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Original Article

Two-step induction of primitive erythrocytes in *Xenopus laevis* embryos: signals from the vegetal endoderm and the overlying ectoderm

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ABSTRACT Primitive blood cells differentiate from the ventral mesoderm blood islands in *Xenopus* embryos. In order to determine the tissue interactions that propagate blood formation in early embryogenesis, we used embryos that had the ventral cytoplasm removed. These embryos gastrulated normally, formed a mesodermal layer and lacked axial structures, but displayed a marked enhancement of α–*globin* expression. Early ventral markers, such as *msx-1, vent-1* and *vent-2* were highly expressed at the gastrula stage, while a dorsal marker, *goosecoid*, was diminished. Several lines of experimental evidence demonstrate the critical role of animal pole-derived ectoderm in blood cell formation: 1) Mesoderm derived from dorsal blastomeres injected with β–*galactosidase* mRNA (as a lineage tracer) expressed α–*globin* when interfaced with an animal pole-derived ectodermal layer; 2) Embryos in which the animal pole tissue had been removed by dissection at the blastula stage failed to express α–*globin*; 3) Exogastrulated embryos that lacked an interaction between the mesodermal and ectodermal layers failed to form blood cells, while muscle cells were observed in these embryos. Using dominant-negative forms of the BMP-4 and ALK-4 receptors, we showed that activin and BMP-4 signaling is necessary for blood cell differentiation in ventral marginal zone explants, while FGF signaling is not essential. In ventralized embryos, inactivation of the BMP-4 signal within a localized area of the ectoderm led to suppression of *globin* expression in the adjacent mesoderm layer, but inactivation of the activin signal did not have this effect. These observations suggest that mesodermal cells, derived from a default pathway that is induced by the activin signal, need an additional BMP-4-dependent factor from the overlying ectoderm for further differentiation into a blood cell lineage.

KEY WORDS: *VBI, BMP-4, activin, ectoderm.*

Introduction

In amphibian embryos, mesodermal derivatives are believed to appear in consequence of the interaction between ectodermal and endodermal cells. The vegetal organizing center (Nieuwkoop center) appears at the opposite site from the point of sperm entrance (Nieuwkoop, 1973), and anterior-dorsal structures develop in accordance with involution of mesodermal cells from the dorsal lip (Keller, 1976; Gerhart et al., 1989). On the other hand, the ventral tissues, such as blood cells, were previously thought to differentiate mainly from the equatorial region of the same side as that of sperm entrance (Keller, 1976; Kessler and Melton, 1994). However, recent studies have redrawn the origin of blood cells; namely, the cells forming ventral blood islands at the tailbud stage arose from the "leading edge mesoderm" at the vegetal limit of the marginal zone (Lane and Smith, 1999; Kumano et al., 1999; Ciau-Uits et al., 2000). Hypothetically, according to the "three signal theory" of Slack et al. (1987), differentiation of the mesoderm is regulated by secretory factors from the surrounding vegetal endoderm (*i.e.*, mesoderm-inducing factors). Candidates of mesoderm-inducing factors are activin (Asashima et al., 1990; Green and Smith, 1990; Thomsen et al., 1990), vg-1 (Thomsen and Melton, 1993), nodal-related factors (Jones et al., 1995; Osada and Wright, 1999) and FGF (Slack et al., 1987; Slack, 1990). These factors can induce various mesodermal

Abbreviations used in this paper: ALK-4, activin receptor-like kinase-4; BMP-4, bone morphogenetic protein-4; DAI, dorsoanterior index; DMZ, dorsal marginal zone; FGF, fibroblast growth factor; VMZ, ventral marginal explant.

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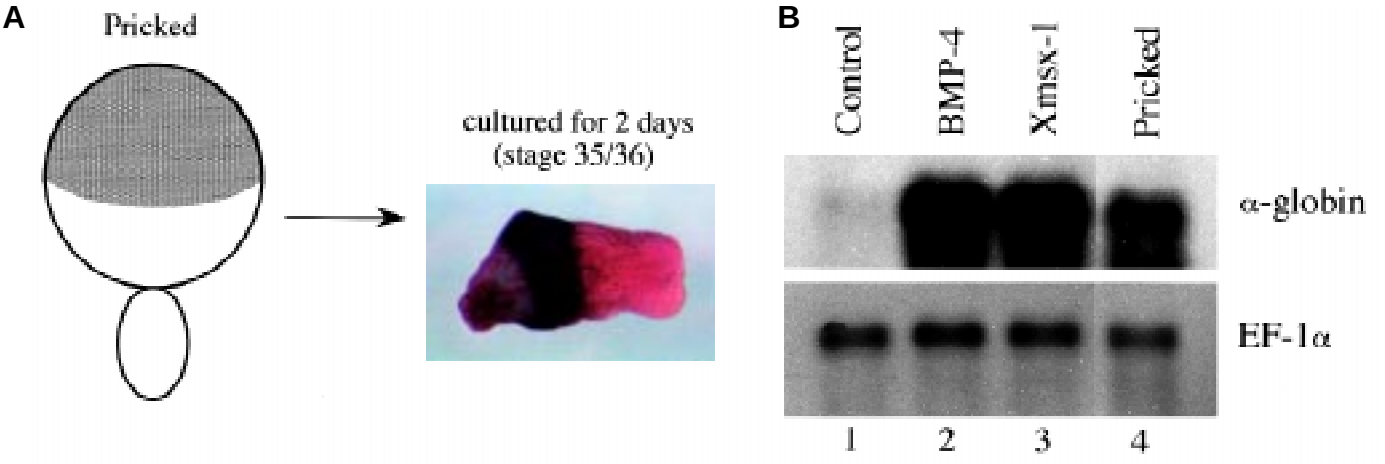

Fig. 1. Removal of the vegetal pole cytoplasm caused an enhanced expression of *α-globin* in the embryo. (A) *Schematic image of the depletion experiment. The vegetal pole cytoplasm was removed by pricking the embryo at about 0.3 NT. Such embryos showed a radial symmetrical shape (DAI=0), (Kao and Elinson, 1988) and expressed a large amount of α-globin.* **(B)** *Northern blot analysis showing that expression of α-globin in the cytoplasm-removed embryos (lane 4) was drastically increased in comparison with that in untreated normal embryos (lane 1). The same effect was also observed in BMP-4 (lane 2)- or msx-1 (lane 3) -injected embryos (5 ng RNA/embryos). The expression of EF-1α served as a loading control.*

tissues in the animal cap explant, but none of them has been proven to induce the differentiation of functional erythropoietic cells. Thus, it is still ambiguous whether factors from the endoderm are sufficient for determination and specification of the ventral mesoderm-derivatives.

