 1994) and functions late in hematopoiesis.

In contrast to GATA-1, other members of the GATA family such as *GATA-2* function early in hematopoiesis during specification and proliferation of progenitor cells. The expression pattern of *GATA-2* in *Xenopus* correlates with the fact that it functions prior to GATA-1 (Bertwistle et al., 1996; Kelley et al., 1994; Walmsley et al., 1994). Maternal transcripts of GATA-2 are uniformly distributed. The expression of zygotic GATA-2 starts at gastrulation, predominantly in the ectoderm and less so in the mesoderm. During neurulation, GATA-2 transcripts are detected in the non-neural ectoderm and also in the ventral mesoderm, where blood precursors are located (Walmsley et al., 1994). *GATA-2* expression continues in the ventral region until the blood island is formed, when its expression is blocked by GATA-1. However, *GATA-2*s' expression is also detected in the dorsal lateral plate (DLP), which gives rise to definitive blood cells, where expression of *GATA-1* is absent. Finally, the expression of *GATA-2* is also detected in the central nervous system (Bertwistle et al., 1996; Walmsley et al., 1994). Although it has been established that mesodermal GATA-2 has a role in the development of blood progenitors, until now there has been no clear evidence for a role for ectodermal *GATA-2* in the regulation of primitive hematopoiesis. One of the aims of this thesis is to identify to role of ectodermal GATA-2 in this process.

The GATA transcription factors regulate the transcription of many other factors that are important during hematopoietic development. One such factor is the stem cell leukaemia protein (Scl). An enhancer region that is responsible for

the activation of *Scl* has been identified, and it has been shown that several transcription factors regulate the activation of Scl, including GATA-2 (Gottgens et al., 2002).

The transcription factor SCL

The *Scl* gene (also known as TAL-1) encodes a basic helix-loop-helix (bHLH) protein, and it is expressed in the progenitors of blood and endothelium (haemangioblasts) and in the central nervous system (Davidson and Zon, 2000). During normal hematopoiesis, *Scl* is expressed in red blood cells, mast cells, megakaryocytes and early CD34+ hematopoietic progenitors. *Scl* binds DNA as a heterodimer to an E-box site (CANNTG sequence) and the lim-domain protein-2 (LMO-2) has been shown to heterodimerize with Scl, allowing it to transactivate target genes (Wadman et al., 1994).

Analysis of *Scl* -/- mice and chimeras suggested a crucial role for *Scl* in the regulation of both primitive and definitive hematopoiesis (Robb et al., 1996; Robb et al., 1995; Shivdasani et al., 1995). Morpholino knockdown analysis of Scl in zebrafish suggested a similar role for Scl in hematopoiesis in zebrafish (Juarez et al., 2005; Patterson et al., 2005). Interestingly, in zebrafish Scl hypomorph embryos, erythroid maturation was deficient; however, erythroid progenitors expressing GATA-1 and embryonic globin did develop. This finding suggests that erythroid maturation steps require higher Scl levels than erythroid specification (Juarez et al., 2005).

Another zebrafish mutant called *cloche* has deficits only in hematopoietic and endothelial cells (Stainier et al., 1995). This mutant shows hematopoietic defects at very early stages of blood specification however, the identity of the gene that is defective in this mutant is not known. The *cloche* mutant fails to expresses *Scl*, *GATA-1*, and *GATA-2* (Stainier et al., 1995), suggesting that *cloche* functions upstream of these transcription factors. Consistent with this observation, overexpression of *Scl* in the *cloche* mutant rescues the blood and vascular defects (Liao et al., 1998).

In *Xenopus*, a homolog of *Scl* has been isolated, and during embryogenesis it is express from the late-gastrula until tadpole stages. The expression pattern of *Scl* correlates with its role in hematopoiesis and vasculogenesis. It is expressed in the ventral mesoderm and has been proposed to specify the hematopoietic mesoderm. Later, it is expressed in the VBI and in the nervous system, specifically in the mid-hindbrain boundary (Mead et al., 1998). In animal caps, overexpression of *BMP-4* induces the expression of *Scl*. Overexpression of *Scl* alone is not sufficient to induce hematopoiesis in isolated *Xenopus* ectodermal explants (animal caps), but it can induce expression of hematopoietic specific genes when the explant is treated with bFGF (Mead et al., 1998). Taken together, these data suggest that *Scl* can specify the hematopoietic mesoderm.

CHAPTER 2

GATA-2 functions downstream of BMPs and CaM KIV in ectodermal cells during primitive hematopoiesis.

Gokhan Dalgin, Riffat Ahmed, Devorah C. Goldman and Jan L. Christian

(Manuscript is under revision)

Department of Cell and Developmental Biology

Oregon Health and Science University, School of Medicine

3181 SW Sam Jackson Park Road

Portland, OR 97239-3098

Abstract

In *Xenopus*, primitive blood originates primarily from the ventral mesoderm, but extrinsic signals from the ectoderm are required during gastrulation to enable these cells to differentiate as primitive erythrocytes. The nature of these signals, and how they are transcriptionally regulated, is not well understood. We have previously shown that bone morphogenetic proteins (BMPs) are required to signal to ectodermal cells to generate secondary non-cell autonomous signal(s) necessary for primitive erythropoiesis, and that calmodulin-dependent protein kinase IV (CaM KIV) antagonizes BMP signaling. The current studies demonstrate that *GATA-2* functions downstream of BMP receptor activation in these same cells, and is a direct target for antagonism by CaM KIV. We show, using loss of function analysis in whole embryos and in explants, that ectodermal *GATA-2* is required for the differentiation and/or survival or erythroid progenitors. Furthermore, we provide evidence that acetylation of GATA-2 is required for its function in primitive blood formation in vivo. Our data support a model in which *GATA-2* is a transcriptional target downstream of BMP receptor activation within ectodermal cells, while activation of the CaM KIV signaling pathway alters GATA-2 function posttranslationally, by inhibiting its acetylation.

Keywords: GATA-2, *Xenopus laevis*, hematopoiesis, erythropoiesis, bone morphogenetic protein, CaM KIV, CBP, acetylation

Introduction

In vertebrates, embryonic hematopoiesis occurs in two distinct phases. The first phase, known as primitive hematopoiesis, occurs during early embryogenesis and gives rise primarily to red blood cells (RBCs), but also some white blood cells (WBCs). Primitive blood is generated in the extraembryonic yolk sac in mammalian embryos, and in the intraembryonic ventral blood island (VBI) in *Xenopus laevis* (Galloway and Zon, 2003). The second phase of hematopoiesis is known as definitive hematopoiesis and generates multipotent hematopoietic stem cells. Definitive hematopoiesis is initiated in the aorta, gonads and mesonephros region in mammals (Cumano et al., 1996; Godin et al., 1993; Medvinsky and Dzierzak, 1996; Medvinsky et al., 1993) and in an analogous region, the dorsal lateral plate in *Xenopus* (Kau and Turpen, 1983; Maeno et al., 1985; Weber et al., 1991).

Embryological experiments have shown that signals derived from non-hematopoietic tissues are essential for normal primitive erythropoiesis (Baron, 2003). In *Xenopus*, numerous lines of evidence suggest that ectodermal cells, which come into contact with underlying ventral mesoderm during gastrulation, serve as an important source of such signals (Maeno, 2003). For instance, when embryos are induced to exogastrulate, which prevents the prospective ectoderm from coming into contact with the mesoderm, primitive erythrocytes fail to differentiate although other mesodermal cell types develop normally (Kikkawa et al., 2001). Furthermore, when ventral mesoderm, which gives rise to the bulk of

the posterior VBI (Ciau-Uitz et al., 2000; Lane and Sheets, 2002; Lane and Smith, 1999; Mills et al., 1999), is explanted at the early gastrula stage (stage 10) and cultured in isolation to the tailbud stage, it fails to form blood cells (Maeno et al., 1994b; Maeno M, 1992). Blood formation can be restored, however, by co-culturing the ventral mesoderm with ectoderm isolated from late blastula or gastrula stage embryos (Maeno et al., 1994b). By the early neurula stage (stage 16), ventral mesodermal explants can differentiate as RBCs when cultured in isolation, suggesting that extrinsic signals essential for hematopoiesis are transmitted between early gastrula and neurula stages (Maeno et al., 1994b).

We have shown that cross talk between the bone morphogenetic protein (BMP) and calmodulin-dependent kinase IV (CaM KIV) pathways within ectodermal cells is required for normal primitive erythropoiesis (Walters et al., 2002). When BMP signal reception is blocked within ectodermal cells, or when CaM KIV is constitutively activated within these same cells, hematopoietic mesoderm is specified normally but fewer differentiated RBCs are present, due at least in part to an increase in apoptosis. Further analysis revealed that CaM KIV functions downstream of BMP receptor activation to inhibit hematopoiesis.

Our previous studies suggested a molecular mechanism for cross talk between the BMP and CaM KIV pathways. CaM KIV functions predominantly in the nucleus where it phosphorylates a number of transcription factors, the best characterized of which is CREB (reviewed by (Soderling, 1999). Once phosphorylated, CREB recruits the transcriptional co-activator, CREB binding

protein (CBP), and this binding is sufficient to activate transcription of target genes (Cardinaux et al., 2000). We found, however, that the ability of CaM KIV to inhibit hematopoiesis did not require activation of CaM KIV target genes. Instead, our data support a molecular model in which CaM KIV inhibits the BMP pathway by activating a substrate that competes with transcriptional complexes downstream of BMP receptor activation for binding to limiting amounts of CBP.

Given that CBP is an essential cofactor for the BMP receptor activated transcription factors, Smad 1, 5 and/or 8 (Massague and Wotton, 2000), one simple possibility is that a CaM KIV substrate competes directly with these Smads for binding to CBP, thereby blocking transcription of all BMP target genes. Inconsistent with this scenario, however, constitutive activation of the CaM KIV pathway in *Xenopus* embryos selectively inhibits the ability of BMP to transduce signals that are required for hematopoiesis, but does not interfere with BMP-mediated specification of epidermal fate (Walters et al., 2002), which is known to require Smad/CBP interactions (Kato et al., 1999). These findings suggest the involvement of a distinct, hematopoietic specific transcription factor that functions downstream of BMP receptor activation and is the direct target for CaM KIV mediated antagonism.

