The Role of BMP-4 and GATA-2 in the Induction and Differentiation of Hematopoietic Mesoderm in Xenopus Laevis

By Mitsugu Maeno, Paul E. Mead, Clair Kelley, Ren-he Xu, Hsiang-fu Kung, Atsushi Suzuki, Naoto Ueno, and Leonard I. Zon

Vertebrate embryonic blood formation is regulated by factors that participate in dorsal-ventral patterning and mesoderm induction. The GATA-binding transcription factors are required for normal hematopoiesis and are expressed during gastrulation when ventral mesoderm (VM) is induced to form blood. Based on the recent demonstration that bone morphogenetic protein (BMP-4) is a potent ventralizing factor and inducer of hematopoietic tissue, we hypothesized that GATA-2 could be induced or activated by BMP-4. Here we demonstrate that BMP-4 can stimulate GATA-2 expression, and that expression of a dominant negative BMP-4 receptor can suppress GATA-2 induction by BMP-4 in ventral mesoderm. Over-expression of GATA-2 in ventral mesoderm leads to increased globin production and forced expression of GATA-2 in primitive ectoderm adjacent to ventral mesoderm also stimulates globin expression. Our results suggest that BMP-4 and GATA-2 can function in two adjacent germ layers, mesoderm and ectoderm, to participate in blood cell formation during embryogenesis.

© 1996 by The American Society of Hematology.
stage embryo. GATA-2 is expressed in both the VM and ectoderm during gastrula stages and may direct hematopoiesis in these two germ layers, similar to BMP-4. Here, we demonstrate that BMP-4 induces increased levels of GATA-2 and the dominant negative BMP-4 receptor suppresses GATA-2 expression in the VM. We also demonstrate that forced expression of GATA-2 in the animal pole stimulates VM to form globin. Taken together, these results suggest that GATA-2 not only functions in a cell autonomous manner during the induction of the blood program, but may also have a role in the animal pole to regulate the differentiation of blood.

MATERIALS AND METHODS

Immunohistochemistry and in situ hybridization methods. Whole embryo immunohistochemistry was performed as described.2 Embryos previously fixed in 3.7% formaldehyde and stored in methanol were gradually rehydrated in phosphate buffered saline (PBS). These were then incubated in a 1/5 dilution of monoclonal antibody, L4-27, supernatant (provided by C. Katagiri, Hokkaido University, Sapporo, Japan). This antibody recognizes larval globins. Peroxidase conjugated secondary antibodies (Jackson labs No. 115036062) were used at a 1:250 dilution. Detection was performed with H2O2 and DAB (Polysciences, Warrington, PA). Whole embryo in situ analysis for GATA-2 expression was performed as previously described using antiserum, digoxigenin-labeled, full-length GATA-2 RNA as probe.2

Western blot analysis. Western blot analysis for globin expression was performed as previously described,2 using the monoclonal antibody L5.41. In each experiment, explants from at least five embryos were pooled, and an equivalent of one embryo was loaded onto a 3M urea-18% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Detection used the ECL system (Amersham).

RNA injections. Embryos were injected at the two or four cell stage with the vectors: BMP-4/pSP64T, delta TRF11/pSP64T (the dominant negative BMP-4 receptor), and GATA-2/PGEMHE. The TRF11 receptor is the mouse ALK-3,22 and the dominant negative construct and its effect on Xenopus development has been described previously.24,25 BMP-4 and BMP-2 can compete for binding to the TRF11 receptor, but activin and TGFβ1 cannot. The BMP-4 and the delta TRF1 plasmids were each linearized with EcoRI and used SP6 polymerase (Ambion). The GATA-2 plasmid was linearized at XbaI and used T7 RNA polymerase (Ambion) for RNA transcription.

RT-PCR analysis. RT-PCR analysis for gene expression was
performed according to standard protocols. RNA was isolated from ten or more animal caps or ventral marginal zone recombinants. A total of 0.5 μg RNA was subjected to reverse transcription. One tenth volume of this cDNA was amplified with primers specific for BMP-4 and for GATA-2 as published, half of this product was loaded on a 5% polyacrylamide gel. To quantify the RNA amount used for each analysis, EF1α expression was examined by Northern blot analysis. Densitometry was performed with a scanning imager (Personal Densitometer; Molecular Dynamics Japan, Tokyo, Japan). For the demonstration of GATA-2 RNA quantification experiment, RNA was synthesized in vitro from the linearized GATA-2/PGEMHE vector and an optical density measurement was obtained to quantify the amount of synthesized RNA. The RNA was serially diluted and RT-PCR analysis was performed as described above.