The other important factor involved in the blood-inducing program is bone morphogenetic protein-4 (BMP-4). Transcripts of *BMP-4* are broadly expressed in the prospective ventral and lateral regions at the gastrula stage, crossing over the ectodermal and the mesodermal areas (Fainsod et al., 1994; Schmidt et al., 1995). Overexpression of *BMP-4* RNA in the dorsal marginal zone or animal cap explants led to the induction of an erythrocyte-specific marker gene (*α–globin*) (Maéno et al., 1994b; Maeda et al., 1997), and a dominant-negative form of the BMP-4 receptor was able to induce a secondary axis when expressed in the prospective ventral blastomeres (Graff et al., 1994; Suzuki et al., 1994). Although there seems to be no doubt that BMP-4 is essential in ventralization and blood cell differentiation in embryogenesis, the mutual regulation between BMP-4 and the mesoderm-inducing factors has not yet been studied. For instance, it is not known whether the mesoderm-inducing factors can trigger *BMP-4* expression in the ectodermal and mesodermal areas in the embryo.

A recent study by Kumano and Smith (1999) has provided us with an important insight into blood cell formation. They showed that the

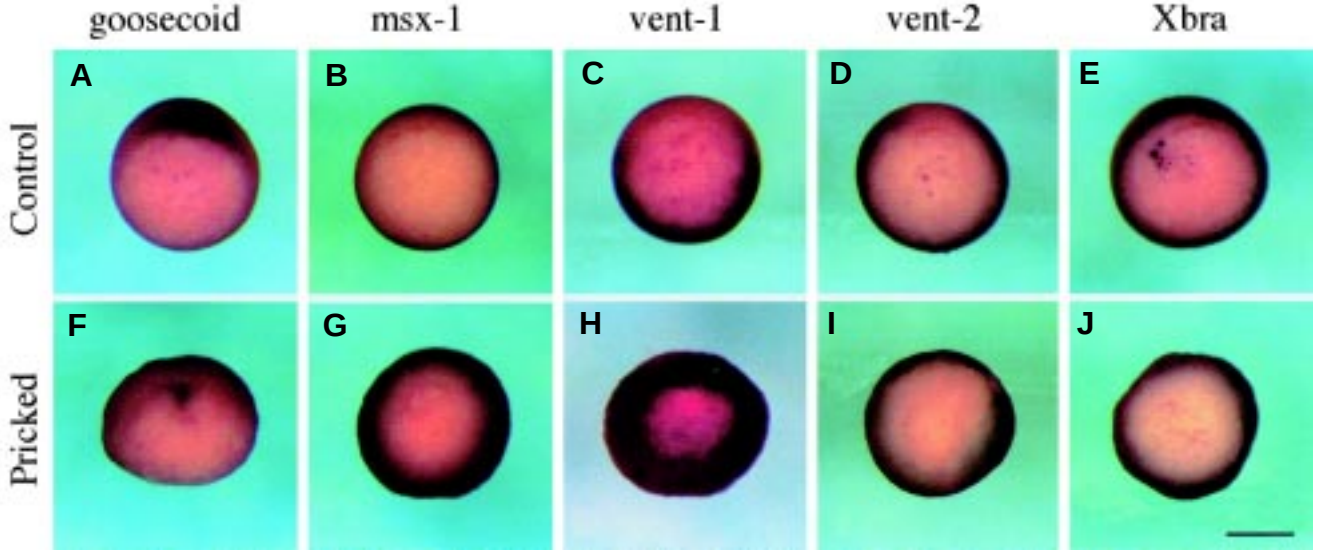

Fig. 2. Whole-mount *in situ* hybridization in control and cytoplasm-removed embryos showing the expression of early dorsal, ventral and panmesodermal markers. *Expression of goosecoid* **(A,F)**, *Xmsx-1* **(B,G)**, *vent-1* **(C,H)**, *vent-2* **(D,I)** *and Xbra* **(E,J)** *was examined in control* **(A-E)** *and cytoplasm-depleted embryos at the early gastrula stage (stage 10+). In treated embryos, the expression level of a dorsal marker (goosecoid) was decreased and expression levels of the ventral markers, Xmsx-1, vent-1, vent-2, were increased. Expression of the pan mesodermal marker (Xbra) was not affected by this treatment. All pictures are vegetal views with the dorsal region to the top. Scale bar in J indicates 0.5 mm.*

