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[REVIEW]

Regulatory Signals and Tissue Interactions in the Early Hematopoietic Cell Differentiation in *Xenopus laevis* Embryo

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ABSTRACT—Bone morphogenetic protein-4 (BMP-4) has been shown as an essential factor in differentiation of the primitive blood cells in *Xenopus laevis* embryo. Organizer factors, in contrast, function as a negative regulator for the blood cell differentiation. Differentiation of both blood cells and muscle tissue are negatively regulated by the organizer activity. However, blood cells but not muscle tissue can differentiate in the organizer-depleted embryos produced by removal of vegetal cortex cytoplasm at the one-cell stage. Thus the blood cells are totally independent cells from the organizer activity. The down-stream target molecules of the BMP-4 signaling, such as vent-1/2 and msx-1/2 are the positive regulators for muscle tissue differentiation, whereas these factors do not promote blood cell formation. It has not yet been elucidated how the BMP-4 signaling promotes the differentiation of blood cells, but it is likely that transcription factors such as GATA-2, SCL, LMO-2, biklf and GATA-1 are at least involved in the initial blood program. Experiments using combined germ layer explants suggest that interaction between ectoderm and mesoderm at the gastrula stage is important for the blood cell formation in mesoderm. BMP-4 produced in the ectodermal cells is essential for this interaction. For understanding the whole program in the embryonic blood cell differentiation, it is important to elucidate the molecular mechanisms underlying the tissue-tissue interaction, in addition to the analysis of the regulatory cascade that takes place in the mesodermal cells.

Key words: BMP, animal pole, induction, blood, frog

INTRODUCTION

The classical transplantation studies in amphibian embryos revealed that they have dual embryonic origins of hemopoietic cells that contribute to distinct populations in ontogeny (Kau and Turpen, 1983; Flajnik *et al*., 1984; Maéno *et al*., 1985a; Zon, 1995). The first population of hemopoietic cells arises from the ventral blood islands in the tailbud embryo, and the second population comes from the dorsal-lateral plate mesoderm where the dorsal aorta, gonad and mesonephros are to differentiate. The ventral blood islands mainly consist of erythroid precursor cells but also they have precursor cells of lymphocytes that differentiate later in thymus and spleen (Maéno *et al*., 1985b). Although the origins of two distinct populations are refined recently (Lane and Smith, 1999; Ciau-Uitz *et al*., 2000), it still remains to be determined how the default mesodermal cells are fated to the hemopoietic lineages in molecular basis. This review describes the efforts to elucidate the molecular mechanisms of how the organizer and anti-organizer factors participate in the hemopoietic cell differentiation in early development of *Xenopus laevis* embryo. In particular, significance of interaction between different germ layers in determination and specification of hemopoietic cells from the ventral mesoderm is emphasized.

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Organizer activity and red blood cell differentiation

In 1985, Smith and colleagues first showed that the organizer activity-baring tissue predominantly inhibits the differentiation of ventral tissues including blood cells (Smith *et al*., 1985). They combined the dorsal marginal zone (DMZ) tissue from the gastrulating embryo with the ventral marginal zone (VMZ) tissue and cultured them *in vitro*. The lineage-tracing experiment revealed that the dorsal tissues such as notochord and muscle differentiated from the VMZ-derived cells. The dorsalizing activity was ascribed to a com-

ponent of Spemann's organizer and was clarified as one of the three essential signals for body plan in amphibian early embryogenesis (Slack *et al*., 1987). Molecular characterization studies of organizer factors in the early 90s revealed that the secretory molecules, such as noggin, chordin and follistatin, that can bind to BMP-4 and inhibit its activity, carry on the dorsalizing activity (Smith and Harland, 1992; Sasai *et al*., 1994; Hemmati-Brivanlou *et al*., 1994; Piccolo *et al*., 1996; Zimmerman *et al*., 1996: Iemura *et al*., 1998). If a dominant-negative form of BMP-4 receptor that lacks the cytoplasmic region of type I receptor was expressed in the prospective ventral blastomeres, differentiation to blood or mesenchymal cells was cancelled, and instead, these cells differentiated to muscle and notochord (Maéno *et al*., 1994a; Suzuki *et al*., 1994; Graff *et al*., 1994). Thus, the organizer activity apparently affects negatively on the differentiation program of blood cells.

