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Original Article

Establishment of the organizing activity of the lower endodermal half of the dorsal marginal zone is a primary and necessary event for dorsal axis formation in *Cynops pyrrhogaster*

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ABSTRACT The formation of the head and trunk-tail organizers in the dorsal marginal zone (DMZ) of an amphibian embryo is thought to require spatial and temporal interactions between the Nieuwkoop center and the DMZ. Recent studies of the *Xenopus* embryo suggested that intra-DMZ interaction is also needed to establish the regional specificity of the DMZ. However, it is not yet clarified when and how the final pattern of the head and trunk-tail organizers is established. To analyze the intra-DMZ interactions, we injected suramin into the blastocoel of the mid-blastula of the urodele, *Cynops pyrrhogaster*, at 6 h prior to the onset of gastrulation. The pigmented blastopore formed normally, but the convergent extension and involution of the DMZ and dorsal axis formation of the embryo were completely inhibited. Expression of *gsc, chd* and *Lim-1* were not maintained, but *noggin* was unaffected in the suramin-treated embryos. Dorsal axis formation and the expression of these genes of the suramin-treated embryos were rescued by replacing the lower endodermal half of the DMZ (LDMZ) with normal LDMZ. The present results of embryological and molecular examinations indicate that organizing activity of the early *Cynops* gastrula DMZ is restricted to the LDMZ, and that the organizing activity of the LDMZ is established during the late blastula stages. The results also indicate that LDMZ triggers the sequential interaction within the DMZ that establishes the final pattern of the regional specificity of the DMZ, and that the formation of the LDMZ is a primary and necessary event for dorsal axis formation.

KEY WORDS: *organizing center, dorsal mesoderm-inducing activity, LDMZ, Nieuwkoop center, Cynops pyrrhogaster*

Introduction

By the late gastrula stage, the organizer contains at least two functional regions: a head organizer and a trunk-tail organizer. Recent molecular analysis of the formation and regional specification of the organizers has demonstrated the significance of several peptide growth factors, such as the FGF and TGF-β families, as the signals for the formation of the organizer (reviewed by Kimelman *et al.*, 1992; Harland and Gerhart, 1997; Gerhart, 2001) or the dorsal-vegetal region of the embryo, the so-called Nieuwkoop center (Kurth and Hausen, 2000; Takahashi *et al.*, 2000).

Stewart and Gerhart (1990) suggested that prior to the onset of gastrulation, the DMZ of the early *Xenopus* gastrula consists of two domains: an upper (future posterior) and a lower (future anterior) domain. Gerhart *et al.* (1991) termed the lower domain the late blastula organizer, which is induced by the Nieuwkoop center. Thus, they defined the Nieuwkoop center as that part of the blastula stage embryo that induces the organizer, whereas Kimelman *et al.* (1992) maintained that the Nieuwkoop center and the late blastula organizer might be located in the same region.

In addition to the upper and lower domains of the *Xenopus* DMZ, because of the double-layered germ structure, and the internal

Abbreviations used in this paper: BC, bottle cells; DMZ, dorsal marginal zone; FDA, fluoresceinated-lysin dextran amine; FGF, fibroblast growth factor; LDMZ, lower half of the dorsal marginal zone; SE, suramin-treated embryo; TGF, transforming growth factor; UDMZ, upper half of the dorsal marginal zone.

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gastrulation and vegetal rotation that occurs prior to the onset of gastrulation (Nieuwkoop and Florshutz, 1950; Keller, 1976; Winklbauer and Schurfeld, 1999), it has been further demonstrated that the *Xenopus* DMZ is subdivided into outer- and inner-DMZ that have different prospective fates and gene expression patterns (Shin and Keller, 1992; Vodicka and Gerhart, 1995; Zoltewicz and Gerhart, 1997). Also, the lower domain, especially the outer endodermal epithelium, has been shown to possess organizer activity that induces notochord during gastrulation (Stewart and Gerhart, 1990; Shih and Keller, 1992; Domingo and Keller, 1995; Lane and Keller, 1997). More recently, Harland and Gerhart (1997) and Gerhart (2001) discussed the spatial and temporal steps for the formation of the organizer in *Xenopus*, and proposed that the Nieuwkoop center itself has several spatially separated subregions of autonomous and non-autonomous parts, including the future head and trunk-tail organizers. They also discussed the significance of the intra-organizer interactions that establish the regional specificity of the head and trunk-tail organizers during the late steps. Thus, it is necessary to analyze the spatial and temporal interactions between the upper-, lower-, outer- and inner-DMZ within the organizer in *Xenopus*. However, it remains to be clarified how the final pattern of the head and trunk-tail organizers is established by these intra-organizer interactions.

In the urodelean early gastrula, especially in *Cynops*, the DMZ has a single-layered structure and all the constituents of the future head and trunk-tail organizers or the prospective dorsal axial mesoderm are located on the egg surface (Kaneda and Hama, 1979; Hama *et al.*, 1985; Imoh, 1988; Nieuwkoop, 1996). In *Cynops*, it has been demonstrated that the DMZ of the early gastrula consist of a lower endodermal domain (LDMZ) and an upper ectodermal domain (UDMZ) of the future trunk-tail organizer (Kaneda, 1981; Kaneda and Suzuki, 1983; Hama *et al.*, 1985), and that secondary axis inducing activity is restricted to the LDMZ (Yamamoto and Suzuki, 1994). It has also been shown that the LDMZ and UDMZ interact in a planar fashion during gastrulation to form the head and trunk-tail organizers (Kaneda, 1981; Kaneda and Suzuki, 1983) and furthermore, the autonomous and temporal changes of the LDMZ properties are essential for patterning the head and the trunk-tail organizers (Kaneda *et al.*, 2002). These facts suggest, at least in the *Cynops* embryo, that the LDMZ acts as the source of dorsal mesoderm-induction and triggers the sequential intra-organizer interaction in the DMZ during gastrulation. Thus, it is possible to more easily analyze the intra-organizer interaction in *Cynops* than in *Xenopus* by subdividing the DMZ into its upper and lower regions. However, it is still obscure when and how the dorsal mesoderm-inducing activity of the LDMZ is determined.