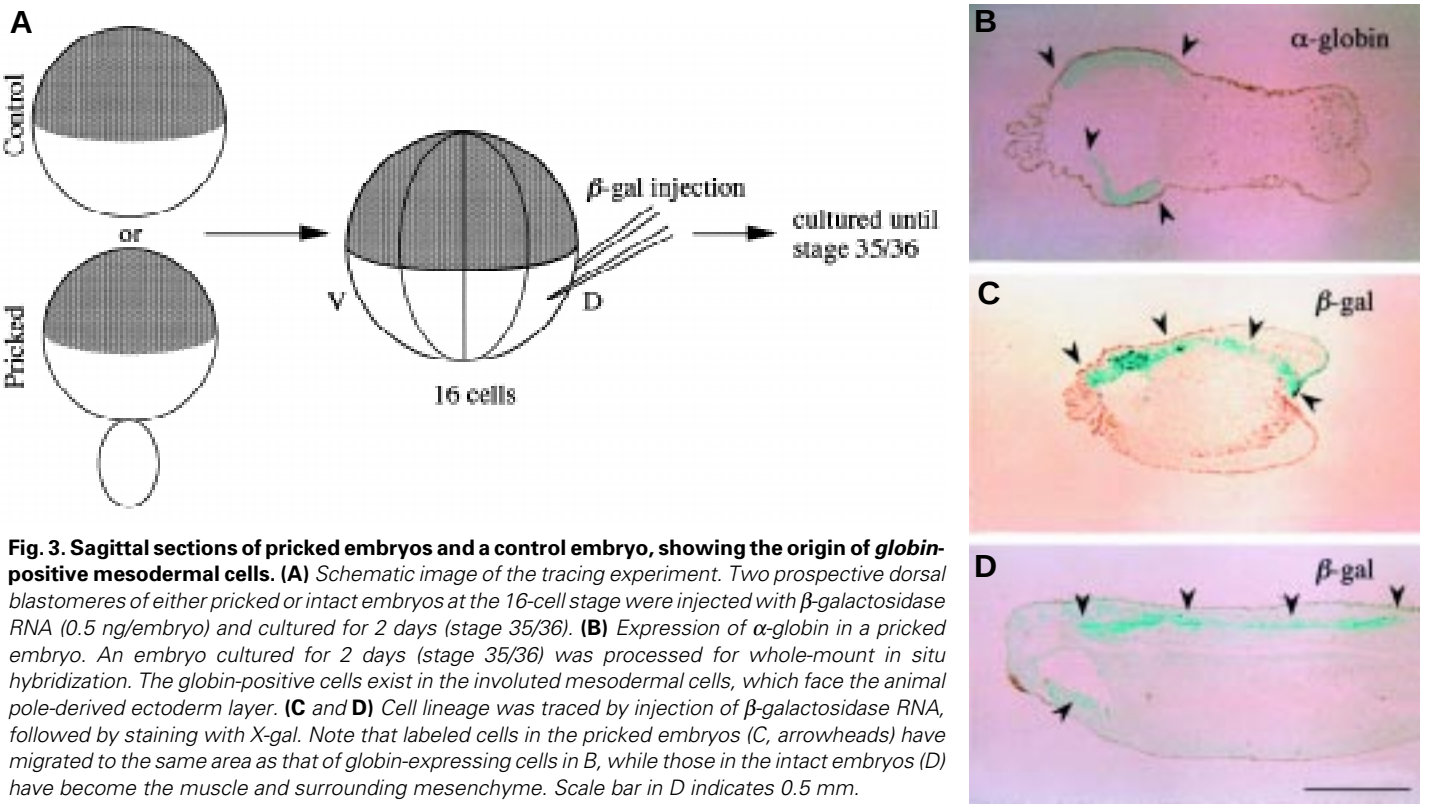

Fig. 3. Sagittal sections of pricked embryos and a control embryo, showing the origin of *globin*-positive mesodermal cells. (A) *Schematic image of the tracing experiment. Two prospective dorsal blastomeres of either pricked or intact embryos at the 16-cell stage were injected with β-galactosidase RNA (0.5 ng/embryo) and cultured for 2 days (stage 35/36).* **(B)** *Expression of α-globin in a pricked embryo. An embryo cultured for 2 days (stage 35/36) was processed for whole-mount in situ hybridization. The globin-positive cells exist in the involuted mesodermal cells, which face the animal pole-derived ectoderm layer.* **(C** *and* **D)** *Cell lineage was traced by injection of β-galactosidase RNA, followed by staining with X-gal. Note that labeled cells in the pricked embryos (C, arrowheads) have migrated to the same area as that of globin-expressing cells in B, while those in the intact embryos (D) have become the muscle and surrounding mesenchyme. Scale bar in D indicates 0.5 mm.*

downstream factors of BMP signaling, such as vent-1, vent-2 and wnt-8, do not function in blood cell differentiation and that the BMP-4 signal from the ectoderm layer may be important at the later stage. This is consistent with the results of our previous studies (Maéno et al., 1994a; 1994b) showing that the ectodermal cells enhanced erythropoietic differentiation in the associated mesodermal cells in the combination explants. In order to further elucidate the blood-inducing program, "vegetal cytoplasm-removed embryos" (Kikkawa et al., 1996) were used in the present study. Removal of small volumes of cytoplasm from the vegetal pole at the one-cell stage causes loss of the axial structure. Such embryos pass through the gastrulation stage normally and a mesodermal layer is formed. We consider this experimental system to be more useful than the explant system used in our previous studies for study of the blood program (Maéno et al., 1994a; 1994b), because the cytoplasm-removed embryo retains the mutual interaction between germ layers through the gastrulation stage. At first, we investigated the erythropoietic differentiation in the treated embryos by analyzing *α–globin* as a differentiation marker of erythrocytes. Subsequent studies of cell lineage-tracing and microsurgical experiments clearly demonstrated that mesodermal cells can differentiate into blood cells under the specific interaction with the overlying ectodermal cells.

Results

Expression of early and late mesodermal markers in pricked embryos

We have shown that removal of the cytoplasm from the vegetal pole area causes complete loss of the embryonic axis in the resultant embryos (Kikkawa et al., 1996). Such embryos exhibited a spherical structure without any dorsal tissues. *In situ* hybridization analysis showed that tadpole *α-globin*, a marker of red blood cells, was strongly positive in embryos cultured for 2 days (Fig. 1A). We thought that this experimental system would contribute to the understanding of the blood cell differentiation program in early embryogenesis, and we first investigated the expression of *α-globin* in the embryos by Northern blot analysis to quantify the message. As shown in Fig. 1B, the embryos from which the vegetal cytoplasm had been removed by pricking expressed a large amount of *α-globin* mRNA. An increase in the level of *α-globin* expression was also observed in embryos in which *BMP-4* or *msx-1* RNA had been injected into dorsal blastomeres. This indicates that the fate of mesodermal cells in the cytoplasm-removed embryos had been modified.

Pricked embryos developed normally until the early gastrula stage after healing in Ficoll-containing medium. In order to examine the effect of pricking on mesodermal differentiation at the gastrula stage, expression of dorsal (*goosecoid*), ventral (*msx-1*, *vent-1*, and *vent-2*) and pan (*Xbra*) mesoderm markers was analyzed in the treated embryos by whole-mount *in situ* hybridization. Externally, the dorsal lip was observed in both pricked and control embryos at stage 10. As shown in Fig. 2, however, *goosecoid* expression in the dorsal marginal zone was clearly diminished. In contrast, all of the three ventral markers (*msx-1*, *vent-1*, and *vent-2*) were expressed in a larger area and their signals extended toward the dorsal marginal zone, whereas the expression of *Xbra* remained constant. Among the ventral markers, expression of *vent-1* in the marginal zone was highly affected by this treatment.

