Cytoplasmic and molecular reconstruction of *Xenopus* embryos: synergy of dorsalizing and endo-mesodermalizing determinants drives early axial patterning

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Summary

Ablation of vegetal cytoplasm from newly fertilized *Xenopus* eggs results in the development of permanent blastula-type embryos (PBEs). PBEs cleave normally and develop into a very simple tissue consisting only of atypical epidermis. We tried to restore complete embryonic development in PBEs by cytoplasmic transplantation or by mRNA injection. We show a two-step reconstruction of the body plan. In the first step, PBEs injected with either marginal cytoplasm or synthetic VegT RNA restored gastrulation and mesoderm formation, but not axial patterning. Injection of Xwnt8 mRNA (acting upstream of β-catenin and thus substitutes for the dorsal determinant) did not restore axial development in PBEs. Simultaneous injections of Xwnt8 and VegT into PBEs resulted in dorsal axis development, showing the synergy of these molecules in axial development. These results suggest that the mixing of two cytoplasmic determinants, i.e. the dorsal determinant in the vegetal pole and the endo-mesodermal determinant in the whole vegetal half, triggers the early axial developmental process in *Xenopus* embryos.

Key words: *Xenopus*, Cytoplasmic determinants, VegT, Xwnt8, Organizer

Introduction

Cytoplasmic determinants, which are located in particular regions of the egg, drive the cell fate during development. Although the non-cell autonomous process acting through cell-cell interaction has been emphasized in the early development of chordates (Gilbert, 1994), cell-inheriting factors also have recently drawn attention. In the African clawed frog, *Xenopus laevis*, vegetal pole cytoplasm plays a key role for the formation of the Spemann organizer. Cytoplasmic transplantation from a dorsal-vegetal blastomere at 16-cell stage *Xenopus* embryos into ventro-vegetal blastomere revealed the presence of a dorsal determinant(s) at this stage (Yuge et al., 1990). The dorsal determinant(s) is localized in the vegetal pole at the early one-cell stage (Fujisue et al., 1993; Holowacz and Elinson, 1993), thereafter it disappears from the vegetal region and reappears in the dorsal region, without any dorsal inducing activity being detected in the ventral subequatorial region at any stage (Yuge et al., 1990; Fujisue et al., 1993). Furthermore, we have shown that deletion of 20-40% vegetal cytoplasm always results in gastrulating non-axial embryos (GNEs), which do not form any dorsal/neural structures (Sakai, 1996; Fujii et al., 2002). This axis deficiency can be fully restored by the transfer of the vegetal cytoplasm, providing a strong proof for dorsal determinants in the vegetal pole (Sakai, 1996).

However, dorsal determinants are not sufficient for the formation of the Spemann organizer. When *Xenopus* eggs are UV-irradiated, the resulting embryos lack entire dorsal structures (Malacinski et al., 1977; Scharf and Gerhart, 1980; Scharf and Gerhart, 1983). This is not because the dorsal determinants are destroyed, because the vegetal pole cytoplasm of the UV-irradiated eggs still possesses transplantable dorsal determinants (Fujisue et al., 1993; Holowacz and Elinson, 1993). UV-irradiation is thought to interfere with the cortical movement (Vincent et al., 1986; Gerhart et al., 1989), which is necessary for the transportation of the dorsal determinants from the vegetal pole to the marginal region. Therefore, we have proposed that in *Xenopus* eggs the organizer forms as a result of mixing of two cytoplasmic determinants, i.e. the dorsal determinant in the vegetal pole and the endo-mesodermal determinant in the whole vegetal half, triggers the early axial developmental process in *Xenopus* embryos.

Key words: *Xenopus*, Cytoplasmic determinants, VegT, Xwnt8, Organizer
is probably because PBEs do not have competent marginal cytoplasm to respond to LiCl treatment. Furthermore, cortex-transplantation experiments have revealed that dorsal determinants are active only when the cortex is transplanted into the sub-equatorial (marginal) region but not into the animal or vegetal region (Kageura, 1997). These results support the notion that putative marginal determinants are responsible for gastrulation, mesoderm formation and dorsal axial development. However, for a certain determinant, ‘the only really satisfactory proof is to transfer cytoplasm from one place to another by microinjection and show that cells inheriting the ectopic cytoplasm become structures normally formed by the egg region from which the cytoplasm came’ (Slack, 1991). Manes et al. (Casal and Manes, 1999; Manes and Campos Casal, 2002) isolated the four animal cells from an eight-cell Bufo embryo, which in this species only makes epidermis. Cytoplasmic transfers generated mesoderm, with notochord and somite arising only with a cytoplasmic combination of the ventroequatorial and the vegetal pole area. Further, Shinagawa and Kobayashi (Shinagawa and Kobayashi, 2000) transferred marginal cytoplasm into the animal pole region of Xenopus embryos and found a blastopore-like structure in the injected region. We tried to show more solid evidence of marginal determinant(s) by mRNA injection and cytoplasmic transfer experiments.