Organizer factors are expressed at the prospective dorsal marginal zone in the gastrulating amphibian embryos (Fig. 1A). The expression of organizer genes is regulated by wnt and nodal signals, which trigger the expression of the transcription factors, such as goosecoid, lim-1 and siamois, in the organizer region (Cho *et al*., 1991; Taira *et al*., 1991; Watabe *et al*., 1995; Lemaire *et al*., 1995). *In situ* hybridization analysis indicated that BMP-4 message is enriched in the prospective ectoderm and mesoderm areas except the organizer region at the gastrula stage (Fainsod *et al*., 1994; Schmidt *et al*., 1995). BMP-4 signaling is maintained in the prospective ventral and lateral regions by the existence of transcription factors, vent-1/2 and msx-1/2 (Gawantka *et al*., 1995; Onichtchouk *et al*., 1996; Maeda *et al*., 1997; Suzuki *et al*., 1997; Onitsuka *et al*., 2000). It has been shown that these transcriptional repressor proteins restrict the expansion of organizer activity at the dorsal region, but they are not related with blood program (Kumano *et al*., 1999; Takeda *et al*., 2000) (Fig. 1B).

The ventralizing activity of the frog BMP-4 was described in 1992 (Dale *et al*., 1992; Jones *et al*., 1992). The embryo injected with BMP-4 RNA exhibited a spherical structure without any embryonic axis and blood-like cells appeared inside. However, it was not shown whether BMP-4 directly or indirectly stimulates red blood cell formation. In the latter case, BMP-4 may deplete organizer activity from the embryo so that lack of organizer indirectly activates the blood differentiation program. The explant system in amphibian embryos made it possible to assess the activity of molecules in a particular type of tissues. The dorsal marginal zone (DMZ) explant after 2-day-culture contains dorsal and lateral tissues, such as notochord and muscle. Such explant, if injected with BMP-4 RNA, differentiates mainly into blood cells as revealed by the expression of red blood cell-specific marker, α-globin (Maéno *et al*., 1994a). Likewise, animal cap explant, which differentiates to atypical ectodermal tissue without treatment, also produces α-globin after the injection of BMP-4 RNA (Clement *et al*., 1995; Hemmati-Brivanlou and Thomsen, 1995). These results revealed that BMP-4 is not merely antagonizing the organizer activity but also stimulating blood program directly.

The notion that the BMP signal is essential for the development of blood cells from undifferentiated embryonic cells was also examined in zebrafish and mammalian systems (Johansson and Wiles, 1995; Kishimoto *et al*., 1997; Nakayama *et al*., 2000; Adelman *et al*., 2002). In mice, the embryonic stem cells isolated from blastocysts were shown to have capacity to differentiate into ventral mesoderm if exposed to the differentiation medium that includes the