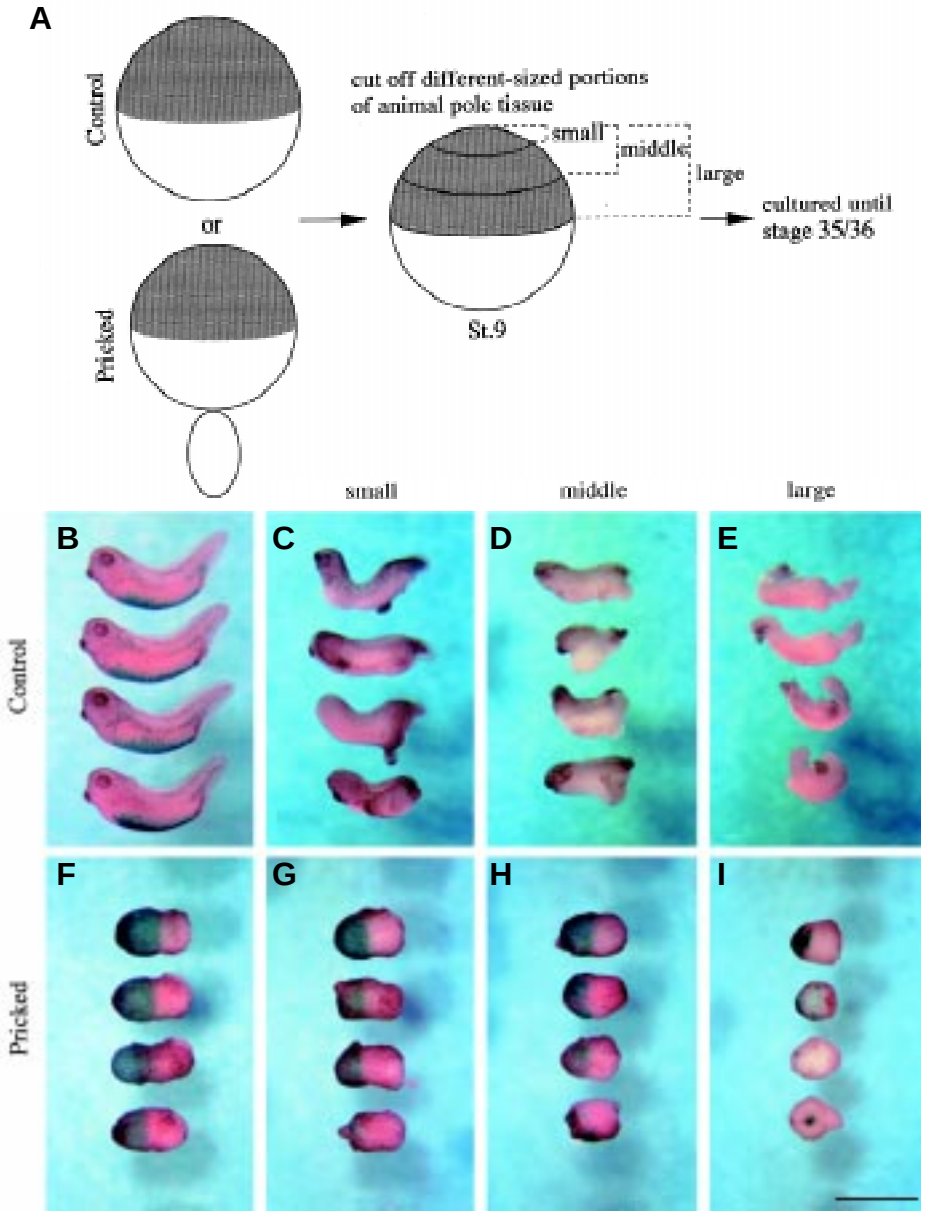

Fig. 4. Effect of animal pole tissue on *α-globin* expression in control and cytoplasm-depleted embryos. (A) *Schematic image of the experimental procedure. Pricked and untreated embryos were allowed to develop until stage 9, and a small (20% of the animal hemisphere)* **(C,G)**, *middle (50% of the animal hemisphere)* **(D,H)**, *or large (100% of the animal hemisphere)* **(E,I)** *part of the animal pole tissue was removed as described in Materials and Methods. Such embryos were further cultured for 2 days and were used for the whole-mount in situ hybridization analysis to detect the expression of α-globin.* **(B-I)** *Typical examples from each experimental group are shown. Note that removal of the animal pole tissue caused a drastic reduction in α-globin expression in both control* **(B,C,D,E)** *and cytoplasm-depleted* **(F,G,H,I)** *embryos. Scale bar in I indicates 2 mm.*

These results suggest that the presumptive mesoderm had been already ventralized by this treatment at the early gastrula stage.

Origin of globin-positive cells in pricked embryos

Histological sections through a fully ventralized embryo showed that it contained three well-differentiated germ layers. As shown in *in situ* hybridization analysis (Fig. 3B), mesodermal cells, which were facing toward the animal pole-derived ectoderm, expressed *globin* mRNA. In order to examine whether prospective dorsal blastomeres contribute to the formation of red blood cells, we performed lineage tracing. The two dorsal-vegetal blastomeres of 16-cell-stage embryos, from which their vegetal cytoplasm had been removed, were injected with *β-galactosidase* RNA for lineage labeling, and these embryos were cultured for 2 days. A comparison of the *globin*-positive cells with Xgal-stained cells revealed that blastomeres, which were located in the dorsal marginal zone, formed a mesodermal layer and contributed mainly to the red blood cells (Fig. 3 C,D). This finding implies that the fate of presumptive dorsal mesodermal cells, the ancestors of notochord, muscle, and anterior pharyngeal mesoderm were converted uniformly into *globin*-positive cells in the pricked embryos.

Indispensable role of the ectodermal layer in the blood-inducing program

We further investigated the mechanism by which the invaginated mesoderm can differentiate into the red blood cells. Since the results of our previous study (Maéno et al., 1994a) suggested the existence of a hematopoietic stimulator(s) in the animal pole tissue, we investigated the significance of the ectodermal layer in blood cell differentiation. Control embryos and embryos depleted by pricking were allowed develop, and various degrees of animal pole cells were manually removed at the blastula stage using fine forceps and a glass needle. After 2 days of culture, expression levels of *α-globin* in the embryos were examined by *in situ* hybridization analysis. As shown in Fig. 4, the amounts of *α-globin* decreased in accordance with increases in the amount of subsequent removal of the animal pole tissue at the blastula stage in the pricked embryos (Fig. 4 G,H,I). Since the excised animal pole cells did not contain blood precursor cells (Fig. 3), we thought that the existence of animal pole cells is indispensable for the blood precursor cells to differentiate. On the other hand, removal of the animal pole tissue also led to severe effects on morphogenesis in the control (non-pricked) embryos (Fig. 4 C,D,E). These embryos exhibited defective structures in ectodermal lineages, such as the brain, eye capsules, cement gland, and epithelium. Surprisingly, the embryos with defects in dorsal axial structure did not express *α-globin* at all. Although it could not be determined why removal of such a small portion (approximately 6% in volume) of the animal pole area caused compete loss of blood cells in embryos, these results strongly suggest the importance of the overlying epidermis in blood cell differentiation.

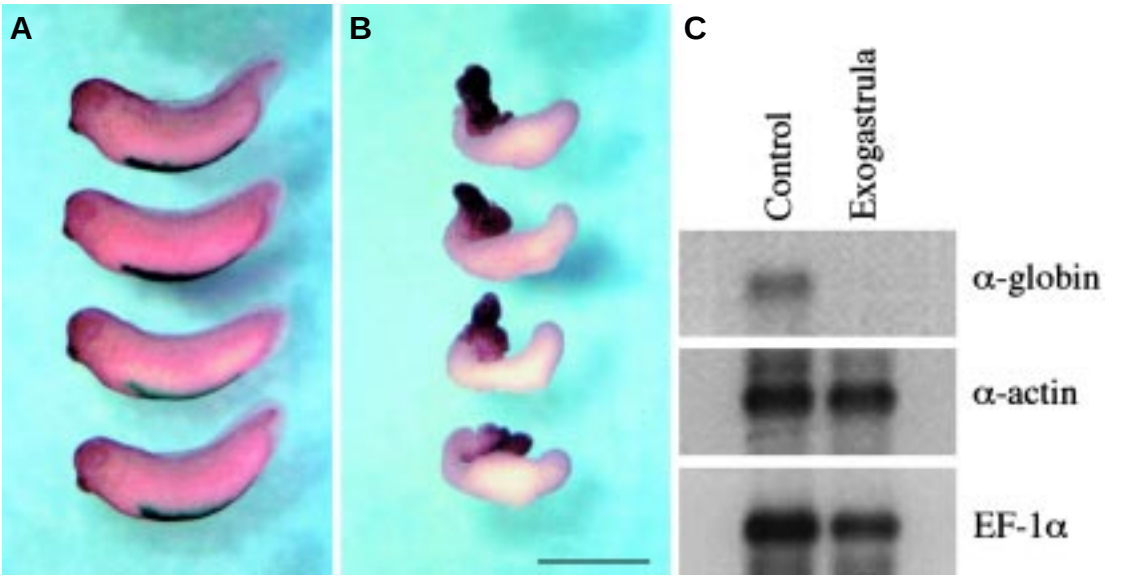

Fig. 5. Effect of animal pole tissue on *α-globin* expression in control and exogastrula embryos. *Treated embryos* **(B)** *and untreated embryos* **(A)** *were allowed to develop until stage 7 and then cultured in 1.4Å~MMR as described in Materials and Methods. Such embryos were further cultured for 2 days (stage 35/36) and were used for whole-mount in situ hybridization analysis (A,B) and Northern blotting analysis* **(C)** *to detect the expression of α-globin. In exogastrula embryos (B), no expression of α-globin was observed. Scale bar in B indicates 2 mm. In addition, the expression of α-actin was not affected in the exogastrula embryos. The expression of EF-1α served as a loading control.*