As recipients for cytoplasmic transfer, we used PBEs, which were made by ablating more than 60% of the vegetal egg surface from an early one-cell stage embryo (Fujii et al., 2002). PBEs do not gastrulate, or express dorsal, neural and endomesodermal genes. They express the epidermal marker EpK, and develop into simple epidermal tissues (Fujii et al., 2002). Therefore, PBEs most probably lack two types of determinants: the dorsal determinants in the vegetal pole and the marginal determinants. Using PBEs as starting materials, we first tried to restore gastrulation, mesoderm formation and finally the entire axial development.

Materials and methods
Permanent blastula-type embryos (PBEs)
Xenopus eggs were inseminated in 10% modified Steinberg’s solution (10% MS) (Sakai, 1996). After 20-25 minutes in diluted sperm suspension, eggs were dejellied using 1% sodium thioglycolate (pH 9.10) and rinsed with 10% MS. The vitelline membrane was removed with fine forceps. The denuded egg was inclined 90° off its vertical axis. A glass rod (diameter 350 μm, length 2 cm) was drawn from a 3 mm diameter glass rod (Kokura glass), soaked in 5 N HCl for 30 minutes and washed three times in 10% MS. The glass rod was placed on the egg so as to divide it into animal and vegetal fragments. After complete separation, both fragments maintained a spindle shape. The lengths of their long and short axes were measured using a micrometer attached to the eyepiece of a microscope. The surface area of the fragments was calculated assuming that they were spheroids. All operations were done on an agar-coated plastic dish containing 10% Steinberg’s solution (50 mg/l Gentamycin, supplemented with 3.0 mM HEPES).

Microinjection
Micropipettes for cytoplasm transfer was manually drawn from hard glass capillary tubes (Drummond), cut to a flat, blunt end (tip external diameter: 40 μm). For mRNA injection, thinner micropipettes (5-7 μm) were used. Micropipettes were held in a Narishige micromanipulator. Microinjections were performed using air pressure from a tuberculin syringe connected to 1 mm diameter polyethylene tubing. For withdrawal of the cytoplasm, the micropipette tip was placed on the cell surface and then negative pressure was increased to break the cell surface. The suction was carried out very slowly. It took about 5 minutes to withdraw 50 nl of cytoplasm. Soon after the suction, the cytoplasm was injected into the host PBEs.

For fluorescent microscopy, the cytoplasmic donor was soaked in 0.01% Neutral Red for 5 minutes to stain donor cytoplasm. This stain could be clearly seen under a fluorescent microscope optimized for rhodamine.

Histology and in situ hybridization
Embryos at various stages were fixed with Bouin d’Holland (2-3 hours) and embedded in paraffin wax after dehydration. Serial 6 μm sections were stained for 10 minutes with 0.5% aniline blue-2% orange G and for 15 minutes with 0.5% Aniline Blue.

Whole-mount in situ hybridization was carried out following the method of Shain and Zuber (Shain and Zuber, 1996). Embryos were fixed in MEMFA for 90 minutes at 20°C, washed, and stored in –20°C 100% methanol. Before addition of anti-DIG antibody, embryos were treated overnight in 10% hydrogen peroxide to bleach out the pigment. In situ hybridization on sections was performed using 6 μm paraffin wax sections based on the method of Endo et al. (Endo et al., 2002). Embryos were fixed in MEMFA, embedded in paraffin wax and sectioned at 6 μm. Sections were mounted on silan-coated slides pretreated with Vectabound. Before hybridization, the slides were soaked in 1% gelatin. Detailed protocols are available on request.

Results
VegT mRNA was entirely absent in PBEs
When more than 60% of vegetal cytoplasm and vegetal egg surface was deleted, the resulting embryos did not gastrulate but formed simple bag-like embryos (Fig. 1A; Table 1) called permanent blastula-type embryos (PBEs). PBEs did not express mesodermal (Xbra) or dorsal (chordin) genes (Fig. 1C,E; Table 2), as shown previously (Fujii et al., 2002). LiCl treatment (0.3 M for 6 minutes at 32-cell stage) of PBEs did not induce chordin expression (0/17, data not shown). These characteristics of PBEs are most probably the result of the absence of maternal VegT. Therefore we analyzed whether PBEs contain VegT mRNA or not. Immediately after the deletion of vegetal cytoplasm, the resulting PBEs were fixed and examined for VegT. PBEs did not contain maternal VegT at all though GNEs contained maternal VegT (Fig. 2; Table 2). With PBEs we have an ideal recipient system for cytoplasm/mRNA transplantation: PBEs do not have VegT and form only epidermal tissue, so it is relatively easy to detect the effect of transplantation sensitively.