Suramin ventralized the dorsal mesodermal differentiation of the DMZ of the early *Xenopus* gastrula (Grunz, 1992, 1993; Oschwald *et al.*, 1993), or aspecifically inhibited dorsal-mesoderm induction in the DMZ (Cardellini *et al.*, 1994), although these results did not indicate which subregions of the DMZ were targeted by suramin. In the present study, using the suramin-treated *Cynops* embryo, we examined the timing of the establishment of the organizing activity of the LDMZ and its role in dorsal axis formation.

Results

Development of the Suramin-Treated Embryo

Suramin was microinjected into the blastocoel of the mid-blastula at 6 h prior to the onset of gastrulation (stage 11 of Okada and Ichikawa's table, 1947). The suramin-treated embryo (SE) continued to develop normally and formed the pigmented blastopore at the same site as the control embryo at stage 11 (Fig. 1 A,D, *white arrowheads*). However, the convergent extension and involution of the DMZ never occurred. By the time the control embryo reached the mid- or late-gastrula stage, the previously formed blastopore of the SE had disappeared (Fig. 1E). Finally, the SE developed into a non-axis embryo with atypical epidermis on the animal side (Fig. 1F), and although blood-like cells were frequently formed, the embryos did not show any dorsal axial mesodermal or neural structures (see Fig. 6E).

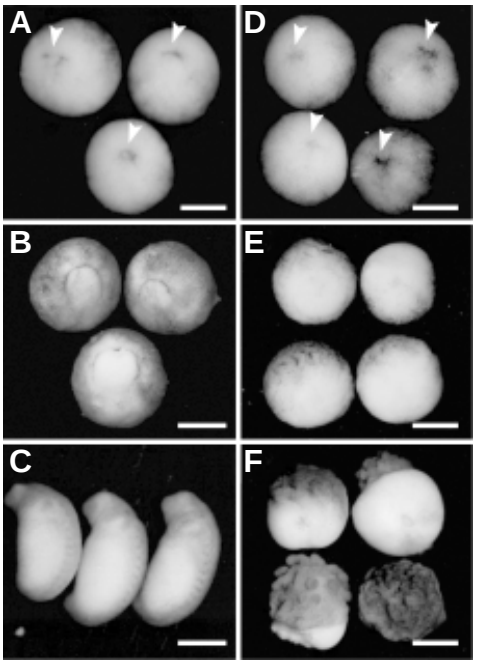


Fig. 1. Effects of suramin on gastrulation and body axis formation. *Suramin was injected at 6 h prior to blastopore formation and the suramin injected embryos (D-F) were observed when the control embryos (A-C) had developed to the early gastrula* **(A)** *stage 11, 0 h; mid-gastrula* **(B)** *stage 12b, 12 h from stage 11; or tail bud embryo* **(C)** *stage 27, 72 h from stage 11. Normal pigmented blastopore (white arrowheads in A and D) formed in the control and suramin-injected (SE) embryos, but once formed it disappeared from the SE at mid-gastrula* **(E)**. *Axis formation was not observed in these embryos* **(F)**. *Bars, 1 mm.*

As shown in Fig. 2, the bottle cells (BC) and their apical contraction were normal in the SE (Fig. 2C) when compared with a normal gastrula (Fig. 2A). Histological examination indicated, however, that the SE did not begin either invagination of the blastopore or involution of the DMZ, even 6 h later when the sibling embryo reached the early mid-gastrula stage (Fig. 2B).

To observe the movement of the blastopore that formed in the SE, FDA was injected into the C1 blastomere of 32-cells embryos in order to label the prospective blastopore (Suzuki *et al.*, 2002), after which suramin was injected into the FDA-labeled blastula. The early gastrula labeled with FDA on the blastopore was selected, and the FDA signals were traced during the further development (Fig. 3). In the control embryo, the FDA signals observed on the blastopore involuted and moved anteriorly with the convergent extension and involution of the archenteron roof (Fig. 3 A-D). On the other hand, the FDA signals in the blastopore of the SE were

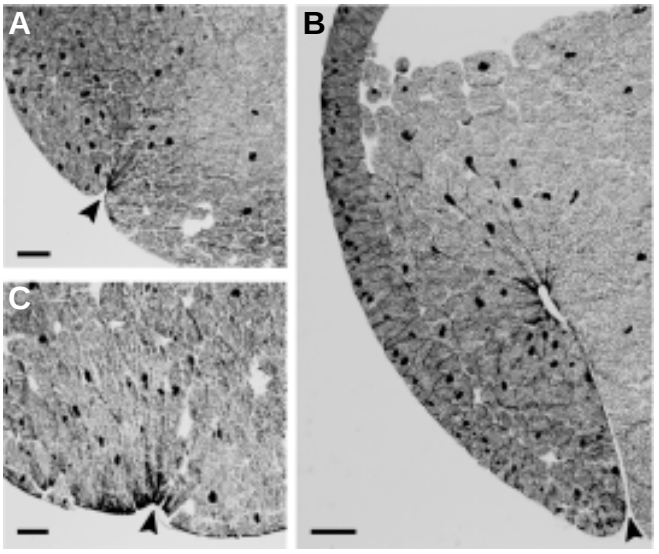
still anchored on the egg surface even when the control embryo had developed into the mid-gastrula (Fig. 3 E-H). These results confirmed that suramin did not inhibit the BC formation of the blastopore, but did arrest both the convergent extension and involution movements.