GATA-2 is an excellent candidate for a transcription factor that functions in ectodermal cells in concert with BMPs and CaM KIV during hematopoiesis. GATA-2 is a zinc finger transcription factor that recruits CBP as a cofactor to activate target genes (Blobel et al., 1998). Loss of function studies in mice have

demonstrated that *GATA-2* is essential for both primitive and definitive hematopoiesis (Fujiwara et al., 2004; Tsai et al., 1994). In *Xenopus*, zygotic expression of *GATA-2* is first detected at the early gastrula stage in ectodermal cells and later expands into the ventral mesoderm (Kelley et al., 1994; Walmsley et al., 1994; Zon LI et al., 1991).

Several lines of evidence suggest that *GATA-2* is a direct target gene downstream of BMPs. First, inhibition of BMP activity in whole *Xenopus* embryos leads to downregulation of *GATA-2* expression in the mesoderm, and less so in the ectoderm (Maeno et al., 1996; Walmsley et al., 1994). Second, BMP-4 can induce expression of *GATA-2* in whole *Xenopus* embryos in the absence of new protein synthesis suggesting a direct transcriptional mechanism, although it is not clear whether this induction occurs in mesodermal cells, ectodermal cells or both (Friedle and Knochel, 2002).

A potential role for ectodermally derived *GATA-2* in blood development has been suggested based on ectopic expression studies. Specifically, ventral mesoderm explanted from a gastrula stage embryo can differentiate as blood if it is co-cultured with ectoderm isolated from a mid-blastula stage (stage 8) or older embryo but not if it is co-cultured with ectoderm isolated at stage 7 (Maeno et al., 1996; Maeno et al., 1994b). Ectopic expression of GATA-2, however, is sufficient to make ectodermal cells isolated from stage 7 embryos competent to support blood formation in co-cultured ventral mesoderm (Maeno et al., 1996).

In the present study, we present loss of function data in whole embryos and explants showing that ectodermal *GATA-2* is required to activate a secondary signal that acts across germ layers and is essential for the differentiation and/or survival or erythroid progenitors. Furthermore, we provide evidence that *GATA-2* functions downstream of BMP receptor activation and is a direct target for antagonism by CaM KIV. Our results support a model in which CaM KIV activates a substrate that binds to, and prevents CBP from acetylating, GATA-2 thereby inhibiting its ability to activate transcription of target gene(s).

Materials and Methods

Embryo culture and manipulation

Ovulation was induced by injecting female frogs with human chorionic gonadotropin (Sigma). Embryonic stages are according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967). Capped synthetic mRNA was synthesized by in vitro transcription of linearized template cDNAs using a MegaScript kit (Ambion) and injected into embryos as described previously (Moon and Christian, 1989). Embryo explants and recombinants were generated and cultured in half strength normal amphibian medium (NAM/2; Peng et al., 1991) as described (Goldman et al., 2006).

Morpholinos and cDNA constructs

Morpholino antisense oligonucleotides (MOs) complimentary to two different alleles of GATA-2 (GATA-2a: 5'-CTTCCATCGCAGGAGCAAAGTTC TC and GATA-2b: 5'-GGTCAGTAGCCACTTCCATTGCAGG) and a standard control MO were purchased from Gene Tools, LLC (Philomath, OR). Sequence encoding an HA epitope tag was appended to the 3' end of the GATA-2 open reading frame by PCR mediated amplification. The GATA-2 HA cDNA was amplified by PCR using an oligonucleotide containing silent mutations (5'*ATG*GAGGTTGCCACCGAT; silent mutations in bold and translation start site in italic) that prevent annealing of the GATA-2 MOs to generate rGATA-2 HA. cDNAs were subcloned into pCS2+ for RNA transcription. Mutation of Serine 385 and of putative acetylated lysine residues was accomplished using the PCR-based splicing by overlap extension technique (Horton et al., 1990) and appropriate primers. Regions of cDNAs generated by PCR were sequenced.

Northern blot and protein analysis

Total RNA was isolated and Northern blots were hybridized with antisense riboprobes as described previously (Christian and Moon, 1993). Bands were visualized with a phosphoimager and quantified using the Macintosh IP lab gel program.

Xenopus embryos were lysed and subject to Freon extraction as described (Moon and Christian, 1989). Proteins were separated by

electrophoresis on an 11% polyacrylamide gel and transferred onto PVDF membrane. Membranes were probed with anti-HA (3F10; Roche) antibody and immunoreactive proteins detected by chemiluminescence. Whole mount immunostaining was performed as described (Moon and Christian, 1989).

Collection and analysis of peripheral blood samples.

Tails were severed from tadpoles and blood collected into medium containing 0.7xPBS, 0.5% BSA and 10 IU/ml of heparin. Cells were concentrated onto slides using a cytocentrifuge and stained with a Hema 3 stain set (Fisher Diagnostics). A minimum of 24 embryos were bled per experimental group and, for each embryo, the number of cells in five random fields was counted at 40X magnification. Each experiment was repeated a minimum of two times and results were pooled. Statistical analysis was performed as described (Wayman et al., 2000). 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 cell number.

Results

Downregulation of GATA-2 inhibits primitive hematopoiesis.

To begin to test whether ectodermal *GATA-2* is required for primitive hematopoiesis, we designed antisense morpholino oligonucletides (MOs) that could be used to block translation of endogenous *GATA-2* mRNAs. Western blot

analysis showed that GATA-2 MOs, but not control MOs, efficiently blocked translation of RNA encoding an HA-epitope tagged version of GATA-2 (GATA-2 HA) when the two were co-expressed in *Xenopus* embryos (Fig. 1A). By contrast, GATA-2 MOs did not block translation of a rescue construct (rGATA-2 HA) containing silent mutations that prevent annealing of the MOs (see Materials and Methods).

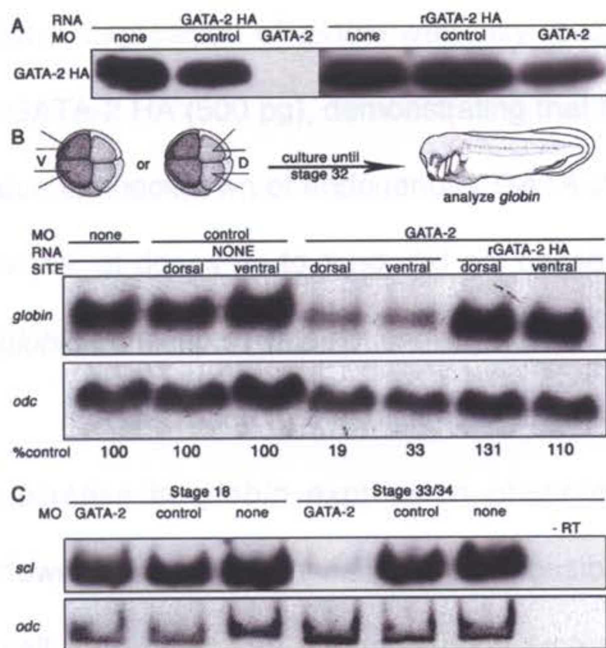

Figure. 1. GATA-2 function is required for differentiation but not specification of erythrocytes in *Xenopus*. (A) Anti-HA immunoblot showing that GATA-2 MOs can block the translation of GATA-2 HA mRNA in vivo but have no effect on translation of rGATA-2 HA RNA that has been modified to prevent the MOs from annealing. Three embryo equivalents were loaded in each lane. (B) Two dorsal or ventral animal pole blastomeres of eight-cell embryos were injected with GATA-2 or control MOs alone or together with rGATA-2 HA RNA as illustrated and expression of *globin* was analyzed by Northern blot at stage 32. Levels of *globin* transcripts, normalized relative to levels of *odc* transcripts, are expressed as a percentage of control below each lane. (C) GATA-2 morpholinos were injected into dorsal animal pole cells of eight-cell embryos and expression of *scl* was analyzed by RT-PCR at stages 18 and 33. Reverse transcriptase was omitted from one set of samples (-RT) as a control for genomic contamination.

GATA-2 or control MOs (6 ng) were then targeted to prospective dorsal or ventral ectodermal blastomeres of eight-cell embryos, as illustrated in Fig. 1B. These blastomeres make only a minor contribution, if any, to erythroid cells in the VBI (Lane and Smith, 1999; Mills et al., 1999). Embryos were cultured until stage 32 at which time expression of *globin*, a marker of differentiated RBCs, was assayed by Northern blot analysis. Embryos injected with GATA-2 MOs showed a severe reduction in expression of *globin* relative to those injected with control MOs (Fig. 1B). Expression of *globin* was fully rescued by coinjection of RNA encoding rGATA-2 HA (500 pg), demonstrating that the observed phenotype is specifically due to knockdown of endogenous *GATA-2* translation. Injection of rGATA-2 HA alone, at doses up to 1 ng, did not cause an increase or decrease in levels of *globin* transcripts (data not shown).

A decrease in the initial specification of blood cells could account for the decrease in *globin* expression observed when expression of *GATA-2* is downregulated. To investigate this possibility, we analyzed expression of *stem cell leukemia* (*scl*), a transcription factor that is expressed in hematopoietic mesoderm early in embryogenesis and is proposed to specify hematopoietic fate (Mead et al., 1998). GATA-2 MOs were injected into dorsal animal pole blastomeres of eight-cell embryos and expression of *scl* was analyzed by RT-PCR. GATA-2 morphants showed normal levels of *scl* transcripts at neurula stage 18 (Fig. 1C) but by tailbud stage 33, morphants showed a severe loss of expression of *globin* (data not shown) and *scl* (Fig. 1C) relative to uninjected or

control MO injected siblings. Taken together, these data suggest that expression of *GATA-2* within ectodermal cells is required for normal differentiation of primitive RBCs in *Xenopus*, but not for the specification of hematopoietic fate

GATA-2 function is required non cell-autonomously in the ectoderm to regulate erythropoiesis.