Transplantation of marginal cytoplasm translocated maternal VegT
First, we transplanted marginal cytoplasm into PBEs. Marginal cytoplasm (MC) was withdrawn with a 40 μm tip micropipette from a GNE (Fujii et al., 2002), an embryo deleted of 20-40% vegetal pole cytoplasm (Fig. 3C). We used GNEs as cytoplasm donors because GNEs gastrulate and express endo-mesodermal markers in the entire absence of dorsal gene expression (Fig. 1D,F; Table 2) (Fujii et al., 2002). In situ hybridization revealed that GNEs contained VegT mRNA (Fig. 2; Table 2); however, the cytoplasm of GNEs does not have dorsalizing activity (Sakai, 1996).

MC was carefully withdrawn from sub-equatorial surface of

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MC was carefully withdrawn from sub-equatorial surface of
GNEs and injected into sub-surface regions of PBEs, which were inverted upside-down at the time of injection. After injection, recipient PBEs were reoriented as before. Some embryos were fixed just after MC injection and examined histologically. Interestingly, the MC was observed as a consistent spherical yolk-rich area in the bottom surface of recipient PBEs (Fig. 3A). When we stained MC by soaking donor embryos in 0.01% Neutral Red, the transplanted MC was observed as a red circular area in the PBE under epifluorescent microscope (Fig. 3E-H).

Some MC-injected PBEs were fixed for in situ hybridization soon after the injection. VegT was seen in 13 out of 15 MC injected PBEs (Fig. 4; Table 2). In these cases, the VegT stain had a spherical shape, resembling the shape of the donor cytoplasm in the epifluorescent view and in the histological section (Fig. 3A,E,F). Negative controls that were injected with PBE-animal cytoplasm (AC) showed no positive stain for VegT (Fig. 4; Table 2).

### Table 1. Injection of cytoplasms/mRNAs resulted in gastrulation and dorsal axis formation in PBEs

<table>
<thead>
<tr>
<th>Embryo type</th>
<th>Stage 17</th>
<th>Stage 38</th>
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<tbody>
<tr>
<td></td>
<td>Gastrulation</td>
<td>Proboscis</td>
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<tr>
<td>GNE</td>
<td>24/24</td>
<td>0/24</td>
</tr>
<tr>
<td>GNE+Xwnt8*</td>
<td>24/24</td>
<td>0/24</td>
</tr>
<tr>
<td>PBE</td>
<td>0/39</td>
<td>0/39</td>
</tr>
<tr>
<td>PBE+MC†</td>
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<td>0/55</td>
</tr>
<tr>
<td>PBE+MC+Xwnt8‡</td>
<td>14/16</td>
<td>0/16</td>
</tr>
<tr>
<td>PBE+MC+VPC§</td>
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<td>0/12</td>
</tr>
<tr>
<td>PBE+VPC (18 nl)†</td>
<td>0/11</td>
<td>7/11</td>
</tr>
<tr>
<td>PBE+VPC (50 nl)†</td>
<td>ND</td>
<td>7/8</td>
</tr>
<tr>
<td>PBE+AC†</td>
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<td>0/15</td>
</tr>
<tr>
<td>PBE+VegT‡</td>
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<td>0/16</td>
</tr>
<tr>
<td>PBE+Xwnt8§</td>
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</tr>
<tr>
<td>PBE+Xwnt8+VegT×2‡</td>
<td>15/18</td>
<td>0/18</td>
</tr>
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</table>

*Xwnt8 (3 pg) was injected at the eight-cell stage.
†MC (50 nl), AC (50 nl) or VPC (18 or 50 nl) was injected at the one-cell stage.
‡MC (50 nl) was injected at the one-cell stage and Xwnt8 (3 pg) or VPC (18 nl) was injected into a blastomere showing the trace of MC injection at the eight-cell stage.
§VegT (12 pg) or Xwnt8 (3 pg) was injected at the four-cell stage.
¶A mixture of VegT (12 pg) and Xwnt8 (3 pg) was injected into one blastomere of a four-cell stage PBE. Furthermore, additional VegT (12 pg) was injected into an adjacent blastomere.