Suramin inhibited the Secondary Axis-Inducing Activity of the LDMZ

Yamamoto and Suzuki (1994) and Imoh *et al.* (1998) demonstrated that a complete secondary axis was induced when together the LDMZ and the BC (LDMZ+BC) were transplanted into the ventral marginal zone. They also reported that the activity was restricted within the supra-blastoporal marginal zone, an area 30º from the pigment line along the animal-vegetal axis and 60º laterally (Yamamoto and Suzuki, 1994), which corresponds to the LDMZ. To examine the effects of suramin on the organizing activity of the LDMZ, LDMZ+BC of the SE were transplanted into the ventral marginal zone of the normal early gastrula and although well-differentiated secondary axis was induced by the LDMZ+BC of the normal embryo, the secondary axis was not induced by the LDMZ+BC of the SE (0 of 7 cases) (Fig. 4A). The transplanted graft was incorporated into the host endoderm. When the LDMZ, either from normal gastrula (10 cases) or from SE (11 cases), was isolated and cultured alone, it formed a rounded mass of endodermal cells (Fig. 4B) and histologically, none of the neural and mesodermal structures developed (Fig. 4 C,D). These results indicate that suramin does not affect the autonomy for tissue differentiation of the LDMZ itself, but rather its secondary axis-inducing activity.

Gene Expression Patterns in the SE

When the SE and the control embryo reached the required developmental stages, the expressions of the organizer- and tissue-specific genes were analyzed (Fig. 5). At the early gastrula stage (stage 11), the expressions of *gsc*, *chd*, *noggin* and *Lim-1* were detected in the control embryo, and their expressions were maintained during gastrulation. In the SE, the expressions of *gsc*, *chd*, *Lim-1* and *noggin* were detected in the early gastrula, but except for *noggin* were not maintained during further development (Fig. 5A). In the control embryo, *bra* expression was not observed at the early gastrula stage, but was induced at the mid-gastrula stage (12 h from stage 11) onward. On the other hand, *bra* expression was never observed in the SE (Fig. 5A). As shown



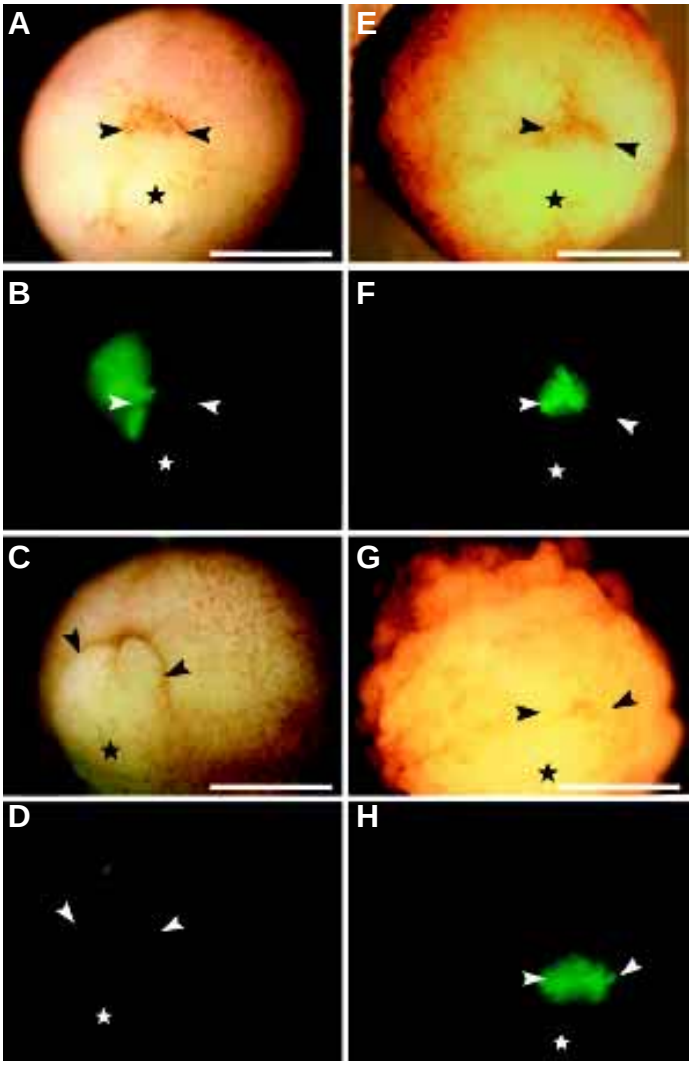


Fig. 2. (Left) Fine structure of the blastopore. (A) *Blastopore of the control early gastrula (stage 11).* **(B)** *Control gastrula that developed for 6 h from stage 11 showed involution of the archenteron.* **(C)** *Blastopore of the SE at the same stage as (B). Note that bottle cell formation and their apical contraction were normal in the SE. Dorsal is to the left. Arrowheads show pigmented blastopore. Bars, 0.1 mm.*

Fig. 3. (Right) Involution of the blastopore in the suramin-injected embryo. *FDA was injected into the C1 blastomere at the 32-cell stage and the embryos in which the blastopore was labeled with FDA were observed. Control early gastrula (stage 11) with pigmented blastopore* **(A)** *and the epifluorescent microscope* **(B).** *FDA signals were detected around the blastopore. At the mid-gastrula stage (12 h after stage 11), FDA signals involuted and moved toward the animal pole* **(C,D).** *In the SE, the pigmented blastopore formed in the same position and shape as the control embryo* **(E,F)**. *When the sibling control embryos reached mid-gastrula, the FDA signals around the blastopore were still anchored on the egg surface of the SE, even after the blastopore had disappeared* **(G,H).** *Arrowheads, lateral edge of the blastopore. Stars, vegetal pole. Bars, 1 mm.*

in Fig. 1, the SE developed into a non-axis embryo with atypical epidermis and an endodermal mass. These embryos expressed epidermis-specific *ep37* and *EF1*-α, but did not express the neural-specific genes (*en-2*, *pax-6* and *NCAM*) or the muscle-specific α-*actin* (Fig. 5B).