A tissue recombination assay was used to more stringently test whether GATA-2 function is required in ectodermal, as opposed to mesodermal cells for normal primitive erythropoiesis. Previous studies have shown that explanted gastrula stage ventral mesoderm cannot differentiate as RBCs unless it is co-cultured with ectoderm (Maeno et al., 1996; Maeno et al., 1994b). To determine whether GATA-2 function is required within these ectodermal cells, we recombined gastrula stage ventral mesoderm with ectoderm isolated from embryos injected with either control, or GATA-2 MO's (10 ng), and analyzed expression of *globin* at the tailbud stage, as illustrated in Fig. 2A. *Globin* was not expressed in ventral mesodermal explants cultured in the absence of ectoderm (Fig. 2B). *Globin* was robustly expressed in recombinants of ventral mesoderm co-cultured with ectoderm isolated from control embryos, but was barely detectable in explants of ventral mesoderm cultured with ectoderm isolated from GATA-2 morphants. Downregulation of GATA-2 within the ectodermal component of recombinants had no effect on expression of the mesodermally derived myeloid marker gene, *xpox2* (Smith et al., 2002), demonstrating that

ectodermally derived GATA-2 is not required for mesoderm differentiation per se, or for normal differentiation of myeloid cells. These data demonstrate that *GATA-2* function is required within ectodermal cells to enable ventral mesoderm to differentiate as RBCs.

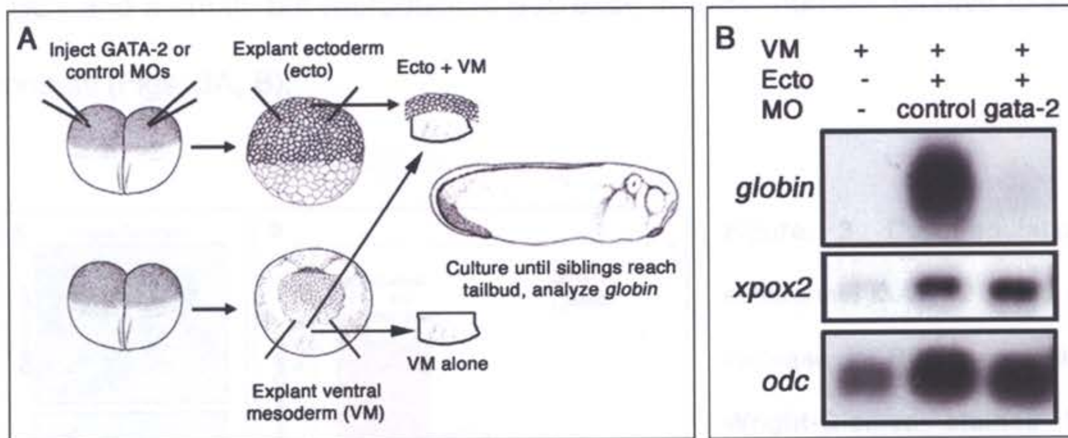

Figure 2. Ectodermally derived GATA-2 is required for normal erythropoiesis. (A) Experimental design is illustrated schematically. (B) Ectoderm (ecto) explanted from embryos injected with either control or GATA-2 MOs was co-cultured with explanted ventral mesoderm (VM) until siblings reached the tailbud stage at which time expression of *globin, odc* and *xpox2* was analyzed by Northern hybridization.

Downregulation of ectodermal GATA-2 leads to an increase in RBC apoptosis

Peripheral blood samples from GATA-2 deficient tadpoles were examined to determine whether downregulation of GATA-2 phenocopies the loss of RBCs and increased apoptosis observed when BMP function is blocked, or CaM KIV is constitutively activated in ectodermal cells (Walters et al., 2002; Wayman et al.,

2000). GATA-2 or control MOs (6 ng) were injected into two dorsal or ventral animal pole blastomeres of eight-cell embryos to preferentially target ectodermal cells. Embryos were cultured to stage 42 and peripheral blood cells from a minimum of 24 embryos in each experimental group were collected onto slides and counted. GATA-2 morphants showed a dramatic decrease in the number of RBCs and a small, but reproducible decrease in WBC number relative to sibling controls (Figs. 3A, B).

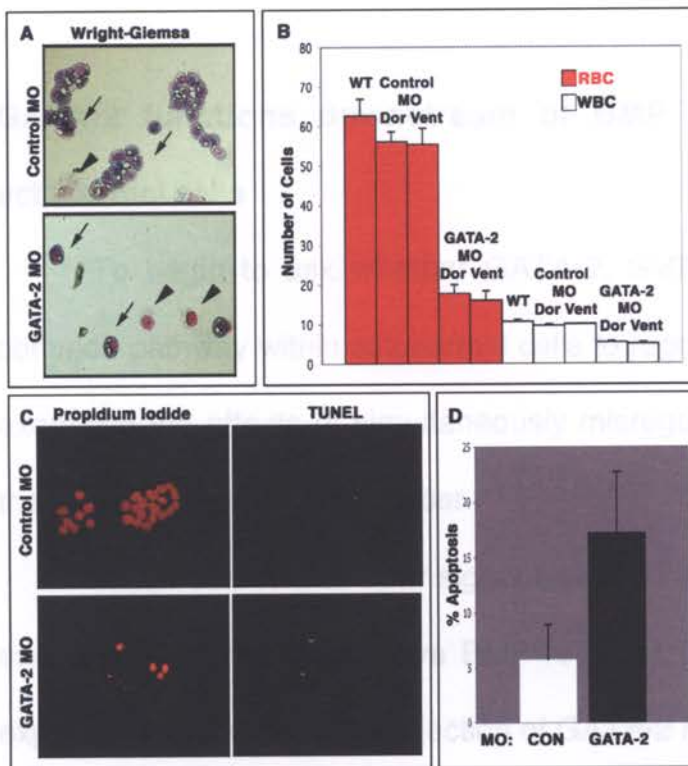

Figure. 3. Downregulation of ectodermal GATA-2 leads to an increase in RBC apoptosis (A) Wright-Giemsa stained blood collected from control or GATA-2 morphant tadpoles. Arrows indicate RBCs, arrowheads indicate WBCs. (B) Mean number (+/- s.e.m.) of RBCs (red bars) and WBCs (white bars) present in control tadpoles or tadpoles derived from embryos in which GATA-2 MOs had been injected into dorsal (dor) or ventral (vent) animal pole blastomeres at the eight-cell stage. Results are pooled from three independent experiments. (C) TUNEL analysis of apoptotic cells. Nuclei were stained with propidium iodide and TUNEL positive cells are in green. (C) Percentage apoptotic cells (mean +/- s.e.m.) determined by counting total and TUNEL-positive cells. Results are pooled from two experiments.

To test the possibility that the decrease in RBC number was due to an increase in programmed cell death, we used the TUNEL assay to quantitate apoptotic blood cells. As shown in Figs. 3C and D, inhibition of GATA-2 expression caused a significant increase in the fraction of blood cells that were apoptotic. Thus, loss of GATA-2 phenocopies the hematopoietic defects observed following downregulation of BMP signaling, or upregulation of CaM KIV activity, consistent with our hypothesis that GATA-2 functions in the same, or a parallel pathway to these signaling cascades.

GATA-2 functions downstream of BMP and CaM KIV signaling in ectodermal cells

To begin to ask whether GATA-2, BMPs and CaM KIV function in a common pathway within ectodermal cells to regulate primitive erythropoiesis, we examined the effects of simultaneously misregulating expression or function of these molecules on RBC number.

When ectodermal BMP signal reception was blocked by injection of RNA encoding a dominant negative BMP receptor (tBR) (400pg) or when GATA-2 expression was reduced by injection of GATA-2 MOs into two dorsal blastomeres of eight-cell embryos, the number of circulating RBCs was reduced by approximately 50% in each case (Fig. 4A). Simultaneous inhibition of BMP function and GATA-2 expression led to an additive decrease in RBC number to approximately 25% of controls. A similar additive decrease in RBC number was

observed when CaM KIV was upregulated and GATA-2 was simultaneously downregulated. As shown in Fig. 4B, co-injection of RNAs encoding constitutively active forms of CaM KIV (50 pg) and its upstream kinase, CaM KK (together referred to as KIVc) (50 pg) together with GATA-2 MOs (6ng) led to an additive loss of RBCs relative to that observed when either pathway was perturbed individually. These data are consistent with the possibility that GATA-2 acts in the same or a parallel pathway to BMPs and/or CaM KIV to regulate ectodermally derived signals that are essential for primitive erythropoiesis.

To test the possibility that GATA-2 functions downstream of BMPs, we asked whether overexpression of GATA-2 in ectodermal cells could rescue hematopoietic defects caused by blockade of BMP signaling in the same cells. Embryos injected with RNA encoding tBR (400pg) showed a greater than 50% reduction in RBC number and this was completely rescued by co-injection of RNA encoding GATA-2 HA (500 pg) (Fig. 4C).

The ability of GATA-2 to rescue erythropoiesis in BMP deficient embryos is not compatible with a model in which ectodermal GATA-2 functions as an obligatory hematopoietic specific component of a transcriptional complex together with Smad1, 5 and/or 8 downstream of BMP receptor activation. A more likely possibility is that BMPs activate transcription of *GATA-2* within ectodermal cells, and that GATA-2 then activates transcription of a unique subset of genes that are required for mesoderm to differentiate as blood.

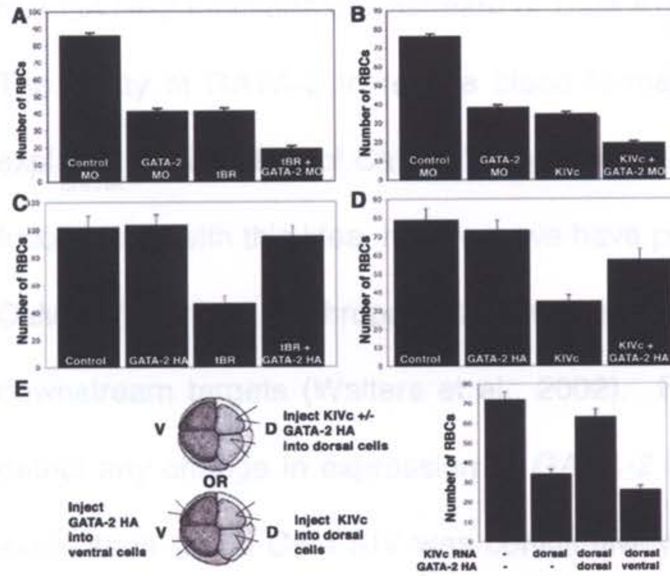

Fig. 4. GATA-2 functions downstream of BMP and CaM KIV signaling in ectodermal cells (A-D) Mean number (+/- s.e.m.) of RBCs present in control tadpoles, or tadpoles made to express GATA-2 MOs, a dominant mutant form of the BMP receptor (tBR), constitutively active CaM KIV (KIVc) and/or GATA-2 HA RNA as indicated. Combined results from at least two independent experiments are shown. (E) Experimental design is shown schematically. Mean number (+/- s.e.m.) of RBCs present in control tadpoles, or tadpoles in which CaM KIV signaling was upregulated in dorsal cells followed by injection of GATA-2 RNA into either the same cells, or cells on the opposite side of the embryo as indicated. Results are pooled from three independent experiments.