**Fig. 1.** Permanent blastula-type embryos (PBEs) are simple organisms consisting only of epidermal tissue. All embryos are at control stage 17. (A,B) Midline sections of a PBE (A) and a GNE (B). (C) PBEs did not show Xbra expression. (D) GNEs expressed Xbra in their vegetally shifted marginal zones. (E,F) PBEs (E) and GNEs (F) showed no chordin expression. Scale bars: 250 μm in A,B; 1 mm in C-F.

**Fig. 2.** VegT mRNA was absent in PBEs but was present in GNEs. Whole-mount in situ hybridization for VegT. Top, PBEs; middle, GNEs; bottom, controls. Scale bar: 1 mm.

**Fig. 2.** Reconstruction of Xenopus embryos 1137
cytoplasm showed that the blastopore formed at the position of injection (Fig. 5B). The presence of large yolk platelets also showed that the blastopore formed in the center of transplanted cytoplasm (Fig. 5C). The MC-injected PBEs also recovered zygotic expression of *Xbra* and *Veg T*, although these embryos did not show any morphological sign of axial patterning or *chordin* expression (Fig. 5E-G; Table 2). In situ hybridization on sections revealed that *Xbra* expression was restricted to a circular marginal area surrounding the injected cytoplasm, which is distinguishable from the host cytoplasm by its large yolk platelets (Fig. 5I,J). We also found that synthetic *Veg T* RNA (12 pg in one blastomere of the four-cell stage PBEs) restored gastrulation and mesoderm formation with an absence of dorsalization (Fig. 5D,H; Table 2). In summary, PBEs developed into GNE-like embryos in response to the injection of *Veg T* or *Veg T* containing MC. These results show direct evidence for the presence of the marginal determinant(s), and show that *VegT* mRNA is a marginal determinant molecule that is both necessary and sufficient for PBEs to form endomesodermal tissues.

Transplantation of vegetal pole cytoplasm resulted in hyperdorsal embryos

We have proposed that the axial patterning of amphibian embryos requires the mixing of two determinants (Sakai, 1996; Doi et al., 2000); the dorsal determinant in a narrow region of vegetal pole cytoplasm (VPC) and the marginal determinant in the marginal cytoplasm (MC). However, given that the marginal determinant is *VegT* mRNA, which is present in the whole vegetal hemisphere (Zhang and King, 1996), the vegetal pole cytoplasm must have both dorsal and marginal determinants. Vegetal pole cytoplasm (VPC, 18 to 50 nl) was withdrawn from a vegetal-pole-containing egg fragment (counterpart to GNE), and then injected into the bottom region of a PBE. As expected, the VPC-injected PBEs resulted in hyperdorsalized embryos (Fig. 6A), forming a proboscis and a radial cement gland (Table 1). In situ hybridization revealed that VPC injected PBEs contained *VegT* mRNA (Table 2).
Injection of Xwnt8 mRNA into PBEs did not result in dorsal axis formation

We examined the independent effect of vegetal-pole-localized dorsal determinant(s) in the absence of the marginal determinants. To this end, we injected Xwnt8 mRNA in place of a pure ‘dorsal determinant’, because Xwnt8 rescues dorsal axis of UV-ventralized embryos (Christian et al., 1991) and acts upstream of β-catenin (Heasman et al., 1994) at the early cleavage stages. We hypothesized that Xwnt8 would act as a putative dorsal determinant in the vegetal pole, which would require the marginal determinant to produce the Spemann organizer.

As we had expected, injection of Xwnt8 alone did not dorsallize PBEs, though GNEs formed an almost normal axis in response to Xwnt8 injection (Fig. 6B,D). Most of the Xwnt8 injected GNEs formed a cement gland, two eyes, melanocytes and a tail fin (Table 1), and thus showed an almost normal appearance (Fig. 6D). These embryos expressed the organizer marker gene chordin at the upper blastopore region (Fig. 6C, Table 3). By contrast, PBEs injected with Xwnt8 showed no morphological signs of dorsalization except for cement gland formation at a low frequency (Table 1). These embryos never showed chordin expression (Fig. 6C; Table 3).

Double injections of VegT and Xwnt8 into PBEs resulted in dorsal gene expression in a cell-autonomous manner

The observed incompetence of PBEs to Xwnt8 is most probably because PBEs do not have the marginal determinant. Transplantation of MC into PBEs should make PBEs to respond to injected Xwnt8. In a preliminary experiment, we treated MC-injected PBEs with 0.3 M LiCl and found that the resulting embryos expressed chordin (12/16) and NCAM (6/6).