Cell culture. Animal cap explants or recombinants of animal pole tissue with ventral mesoderm were prepared as previously described. The restricted area of prospective ventral mesoderm (VM in Fig 1) was prepared by removing all the cells above the blastocoel floor level. The explants excised were cultured in Steinberg’s solution for the designated periods. Each experiment was repeated multiple times to ensure reproducibility of results. The repeated experiments involved deriving new embryonic explants and subjecting these tissues to either RT-PCR or Western blot analyses. In all cases, similar results were obtained compared with those that are presented in each figure. We have stated the number of repeat experiments performed in each figure legend.

RESULTS

BMP-4 expression leads to extensive globin expression in the embryo. BMP-4 induces ventralization of the whole embryo and has been shown to activate globin RNA expression in the animal pole, which normally gives rise to ectoderm. To determine the effect of BMP-4 on globin protein expression in the whole embryo, BMP-4 RNA was injected at the one cell stage and embryos were allowed to reach stage 30. Whole embryo immunohistochemistry with a monoclonal antibody to embryonic globin demonstrated that embryos injected with BMP-4 exhibited globin expression throughout the embryo except in the most dorsal dome (Fig 2, the arrows delineate the dorsal border of the blood island). This is in contrast to a typical V-shaped blood island seen in embryos injected with a control RNA (β-galactosidase). This data suggests that BMP-4 expression leads to the commitment of more mesoderm of the embryo to form hematopoietic tissue.

Spatial regulation of GATA-2. The equatorial or marginal zone (MZ) of the gastrula gives rise to the mesodermal tissues. GATA-2 is first expressed in the ectodermal animal pole (AP) and subsequently in both the ventral and dorsal marginal zones of embryos during gastrulation. Because these MZ explants included an outer cell layer that could be contaminated with animal pole cells, we studied GATA-2 expression in VM and dorsal mesoderm (DM) that has been completely separated from these outer cell layers. A sensitive semi-quantitative RT-PCR analysis that can detect levels of GATA-2 RNA as low as 0.8 pg was used. The level of GATA-2 RNA expression detected in whole embryos by this assay correlates with the expression detected by whole embryos in situ analysis. For instance, the level of GATA-2 RNA increases substantially during neurula stages and then decreases during further development (Fig 3B). In the early gastrula embryo, GATA-2 is expressed in the AP and VM, and at a low level in the DM (Fig 3C).

To understand the regulation of GATA-2 in the AP cells, GATA-2 expression was examined by RT-PCR in isolated AP ectoderm at various stages of development. AP from the early blastula (stage 7) express very low levels of GATA-2 (Fig 3D) in contrast to the level expressed in a stage 10 animal pole explant. The removal of the AP at stage 7 may prevent the cells from being stimulated by factors from the rest of the embryo that regulate GATA-2 expression. To examine this, stage 7 AP explants were explanted and cultured until stage 10. GATA-2 in the cultured AP was expressed at a low level, comparable with that of stage 7 AP. This suggests that GATA-2 expression is induced by other regions of the embryo during the blastula and gastrula stages.

Relationship between BMP-4 and GATA-2. Based on the
putative role of BMP-4 as a VM inducer, we studied whether BMP-4 could induce GATA-2 expression. Embryos were injected at the one cell stage with BMP-4 RNA and grown until tailbud stage 28. In situ analysis of these ventralized embryos showed GATA-2 expression throughout the mesodermal and ectodermal layers, except where small remnants of dorsal tissue are evident (Fig 4A). Embryos ventralized by UV-irradiation also have expanded GATA-2 expression, demonstrating that GATA-2 expression reflects the ventral character of the embryo.²

To define the regulation of GATA-2 expression, AP regions derived from embryos injected with BMP-4 RNA were removed at stage 7 and cultured until early stage 10. BMP-4 leads to a marked increase in GATA-2 RNA level compared with the level detected in AP from uninjected embryos or from embryos injected with RNA encoding BMP1B (Fig 4B).²⁴ Thus, BMP-4 can induce GATA-2 expression in animal pole ectoderm.

To examine a potential feedback mechanism, the ability of GATA-2 to induce BMP-4 was evaluated in AP explants.
BMP-4 was detected in untreated AP explants, as previously described, while injection of GATA-2 RNA did not lead to increased BMP-4 RNA levels in the AP. Thus, GATA-2 is activated downstream of BMP-4 signaling during normal development.