Consistent with this possibility, previous studies have shown that BMPs are required for transcription of *GATA-2* in the ventral mesoderm, and possibly also in the ectoderm (Friedle and Knochel, 2002; Maeno et al., 1996; Walmsley et al., 1994).

To ask whether GATA-2 also functions downstream of CaM KIV, we injected embryos with RNA encoding constitutively active components of the CaM KIV signaling cascade (50 pg each) either alone, or together with RNA encoding GATA-2 HA (500 pg). As shown in Fig. 4D, GATA-2 partially rescued the loss of RBCs caused by constitutive activation of CaM KIV, demonstrating

that GATA-2 functions downstream of CaM KIV during primitive hematopoiesis. The ability of GATA-2 to rescue blood formation in these embryos might be explained if activation of CaM KIV negatively regulates transcription of *GATA-2*. Inconsistent with this idea, however, we have previously shown that the ability of CaM KIV to inhibit erythropoiesis is independent of transcription mediated by its downstream targets (Walters et al., 2002). Furthermore, we were unable to detect any change in expression of *GATA-2* in whole embryos or ectodermal explants in which CaM KIV was constitutively activated (data not shown). An alternate possibility, that is consistent with our previously published data, is that CaM KIV activates a substrate that competes with GATA-2 for binding to limiting amounts of CBP. The inability to recruit CBP to the promoter of GATA-2 target genes would then lead to a loss of histone acetylation, and thus gene silencing. This scenario also seems unlikely, however, since only a small fraction of overexpressed GATA-2 HA would be bound to the promoters of target genes and this small pool would not effectively compete with unbound GATA-2 for the limiting amounts of CBP that are available in the nucleus. A final possibility, which we favor based on our results presented below, is that activation of CaM KIV causes GATA-2 to be post-translationally modified in a way that negatively regulates its activity, and excess GATA-2 is able to overcome this negative regulation.

If GATA-2 is a direct target of CaM KIV as proposed, then the ability of ectopic GATA-2 to rescue hematopoietic defects caused by activation of CaM

KIV should be strictly cell autonomous. To test this possibility, CaM KIV activity was upregulated in two dorsal ectodermal cells of eight-cell embryos and RNA encoding GATA-2 HA was subsequently injected into these same two cells, or into two ventral ectodermal cells, as illustrated in Fig. 4E. GATA-2 was able to rescue the loss of RBCs caused by constitutive activation of CaM KIV only when it was expressed in the same cells in which CaM KIV signaling was upregulated. Taken together, these data demonstrate that GATA-2 functions downstream of both BMPs and CaM KIV. Futhermore, our results imply that GATA-2, rather than the BMP pathway, is the direct target of negative regulation by CaM KIV.

A putative CaM KIV phosphorylataion site in GATA-2 does not appear to regulate nuclear localization or function

Recent studies have shown that phosphorylation of human GATA-2 at a potential CaM KIV phosphorylation site (serine 401; illustrated in Fig. 5A) impairs its nuclear translocation and DNA binding activity (Menghini et al., 2005), raising the possibility that CaM KIV can also phosphorylate this site and thus negatively regulate GATA-2 function during hematopoeisis. To begin to test this possibility, we examined nuclear localization of ectopic GATA-2 in ectodermal cells of *Xenopus* embryos in the presence or absence of constitutively active CaM KIV. RNA encoding GATA-2 HA (200 pg) was injected into a single blastomere of two-cell embryos either alone, or together with RNA encoding constitutively active components of the CaM KIV cascade (50 pg each).

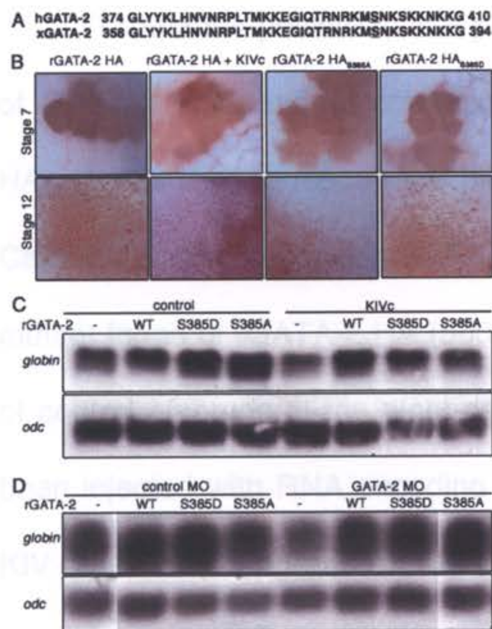

Fig. 5. Mutation of a putative CaM KIV phosphorylataion site in GATA-2 does not affect nuclear localization or function (A) Alignment of sequence of human (hGATA-2) and *Xenopus* Gata-2 (xGATA-2) surrounding the putative phosphorylated serine which is underlined. (B) Immunolocalization of GATA-2 HA in stage 7 or stage 12 embryos that had been injected with the RNA(s) indicated above each panel. (C-D) Northern blot analysis of expression of *globin* in stage 32 control embryos or in embryos that had been injected with wild type (WT) or mutant GATA-2 RNA, constitutively active forms of CaM KIV and its upstream kinase (KIVc) and/or MOs as indicated above each lane.

The subcellular localization of GATA-2 was determined by immunostaining whole embryos with an anti-HA antibody. As shown in Fig. 5B, GATA-2 HA was distributed throughout the entire cell at the blastula stage (stage 7) but began to concentrate in nuclei at the start of gastrulation (stage 10, data not shown) and was found exclusively in the nucleus by stage 12. Constitutive activation of the CaM KIV cascade had no effect on nuclear translocation of GATA-2 HA. Furthermore, mutant forms of *Xenopus* GATA-2 HA in which Ser385 (analogous to Ser401 of human GATA-2) was substituted with aspartate (GATA-2 HA_{S385D}) to mimic constitutive activation, or with a nonphosphorylable alanine residue (GATA-2 HA_{S385A}), showed an identical temporal pattern of cytoplasmic localization followed by nuclear translocation (Fig. 5B).

To test the possibility that phosphorylation of Ser385 impairs the function of GATA-2 independent of nuclear localization, we asked whether GATA-2 HA_{S385D} was able to rescue the blood defects caused by either upregulation of CaM KIV or downregulation GATA-2. RNA encoding wild type or phosphorylation mutant forms of rGATA-2 HA (500 pg) was injected into two dorsal blastomeres of control embryos at the eight-cell stage, or into embryos that had previously been injected with RNA encoding constitutively active components of the CaM KIV cascade (50 pg each) or GATA-2 MOs (6 ng). Embryos were cultured to stage 32 at which time expression of *globin* was analyzed by Northern blot. As shown in Fig. 5C and D, overexpression of wild type or phosphorylation mutant forms of GATA-2 HA did not perturb expression of *globin* in control embryos, thereby ruling out a dominant mutant effect. Furthermore, we found no difference in the ability of GATA-2 HA_{S385D} to rescue blood formation in embryos in which CaM KIV was hyperactivated or GATA-2 expression downregulated relative to wild type GATA-2 HA or GATA-2 HA_{S385A} (Fig. 5C and C). Taken together, these data demonstrate that CaM KIV does not impair nuclear translocation of ectopically expressed GATA-2. Furthermore, whereas a mutation that mimics constitutive phosphorylation of Ser401 in human GATA-2 impairs its nuclear translocation and function in cultured preadipocytes (Menghini et al., 2005), the analogous mutation has no effect on ectodermal GATA-2 nuclear localization or function in the context of *Xenopus* primitive hematopoiesis.

Mutation of putative acetylated lysines inactivates GATA-2 in vivo

An alternate mechanism by which CaM KIV could negatively regulate GATA-2, which is consistent with our previous studies suggesting that constitutive activation of CaM KIV reduces the availability of limiting amounts of CBP (Walters et al., 2002), is by inhibiting CBP mediated acetylation of GATA-2. Recent studies have shown that endogenous GATA-2 is acetylated in vivo and mutation of a subset of lysine residues that are normally acetylated by the CBP homolog, p300, causes a severe loss of the transactivation potential of GATA-2 (Hayakawa et al., 2004).

To begin to test the possibility that acetylation of ectodermally derived GATA-2 is required for primitive hematopoiesis, we generated a series of GATA-2 mutants in which lysine residues that have been shown to be acetylated in vivo (Hayakawa et al., 2004) were substituted with arginine. The location of substituted lysine residues relative to the amino- (N) and carboxy (C)-terminal zinc finger motifs is illustrated in Fig. 6A. A compound mutant (M1 + M2) that includes substitutions of all lysines present in M1 and M2 was also generated. To rule out the possibility that the amino acid substitutions caused gross misfolding, which might destabilize or block nuclear translocation of GATA-2, wild type and lysine mutant forms of GATA-2 HA were overexpressed in *Xenopus* embryos and the steady state level and subcellular distribution of each isoform was analyzed by Western blotting and whole mount immunostaining, respectively. All mutant

GATA-2 HA proteins were present at levels equivalent to wild type, and were localized to the nuclei of gastrula stage (stage 12) embryos (data not shown).