We therefore co-injected Xwnt8 and MC into a single cell of eight-cell stage PBEs. MC was first injected into the bottom of PBEs at the one-cell stage. At the eight-cell stage, the injected MC was seen as a white area encompassing several blastomeres in the bottom region. Xwnt8 mRNA was injected into the white/black border in one blastomere of MC-injected PBEs. The resulting embryos had regenerated axis formation.
and chordin expression (Fig. 7A,D). NCAM and Krox20 expression showed near-normal patterns in MC/Xwnt8 injected embryos (Fig. 7E,F). Negative control embryos receiving animal pole cytoplasm (AC) instead of MC and then injected with Xwnt8 did not form dorsal structures nor express NCAM or Krox20 (Fig. 7G).

Co-injection of VegT and Xwnt8 also caused dorsal structures and expression of chordin (Fig. 7B,C; Tables 1 and 3). Interestingly, when these two mRNAs were injected into separate blastomeres of the four-cell stage PBEs, the embryos did not dorsalize or express chordin (0 out of 16 cases; Fig. 7C; Table 3).

Double injections of marginal cytoplasm and vegetal-pole cytoplasm into PBEs restored normal axial structures

In our final experiment, we tried to restore normal embryos by double injections of marginal cytoplasm (MC) and vegetal pole cytoplasm (VPC). As described above, injection of VPC into PBEs resulted in the formation of hyperdorsalized embryos. This is probably because these embryos had circular dorsalizing region, the situation similar to LiCl-treated embryos (Kao and Elinson, 1988). Therefore, we tried to create a posteriorizing domain outside of the VPC injected dorsalizing region. To this end, PBEs at the one-cell stage were first injected with 50 nl MC into the bottom, and at eight-cell stage injected with 18 nl VPC into the white/black border in one blastomere.

Most of the resulting embryos formed near-normal body axes, with a cement gland, two eyes, melanocytes, muscle tissue and a tail fin (Fig. 7H; ‘PBE+MC+VPC’ in Table 1). Interestingly, the overall shape of these embryos was more normal when compared with mRNA-injected embryos (compare Fig. 7H with 7A,B). Naturally, these embryos expressed chordin (Fig. 7D, Table 3).

Table 3. Co-injection of Xwnt-8 and MC/Veg-T resulted in dorsal gene expression in PBEs

<table>
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<th>Embryo type</th>
<th>Stage 17</th>
<th>Stage 1</th>
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<tr>
<td></td>
<td>NCAM</td>
<td>Krox20</td>
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<tr>
<td>PBE</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GNE+Xwnt8*</td>
<td>6/6</td>
<td>7/7</td>
</tr>
<tr>
<td>PBE+Xwnt8*</td>
<td>7/8</td>
<td>5/6</td>
</tr>
<tr>
<td>PBE+MC+Xwnt8†</td>
<td>0/7</td>
<td>0/4</td>
</tr>
<tr>
<td>PBE+AC+Xwnt8‡</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PBE+MC+VPC</td>
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<td>ND</td>
</tr>
<tr>
<td>PBE+Xwnt8+VegT§</td>
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</tr>
<tr>
<td>PBE+Xwnt8+VegTx2¶</td>
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*Xwnt8 (3 pg) was injected at the eight-cell stage.
†MC/AC (50 nl) was injected at the one-cell stage and Xwnt8 (3 pg) or VPC (18 nl) was injected into a blastomere showing the trace of MC/AC injection (see Fig. 3E) at the eight-cell stage.
‡Veg-T (12 pg) was injected into two adjacent blastomeres at the four-cell stage.
§Veg-T (12 pg) was injected into one blastomere at the four-cell stage; thereafter, Xwnt8 (3 pg) was injected into a separate blastomere.
¶A mixture of VegT (12 pg) and Xwnt8 (3 pg) was injected into one blastomere of a four-cell stage PBE. Furthermore, additional VegT (12 pg) was injected into an adjacent blastomere.

Fig. 7. Double injections of dorsalizing and endo-mesodermalizing determinants restored the dorsal axis. (A,B,H) Stage 38. (C,D) Stage 11. (E-G) Stage 17. (A) A PBE injected with MC (50 nl) and Xwnt8 (3 pg). (B) A PBE injected with VegT and Xwnt8. A single blastomere of four-cell stage PBE was injected with a mixture of 12 pg VegT and 3 pg Xwnt8, thereafter an adjacent cell was injected with 12 pg VegT only. With this protocol, we aimed to make an organizer and a posteriorizing center in the host PBE. (C) Expression of chordin in PBEs. (Top) VegT- and Xwnt8-injected embryos as shown in B. (Middle) These embryos also received 12 pg VegT and 3 pg Xwnt8 at the four-cell stage, but in separate blastomeres. (Bottom) PBEs injected with 12 pg VegT into two adjacent cells of a four-cell stage PBE. (D) Injection of MC and Xwnt8 (top) and MC and VPC (bottom, see text) restored chordin expression in PBEs. (E) NCAM expression in PBEs injected with MC (50 nl) and Xwnt8 (3 pg). (F) Krox20 expression in PBEs injected with MC (50 nl) and Xwnt8 (3 pg). Note the two-band structures in the upper-left sample. (G) Negative controls in which 50 nl animal pole cytoplasm (AC) and Xwnt8 (3 pg) was injected into PBEs. (Top) In situ hybridization for NCAM. (Bottom) In situ hybridization for Krox20. (H) A normally looking embryo derived from a PBE injected with 50 nl MC and 18 nl VPC. Scale bars: 1 mm.