LDMZ rescued Dorsal Axis Formation of the SE

The LDMZ+BC of the SE were removed and replaced with their equivalent from a normal early gastrula (Fig. 6A). The LDMZ-replaced embryo began the convergent extension and involution movements of the DMZ, and finally dorsal axis formation occurred. Typical profiles of the rescued embryos are shown in Fig. 6D. Although these rescued embryos showed mainly trunk-tail or tail structures, 15 of 18 (83%) LDMZ-replaced embryos formed a normal dorsal axis. Histologically, most (11 of 15) of the rescued embryos formed the body axis with well-differentiated neural and dorsal axial mesodermal structures, and 7 of 15 formed the hindbrain with an ear vesicle (Fig. 6 F,G).

Imoh *et al.* (1998) reported that LDMZ+BC grafted onto the ventral marginal zone induced a complete secondary axis, and they clearly showed that the grafted LDMZ+BC itself formed the roof and floor of the anterior archenteron and developed into the anterior endodermal organs only, such as pharynx, gill rudiment and some parts of the head mesenchyme. In the present study, when the FDA-labelled LDMZ+BC of the normal gastrula was grafted onto the SE, we confirmed that it formed endodermal tissues such as pharynx and the roof and floor of the anterior archenteron. In these rescued embryos, notochord, somites and neural structures were entirely derived from the host SE (Fig. 6 H,I). In addition, the effects of suramin on the reactivity of the animal cap ectoderm of the SE were assayed by a sandwich culture method. When the normal LDMZ was wrapped in animal cap ectoderm of the SE, the sandwich explants formed trunk-tail or tail structures (82%, 14/17 cases), which indicates that suramin does not inhibit the reactivity of the host cells, but does inhibit the organizing activity of the LDMZ.

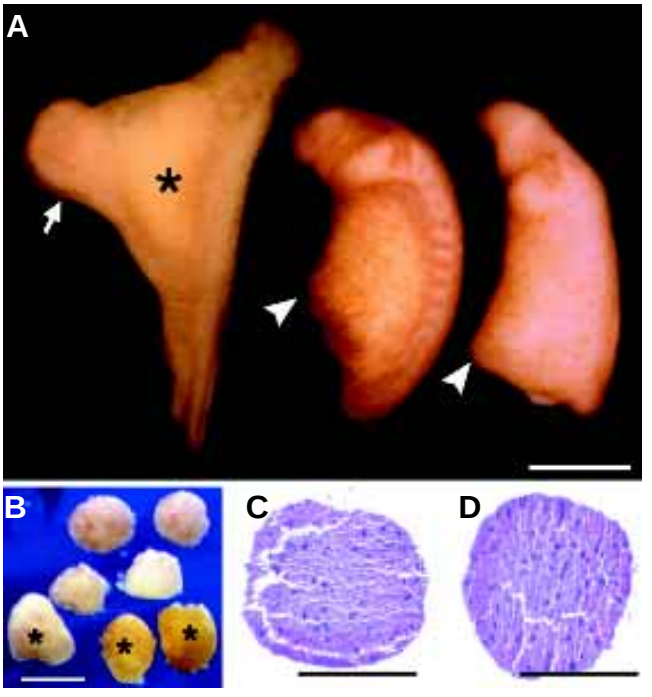


Fig. 4. Secondary axis-inducing activity of the LDMZ. (A) *Control embryo (asterisk) that has been grafted with LDMZ+BC of the normal gastrula onto the ventral marginal zone formed a complete secondary axis (arrow). When the LDMZ+BC of the SE was grafted, the secondary axis was not induced. White arrowheads indicated the implanted LDMZ+BC of the SE. Bar, 1 mm.* **(B)** *LDMZ isolated from either normal gastrula or SE (asterisk) and cultured for 1 week formed a rounded mass of endodermal cells. Histologically, both the LDMZ of the normal gastrula* **(C)** *and the SE* **(D)** *formed a mass of endoderm, but none of the mesodermal or neural structures developed. Bars, 0.5 mm.*