To ask whether these GATA-2 variants retained activity in vivo, we analyzed their ability to rescue RBC number in GATA-2 morphant tadpoles. GATA-2 MOs (6 ng) were injected into two dorsal cells of eight-cell embryos followed by injection of RNA encoding wild type or mutant forms of GATA-2 HA. Peripheral blood was collected and analyzed when embryos reached stage 42. As shown in Fig. 6B, M1 and M2 mutants rescued RBC number to the same levels as wild type GATA-2, whereas the compound M1+M2 mutant, and the M3 mutant completely lacked rescue activity in this assay. The M3 mutant did not perturb blood development when overexpressed in wild type embryos (Fig. 6B, C). A similar result was obtained when we analyzed the ability of the putative acetylation mutants to rescue hematopoietic defects caused by constitutive activation of Cam KIV with two exceptions: the M1 mutant showed slightly reduced activity and the M2 mutant was unable to rescue blood formation in embryos in which Cam KIV was hyperactivated (Fig. 6C). This observation was unexpected given that the M2 variant completely rescued the loss of RBCs caused by knock down of GATA-2 expression (Fig. 6B). Taken together, our data are consistent with the hypothesis that acetylation of GATA-2 is essential for its function in regulating transcription of ectodermal target genes that are required for normal erythropoeisis and that activation of CaM KIV inhibits CBP mediated acetylation of endogenous GATA-2.

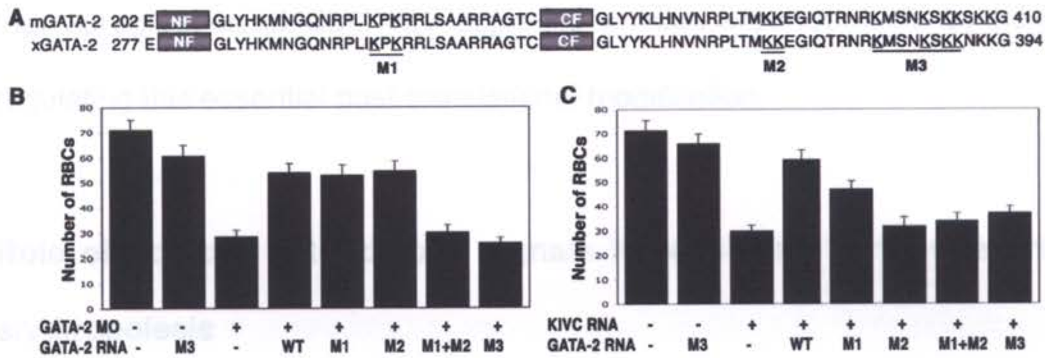

Fig. 6. Mutation of putative acetylated lysines inactivates GATA-2 in vivo. (A) Alignment of sequence surrounding the N- and C-terminal zinc fingers (NF; N-Finger, CF; C-Finger) of mouse (mGATA-2) and *Xenopus* Gata-2 (xGATA-2). Acetylated lysine residues that were mutated in the studies of Hayakawa et al (Hayakawa et al., 2004) or in the current studies are underlined. (C-D) Mean number (+/- s.e.m.) of RBCs present in control tadpoles, or in tadpoles derived from embryos that had been injected with the RNAs and/or MOs indicated below each bar. Results are pooled from a minimum of 4 independent experiments.

Unfortunately, the lack of antibodies that recognize endogenous GATA-2 prevent us from definitively testing this hypothesis at present.

Discussion

In the current study, we have shown that ectodermal GATA-2 is required for the differentiation and survival, but not for the specification, of RBC progenitors. Our data are consistent with previously published work suggesting that *GATA-2* is a transcriptional target of BMPs (Friedle and Knochel, 2002; Maeno et al., 1996; Walmsley et al., 1994). Furthermore, our data suggest that acetylation is important for the biological function of GATA-2 in the ectoderm, and

that upregulation of CaM KIV signaling inhibits erythropoiesis by negatively regulating this essential post-translational modification.

Role of non-cell autonomous signals in regulating vertebrate primitive erythropoiesis

Signaling between neighboring tissues is crucial for proper embryonic development. In vertebrates, blood originates from the mesoderm but extrinsic signals derived from adjacent tissues are known to be essential for normal hematopoiesis. In *Xenopus*, for example, signals produced by ectodermal cells must be transmitted to the mesoderm during gastrulation to enable these cells to form blood (Maeno, 2003). Our data demonstrate that BMPs (Walters et al., 2002) and GATA-2 are required to generate these signals. In mice and chick, primitive (visceral) endoderm (VE), which is a secretory epithelium that signals to the nascent mesoderm, may play a role analogous to the ectoderm in *Xenopus* since it has been shown to be crucial for the development of primitive blood (reviewed by (Baron, 2003). Specifically, when pre- or early-gastrula stage mouse embryos are stripped of their VE, the adjacent posterior mesodermal cells, which normally differentiate as primitive erythrocytes, are unable to do so (Belaoussoff et al., 1998). Recombination with VE, however, restores their ability to form blood. By the midstreak stage (embryonic day 6.75), mesodermal explants can differentiate as RBCs when cultured in isolation, suggesting that the blood-inducing signal from the VE is transmitted prior to this time (Belaoussoff et

al., 1998). Whether the non-cell autonomous signals necessary for blood formation in mouse are identical to those in *Xenopus*, and whether BMPs and/or GATA-2 play any role in activating these signals is unknown. Intriguingly, BMPs are required to signal to the VE to generate a non-cell autonomous signal that enables adjacent mesoderm in the posterior epiblast to form primordial germ cells (de Sousa Lopes et al., 2004; Gu et al., 1999). Expression of *GATA-2* has been reported as early as E7.5 in the lateral mesoderm and extraembryonic ectoderm (Minegishi et al., 1999), but it is not known if it is expressed in the VE at earlier stages, or whether its expression is dependent on BMPs. *GATA-2* is required for both primitive and definitive hematopoiesis, and chimeric analysis, as well as in vitro differentiation assays clearly reveal a cell autonomous role for this transcription factor in definitive blood progenitors (Tsai et al., 1994). Whether it also plays a non-cell autonomous role, analogous to that of *Xenopus GATA-2*, remains to be investigated.

Despite strong evidence that non-cell autonomous signals are required for mesodermal cells to differentiate as blood during vertebrate embryogenesis, the molecular nature of these signal(s) remains obscure. We have recently shown that depletion of *Xenopus* steel/stem cell factor (SCF) from ectodermal cells, or introduction of a dominant mutant form of its cognate receptor (c-kit) into mesodermal cells, blocks normal primitive erythropoiesis (Goldman et al., 2006). Thus, SCF is an excellent candidate for a cytokine that functions downstream BMPs or GATA-2 to regulate blood development. Preliminary studies, however,

have failed to provide evidence that expression of SCF requires BMP or GATA-2 function (Goldman et al., 2006). Further studies will be required to identify transcriptional targets of GATA-2 within ectodermal cells that regulate primitive erythropoieisis.

BMPs function in different tissues at multiple developmental stages to regulate hematopoiesis

Commitment of cells to the hematopoietic lineage is initiated by ventral patterning of the mesoderm during gastrulation, a process that requires BMPs (Nieto, 1999). When endogenous BMP signaling is blocked by introduction of BMP inhibitors into ventral cells of *Xenopus* embryos, ventral tissues, including blood, fail to differentiate and a secondary dorsal axis is induced (Kumano et al., 1999; Maeno et al., 1994a; Zhang and Evans, 1996). Furthermore, BMP-4 mutant mice lack posterior mesoderm and primitive blood cells (Winnier et al., 1995) while zebrafish embryos mutant for BMP-2b (Kishimoto et al., 1997; Mullins et al., 1996; Nguyen et al., 1998 or BMP7 {Dick, 2000 #398; Schmid et al., 2000) show a dorsalized phenotype accompanied by a decrease in RBCs. Conversely, *Xenopus* embryos made to ectopically express BMP-4, or zebrafish embryos mutant for BMP antagonists, show an expanded domain of expression of erythroid-specific genes in whole embryos. Thus, high levels of BMP signaling are required during gastrulation to specify ventroposterior fates, including blood, (reviewed by (Davidson and Zon, 2000).

GATA-2, or another member of the GATA family, has been proposed to cooperate with BMP-4 in ventral patterning of the mesoderm (Sykes et al., 1998). This hypothesis is based on studies showing that overexpression of a dominant interfering GATA factor, consisting of the GATA-2 DNA binding domain fused to the engrailed repressor domain, phenocopies the dorsalization observed when BMP function is blocked in *Xenopus* embryos (Sykes et al., 1998). We find, however, that overexpression of this fusion protein in ectodermal cells of *Xenopus* embryos does not phenocopy the hematopoietic defects observed in GATA-2 morphants, and GATA-2 morphants do not show any signs of dorsalization (Dalgin and Christian, unpublished data). These results demonstrate that *GATA-2* is not required for ventral mesodermal fate and raise questions as to whether the GATA-engrailed fusion protein specifically targets, and/or is capable of targeting all GATA family members. Chimeric proteins consisting of the engrailed repressor domain fused to the DNA binding domain of a number of different transcription factors, including multiple GATA family members (Sykes et al., 1998), STAT1, 3 or 5 (Nishinakamura et al., 1999), Xlmo4 (de la Calle-Mustienes et al., 2003), CBTF (Scarlett et al., 2004) and Xpcl1 (Yoshitake and Christian, unpublished data) can all induce dorsalization when overexpressed in *Xenopus* embryos. Given these findings, the specificity of this phenotype must be interpreted with caution.

A number of recent studies, in addition to the current one, have identified roles for BMPs in hematopoiesis that are independent of their early function in

specifying ventroposterior fate in the mesoderm. BMPs, for example, have been shown to be required during mid- to post-gastrula stages for specification of myeloid fate. Specifically, zebrafish embryos mutant for the BMP receptor, Alk8, or embryos in which BMP function is removed at the bud stage, well after dorsoventral pattern is established, lack the pool of myeloid cells that are normally derived from anterior lateral plate mesoderm (Hogan et al., 2006). Conversely, embryos in which BMP signaling is upregulated at doses that do not affect dorsal patterning show an increase in the number of myeloid cells (Hogan et al., 2006; Walters et al., 2002). Finally, recent studies have shown that BMP signaling negatively regulates hematopoietic fate within the lateral mesoderm during somitogenesis (Gupta et al., 2006; Pyati et al., 2005). Although we, and others (Rhodes et al., 2005) find no evidence that GATA-2 is required for myeloid fate, it remains possible that it functions downstream of BMPs in the lateral mesoderm.