Discussion

Entire absence of VegT mRNA in PBEs results in the absence of endo-mesoderm

The present study reveals that PBEs do not contain VegT mRNA. The entire absence of VegT in PBEs most probably causes the absence of zygotic VegT and Xbra expression and
the absence of gastrulation (Tables 1, 2). PBEs did not express the endodermal marker Xsox17 (data not shown), the ventral endo-mesodermal marker Xwnt8 or several dorsal/neutral marker genes examined (Fuji et al., 2002). PBEs develop into epidermal bag-like structure and express the epidermal marker EpK (Fuji et al., 2002). Thus, our PBEs are simple organisms consisting only of ectoderm and are therefore an ideal material for studying cytoplasmic determinant and for studying maternally and zygotically expressed gene products. We can detect the results of experimental manipulation in the absence of intrinsic endo-mesodermal and neural gene expression.

Previous studies have shown that when maternal VegT mRNA is depleted by antisense oligonucleotides, the vegetal masses of the resulting embryos do not produce mesoderm-inducing signals. Mesoderm formation in these embryos occurred ectopically in the vegetal region rather than the equatorial region (Zhang et al., 1998; Kofron et al., 1999). However, these VegT-depleted embryos include ~10% maternal VegT, probably causing zygotic expression of VegT and Xbra and gastrulation in the vegetal pole region. Furthermore, these embryos form some axial structures. In contrast to VegT-depleted embryos, PBEs never express zygotic VegT or Xbra nor form dorsal axial structures.

The Xenopus cell fate is characterized by variable cell lineages. Lineage tracing experiments show that Xenopus blastomeres develop variably (Dale and Slack, 1987; Moody, 1987; Masho, 1990) so that it is impossible to forecast the specific fate of a certain cell. This is quite different from the invariant development of ascidian embryos in which the fate of a cell is identical in all individual embryos (Nishida and Satoh, 1985; Nishida, 1987). The variable fate of early Xenopus embryos makes it difficult to assess the results of experimental manipulations. The fate of PBEs is always atypical epidermis although they can make a full spectrum of embryonic structures. Using PBEs as starting materials, we can assess the effects of experimental manipulations by comparing the development of the resulting embryos with the epidermal fate of PBEs.

**VegT mRNA is a marginal determinant**

When MC was injected into PBEs we found, by in situ hybridization, the conspicuous presence of VegT mRNA. As VegT mRNA was entirely absent in PBEs, the observed VegT in the MC-injected PBEs must have come from the injected MC. To our knowledge, this is the first visualization that an injection of any cytoplasm transfers a specific mRNA into chordate host cells. The MC-injected PBEs gastrulated and expressed zygotic VegT and Xbra at high frequencies. Furthermore, we found that injection of synthetic VegT mRNA also resulted in gastrulation and Xbra expression. These results show that the maternal VegT is a marginal determinant, although VegT localization is not restricted to the marginal zone.

It should be noted that the dose of injected VegT (12 pg) was very low compared with previous studies. Zhang and King (Zhang and King, 1996) injected 0.7 or 2.5 ng of VegT mRNA into the ventral marginal zone and found secondary axis formation. Kurth and Hausen (Kurth and Hausen, 2000) injected 300 pg into animal pole, which resulted in weak gastrulation. In comparison with the animal pole of normal embryos, PBEs are very sensitive to VegT injection. We injected 12 pg VegT into the animal pole of one-cell stage embryos and found no morphological signs of gastrulation (data not shown), though the same dose induced gastrulation in all 16 PBEs examined (Table 1). We did not observe dorsal axis formation or chordin expression in PBEs injected with 12 pg of VegT. When an excess amount of VegT (60-250 pg) was injected, the resulting embryo elongated and formed axial structures (data not shown). We conclude that 12 pg of VegT is sufficient for PBEs to develop into GNE-like embryos. Similarly, Darras et al. (Darras et al., 1997) found that injection of vegetal pole cytoplasm into animal pole of Xenopus embryos led to expression of both siamois and Xnr3 but not Xbra in animal caps. Injection of vegetal pole cytoplasm into PBEs resulted in a hyperdorsalized phenotype, indicating the activation of both dorsalizing and mesodermalizing activity. The different results obtained by our study and Darras et al. (Darras et al., 1997) may be due to a difference in sensitivity between PBEs and animal caps.