Similar results were obtained in the exogastrulated embryo, a conventional experimental model for investigating mesoderm-ectoderm interaction. Such embryos incubated in a high-salt medium during the blastula stage cause failure of mesoderm involution. In the resultant embryos, mesodermal derivatives differentiate without interaction with the overlying epidermis (Holtfreter, 1933; Ruiz i Altaba, 1992). We investigated the expression levels of *α-globin* in the exogastrulated embryos. As shown in Fig. 5 B,C, only trace levels of *α-globin* expression were observed in the treated embryos, although muscle differentiated autonomously (Fig. 5C). Furthermore, in the exogastrulated embryos whose vegetal cytoplasm had been removed at the one-cell stage, a marked reduction in *globin*-positive cells was observed (data not shown). These results are again consistent with the hypothesis that specific induction from epidermal cells is involved in blood cell differentiation.

Roles of BMP-4 and activin signals in erythrocyte differentiation in ventral marginal zone explants

To examine the importance of BMP-4, activin and FGF signals in the ventral marginal zone (VMZ) explants, from which red blood cells actually differentiate, we attempted to inhibit each signal using a dominant negative mutant of the receptor in VMZ blastomeres. In these experiments, we used RNAs encoding a dominant negative mutant of the BMP receptor (*tBR*), dominant negative mutant of the activin receptor (*tALK4*), and a dominant negative mutant of the FGF receptor (*XFD*) (Suzuki et al., 1994; Chang et al., 1997; Amaya et al., 1991).

RNA was injected into two prospective ventral cells at the 4-cell stage, and the VMZ region was dissected at stage 10+. These explants were cultured for 2 days to detect α-globin protein by Western blot analysis (Fig. 6A). As shown in Fig. 6B, α-globin expressions of *tBR*--injected and *tALK-4*-injected VMZ explants were completely inhibited. However *XFD* did not affect the α-globin expression in these explants. The same dose of *XFD* was enough to see the posterior truncation phenotypes if it was injected into prospective dorsal cells at the 4-cell stage (Amaya et al., 1991). It seems, therefore, that endogenous FGF signaling is inhibited by the RNA doses used in the present experiment.

Histological observation of the RNA-injected explants showed that control VMZ explants formed three well-differentiated germ layers (Fig. 6C). *tBR*-injected VMZ explants contained dorsal tissues such as neural cells and muscle, as was reported previously (Graff et al., 1994; Suzuki et al., 1994; Maéno et al., 1994). There was no obvious germ layer structure in the*tALK-4*-injected VMZ, suggesting that no mesoderm had been induced in the explants. In contrast to the *tBR*- and *tALK-4*-injected explants, *XFD*-injected VMZ explants contained blood-like cells. The epidermis layer was very thick and melanin granules were accumulated in the XFD-injected explants. These structures resembled that of an atypical ectoderm, as was also observed in the animal cap without any treatment. These results indicate that BMP-4 and activin signalings are definitely required but that FGF signaling is not important for red blood cell differentiation.

In order to assess the importance of BMP-4 and activin signalings further, we injected dominant-negative forms of receptor RNAs into one animal pole cell of 16-cell- stage embryos that had been pricked at tha one-cell stage to remove the vegetal cytoplasm (Fig. 7A). The resultant embryos were allowed to develop until the tailbud stage, and the expression of *α–globin* was examined by whole-mount *in situ* hybridization. Co-injection of *β-galactosidase* RNA with *tBR* or *tALK-4* RNA enabled us to trace the injected cells. As shown in Fig. 7B, the β–galactosidase-positive cells (indicated as blue) are overlying the *α–globin*-positive cells (indicated as red) in the control (*β-galactosidase* alone) and *tALK-4*-injected embryos. In contrast, the β–galactosidase-positive cells exist apart from the *α–globin*-positive cells in *tBR*-injected embryos. We often observed that the surface of epidermis where *tBR* RNA had been injected forms a small protrusion. In five independent experiments, 23% and 5% of embryos injected with *tBR* (0.625 and 1.25 ng/embryo, respectively) exhibited overlapped distributions of β–galactosidase- and *α–globin*-positive cells. These findings are in