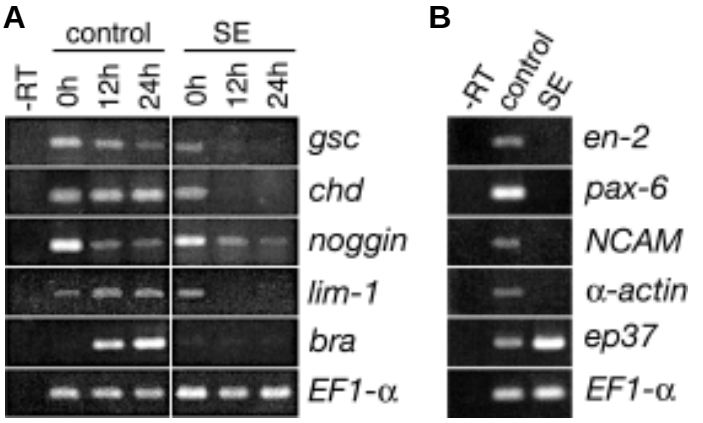


Fig. 5. Gene expression patterns of the suramin-injected embryo. (A) *Expression of the organizer-specific genes in control and SE embryos. Total RNA was isolated from individual embryos when the control embryos reached the early gastrula (stage 11, 0 h), mid-gastrula (stage 12b, 12 h) or late gastrula (stage 13c, 24 h). At the early gastrula stage (0 h), the SE expressed organizer-specific genes but their expression was not maintained during further development.* Bra *expression was induced from mid-gastrula stage onward in the control, but not in the SE.* **(B)** *Expression of tissue-specific genes. RNA was isolated from the tail-bud stage embryo (stage 27, 72 h from stage 11) and analyzed. SE expressed epidermis specific* ep37 *but not neural- or muscle-specific genes. In each analysis,* EF1-α *was used as an internal control. -RT is a negative control in which RNA from the 0 h control was reacted without reverse transcriptase.*

LDMZ rescues Gene Expression in the SE

As shown in Fig. 5, the expression of *gsc*, *chd*, *noggin* and *Lim-1* were detected at the early gastrula stage, but these gene expressions were not maintained in the SE (Fig. 5A). In the rescued embryo, however, the expression of all these genes recovered to the same level as the control embryo, when examined in the mid- (12 h after stage 11) and late- (24h after stage 11) gastrula (Fig. 7). *Bra* expression was also recovered in the rescued embryos. To identify the antero-posterior regional characteristics of the neural differentiation in the rescued embryos, the expressions of the tissue-specific genes were examined in individual rescued embryos. As shown in Fig. 8, 1 of 5 rescued embryos (#1 embryo of Fig. 8 B,D,) expressed *en-2* in addition to *pax-6*; it also expressed *otx-2* (data not shown). The other four rescued embryos expressed *pax-6* and *NCAM* (Fig. 7D), but not *en-2*. Muscle specific α-*actin* expression was detected in all rescued embryos. Comparing the histological and RT-PCR analy-