**Effects of the dominant negative BMP-4 receptor on GATA-2 expression.** To determine whether BMP-4 signaling is required for the induction of GATA-2, the effect of a dominant negative BMP-4 receptor was examined using VM or AP explants. The dominant negative receptor (delta TRF1) is derived from the mouse ALK-3, and the dominant negative construct and its effect on Xenopus development has been described previously. BMP-4 and BMP-2 can compete for binding to the TRF1 receptor, but activin and TGFβ1 cannot. Expression of the dominant negative BMP-4 receptor in VM significantly decreased GATA-2 expression by 21-fold (based on densitometry). Thus, BMP-4 signaling is required to stimulate GATA-2 expression and hematopoietic mesoderm formation (Fig 5). In contrast, the dominant negative BMP-4 receptor slightly suppressed endogenous GATA-2 RNA expression in AP explants by approximately three-fold. These findings suggest that regulation of GATA-2 expression is distinctly different in the animal pole compared with that in the VM.

**Role of GATA-2 during early development.** To determine whether forced GATA-2 expression could stimulate hematopoiesis in Xenopus, GATA-2 RNA was injected into both cells of two cell embryos, VM was explanted at stage 10, and globin expression was determined at 48 hours of culture by Western blot analysis using a specific embryonic globin monoclonal antibody. As shown in Fig 6, GATA-2 is able to stimulate low levels of globin expression in VM.

Previous studies have demonstrated a stimulation of hematopoietic differentiation in the VM by AP ectoderm (Fig 1). This AP ectoderm expresses GATA-2 and becomes juxtaposed to the VM during gastrulation, adjacent to mesoderm that will give rise to blood. In addition to the role of GATA-2 in hematopoietic progenitors, GATA-2 may also regulate an ectodermal program that promotes globin expression. To test this hypothesis, GATA-2 RNA was injected into embryos at the two cell stage, APs were explanted at stage 7, and co-cultured with stage 10 VM. While uninjected stage 7 AP explants do not promote globin expression, surprisingly, stage 7 AP from embryos injected with very low levels of GATA-2 RNA (8 pg) stimulated globin protein synthesis. The level of globin detected was comparable with that induced by stage 10 animal caps (Fig 1 and data not shown) and was much higher than in VM explants from embryos injected with GATA-2 RNA. These studies suggest a role for GATA-2 in adjacent ectoderm for the production of globin.
DISCUSSION

BMP-4 and hematopoietic mesoderm. The induction of blood formation involves both the commitment of mesoderm to form hematopoietic tissue and the subsequent differentiation of hematopoietic lineages. VM is induced during or before gastrulation and BMP-4 appears to have a prominent role in this process. BMP-4 is first expressed in the AP, and then localized to the ventral and lateral MZ during gastrulation. Forced expression of BMP-4 ventralizes whole embryos and BMP-4 can rescue partially dorsalized embryos caused by lithium chloride treatment. Furthermore, BMP-4 has been shown to induce globin RNA synthesis in the AP. Based on our studies of VM and AP recombinants, BMP-4 expressed in the AP can promote globin production of adjacent hematopoietic tissue. In higher vertebrates, BMP-4 can stimulate globin expression in ES cell cultures. BMP-4 and its receptor have also been shown to be required for normal VM formation. BMP-4 thus appears to participate in both the induction and differentiation of VM.

The role of GATA-2. Disruption of GATA-2 in mice leads to a substantial proliferative defect in developing primitive and definitive hematopoietic progenitors. In vitro cultures of homozygous GATA-2 ES cell lines demonstrate a substantial decrease in progenitor number and these homozygous lines fail to contribute to hematopoiesis in vivo in chimeric mice. In support of this important role of GATA-2 in embryonic blood formation, we have demonstrated that GATA-2 is expressed in the early VM that will become blood. Furthermore, forced expression of GATA-2 in the VM leads to a slight increase of globin protein expression after culture for 48 hours. Thus, as shown in the mouse, GATA-2 acts in a cell-autonomous manner during normal blood formation.

In addition to expression in the VM, GATA-2 is abundantly expressed in the ventral ectoderm. This ectodermal expression pattern is also exhibited in the zebrafish, in which the ventral yolk syncytial layer expresses high levels of GATA-2 RNA. Early hematopoietic progenitors contact this layer, similar to the contact of ventral hematopoietic mesoderm and ventral ectoderm in Xenopus. Previous studies demonstrate that the ventral ectoderm stimulates hematopoietic differentiation. Our studies, using AP and VM co-cultures, showed that GATA-2 expression in the AP ectoderm increased globin expression within the VM. Thus, in addition to a hematopoietic cell autonomous role, GATA-2
may also function in this ectodermal (or stromal) layer in a non-cell autonomous manner to promote hematopoietic differentiation. Because the functional equivalent to this ventral ectoderm is not obvious in higher vertebrates, it is not easy to determine whether GATA-2 has a non-cell autonomous role in the hematopoietic development of higher species. Although difficult to demonstrate, stromal cell lines from the GATA-2(−/−) embryos may have a decreased efficiency of ability to support hematopoiesis or hematopoietic stem cell development.