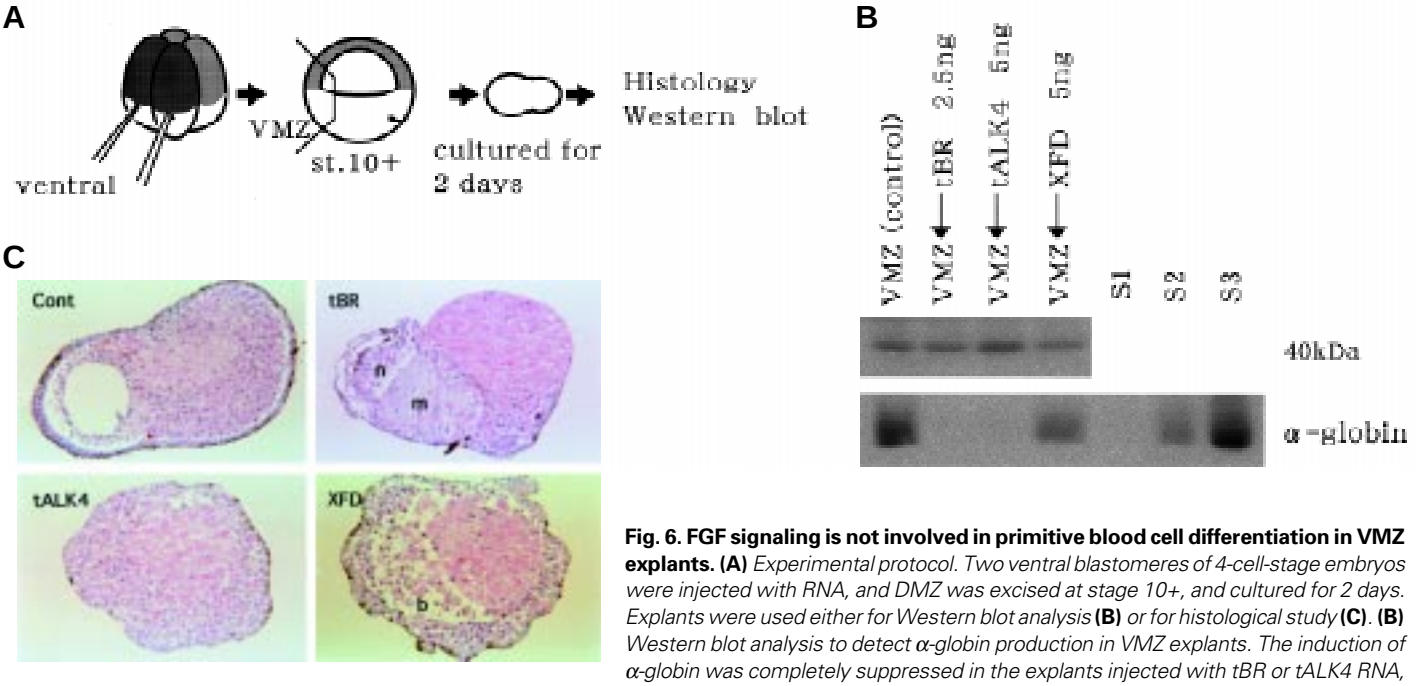

Fig. 6. FGF signaling is not involved in primitive blood cell differentiation in VMZ explants. (A) *Experimental protocol. Two ventral blastomeres of 4-cell-stage embryos were injected with RNA, and DMZ was excised at stage 10+, and cultured for 2 days. Explants were used either for Western blot analysis* **(B)** *or for histological study* **(C)**. **(B)** *Western blot analysis to detect α-globin production in VMZ explants. The induction of α-globin was completely suppressed in the explants injected with tBR or tALK4 RNA, but retained in the explants injected with XFD RNA.* **(C)** *Histological observation of RNA-injected explants. A tBR-injected VMZ explant contained well-differentiated axial structures such as neural tissue (N) and muscle (M). A tALK4-injected explant contained homogenous undifferentiated cells. Blood-like cells (B) are shown in the explants injected with XFD. A thick epidermis layer with many melanin granules is also characteristic of XFD-injected explants.*

sharp contrast to the results showing that β–galactosidase- and *α–globin*-positive cells were mostly overlapped in *tALK-4*-injected embryos (Table 1).

Histological observation of the stained embryos showed that the *α-globin*-positive cells are located in the mesodermal layer. The superficial layer injected with *tBR* RNA formed an atypical thick epidermis. Cement gland or neural tissue was not observed in the examined specimens. β–galactosidase- and *α–globin*-positive cells were in direct contact with each other in the *tALK-4*-injected embryos, whereas they were spatially separated in *tBR*-injected embryos (data not shown).

Discussion

Pricked embryos provide an ideal experimental model for studies of the blood-inducing program

We have pointed out the importance of animal pole tissue in stimulation of primitive erythropoietic differentiation in the ventral blood island mesoderm (Maéno et al., 1994a). This conclusion was obtained from the results of experiments using a combination of explants of the ventral mesoderm and animal pole-derived ectodermal cells. We have shown that the prospective ventral mesoderm excised from the early- gastrula embryo could not fully differentiate into erythrocytes and that the association of ectodermal cells dramatically enhanced the erythropoietic differentiation. However, the possibility of slight contamination of the animal pole cells (ectodermal cells) in the mesodermal explant can not be ruled out, and it was difficult to conclude that ventral marginal cells are non-committed mesodermal cells at the early gastrula stage. The experimental system used in the present study is superior to the previous one with respect to the following two points. First, the majority of marginal cells, including prospective dorsal mesodermal cells, differentiated into erythrocytes in the present study, whereas only prospective ventral cells were used in the previous studies. This implies that animal pole cells can induce blood cells from the prospective dorsal mesoderm. Second, the present system allowed us to prevent contact of the ectoderm to the mesoderm by a relatively simple method. We demonstrated, in this way, the importance of ectodermal cells in contact with mesodermal cells for blood cell differentiation.

Association of endodermal cells is not sufficient for blood cell differentiation

Although it is believed that mesodermal derivatives in amphibian embryos differentiate as a consequence of interaction between ectodermal and endodermal layers, the mechanisms underlying the differentiation of mesoderm derivatives are still a matter of conjecture. The ventral mesodermal derivatives can be induced from animal cap cells by the association of ventro-vegetal prospective endodermal cells at the blastula stage (Nieuwkoop, 1969) or by the addition of FGF or low doses of activin to the culture medium (Slack, 1990; Ariizumi et al., 1991). A recent study has shown that a combination of FGF and activin synergistically enhanced the differentiation of *globin*-positive erythrocytes in a cap assay (Miyanaga et al., 1999). From these results, an inductive signal from endodermal cells could be predicted. On the other hand, however, there is no direct proof of *globin*-positive cells being induced from the animal cap in a combination with vegetal cells. In fact, we could not detect any *globin* message in the Nieuwkoop's combination explant (data not shown). In the present study, as shown in Fig. 4, removal of animal pole tissue from the vegetal cytoplasm-depleted embryo caused decreased expression of