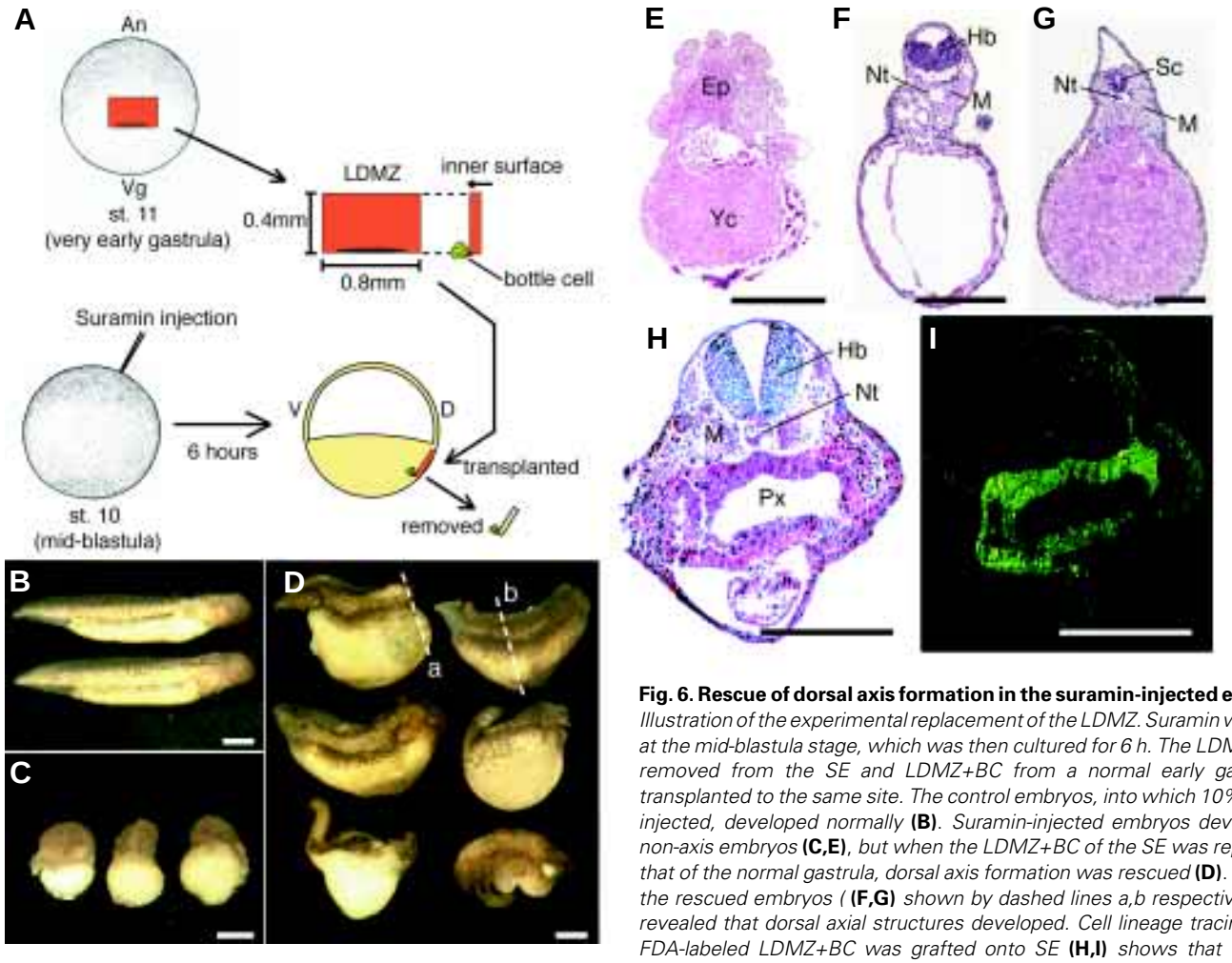


Fig. 6. Rescue of dorsal axis formation in the suramin-injected embryo. (A) *Illustration of the experimental replacement of the LDMZ. Suramin was injected at the mid-blastula stage, which was then cultured for 6 h. The LDMZ+BC was removed from the SE and LDMZ+BC from a normal early gastrula was transplanted to the same site. The control embryos, into which 10% MSB was injected, developed normally* **(B)**. *Suramin-injected embryos developed into non-axis embryos* **(C,E)**, *but when the LDMZ+BC of the SE was replaced with that of the normal gastrula, dorsal axis formation was rescued* **(D)**. *Sections of the rescued embryos (* **(F,G)** *shown by dashed lines a,b respectively, in* **(D)** *) revealed that dorsal axial structures developed. Cell lineage tracing in which FDA-labeled LDMZ+BC was grafted onto SE* **(H,I)** *shows that the grafted LDMZ+BC formed the anterior archenteron roof and pharyngeal endoderm. Entire neural and mesodermal structures were the induction products. Ep, atypical epidermis; Yc, yolk cell mass; Hb, hindbrain; M, somite; Nt, notochord; Sc, spinal cord; Px, pharyngeal endoderm. Bars, 1 mm.*

ses, these results indicate that, although the anterior head neural axis was not always rescued, substitution with the LDMZ+BC of the normal embryo re-established dorsal axis formation in the SE.

Discussion

In *Xenopus*, it has been shown that DMZ of the early gastrula consists of multiple domains with different prospective fates and gene expression patterns (Stewart and Gerhart, 1990; Domingo and Keller, 1995; Vodicka and Gerhart, 1995; Lane and Keller, 1997; Zoltewicz and Gerhart, 1998). Organizer formation or dorsal mesoderm induction occurs during the blastula stages (see review, Harland and Gerhart, 1997), although Grunz (1992 1993) demonstrated that dorsalization of the *Xenopus* organizer occurs during gastrulation. Thus, Harland and Gerhart (1997) and Gerhart (2001) proposed that the intra-organizer interaction, or intra-DMZ interaction, that occurs in the later stages plays an essential role in the final patterning of the region specific organizers. We report that the LDMZ is essential to trigger the sequential intra-DMZ interactions that lead to dorsal axis formation and the final patterning of the DMZ.

Suramin directly inhibits the Dorsal-Mesoderm Inducing Activity of the LDMZ

Suramin inhibits the dorsal mesodermal differentiation or the dorsal mesoderm induction of the dorsal lip of *Xenopus* gastrulae (Grunz, 1992, 1993; Cardellini *et al.*, 1994; Fainsod *et al.*, 1994), but because the 'dorsal lip' is a mixture of outer and inner DMZ, it remains unclear which component of the dorsal lip are targeted by suramin. The present study revealed that suramin does not inhibit blastopore formation (Figs. 1- 3), but does inhibit the secondary axis-inducing activity of the LDMZ (Fig. 4). The present results also demonstrate that the convergent extension and involution movements of the DMZ and dorsal axis formation of the SE are rescued only by replacement with LDMZ+BC from a normal embryo (Fig. 6). The LDMZ of the early *Cynops* gastrula possesses dorsal mesoderm (notochord)-inducing activity (Kaneda, 1981; Kaneda and Suzuki, 1983; Suzuki *et al.*, 1984; Yamamoto and Suzuki, 1994; Kaneda *et al.*, 2002), although the region itself differentiates into endodermal organs, including the pharyngeal endoderm (Figs. 4 and 6). Using a sandwich assay, the reactivity of the animal cap ectoderm of the SE was shown to be unaffected by suramin, which concurs with the results of Grunz (1992; 1993). In addition, when

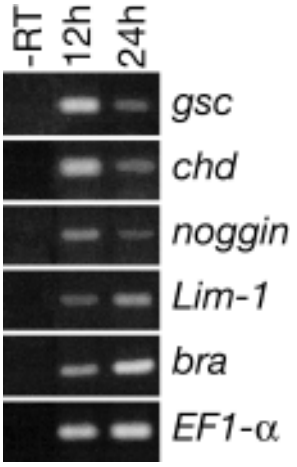


Fig. 7. Expression of organizer-specific genes in the rescued embryo. *LDMZ+BC were grafted onto SE and the embryos were developed for 12 and 24 h at 20°C until they reached the mid- (12 h, stage 12b) or late-gastrula (stage 13c, 24 h). RNA was isolated and analyzed by RT-PCR. -RT is a negative control in which RNA from the 24h rescued embryo was reacted without reverse transcriptase.*

suramin is injected into the mid- or late-*Cynops* gastrula, notochord differentiation is not inhibited (data not shown). These facts and the present results indicate that suramin does not inhibit dorsal mesoderm differentiation, but rather the dorsal mesoderm-inducing activity of the LDMZ.

In the present study, the expressions of *gsc*, *chd*, *noggin*, and *Lim-1* were detected at the early gastrula stage, but apart from *noggin* were not maintained in the SE; *bra* expression was not detected at all at stage 11 of either the SE or the normal gastrula. We previously reported that LDMZ itself expressed *noggin*, *gsc*, *Lim-1* but not *bra*. Gene expression was not affected when the LDMZ was isolated and treated *in vitro* with suramin for 3 h. However, when the LDMZ was cultured in sandwich with animal cap ectoderm, the LDMZ induced notochord and *bra* expression and the inducing activity of LDMZ were inhibited by suramin (Kaneda *et al.*, 2002). Thus, complete inhibition of *bra* expression in the SE is the result of the lack of dorsal mesoderm (notochord) induction in the DMZ, because *bra* is known to be activated by the dorsal mesoderm induction during gastrulation (Conlon and Smith, 1999; Tabata-Sakaguchi *et al.*, 2001).