**Regulation of GATA-2 by BMP-4.** BMP-4 is sufficient to induce AP cells to express abundant GATA-2; however, our studies with the dominant negative BMP-4 receptor demonstrate that BMP-4 signaling is not necessary for GATA-2 expression in AP cells. Thus, the mesodermal and ectodermal expression of GATA-2 is regulated by separate mechanisms. Our AP explant experiments in Fig 3 suggest that the ectodermal expression appears to be stimulated by factors supplied by the embryo between stages 7 and 10. These inducing factors are likely to be distinct from BMP-4, based on our results that the dominant negative BMP-4 receptor does not suppress endogenous GATA-2 expression in the AP. The regulation of GATA-2 in the ectodermal and mesodermal germ layers may be regulated by different signaling cascades or alternative gene regulation, such as the activation of distinct GATA-2 promoter elements in each germ layer.

**BMP-4 and GATA-2 each function in adjacent mesoderm and ectoderm to promote hematopoietic differentiation.** BMP-4 has been shown to have two effects on the induction of blood formation. First, it can induce VM formation, and second, it can direct AP ectoderm to promote globin expression in VM. These direct and indirect roles for BMP-4 may be similar to the activity of activin on erythroid progenitors in the bone marrow of higher vertebrates. Activin induces hemoglobin production in purified normal erythroid progenitors and in erythroleukemia cells. Recently, a region of the β-globin promoter was mapped which is "responsive" to activin. These studies support a role for activin to induce differentiation as a direct consequence of receptor stimulation on hematopoietic cells. Activin A also significantly potentiates colony forming unit-erythroid-(CFU-E) and burst-forming unit (BFU)-E growth in the presence of erythropoietin; however, activin alone does not stimulate colony formation. In hematopoietic cultures, this modulatory action of activin is mediated indirectly through the action of T cells and monocytes, potentially inducing the release of growth factors from these accessory cells.

Activin is produced by the bone marrow stromal cells and monocytes, and its production is stimulated by factors such as interleukin 1 (IL-1), tumor necrosis factor (TNFα), LPS, and granulocyte macrophage colony-stimulating factor (GM-CSF). Thus, activin regulates erythropoiesis by directly inducing a differentiation program in erythroid progenitors and by stimulating accessory cells to produce factors that lead to increased proliferation in the presence of erythropoietin. The activities of activin on marrow erythroid progenitors may be similar to the "direct" action of BMP-4 on VM and the "indirect" stimulation of BMP-4 on hematopoietic differentiation by the AP cells.

BMP-4 and activin are members of the TGF-β family. Their receptors are formed as multimeric complexes consisting of type I and II receptors. Recent studies have demonstrated that activin and BMP-4 can bind to the same type I and possibly type II receptors based on in vitro studies (J. Massague, personal communication, May 1995). It is thus possible that erythroid differentiation may occur on stimulation of either BMP-4 or activin receptor subunits.

During the cell movements of gastrulation, the ventral AP ectoderm and mesoderm become juxtaposed, and BMP-4 apparently acts in these two adjacent layers of tissue to affect hematopoietic induction and differentiation. BMP-4 expression in the MZ stimulates the formation of VM, heralded by the induction of specific regulators of hematopoietic gene transcription such as the GATA-binding proteins. By the end of gastrulation, AP tissue expressing BMP-4 and GATA-2 comes in contact with the VM. BMP-4 and GATA-2 in the AP promotes blood island formation and globin expression in the VM. BMP-4 and GATA-2 may function in both the VMZ and AP ectoderm to stimulate blood formation. This is analogous to sonic hedgehog’s function in the determination of the adjacent notochord and neural floor plate. Our results are complementary to those of Zhang and Evans who demonstrate that zygotic BMP-4 receptor signaling is required for normal hematopoiesis in Xenopus. Future studies will focus on isolating an AP factor stimulated by GATA-2 that regulates hematopoietic differentiation and to define other inducers of GATA-2 in the AP ectoderm.

**REFERENCES**


The role of BMP-4 and GATA-2 in the induction and differentiation of hematopoietic mesoderm in Xenopus laevis
M Maeno, PE Mead, C Kelley, RH Xu, HF Kung, A Suzuki, N Ueno and LI Zon