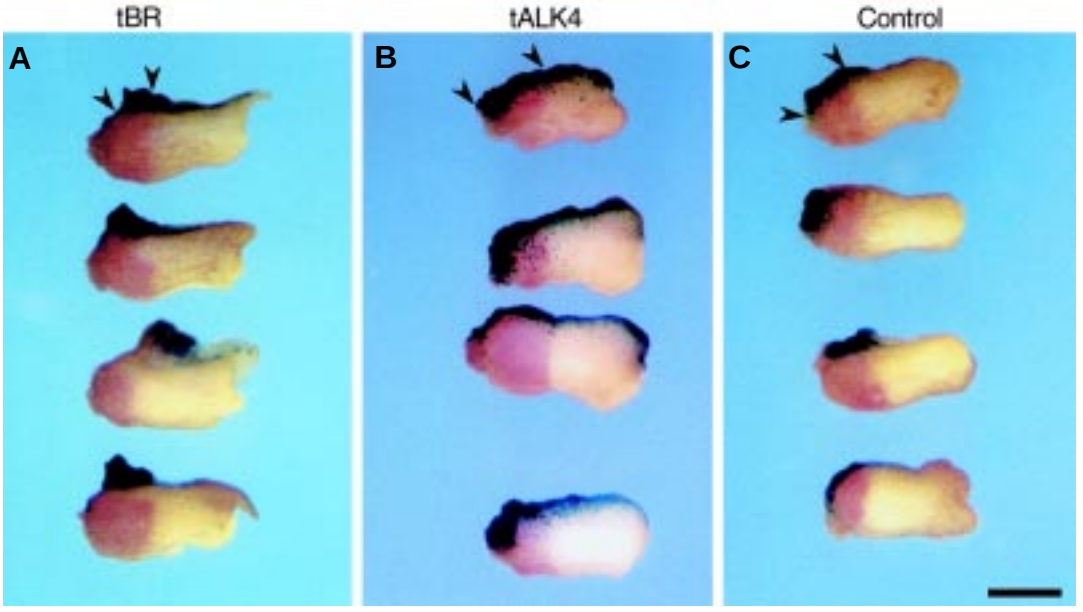

Fig. 7. BMP-4 signaling is involved in the stimulation process from the epidermis to the blood precursor cells in the adjacent mesoderm. *At the 16-cell stage, truncated BMP-4 receptor (tBR) RNA* **(A)** *or truncated activin receptor (tALK4) RNA* **(B)** *was injected into a single animal cell of embryos in which the vegetal pole cytoplasm had been removed (pricked embryos). Each cell lineage was simultaneously traced by co-injection of β-galactosidase RNA. On the second day of culture after injection, embryos were fixed and processed for X-gal staining (blue signals) followed by whole-mount in situ hybridization. The globin-expressing cells were visualized with fast red (red signals). β-galactosidase-positive cells are largely overlying the globin-expressing cells in the control and tALK4-injected embryos, whereas β-galactosidase-positive cells are apart from the globin-expressing cells in tBR –injected embryos. Two arrowheads beside the top embryos of each panel indicate the overlapping regions. Embryo shown in the top of panel A was judged as an overlapped embryo in Table 1. Scale bar = 1 mm.*

the *globin* message, and the exo-gastrulated embryo, in which the ectoderm and mesoderm rarely interact with each other, did not express *globin* at all, although the *actin* message was detected normally (Fig. 5). Taken together with the fact that expression of ventral mesoderm markers was highly enhanced at the mid-gastrula stage after depletion of the vegetal cytoplasm, the results indicate that the action of vegetal cells is not sufficient for erythropoietic differentiation in the ventral mesoderm.

Induction of blood cells by association of ectodermal cells

Figure 8 represents our hypothesis on the inductive regulation of primitive erythropoiesis. The cells located in the marginal area are, at first, induced to form a "default mesoderm" by activin-like signaling probably derived from prospective endodermal cells. Such a default status of the mesoderm is further committed for erythroid lineage by the factor(s) derived from the ectodermal layer. This factor(s) may be BMP-4 itself or a BMP-4-dependent factor(s), since the activation of BMP-4 signaling is essential for the differentiation of ventral tissues. Overexpression of *BMP-4* in the prospective dorsal blastomeres converts the fate of the dorsal phenotype to a ventral one. The disruption of this signal using a dominant-negative receptor in the prospective ventral blastomeres caused impairment of blood cell differentiation and induction of a secondary axis (Graff et al., 1994; Suzuki et al., 1994; Maéno et al., 1994b). On the other hand, in the ectodermal lineage, *BMP-4* suppresses the differentiation of neural tissue differentiation (Wilson and Hemmati-Brivanlou, 1995), and knockout of the BMP-4 signal is sufficient for inducing neural tissue (Xu et al., 1995; Suzuki et al., 1995). Thus, the blood cells differentiate in the area where the BMP-4 signaling is activated in both mesodermal and ectodermal layers. Therefore, at least two steps of induction (or two distinct factors) are necessary for complete differentiation of blood cells. The two-step induction model is in good agreement with a recent observation by Kumano and Smith (1999) that inhibition of the BMP-4 signaling in the epidermis is the most effective to the reduction of globin synthesis in mesodermal tissue. Considering the timing of interaction between mesodermal and ectodermal cells, they suggested that the blood cell induction occurs after the late gastrula stage. Evidence of the importance of ectodermal cells was also obtained in the present study. Our system was able to exclude the effect of the Spemann organizer, a strong inhibitor of blood cell formation.

In the present study, we investigated the regulation of blood cell differentiation in early-stage *Xenopus* embryos, but the regulation of endothelial cell differentiation in the blood islands is also of great interest. Recent studies using mammalian and avian species have suggested that both lineages are derived from a common precursor cell. The results of preliminary studies conducted in our

TABLE 1

SPECIFIC INHIBITION OF *GLOBIN* EXPRESSION BY A TRUNCATED BMP-4 RECEPTOR

RNA	dose/embryo (ng)	% overlapped (number of embryos)
tBR	0.625	23 (39)
tBR	1.25	5 (20)
tALK4	0.625	100 (24)
tALK4	2.5	94 (16)
None	-	100 (26)

Designated doses of *tBR* or *tALK4* RNA were coinjected with *β–galactosidase* RNA into an single animal pole cell of pricked embryos at the 16-cell stage. Control embryos were injected with *β-galactosidase* RNA (0.5 ng/embryo) alone. The embryos were cultured for 2 days and subjected to whole-mount *in situ* hybridization to detect *globin* message (red). Injected cells were lineage-labeled by X-gal staining (blue). The percentage indicates the ratio of embryos showing areas of overlapping expression of *globin* and *β-galactosidase* over the total number of embryos injected (n).

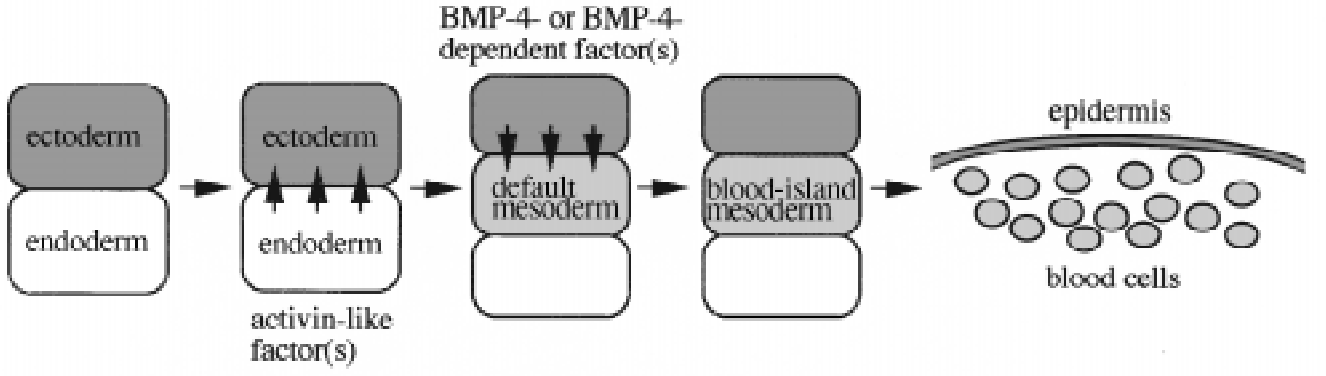

Fig. 8. A model of the blood differentiation program in the ventral mesoderm. *As is generally accepted, endoderm-derived factors, such as activin (or an activin-like factor), induce the formation of a default mesoderm in the marginal zone of the embryo. This default mesoderm needs interaction with the overlaying ectoderm to differentiate into blood cells. This factor(s) is thought to be BMP-4 itself or a secretory factor(s) activated by the BMP-4 signaling (BMP-4-dependent factor).*

laboratory showed that *tie-2*, a specific cellular marker for angiogenesis (Sato et al., 1993), is induced by injection of *BMP-4* RNA in dorsal marginal zone explants in which neither *globin* nor *tie-2* is expressed if cultured without injection. Further studies are needed to elucidate the control mechanisms in mesoderm patterning and tissue specification in early embryogenesis.