The LDMZ is Necessary to organize Dorsal Axis Formation and Morphogenetic Movements of the DMZ

When the LDMZ+BC of the normal embryo was grafted onto the same region of the SE, the convergent extension and involution movements and the dorsal axis formation of the SE were rescued (Fig. 6). In that case, it was clear that dorsal axial structures of the rescued embryos were derived from host cells (Fig. 6 H,I) and the expressions of the organizer-specific genes had also recovered to the same level as the control embryo (Fig. 7). Because the activity of the LDMZ was inhibited when the embryos were treated with suramin at the late blastula stage, it suggests that the activity of the LDMZ was established during the late blastula stage, and also suggests that the arrest of the convergent extension and involution movements in the SE were the result of failure of dorsal mesoderm induction in the DMZ, because these movements are functions of mesodermized cells (Keller, 1991). These facts suggest that the dorsal mesoderm induction in the DMZ occurs during the early phase of gastrulation.

Contrary to expectation, most of the rescued embryos could not form a complete axis, especially the anterior forebrain structure. Gerhart *et al.* (1991) demonstrated that suramin blocked the involution movements of the archenteron roof in the *Xenopus* embryo and thus caused a defect in head formation. Thus, it is possible that residual suramin in the blastocoel inhibited complete involution of the archenteron and thus the anterior head structures were not formed normally. The influence of artificial effects, such as incomplete healing of the wound, and an insufficient number of replaced LDMZ+BC cells would also affect complete head formation. Another possibility is that suramin induced premature expression of *BMP-4* (Fainsod *et al.*, 1994), which prevents anterior head formation (Hartley *et al.*, 2001), because the *BMP-4* and *Wnt*/β-*catenin* signalling gradient in the prospective neural field antagonizes anterior head inducing signals and regulates antero-posterior neural patterning (Kiecker and Niehrs, 2001).

Formation of the LDMZ is a Primary and Necessary Event for Dorsal Axis Formation

It has been suggested that the DMZ of the early *Cynops* gastrula consists of two domains: the upper ectodermal UDMZ of the prospective notochord and the endodermal LDMZ of the prospective pharyngeal endoderm and prechordal endoderm. Notochord induction in the UDMZ occurs during gastrulation by planar induction from the LDMZ (Kaneda and Suzuki, 1983). Several studies in *Xenopus* have demonstrated that the Nieuwkoop center induced head and trunk-tail organizers in the DMZ prior to the onset of gastrulation (Zoltewicz and Gerhart, 1997; Gerhart, 2001), but it

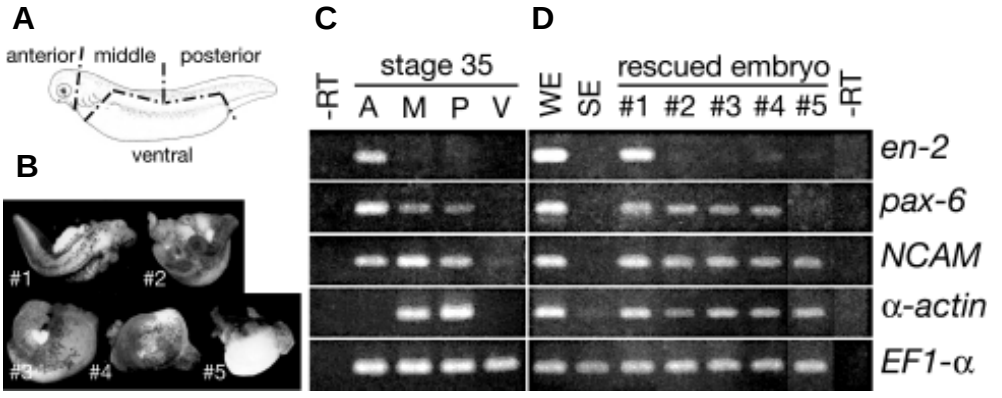


Fig. 8. Expression patterns of neural- and muscle-specific genes in the rescued embryos. (A) *Scheme of stage 35 embryo that has been dissected into four parts for analyzing regional gene expressions.* **(B)** *External profiles of five typical rescued embryos used for RT-PCR analysis.* **(C)** *Regional expression of* en-2, pax-6, NCAM, *and* α-actin *in the four parts of a normal embryo. A, anterior; M, middle; P, posterior; V, ventral corresponding to (A).* **(D)** *Expression patterns of* en-2, pax-6, NCAM *and* α-actin *in the rescued embryos. RNA from whole embryos (WE) or suramin injected embryos (SE) were used as control. The numbers (#1-#5) of the rescued embryos correspond to #1-#5 in (B). -RT is a negative control in which RNA from stage 35 whole embryo was reacted without reverse transcriptase.*