CaM KIV regulates GATA-2 post-translationally

Transcription factors are tightly regulated at transcriptional, posttranscriptional and posttranslational levels, and our results suggest that GATA-2 is posttranslationally modified downstream of CaM KIV. The most obvious modification that might occur in response to upregulation of CaM KIV is phosphorylation. Consistent with this possibility, human GATA-2 is phosphorylated at a potential CaM KIV site (Serine401) in a PI-3K/Akt dependent

manner in transfected HEK293 cells and this negatively regulates its activity (Menghini et al., 2005). Specifically, phosphorylated GATA-2, or a mutant form of GATA-2 in which serine 401 is substituted with aspartate to mimic phosphorylation, is unable to undergo nuclear translocation in HEK293 cells, or to block adipocyte differentiation when overexpressed in a cultured preadipocyte cell line. By contrast, our current data show that ectopic GATA-2 carrying an analogous serine to aspartate substitution undergoes proper temporally regulated nuclear translocation in *Xenopus* embryos, and is able to rescue hematopoietic defects in GATA-2 morphants. Thus, a phosphorylation event that can control the nuclear localization and function of GATA-2 in cultured HEK cells and preadipocytes does not appear to be capable of regulating GATA-2 function in vivo in the context of *Xenopus* primitive hematopoiesis. Phosphorylation of other GATA family members has been shown to either suppress or enhance their ability to regulate expression of specific target genes when overexpressed in cultured cells, but, as with GATA-2, the in vivo importance of phosphorylation is unclear. GATA-1, for example, is phosphorylated at seven different serine residues, either constitutively or in response to extracellular signals, and yet mice carrying serine-to-alanine mutations at three of these sites do not show hematological defects indicative of impaired GATA function (Rooke and Orkin, 2006).

Acetylation represents another important posttranslational modification that is crucial for the activity of GATA family members (reviewed by (Huo and

Zhang, 2005). GATAs recruit histone acetyl transferases (HATs) to chromatin, leading to histone acetylation, and this alters chromatin structure enabling target genes to be transcribed. GATA proteins can also serve directly as substrates for HATs, and this modification is essential for their transactivation potential. GATA-2, for example, is known to bind CBP (Blobel et al., 1998) and has been shown to be acetylated in hematopoietic progenitor (KG1) cells by the CBP homolog, p300 (Hayakawa et al., 2004). Mutant forms of GATA-2 in which the predominant acetylated lysines are substituted with alanines have severely reduced transactivation activity, although they retain the ability to interact with p300 suggesting that the loss of activity is not due to an inability to recruit this transcriptional coactivator to histones. Notably, although several of the lysine mutants retain the ability to bind DNA, all are non-functional as assayed by their ability to inhibit growth of IL-3 dependent hematopoietic progenitor cells (Hayakawa et al., 2004). In the current studies, we provide the first evidence that acetylation of GATA-2 is required for erythropoiesis in a whole animal model.

Model for interactions between BMPs, CaM KIV and GATA-2 during primitive hematopoiesis.

Taken together, our current and previous (Walters et al., 2002) data suggest a molecular mechanism by which BMPs, CaM KIV and GATA-2 interact within ectodermal cells to regulate primitive hematopoiesis (illustrated in Fig. 7). According to our model, GATA-2 is a direct transcriptional target downstream of

BMP receptor activation within ectodermal cells, while activation of the CaM KIV signaling pathway alters GATA-2 function posttranslationally. We hypothesize that under physiological conditions, sufficient CBP is present to acetylate GATA-2 so that it can activate transcription of target gene(s) that encode secondary signaling molecules that are required for mesoderm to form blood (Fig. 7A). When CaM KIV is hyperactivated, however, high levels of phosphorylated substrate accumulate and this phosphorylation event enables them to recruit limiting amounts of CBP away from endogenous GATA-2. This leads to hypoacetylation of GATA-2, and an inability to activate transcription of target genes that are required for hematopoiesis (Fig. 7B).

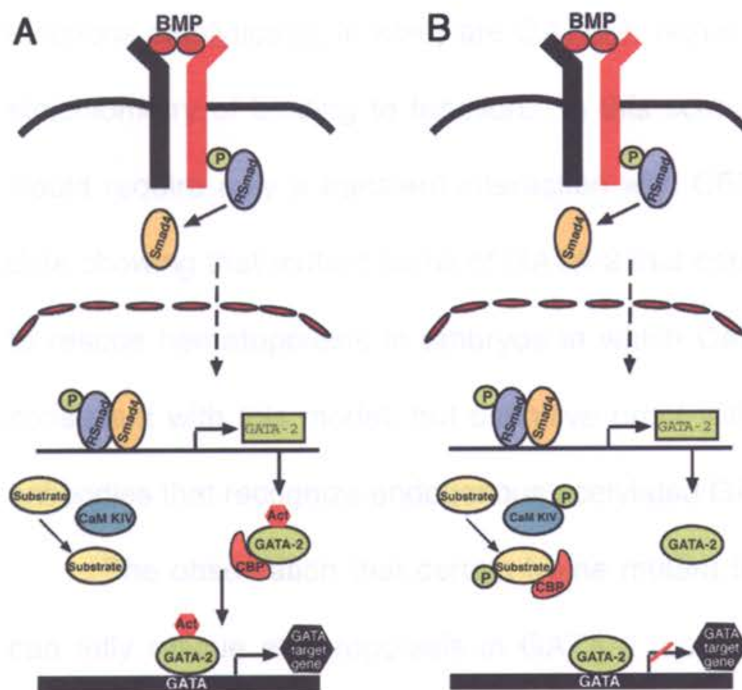

Figure. 7. Model for regulation of ectodermal GATA-2 by BMPs and CaM KIV during primitive hematopoiesis. See text for details

The idea that upregulation of CaM KIV activity leads to sequestration of limiting amounts of CBP is based primarily on our previously published studies but has precedent in that others have shown that competition for binding to limiting amounts of CBP can regulate gene transcription (Chakravarti et al., 1996; Kamei et al., 1996; Takahashi et al., 2000). In our original model, we proposed that CBP bound to and functioned as a co-activator for a hematopoietic specific transcription factor (e.g. GATA-2) by acetylating histones at the promoter of its target genes. The ability of overexpressed GATA-2 to rescue hematopoiesis in embryos in which CaM KIV is activated argues against this possibility, however, since excess free GATA-2 would effectively squelch the activity of the small pool of CBP bound GATA-2. In the current model, we propose instead that CBP functions catalytically, to acetylate GATA-2, rather than requiring a one to one stoichiometry of binding to function. In this scenario, overexpressed GATA-2 would require only a transient interaction with CBP to become functional. Our data showing that mutant forms of GATA-2 that cannot be acetylated are unable to rescue hematopoiesis in embryos in which CaM KIV is hyperactivated are consistent with this model, but definitive proof will require the development of antibodies that recognize endogenous acetylated GATA-2 in vivo.

The observation that certain lysine mutant forms of GATA-2 (M1 or M2) can fully rescue erythropoiesis in GATA-2 morphants, but not in embryos in which CaM KIV is upregulated demonstrates that loss of activity in the latter case is not due to gross structural defects imposed by the amino acid substitutions.

One simple explanation for these findings, which is consistent with our model, is that acetylation of both of the lysines targeted in the M1 mutant, or both of the lysines targeted in the M2 mutant is sufficient for GATA-2 activity whereas loss of acetylation of all four lysines (M1+M2) leads to loss of activity. In GATA-2 morphants, sufficient CBP is available to acetylate all of the available lysines in ectopically expressed GATA-2, whereas in embryos made to express constitutively active CaM KIV, only a subset of available lysines may be acetylated. In these embryos, hypoacetylated wild type GATA-2 may be fully active whereas hypoacetylation, in combination with loss of availability of the M1 or M2 lysines for acetylation may be sufficient to block transactivation potential.

BMPs, CaM KIV, and GATA family members may interact during patterning of additional organs outside of the hematopoietic system. We have shown that inhibition of BMP function, or hyperactivation of CaM KIV in *Xenopus* embryos leads to heart and gut defects that are similar to those observed in animals in which GATA-4, -5 and/or 6 function has been perturbed (Peterkin et al., 2005; Walters et al., 2001; Walters et al., 2002; Wayman et al., 2000). Mice or frogs lacking *GATA-4*, for example, or fish mutant for *GATA-5* display cardia bifida (reviewed by (Molkentin, 2000), and, in mice, this can be rescued by restoring GATA-4 expression in the endoderm (Narita et al., 1997). Inhibition of BMP signaling or activation of CaM KIV in the endoderm of *Xenopus* embryos also leads to cardia bifida (Walters et al., 2001). In addition, *Xenopus* embryos in which BMP signals are blocked, or CaM KIV activity is upregulated show defects

in gut looping and endodermal differentiation (Walters et al., 2002) similar to those observed in GATA-5 and/or 6 morphants (Afouda et al., 2005). Within the endoderm and cardiac mesoderm, GATA factors and BMPs have been shown to regulate each others expression and to cooperate to regulate transcription of target genes. For example, *GATA-4* and *GATA-6* are expressed downstream of BMPs and both of these genes are required to maintain transcription of *BMP-4* (reviewed by (Peterkin et al., 2003). Furthermore, GATA-4 and the intracellular transducers of BMPs, SMAD-1 and SMAD-4, can physically interact and are required for transcription of the cardiac-specific transcription factor *Nkx2.5* (reviewed by (Brown et al., 2004; Peterkin et al., 2005).

Loss of function analyses clearly demonstrate that GATA and BMP pathways interact in multiple organ systems, whereas evidence that endogenous CaM KIV can regulate GATA function is currently lacking. Mice mutant for CaM KIV have defects in maintenance of adult hematopoietic stem cells (Kitsos et al., 2005) and in learning and memory (Ahn et al., 1999), but primitive hematopoiesis has not been evaluated in these mutants. The current studies demonstrate that ectodermal GATA-2 is required for primitive hematopoiesis and clarify the mechanism by which hyperactivation of CaM KIV disrupts this function in vivo.