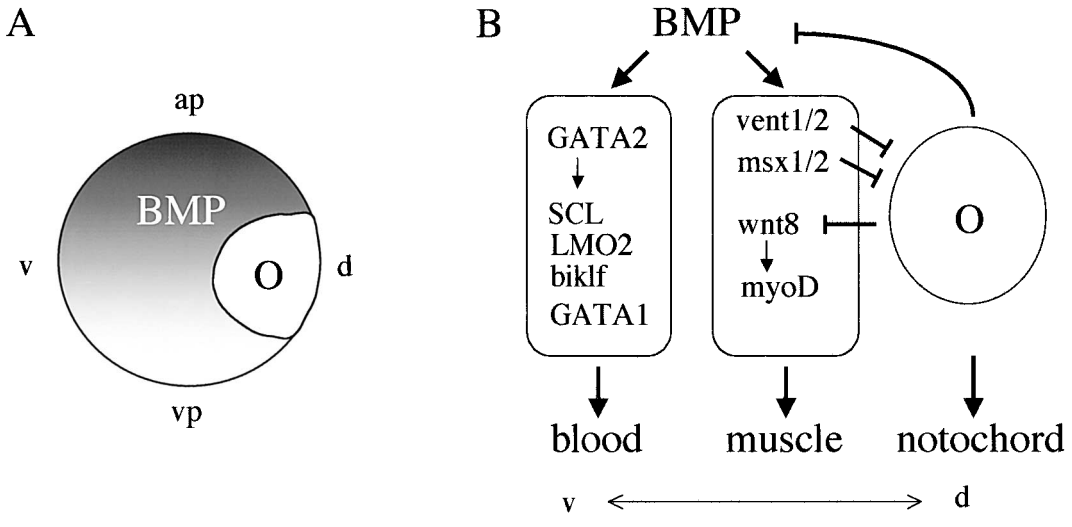

Fig. 1. Factors involved in patterning of mesodermal derivatives along with dorso-ventral axis. (A) Localization of organizer factors (O) and BMP-4 (BMP) in the early gastrula embryo. (B) Ventralizing signaling cascade raised by BMP activity. Vent and msx are the direct target genes of BMP signal. These factors restrict the organizer activity by suppressing the transcription of organizer genes. Wnt8 is a factor essential for the expression of myoD and muscle tissue differentiation. GATA-2 is a candidate of direct target of the BMP signal. SCL, LMO2, biklf and GATA1 are involved in the differentiation of primitive blood formation at the neurula and tailbud stages. ap, animal pole; ve, vegetal pole; v, ventral; d, dorsal.

recombinant BMP peptides (Johansson and Wiles, 1995). It was further shown recently that BMP and vascular endothelial growth factor (VEGF) cooperate each other in formation of the blood cells from the embryonic stem cells *in vitro* and VEGF enhances the differentiation of lymphoid lineages (Nakayama *et al*., 2000). These observations confirm the conserved roles of the BMP signaling in the hemopoietic differentiation in vertebrate embryonic cells.

It is well known that molecules carrying on the organizer activity are unequivocal to establish embryonic axes. Without this activity embryo differentiates into a spherical structure lacking neural and muscle tissues (Fig. 2). The axis-less embryos can be made easily by a brief UV irradiation at the vegetal pole or by removal of a small aliquot of vegetal cortex cytoplasm after fertilization (Fig. 2B). Such embryos are still able to form the mesoderm layer after the gastrulation and the α-globin message is detected in a large area of mesoderm associated with animal pole-derived ectoderm (pigmented area) of these embryos. These hyper-ventralized embryos have no muscle or notochord tissue. It has been shown that the differentiation of muscle tissue depends on wnt-8, a downstream factor of the BMP-4 signaling (Fig. 1B). However, mesoderm cannot differentiate into the muscle tissue without the existence of organizer activity in embryos. This implies that BMP-4 functions in the muscle development by means of modification of organizer signal through wnt-8. In contrast, a major population of primitive blood cells differentiate independent from the organizer activity, although an anterior part of ventral blood islands apparently originates from the organizer-dependent dorsal area as discussed below (Tracey *et al*., 1998; Lane and Smith, 1999).

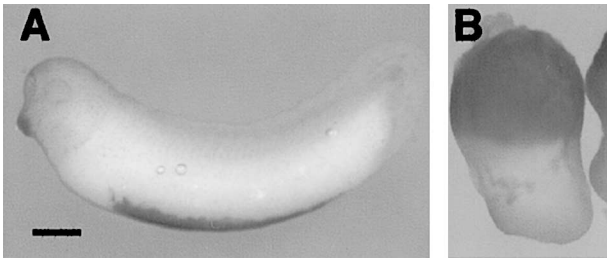

Fig. 2. Whole-mount *in situ* hybridization showing the expression of α-globin in untreated (A) and hyper-ventralized albino embryo (B). Hyper-ventralized embryo was produced by removal of vegetal cortex cytoplasm after fertilization (Iraha *et al*., 2002). Expression of α-globin is observed in the ventral blood islands of untreated embryo and in the one end of hyper-ventralized embryo (upper side of panel B) where the animal pole cells are localized. Both embryos are st. 32. Bar in A indicates 0.5 mm.

Origin of primitive blood cells

Traditional fate map studies suggested that the lateral mesoderm including ventral blood islands originates from the ventral marginal zone (VMZ) which is located at the farthest place from the organizer-forming region at the early gastrula stage (Keller, 1976; Dale and Slack, 1987) (Fig. 3A and 3B). Lane and Smith (1999) proposed a modified fate map by injection of β-gal RNA as a tracer to pursue the descendants of each blastomere of the 32-cell-stage embryo (Fig. 3C). Their results were different from the previous view that the C4 cells (contributing to the ventral marginal zone at the gastrula stage) are the major source for blood islands. The C3 cell contributes mainly to the posterior muscle tissue and the D1-4 cells located at more vegetal area give rise to ventral mesoderm including blood islands (Fig. 3C). This ring-shaped area of vegetal marginal zone was called "leading edge mesoderm (LEM)" (Keller, 1991), where the primitive blood precursor cells are mainly located. Since LEM was closely associated with the organizer region at the prospective dorsal part of embryo (see Fig. 1A), it was necessary to explain how these cells were fated to blood cells under the influence of the organizer activity. Their follow-up study using the explant system showed that nodal and FGF are a positive and a negative regulator, respectively, for red blood cell differentiation (Kumano *et al*., 1999; Kumano and Smith, 2000; Kumano *et al*., 2001). In the animal marginal zone, at which FGF is active, muscle tissue appears, and in the vegetal marginal zone (LEM), at which