**Xwnt8 acts in synergy with VegT in a cell autonomous manner to form dorsal axial structures**

Previously, we have proposed that the organizer forms by the mixing of vegetal pole determinants and marginal determinants. However, the present study shows that the marginal determinant is most probably VegT mRNA scattered all over the vegetal half of the egg. Therefore, vegetal pole cytoplasm (VPC) must have both dorsal and marginal determinants, and injection of VPC should result in dorsal axis formation in recipient PBEs. In fact, VPC-injected PBEs showed VegT translocation and hyperdorsalized phenotypes. We injected Xwnt8 mRNA in place of natural dorsal determinant. Xwnt8 is a dorsalizing factor that acts upstream of β-catenin. β-catenin protein translocates in nuclei of the dorsal side of cleavage stage Xenopus embryos (Schneider et al., 1996), and causes dorsal axial development (Brannon et al., 1997). When β-catenin is depleted early in the cleavage stage, resulting embryos do not develop dorsal axial structures (Heasman et al., 1994). Injection of Xwnt8 does not rescue the β-catenin-deleted non-axial phenotype (Heasman et al., 1994).

The present results show clearly that injection of Xwnt8 alone does not cause the formation of dorsal structures in recipient PBEs, while the same dose (3 pg into a single cell of and embryo at the eight-cell stage) into GNEs resulted in well-organized axial development with chordin expression in the appropriate position. As described above, VegT alone also did not dorsalize PBEs. Double injections of VegT and Xwnt8 into PBEs resulted in chordin expression and development of a near-normal body axis. Although VegT has been proposed to play roles in the early wnt dorsalizing pathway (Zhang et al., 1998; Houston et al., 2002) the present deletion-injection experiments provide the first direct proof that VegT is required for the wnt dorsalizing process. Baker et al. (Baker et al., 1999) reported that animal ectodermal tissue is neutralized by a single injection of Xwnt8; however, the animal tissue in their experiments may contain VegT mRNA. In relation to this, some Xwnt8-injected PBEs formed a cement gland, a most anterior dorsal marker. In these cases, host PBEs might contain a low level of VegT, which might act in synergy with injected Xwnt8 to form a cement gland. Even if this is true, the results presented here still showed that Xwnt8 injected PBEs did not express chordin or NCAM. For strong expression of these
genes, a substantial amount of VegT is necessary. In any case, details of the molecular characteristics of Xwnt8 injected PBEs are not clear at present. For example, gene expression upstream of chordin (e.g. Xnr genes and siamois) and nuclear transportation of β-catenin were not examined in the present study. Although Xwnt8 is thought to be a secreting factor, the present study strongly suggests that Xwnt8 acts in a cell-autonomous manner in synergy with VegT, as the injection of VegT and Xwnt8 into separate cells did not cause dorsal axial development. Furthermore, given that Xwnt8 acts upstream of β-catenin and thus acts before MBT, VegT also should act before MBT. This is in accordance with the fact that the β-catenin-Tcf-dependent transcription of the Xenopus nodal genes Xnr5 and Xnr6 occurs as early as the 256-cell stage (Yang et al., 2002).

In summary, VegT plays dual roles in early Xenopus development. First, VegT acts as an upstream factor of endomesodermal genes such as Xsox17, Xwnt8 and Xbra. Second, VegT acts as a co-factor of dorsal determinant(s) to activate the wnt-dorsalizing cascade.

It has been proposed that cortical rotation drives dorsal determinant(s) to a marginal sector where translocation of β-catenin protein in the dorsal marginal zone nucleus results in siamois expression (Brannon et al., 1997). Possible dorsal determinant molecules are Xdsh and GBP (Yost et al., 1998); however, there has been no evidence for their localization at the vegetal pole. After the translocation of β-catenin, the earliest gene expression has been observed to occur at 256-cell stage (Yang et al., 2002). For this early expression of Xnr5 and Xnr6, the two determinants presented in this paper most probably play crucial roles.