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CHAPTER 3

Both N-terminal deletion and dominant negative GATA-2 do not confer hematopoietic defects.

Gokhan Dalgin and Jan L. Christian

Department of Cell and Developmental Biology

Oregon Health and Science University, School of Medicine

3181 SW Sam Jackson Park Road

Portland, OR 97239-3098

Introduction

The GATA family of transcription factors is critical for proper development of many tissues and cell types. They are zinc-finger transcription proteins, containing two zinc-fingers (N- and C-terminal) and a nuclear localization signal region in their C-terminal domain. At the very beginning of the N-terminus, they have a transactivation domain. While the N-terminal zinc-finger is involved in protein-protein interaction, the C-terminal zinc-finger is responsible for binding to DNA (reviewed by (Lowry and Atchley, 2000).

GATA family proteins are highly conserved in their zinc-finger domain but diverse in their N-terminal region. When GATA-1's DNA binding domain is replaced with GATA-3's DNA binding domain, this chimeric protein is able to rescue the differentiation of the *GATA-1*-null G1E cells (derived from GATA-1-null embryonic stem cells) (Weiss et al., 1997). Moreover, functional analysis of GATA factors in *in vitro* reporter assays suggests that they have an essential transactivation domain in their N-termini (Martin and Orkin, 1990). However, rescue analyses in G1E cells suggest that the N-terminal transactivation domain is dispensable for erythroid and megakaryocytic cell differentiation (Blobel et al., 1995; Visvader et al., 1995; Weiss et al., 1997). Thus, functional differences that have been identified *in vitro* may not be significant *in vivo*.

Analysis of GATA factors suggested that the C-terminal zinc-finger of either GATA-1 or GATA-2 is sufficient to bind to DNA and is able to partially induce megakaryocytic differentiation. However, this domain does not have

transactivation potential (Visvader et al., 1995). Moreover, a mutant lacking the N-terminal zinc-finger domain has all the biological functions of GATA-1, but it has been shown that it binds to DNA less stably (Martin and Orkin, 1990). Since the N-terminal zinc-finger is involved in protein-protein interaction and stabilizing DNA binding, the C-terminal domain might function by changing DNA confirmation or by displacing another factor. Moreover, different domains of GATA factors may have distinct roles. For example, in addition to distinct functions of the zinc-finger domains, deletion of the first 193 amino acids of the GATA-1 protein substantially decreases the transactivation potential of GATA-1, although the DNA binding and induction of megakaryocytic differentiation are normal (Visvader et al., 1995).

As described in the previous chapter, GATA-2 function is essential within ectodermal cells for primitive hematopoiesis. When GATA-2 expression is blocked in ectodermal cells, by injection of morpholino antisense oligonucleotides, primitive RBCs do not form. Early in my thesis work, I attempted to use a previously characterized dominant mutant form of GATA-2 as an alternate approach to blocking GATA-2 function.

Transcriptional activation of a gene is regulated by the basal transcription machinery that is recruited, by sequence-specific transcription factors to regulatory sites on genes. These factors either activate or suppress transcription based on whether they recruit activator or repressor complexes, respectively. One example of a transcriptional repressor is *Drosophila* engrailed (En), a

homeodomain-containing protein that has been shown to have one strong repressor domain. Previous studies have demonstrated that the repressor domain of En blocks the interaction of transcription factors and basal transcription machinery. Furthermore, this domain can repress transcription when it is fused to heterologous DNA-binding domains. Such a chimeric protein was generated for the transcription factor GATA-2 (Sykes et al., 1998). This fusion protein consisted of the DNA-binding domain of GATA-2 (the two zinc-fingers and the C terminal domain) fused to the En repressor domain called GATA-2-Engrailed (G2En).

This dominant negative construct has been shown to inhibit GATA-2 function in *Xenopus* (Sykes et al., 1998). Specifically, when GATA-2 function was inhibited in the ventral site of the *Xenopus* embryo by injecting G2En, this caused axis duplication that was similar to blocking BMP's function in the ventral site of the embryo. Sykes et al. suggested that downregulation of GATA-2 (as well as other GATA family members) with G2En mimics downregulation of BMPs in the ventral blastomeres and that GATA-2 is required for ventral mesodermal fate. This finding raised the possibility that GATA-2 function can be blocked specifically in the blastomeres that confer ectoderm by injecting RNA encoding G2En, a process that is described in the results section of this chapter.

In this chapter I will provide preliminary data suggesting that G2En may not be a specific inhibitor of GATA-2, and it may not be required for ventral mesodermal fate. Moreover, I will provide evidence that this N-terminal region of

GATA-2 may not be necessary for the primitive erythropoietic function of GATA-2.

Material and Methods

Plasmid construction and expression

The DNA binding domain of *Xenopus* GATA-2 (amino acids between 263 and 380) was amplified by PCR and was fused in frame with the engrailed repressor domain. cDNAs were subcloned into pCS2+ for RNA transcription. Capped synthetic mRNA was synthesized by in vitro transcription of linearized template cDNAs using a MegaScript kit (Ambion) and injected into embryos as described previously (Moon and Christian, 1989).

Protein isolation, dephosphorylation and immunobloting

Embryos were injected at the one-cell stage with RNAs encoding the analyzed forms of GATA-2. Protein was isolated by Freon extraction and 40μg of protein was treated with calf intestinal phosphatase (CIP, New England Biolabs) in the buffer provided by the supplier at 37°C for 60 minutes. Protein was separated by electrophoresis on an 11% polyacrylamide gel (SDS-PAGE) and transferred onto PVDF membrane, then probed with anti-HA (3F10) antibody (Roche). The manufacturer's protocol was followed for antibody concentrations and chemiluminescence (Pierce) was used to detect protein.

Results and Discussion

G2En does not mimic the erythropoietic phenotype caused by GATA-2 Morpholinos.

In an attempt to downregulate GATA-2 function in the *Xenopus* ectoderm a dominant negative form of GATA-2 was used (G2En). RNA encoding G2En was targeted into the prospective ectoderm and embryos were raised until the tailbud stage. Erythropoiesis was analyzed by expression of *globin*. Since overexpression of G2En in the ventral site of the embryo caused axis duplication (Sykes et al., 1998) this construct was also injected into the prospective ventral site of the embryo as a control.

While ventral injection of G2En caused axis duplication and confirmed the published data, overexpressing G2En in the prospective ectoderm did not cause any erythropoietic defects. When the same experiments were repeated with GATA-2 MOs, downregulation of endogenous *GATA-2* with GATA-2 MOs in the ventral blastomeres did not duplicate the axis. Contrary to G2En, targeting prospective ectoderm with G2MOs caused severe defects in primitive erythropoiesis. Thus, findings suggest that G2En is not a specific inhibitor of GATA-2 or other GATA factors, and it is not required for ventral mesodermal fate.

We, and others (Sykes et al., 1998; Nishinakamura et al., 1999; dela Calle-Mustienes et al., 2003; Scarlett et al., 2004; Yoshitake and Christian, unpublished data) have shown that fusion of the engrailed repressor domain to a number of different transcription factors, including various GATA and STAT

family members, artificially inhibits BMP function during early embryogenesis. This might be due to the nature of the engrailed repressor, which nonspecifically suppresses the function of other transcription factors required for transducing BMP signals.

Deletion of the N-terminal region of GATA-2 does not affect its hematopoietic function.

While generating an HA epitope tagged *Xenopus* GATA-2, serendipitously, a functional deletion construct was generated (Fig 1a.). This construct was called delta GATA-2 HA and it was missing the amino acids between 136 and 209. Interestingly, delta GATA-2 HA was able to rescue the phenotype caused by MO mediated downregulation of endogenous GATA-2 (Fig 1b.). Specifically, GATA-2 MOs (6 ng) were injected into two dorsal animal pole blastomeres of eight-cell stage embryos alone or together with RNAs encoding either delta GATA-2 HA or GATA-2 HA.

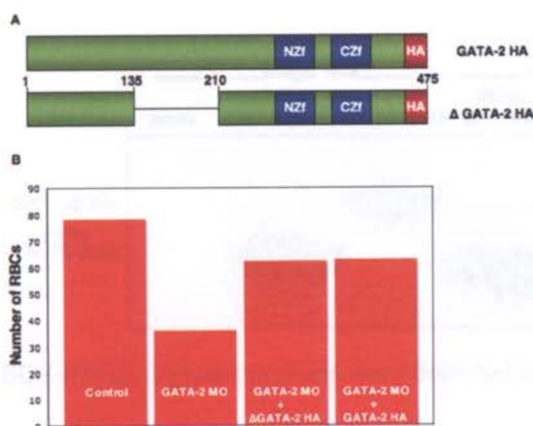

Figure 1. (A) Schematic diagram showing the deleted region in GATA-2. (B) Embryos were injected with GATA-2 MO alone or together either with RNA encoding full length GATA-2 or delta GATA-2. Embryos were raised and peripheral blood was concentrated on the slides and counted. Both full length and the delta form of GATA-2 could rescue the blood defects caused by downregulation of GATA-2 by MOs.

To investigate the possible mechanism that gives the delta GATA-2 HA wild type GATA-2 properties, we first analyzed the sequence that is deleted in delta GATA-2 HA. We observed that in this domain, there is a PEST sequence (a peptide motif rich in proline, glutamate, serine, and threonine). The PEST motif is recognized by the protein degradation machinery, and it targets proteins for proteolytic degradation (Rechsteiner and Rogers, 1996). Therefore, missing this domain might make delta GATA-2 HA more stable.

To test this possibility, RNA encoding either delta GATA-2 HA or GATA-2 HA was injected into *Xenopus* embryos at the one-cell stage. Embryos were raised until the gastrula stage and samples were taken at stages 10.5 and 12.5. Proteins were isolated from embryos and equal amounts of proteins were separated by SDS-PAGE. Translated proteins were detected by HA immunoblotting. Results from two experiments suggested that there was no significant differences between the full length and the deletion version of GATA-2 HA (Fig 2).