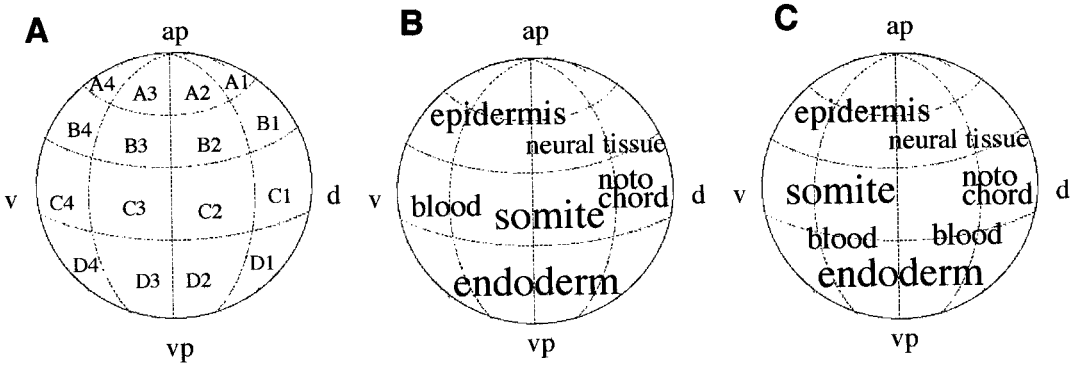

Fig. 3. Fate maps of *Xenopus* 32-cell embryos. Nomenclature of blastomeres (A). A conventional map (B) (see review by Zon, 1995) and a newly proposed map (C) (Lane and Smith, 1999). In the new map, ventral marginal zone mainly comprises prospective somite tissue and the area of vegetal marginal zone, that is called "leading edge mesoderm (LEM)", of both dorsal and ventral blastomeres comprise prospective blood cells. The details of the modification of map is also discussed elsewhere (Lane and Sheets, 2000).

FGF is negative and nodal is positive, then the blood cells appear. This hypothesis is attractive because the expression pattern of brachyury and nodal-related factors is consistent with the distribution of red blood precursor cells in the gastrulating embryo. However, we and other investigators pointed out that the association of ectoderm tissue derived from animal pole cells is important for the extensive blood cell differentiation (Maéno *et al*., 1992; Maéno *et al*., 1994b; Walmsley *et al*., 2002). This interaction should occur during or after the gastrulation process. This view proposes that the determination and specification from the mesoderm to blood cells may occur through interaction between germ layers (Kikkawa *et al*., 2001).

According to the recent tracing experiment as described above, the blastomeres of VMZ excised at the early gastrula stage should differentiate mainly into muscle tissue, since this region includes A4, B4, C4 and D4 cells of the 32-cell embryo (Fig. 3A). However, the VMZ explant after 2-day-culture, in fact, does not contain muscle cell at all, but instead, blood, endothelial and mesenchymal cells are found. What does make different results in lineages between the whole embryo and the VMZ explant? The VMZ explant system provides us with an excellent experimental system for investigating the regulatory mechanism for blood and vascular cells. Thus it is worth to make clear the source of blood cells in the explant of VMZ. Our tracing experiment using β-gal RNA as a tracer indicated that C4 cell of the 32-cell embryo contributes mainly both to blood cells and vascular cells (Iraha *et al*., 2002). The A4 and B4 cells differentiated into epidermis and the D4 cell descendants were mainly incorporated in the endoderm area. These results indicated that the C4 cells, that are fated to muscle lineage in the whole embryo, differentiate into vascular and blood cell lineages in the VMZ explant. It suggests therefore that an inductive event occurred after the gastrula movement is very important for the determination of mesoderm derivatives.