has also been demonstrated that the specificity of the early *Xenopus* gastrula DMZ is quite labile (Grunz, 1992, 1993) and that outer endodermal epithelium is a source for notochord cell induction during gastrulation (Stwart and Gerhart, 1990; Shih and Keller, 1992; Domingo and Keller, 1995; Lane and Keller, 1997). Recently, Gerhart (2001) proposed that Nieuwkoop center is spatially subdivided into several domains, including the future head and trunk-tail organizers, and that intra-organizer interaction is required for the final patterning of the head and trunk-tail organizers. The present results indicate that the dorsal mesoderm (notochord)-inducing activity of LDMZ was inhibited in the SE. In addition, normal LDMZ alone could induce the dorsal axis when grafted onto the SE. It is therefore reasonable to conclude that at the early gastrula stage of *Cynops*, the dorsal mesoderm is not yet induced in the DMZ and furthermore, the autonomous and temporal changes of the organizing activity of the LDMZ patterned the head and trunk-tail organizers (Kaneda *et al*., 2002). This suggests that the LDMZ is the center for dorsal mesoderm induction in the DMZ and triggers the spatial and temporal intra-DMZ interactions that establish the final pattern. Thus it is reasonable to conclude that the formation of the LDMZ is a primary and necessary event for dorsal axis formation. Although both the site of the Nieuwkoop center in *Cynops* and the expression of its gene are still obscure, and there is still the possibility that the Nieuwkoop center induces the LDMZ, the facts and the present results suggest that the LDMZ has Nieuwkoop center activity, at least in its autonomy for endodermal differentiation and function.

Materials and Methods

Embryos

Cynops pyrrhogaster embryos were used. Fertilized eggs were obtained by subcutaneous injection of human chorionic gonadotropin (Teikoku Zoki Co., Japan) into adult females and the eggs were then developed at 20ºC until they reached the required developmental stage. The embryos were staged according to the normal table of Okada and Ichikawa (1947). The very early gastrula in which the pigmented blastopore appeared was designated as stage 11, which in *Cynops* is almost identical to the stage 10– or stage 10 earliest *Xenopus* gastrula (Kaneda *et al*., 2002). Gastrulation was accomplished in approximately 24 h at 20°C (Kaneda and Hama, 1979).

Suramin Treatment

We used blastula embryos that were expected to be 6 h prior to stage 11. The jelly coat was removed and the embryos were transferred to 10% modified Steinberg's solution (100% MSB: 54.2 mM NaCl, 0.67 mM KCl, 0.34 mM Ca $(NO_3)_2$, 0.83 mM $MgSO_4$ and 3.0 mM HEPES-NaOH, pH7.4). Then 20 nl of 10% MSB containing 10 mM suramin (Calbiochem Inc., USA) was injected into the blastocoel to achieve a final concentration of approximately 100 μM. As a control, 20 nl of 10% MSB was injected. After injection, the SE were developed at 20°C, and those embryos that formed the pigmented blastopore 6 h later were selected and used.

Microsurgical Operation, Cell Culture and Histology

Because the LDMZ (0.4 mm by 0.5 mm) alone can not involute (Yamamoto and Suzuki, 1994; Imoh *et al*., 1997), the LDMZ was isolated together with BC to facilitate involution of the grafted LDMZ. The prospective fate of the LDMZ corresponded to the pharyngeal endoderm and anterior prechordal plate endoderm (Hama *et al*., 1985). For analyzing the secondary axis inducing activity, isolated LDMZ+BC was transplanted into the ventral marginal zone of the normal early gastrula and cultured in 10% MBS containing 50 μg/ml gentamycin sulfate. For the rescue experiment, the LDMZ+BC of the SE were removed and replaced with the same size of normal LDMZ+BC (Fig. 6A). The replaced embryos were cultured as before and used for histological or RT-PCR analysis. For histology, embryos were fixed with Bouin's fixative, dehydrated through an ethanol series, and embedded in paraffin wax. Sections (10 μm) were stained with hematoxylin and eosin. For histological examination of the blastopore, embryos were fixed with gulutalaldehyde-containing fixative, embedded in paraffin, sectioned at 6 μm, and stained with molybdic hematoxylin (Imoh, 1988).

FDA Injection and Cell Lineage Tracing

FDA was injected at the 2-cells stage and the FDA-labelled embryos were developed until the desired stage. For cell lineage tracing, FDA-labelled LDMZ+BC was grafted onto SE and the embryos were subsequently fixed with Bouin's fixative, embedded into paraffin and sectioned as before. Serial sections (10 μm) were observed as described (Kaneda *et al*., 2002). For tracing of the blastopore, FDA was injected into the dorsal C1 blastomere at the 32-cell stage (Suzuki *et al*., 2002) and embryos labelled with the pigmented blastopore were selected and the movement of the blastopore was observed.

RT-PCR Analysis

To analyze organizer specific gene expression, the SEs were harvested at stages 11, 12c (mid-gastrula, 12 h after stage 11) and 13c (late gastrula, 24 h after stage 11) of the sibling embryo. For the expression of the tissue-specific genes, tail-bud stage embryos (approx. 72 h after stage 11) were used. Of the rescued embryos, those at 12 and 24 h after LDMZ+BC grafting were used for the organizer specific genes, and at 10 days for the tissue-specific genes.

Total RNA was extracted from individual embryos as described previously (Tabata-Sakaguchi *et al*., 2001; Kaneda *et al*., 2002). Oligo d (T) primed first strand cDNA was synthesized from 1 μg of the total RNA by PowerScript reverse transcriptase (CLONTEC, USA). PCR were carried out in a 50μl reaction volume using recombinant *Taq* DNA polymerase (Invitrogen, USA). The primer sequences for neural specific *NCAM*, *en-2*, *pax-6*, muscle specific α-*actin* and epidermis specific *ep-37* were as described by Mizuno *et al*. (1997). The primer sequence and PCR conditions for *gsc*, *noggin*, *bra* and *Lim-1* were as described previously (Sone *et al*., 1997; Kaneda *et al*., 2002). The PCR products were resolved by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining. Each amplified fragment was subcloned into a pGEM-T easy vector (Promega, USA) and the DNA sequences were confirmed by automated DNA sequencer.

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