Materials and Methods

Preparation of eggs and embryos and RNA injection

Xenopus laevis females were injected with 250 units of human chorionic gonadotropin and kept at 20-23°C for overnight to induce ovulation. Eggs were fertilized with a sperm suspension prepared by mincing testis in 1xMMR (100 mM NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 5 mM Hepes (pH 7.6), 0.1 mM EDTA). After 10-15 minutes, fertilized eggs were dejellied in 2.5% thioglycolic acid (pH 8.3). Eggs were washed twice in 50% Steinberg's solution (29 mM NaCl, 0.035 mM KCl, 0.25 mM $Ca(NO_3)4H_2O$, 2.3 mM Tris-HCl, 4 μg/ml phenol red, pH 7.4) and used for experiments. Stages were determined according to Nieuwkoop and Faber (1967).

Capped mRNAs were made using a MEGAscript kit (Ambion). *tALK4* in pSP64T (Chang et al., 1997), *XFD* (Amaya et al., 1991) in pSP64T, and *tBR* (Suzuki et al., 1994) in pSP64T were linearized with EcoRI, and RNAs were transcribed with SP6 RNA polymerase.

Removal of the vegetal pole cytoplasm

Removal of the vegetal pole cytoplasm was performed as described previously (Kikkawa et al., 1996). Briefly, dejellied eggs were transferred to 50% Steinberg's solution containing 6% Ficoll. Eggs were pricked with a fine glass needle at the vegetal pole. Pricking was done at 30-40% of the time duration of the first cell cycle (0.3-0.4 NT). Pricked eggs were healed in 6% Ficoll solution for 5 minutes. The eggs were then transferred to 50% Steinberg's solution and left in the solution for 5 minutes so that the vegetal pole region was squeezed by the hydrostatic pressure, and then, the eggs were transferred to 6% Ficoll solution and cultured at 23°C until the end of blastula stage. The eggs were again transferred to 50% or 100% Steinberg's solution containing antibiotics and cultured at 23°C until use.

Induction of exogastrula embryos and microsurgery

Exogastrula embryos were obtained as previously described (Ruiz i Altaba, 1992). After manual removal of the vitelline membrane of each embryo in the early blastula stage (stage 7) with forceps, the embryos were inverted (pigment-side down) and kept on an agarose-lined dish in 1.4xMMR until the end of the gastrula stage (stage 13). Embryos were then transferred to a new dish containing Steinberg's solution with antibiotics and cultured until the sibling control embryos had reached stage 35/36.

To examine the role of ectodermal cells in blood cell differentiation, animal pole tissue was cut off either from the control or vegetal cytoplasm-depleted embryos at stage 9. The vitelline membranes of the embryos, and the embryos were placed on an agarose-lined dish. Appropriate sizes of the animal pole areas were then excised using fine forceps and a glass needle. After healing (30-60 min), each embryo was laid in a small depression made in the agarose and cultured for 2 days. The manipulation and culture of embryos were done in sterilized Steinberg's solution containing antibiotics. By measuring the radii of excised animal pole tissues, the sizes of removed tissues were classified into 3 groups: "small-sized" tissue containing approximately 6% of the animal hemisphere, "middle-sized" containing 25% of animal hemisphere, and "large-sized" containing 100% of the animal hemisphere in volume.

Whole-mount in situ *hybridization*

Whole-mount *in situ* hybridization was performed with digoxigenin-labelled probes according to the protocol of Shain and Zuber (1996). Embryos were fixed in MEMFA (0.1 M MOPS, 2 mM EDTA, 1 mM $MgSO_4$, 3.7 % formaldehyde) for 2 hours at room temperature and stored in 100 % methanol at –20°C . Antisense probes were synthesized from the following plasmids: *goosecoid*, a 360 bp fragment in pCR1000 (linearized by HindIII); *msx-1*, an N-terminal fragment (EcoR I /HindIII, 440bp) in pSP72; *Xvent-1*, full-length (Sal I /Not I, 1.0 kbp) in pBS+; *Xvent-2*, full-length (Sal I/Not I, 1.2 kbp) in pBS+; *Xbra*, a C-terminal fragment (Stu I/EcoR I, 682 bp) in pSP72; and *α-globin*, a fragment (Pst I/Pst I, 360 bp) in pBS+. All of the antisense probes were transcribed using T7 polymerase (Ambion). Positive signals were detected by BM purple (Boeringer) as a substrate.

Lineage-tracing by β-galactosidase

To determine the origin of *globin*-expressing cells in the pricked embryos, *β-galactosidase* RNA was used as a tracing marker as described previously (Maéno et al., 1994b). After removal of vegetal pole cytoplasm at the one-cell stage, capped *β-galactosidase* RNA (0.5 ng/embryos) was injected into two vegetal dorsal blastomeres at the 16-cell stage. These embryos were fixed at 2 days of culture in a solution containing 1% paraformaldehyde, 0.2% glutaraldehyde, 0.02% NP40, 1xPBS (pH 7.4) for 1 hour at 4°C After thorough washings, the embryos were incubated in 1 mg/ml X-gal solution to develop blue color (Sanes et al., 1986). The stained embryos were embedded in Paraplast and cut into 7 μm sections for histological analysis.

For double staining with *in situ* hybridization, a truncated version of receptor RNA (*tBR* or *tALK4*) and capped *β-galactosidase* RNA (0.5 ng/embryos) were co-injected into an animal pole cell of 16-cell-stage em-

bryos, from which the vegetal pole cytoplasm had been removed at the one-cell stage. After staining with X-gal (blue), embryos were refixed in MEMFA solution for 2 h and processed for whole-mount *in situ* hybridization to detect *globin* expression. *In situ* signal was visualized with fast red (red). Some stained embryos were frozen in Tissue Tek Compound and cut into 10 μm-thick sections in a cryostat for histological observation.

Northern blot analysis

Total RNA was extracted from control or treated embryos at stage 35/36 as described previously (Chomczynski and Sacchi, 1987). Each RNA sample (2 μg) was loaded on a 1% formaldehyde-containing agarose gel, transferred to a Hybond-N nylon membrane, and hybridized with *Tα-globin* (0.8 kb Pst I fragment from XLG19; Sandmeier et al., 1988), *α–actin* (1.2 kb BamH I/Hind III fragment; Mouhun et al., 1984) or *EF-1α* (0.4 kb Pst I/Sac I fragment; Krieg et al., 1989). Hybridization was performed in Hybrisol I (Oncor), and the membrane was exposed to Fuji RX-U film after washing. The same blot was sequentially hybridized with different probes to detect each message.

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