Two-step activation model for red blood cell formation

In general, tissue interaction is important for the determination and specification of a particular cell type in vertebrata development. The classic studies in avian embryonic tissue culture experiments demonstrated that the association of endoderm cells is necessary for the development of blood island formation (Wilt, 1965; Miura and Wilt, 1969; Zagris, 1986). On the other hand, in recent studies in *Xenopus* embryo, animal pole tissue that differentiates into the epidermal cells stimulates the differentiation of blood cells as indicated by α-globin expression in explants. In this amphibian system, no obvious positive effect was observed in the combination with endodermal tissue (Maéno *et al*., 1992). The stimulation may occur due to transfer of specific molecules from ectoderm to mesoderm rather than non-specific effect of the ectoderm on survival of mesoderm derivatives. In our recent experiment, muscle and notochord tissues but not blood cells differentiated in the exogastrulated embryos in which there was no contact between ectoderm and mesoderm layers. Furthermore, the removal of animal pole tissue from blastula embryos caused severe impairment in blood cell differentiation even though the epithelial tissues developed normally (Kikkawa *et al*., 2001). These results suggested that the precursor cells located in the LEM of the gastrula embryo are still undifferentiated cells that can be influenced by the surrounding environment (Fig. 4).

The differentiation of ventral blood islands in *Xenopus* embryo appears as Y-shaped α-globin expression pattern at the tailbud stage (stage 26-27). Prior to the expression of α-globin, it has been shown that the same region of mesoderm expresses GATA-1, GATA-2, LMO-2, biklf (Neptune), and SCL (Zon *et al*., 1991; Kelley *et al*., 1994; Walmsley *et al*., 1994; Mead *et al*., 1998; Mead *et al*., 2001; Huber *et al*., 2001) (Figs. 1 and 3). SCL (stem cell leukemia), an earliest marker ever known, starts to express in the anterior ventral mesoderm at the neurula stage (stage 15–16), thus the determination for the red blood cells should be done prior to the neurula stage (Mead *et al*., 1998). The red cell marker was detected in the cultured explant of ventral marginal zone that was excised from the early gastrula stage (stage 10). However, the tissue from the restricted area of marginal zone without any cells from the animal pole tissue (pigmented cells) showed a poor differentiation of red blood cells (Maéno *et al*., 1992). If the prospective endodermal region including descendants of D1–D4 blastomeres of the 32-cell-stage-embryo was excised at the early gastrula stage and cultured for two days, no blood cell marker was detected (Maéno *et al*., 1992). All these observations suggested that the essential step for blood cell determination occurs during the late gastrula and the early neurula stages. As shown in Fig. 4, the invaginated mesoderm cells migrate to the ventral side and interact with the ectoderm layer. The molecular nature of the factor derived from ectodermal cells and of the response occurring in the mesodermal cells remains to be discovered, but a candidate involved in this activation is BMP-4 (Fig. 4) since the major site of BMP-4 expression in the gastrula embryo is the animal pole area as shown by *in situ* hybridization (Fainsod *et al*., 1994).

Taken together, I propose the “two step activation model” for the determination of blood cells from the undifferentiated totipotent embryonic cells (Fig. 4, Kikkawa *et al*., 2001). These undifferentiated cells must be induced by mesoderm-inducing factors, such as activin or nodal-related (nr) factors. The expression pattern of nr factors in embryos as well as the responding capacity of animal pole tissue to the mesoderm-inducing factors suggest that the first mesoderm-inducing step occurs at the blastula stage (stage 8–9). The second step involves the activation by the ectodermal cells that makes the default mesoderm cells to differentiate into ventral blood islands.