Absence of gastrulation, mesoderm formation and dorsal gene expression in the vegetal pole

As described above, we propose that the mixing of vegetal half cytoplasm (VegT mRNA) and the vegetal pole determinant(s) (unknown molecules) plays a main role in organizer formation. VegT mRNA in the marginal zone is most likely responsible and the cause of this region-specific gastrulation, as well as Xbra expression, dorsal axis formation in response to dorsal cortex transplantation (not to mention dorsalization by LiCl treatment) and Xwnt8 overexpression. The absence of maternal VegT in the animal pole is the likely cause of the lack of gastrulation. In addition, lack of the competence to cortex transplantation (Kageura, 1997) and the lack of competence to LiCl treatment and Xwnt8 injection (present study) are probably caused by the absence of maternal VegT. If the mixing of the two determinants is necessary and sufficient, the vegetal pole of a UV-irradiated embryo with both determinants should form the Spemann organizer. In support of this idea, the present study shows that vegetal pole cytoplasm with (most probably) both determinants, leads to dorsalized PBEs. Darras et al. (Darras et al., 1997) found that siamois and Xnr3 expression occurs at the UV-irradiated vegetal pole cells, indicating the activation of β-catenin pathway; however, the vegetal pole cells do not have any axis-forming activity at the gastrula stage. Even when siamois is injected into the vegetal pole, the injected cells do not have axis forming activity. In addition, the vegetal pole region do not respond to the transplantation of dorsalizing cortex (Kageura, 1997). These results show that the early dorsalizing process leading to siamois expression can be active in the vegetal pole; however, the later dorsalizing process leading to chordin expression is not active in the vegetal pole. Furthermore, Xbra is not expressed in the vegetal pole region in normal development. The absence of a dorsalizing pathway leading to chordin expression and the absence of Xbra expression in the vegetal pole requires some explanation other than being due to the absence of maternal VegT. Injection of MC into PBEs resulted in the formation of a MC sphere around which Xbra was expressed. VegT is apparently a causal factor for Xbra expression; however, Xbra was not expressed in the VegT-containing region.

We propose that the organizer forms in the margin of VegT containing cytoplasm, where both VegT and dorsal determinant(s) are present. When we injected vegetal pole cytoplasm, the recipient PBE has a margin of VegT cytoplasm where dorsal determinant(s) are also most likely present. It should be noted that the organizer should form in a cell-autonomous manner, as described above. By contrast, the UV-vegetal region does not develop an organizer as this region does not have the VegT margin. However, we do not know why this dorsalizing process occurs only in the VegT margin.

Proposed model

Our model for the formation of Spemann organizer is presented in Fig. 8, which shows a unique intersection (yellow) of the marginal region (broken rectangle) and the dorsal determinant (red) (which comes from the vegetal pole region). Previously, we postulated a specific ‘margin determinant’ as being present in the marginal zone (Sakai, 1996). In the present model, the marginal region is postulated to be a ‘margin’ of VegT containing cytoplasm, which is present all over the vegetal half of one-cell stage egg (green). The present model is not special because this model is based on a tradition of the determinant hypothesis (Elinson and Kao, 1989): For the formation of the Spemann organizer, the importance of the spread of the determinant(s) on the dorsal side has been suggested. Stewart and Gerhart (Stewart and Gerhart, 1990) suggest that inductions to establish the organizer occur after the original cytoplasmic inheritance. There is experimental support for the activity of the Nieuwkoop center on the vegetal dorsal side; however, Kodjabachian and Lemaire (Kodjabachian and Lemaire, 1998) argue that this is dispensable in normal axial development. Rather, they
emphasized a dorsal determinant in the vegetal pole. Furthermore, Sokol and Melton (Sokol and Melton, 1991) indicate that the dorsal ectoderm is different from the ventral ectoderm in the competence to activin.

The present model is similar to other models of recent years that suggest a pathway of VegT to endodermal and mesodermal gene expression, as well as a pathway from the dorsal determinant to nuclear β-catenin to siamois to chordin and other organizer gene expressions (Heasman, 1997; Moon and Kimelman, 1998; Agius et al., 2000; Chan and Etkin, 2001). However, the present experiments show direct evidence that the synergy of these two factors occurs in a cell-autonomous manner. The unique point of our model is that we propose explicitly that the dorsalizing process and the endomesodermalizing process synergize in an intracellular manner, although the molecular details of the synergy have yet to be investigated.

The present deletion/injection experiments provide direct proof for the marginal determinant, which is necessary for the formation of both endo-mesoderm and dorsal axial structures. Rigorous genetic proof for mechanism(s) of the synergy of dorsal and marginal determinants is lacking, but hopefully the present model will provide a basis for further studies. The present PBE system should serve as a good experimental system for investigating the molecular mechanisms of the synergistic process.

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