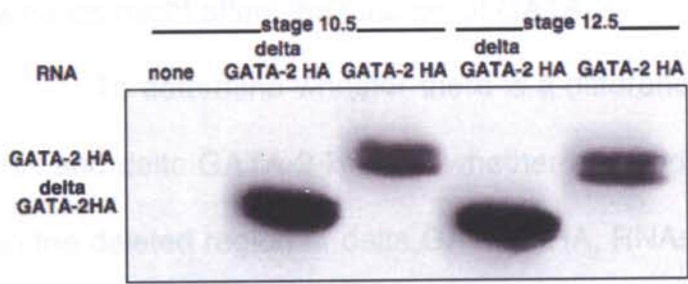

Figure 2. Embryos were injected with RNAs encoding either delta rGATA-2 Ha or rGATA-2 HA. Isolated proteins from stage 10.5 and 12.5 were separated on a SDS-PAGE. Injected proteins were detected by HA immunoblotting.

It has been shown that a deletion mutant form of GATA-1, which lacks the first 193 amino acids of the protein, is still able to induce megakaryocytic differentiation (Visvader et al., 1995). Consistent with this, our delta GATA-2 HA was able to rescue the blood phenotype caused by downregulation of endogenous GATA-2 in the *Xenopus* ectoderm (Fig. 1b). Taken together, these data suggest that the hematopoietic function of GATA-1 and GATA-2 might be independent of the majority of their N-termini.

Interestingly, the deleted sequence also contains consensus motifs for several known kinases (Fig. 3), such as Mitogen-activated Protein kinase (MAPK)/Extracellular Signal-regulated Kinase (Erk), Glycogen Synthase Kinase 3 (GSK-3), Protein Kinase A (PKA) and Casein Kinase I and II. It also contains a consensus sequence for sumoylation. Moreover, it has been shown that GATA factors are phosphorylated (Crossley and Orkin, 1994; Menghini et al., 2005; Partington and Patient, 1999). Consistent with these data, GATA-2 HA is detected as a doublet (Fig. 2) suggesting that GATA-2 might be phosphorylated. These observations raise the possibility that phosphorylation of this domain by kinases might affect the function of GATA-2.

To determine whether there is a difference in phosphorylation of GATA-2 HA and delta GATA-2 HA and whether any phosphorylation sites are contained in the deleted region of delta GATA-2 HA, RNAs encoding either GATA-2 HA or delta GATA-2 HA were injected into *Xenopus* embryos at the one-cell stage and embryos were raised until gastrulation.

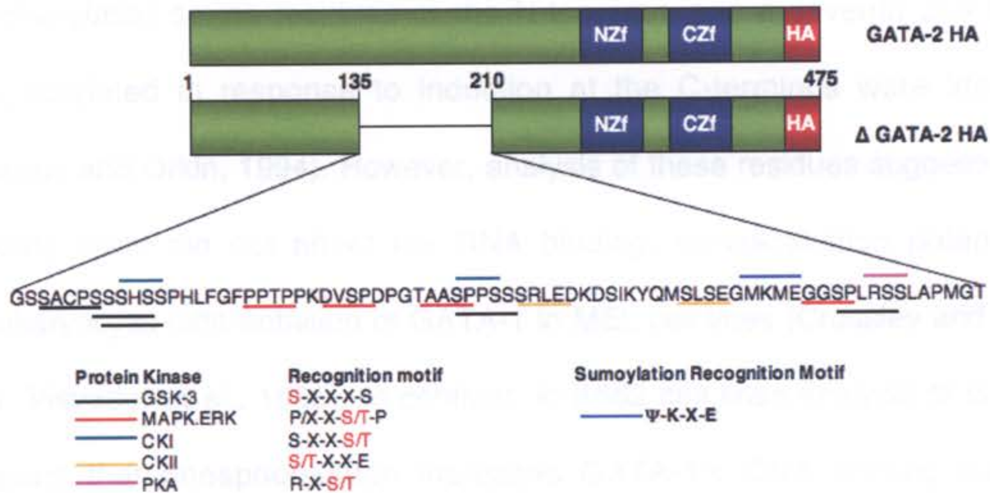

Figure 3. Putative posttranslational modification sites that are missing in delta GATA-2 HA. Extracellular Signal-regulated Kinase (Erk), Glycogen Synthase Kinase 3 (GSK-3), Protein Kinase A (PKA) and Casein Kinase I and II. Phosphorylation residues indicated in red.

Proteins were isolated from two different stages and subjected to a dephosphorylation reaction by using alkaline phosphatase (which dephosphorylates serine, threonine and tyrosine residues). Subsequently, dephosphorylated and non-dephosphorylated proteins were separated by SDS-PAGE and followed by immunoblotting with HA. Results from multiple experiments were not conclusive.

Given the fact that phosphorylation of GATA factors *in vitro* is controversial in the literature, it is possible that phosphorylation may not have a physiological role for hematopoietic function of GATAs. For example, GATA-1 is phosphorylated both in mouse erythroleukaemic cells (MEL) and in human erythroleukaemic cells (K562) (Crossley and Orkin, 1994; Partington and Patient,

1999). The phosphorylation sites were mapped and six constitutively phosphorylated serine residues at the N-terminus and a seventh one that is phosphorylated in response to induction at the C-terminus were identified (Crossley and Orkin, 1994). However, analysis of these residues suggested that mutating them did not affect the DNA binding, transactivation potential or megakaryocytic differentiation of GATA-1 in MEL cell lines (Crossley and Orkin, 1994; Visvader et al., 1995). In contrast, in K562 cell lines analysis of GATA-1 suggests that phosphorylation increases GATA-1's DNA binding capacity (Partington and Patient, 1999). Therefore, there might be differences between the two cell lines, such as a requirement for phosphorylation sites during hematopoiesis. Yet, recently, a triple knock-in mutant of GATA-1 phosphorylation sites was generated and suggested that mutating the phosphorylation sites does not affect hematopoiesis in mice (Rooke and Orkin, 2006). Nevertheless, these knock-in mice do not cover all the possible phosphorylation sites on GATA-1 and other sites may be required for hematopoiesis. Consistent with this idea, GATA-1 has been shown to be a MAPK substrate, and MAPK phosphorylation occurs on the two upstream serine residues that were not a target for mutagenesis in the triple knock-in mice (Towatari et al., 2004). Mutation of these residues weakens GATA-1's ability to interact with the LIM-only protein (LMO-2) in cell lines.

Likewise, it has been shown that in a myeloid progenitor cell line (416b) GATA-2 phosphorylation is regulated by MAPK (Towatari et al., 1995). In addition, as noted in Chapter I, GATA-2 is phosphorylated by PI-3K/Akt on a

serine residue next to its C-terminal zinc-finger, and constitutive phosphorylation of this site promotes adipocyte differentiation due to the inability of GATA-2 to translocate to the nucleus (Menghini et al., 2005). However, as explained in Chapter I, generation of this analogous mutation in *Xenopus* (which is also a consensus CaM KIV target) did not affect GATA-2's hematopoietic function.

Taken together, analysis of GATA factors and their domains *in vitro* gives us precious information about the function of these factors. Very recently, it has been shown that GATA-1 is subjected to degradation by ubiquitination. While acetylation of GATA-1 marks the protein for degradation, it is not degradated until it is phosphorylated (Hernandez-Hernandez et al., 2006). Even though one type of post-translational modification may seem not to affect hematopoietic functions of GATA factors, it is likely that these modifications in combination can modulate GATA function, and different cell types may utilize different properties of GATA factors. Functional analysis of these *in vitro* observations should also be tested *in vivo*.

CHAPTER 4

Thesis summary and future directions

The role of ectodermal GATA-2 in *Xenopus* primitive hematopoiesis

It has long been known that ectodermal signals are required for the proper development of primitive erythrocytes from mesoderm, and overexpression of BMPs or of the transcription factor *GATA-2* in the ectoderm has been shown to be sufficient to induce these factors. In the second chapter of this thesis, I have shown that in fact *GATA-2* is required for regulation of this ectodermal signal by downregulating endogenous *GATA-2* in the ectoderm with morpholinos. However, *GATA-2* is not required for the specification of the primitive blood progenitors. Instead, it is required for their differentiation. In addition, I have provided evidence for the first time in the whole animal that acetylation of GATA-2 is required for its hematopoietic function and that the CaM KIV pathway might modulate this requirement, possibly by competing for CBP.

In the third chapter of the thesis, I provided preliminary data suggesting that a commonly used dominant negative fusion protein (G2En) might maintain its function non-specifically. When I downregulated GATA-2 function in embryos by injecting GATA-2 MOs, I did not see the developmental defects caused by overexpressing this dominant-negative form of GATA-2. Moreover, G2En cannot reproduce the hematopoietic defects caused by GATA-2 MOs. Therefore, when

using this fusion protein for dominant negative purposes results should be analyzed more carefully. I have also provided evidence that the N-terminal region of GATA-2 may not be critical for its hematopoietic function. However, none of these data are definitive and these possibilities require more analysis.

Future directions

The future of this project is very exciting. For example, identification of the timing of the ectodermal signal that is modulated by GATA-2 is important. To identify this, a cDNA encoding a hormone-inducible form of GATA-2 (rGATA-2-HA-GR; glucocorticoid receptor) has been generated in the lab. This construct's nuclear translocation can be controlled by the addition of hormone into the media. Therefore, rGATA-2-HA-GR can be co-injected with GATA-2 MOs and treated at different times with the hormone. The ability of rGATA-2-HA-GR to rescue blood formation when embryos are treated with hormone at different times can then be assessed. Thus, the developmental window during which GATA-2 function is required can be identified.

Once the timing of the ectodermal signal is determined, it will be important to identify the ectodermal factors that are transcriptionally regulated by GATA-2 that are required for the hematopoietic mesoderm to differentiate as primitive erythrocytes. This will provide us with important information about the mechanism by which different germ layers work together to develop certain properties of the embryo. Moreover, identification of the signaling factors that are

regulated by GATA-2 will help us to differentiate the critical steps that regulates the differentiation of red blood cells and may help us to treat blood diseases.

Initial steps are being taken in the lab to identify ectodermal signals and a microarray will be performed using tissues isolated from embryos in which ectodermal GATA-2 is downregulated. Further experiments will be performed by members of the Christian laboratory.

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