Molecular analyses of blood formation program

It has been shown that introduction of BMP-4 is suffi-

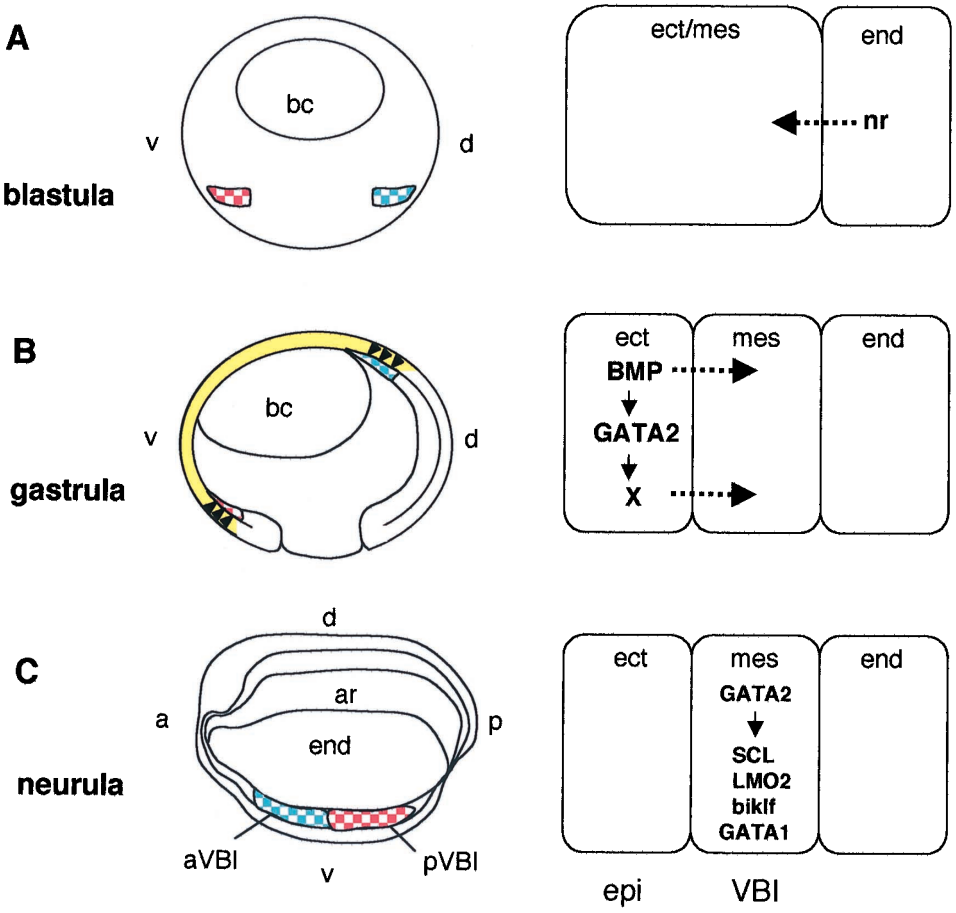

Fig. 4. A model of mechanism underlying the primitive blood formation in *Xenopus* embryo. At the blastula stage, the precursor cells for blood cells (stippled area) are located at the leading edge mesoderm (LEM, Lane and Smith, 1999) at both prospective ventral (red) and dorsal (blue) marginal zone. The cells located in the dorsal marginal zone will form anterior ventral blood islands (aVBI) whereas the cells located in the ventral marginal zone will form posterior ventral blood islands (pVBI). At first, mesodermal cells become competent to blood cell induction by the activation of activin/nodal-related (nr) signaling from the endoderm. Determination of blood cells occurs after specific interaction between ectoderm and mesoderm at the gastrula stage through BMP signaling-dependent pathway (arrowheads). Unknown X factor might be involved in the activation process (Kikkawa *et al*., 2001). Distribution of X factor is indicated by yellow color. Blood cell-specific expression of transcription factors, such as GATA2, SCL, LMO2, biklf and GATA1, starts at the neurula stage at which the fate of blood cells is being determined. bc, blastocoel; ar, archenteron; d, dorsal; v, ventral; a, anterior; p, posterior; end, endoderm; mes, mesoderm; ect, ectoderm; epi, epidermis; VBI, ventral blood islands.

cient for α-globin expression in the animal pole tissue explant. On the other hand, activin or nodal-related factors, known as general mesoderm inducer molecules, do not induce sufficient α-globin expression (Jones *et al*., 1995; Miyanaga, 1998). Thus the BMP-4 signal is essential for the development of blood cells at the initiation step of the program. BMP-4 is a secretory molecule that binds to the BMP-4 receptors and transduces a specific signal to activate the target genes through Smad proteins. If one of direct target genes of the BMP-4 signal encodes for a transcription factor responsible for blood cell differentiation by itself, the BMP-4 signal activates the blood program through this molecule. A few transcription factors are implicated in the differentiation program of blood cells. SCL, isolated from a murine leukemia cell line, was shown to be a most upstream transcription factor functioning in the blood cell lineage (Mead *et al*., 1998; Walmsley *et al*., 2002). GATA-1 and -2 are the essential transcription factors that regulate *globin* gene expression in erythroid lineage (Kellley *et al*., 1994; Walmsley *et al*., 1994). Recently involvement of LMO-2 and biklf in primitive blood cell differentiation was reported respectively (Mead *et al*., 2001; Huber *et al*., 2001). These factors are expressed in the blood islands in the ventral mesoderm of *Xenopus* embryo and play essential roles in blood cell differentiation program. However, the process of determination and specification of blood cell lineage from the undifferentiated mesoderm has not yet been fully understood. Except GATA-2, these transcription factors start to express at the neurula stage. GATA-2 is shown to be a direct target of the BMP signal in the gastrula embryo (Friedle and Knöchel, 2002) and a good candidate molecule for binding DNA at the blood stem cell enhancer found in the 3' flanking region of SCL gene and for inducing SCL (Gottgens *et al*., 2002). However, it is still obscure whether the expression of GATA-2 at

the gastrula stage is related with the initiation of SCL, LMO-2 and GATA-1 expression in blood islands at the tailbud stage.

Differential regulation of red and white blood cell differentiation

In avian and amphibian species it has been shown that the primitive blood cells mainly contribute to the transient population of red blood cells (Zon, 1995). However, our previous studies using the chimeric animals between the cytogenetically labeled individuals revealed that a small population of hemopoietic precursors are involved in the ventral blood island-derived cells and a significant population of adult lymphocytes and erythrocytes arises from this area. The classical transplantation experiments showed that 70–90% of larval thyomocytes and splenocytes, and 20–30% of adult thyomcytes and splenocytes are derived from the ventral blood islands (Maéno *et al*., 1985a). Therefore, it is reasonable to speculate that a small number of hemopoietic stem cells arise at the ventral blood islands and these cells colonize the hemopoietic organs to proliferate and differentiate into peripheral lymphocytes at later stages. A recent study by Walters *et al*. (2002) demonstrated that the BMP signaling in the blood precursor cells and overlying ectodermal cells affects on the following determination of the cell fate to white or red blood cell lineage, but further studies will be necessary to elucidate how the hemopoietic stem cells that arise from the primitive blood-forming region are developmentally regulated.

In addition to the lymphoid lineages, phagocytic cells should be argued in terms of their embryonic origin. Ohinata *et al*. (1989) established a monoclonal antibody against an antigen (XL-1) common to all the white blood cells. XL-1-positive cells appeared in the mesenchyme of the whole body as migrating cells at the late tailbud stage prior to the timing of differentiation of lymphocytes in thymus and spleen. Cell-lineage tracing experiment showed that these macrophage-like cells are not derived from the ventral blood islands. These cells arise even from a head part or a tail part explant. Therefore these macrophage-like cells differentiate everywhere in the tailbud embryo (Ohinata *et al*., 1990). Furthermore, in the recent study to identify the white blood cell lineage by the expression of specific lineage marker in zebrafish, a population of hemopoietic precursor cells was found in the head mesenchyme near the neural tube. These cells in zebrafish express PU.1 transcription factor and differentiate into macrophage-like cells (Lieschke *et al*., 2002). Emergence of macrophage-like cells derived from the anterior ventral mesoderm was also reported in *Xenopus* embryo (Smith *et al*., 2002). These observations suggest an existence of uncharacterized hemopoietic origin in vertebrate embryos. It is not known yet whether these cells contribute to a transient or a definitive population of blood cells. This should be answered by the transplantation technique.

Concluding remarks

This review describes the possibility of ectoderm as a specific inducer of the primitive blood cell formation in *Xenopus* embryo. Although it is likely that expression of BMP-4 in the ectodermal cells is important in differentiation of blood cells in adjacent mesoderm, it is still obscure whether or not BMP-4 is the only factor responsible for the blood cell formation. Since BMP-4 also activates the expression of wnt-8 in dorso-lateral mesoderm and promotes the differentiation of muscle tissue, there must be the mechanisms by which the BMP signaling is separated into these different mesodermal lineages. GATA-2 is a possible target gene of BMP-4 signal at the gastrula and neurula stages in ectodermal cells, but it is expressed in a broad area where both blood and non-blood mesoderms are localized. Therefore, in future, elucidation is warranted to define more factors that are expressed in ectoderm and mesoderm in the prospective ventral blood islands.

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