Development, Growth & Differentiation
The Japanese Society of Developmental Biologists

Develop. Growth Differ. (2014) **56**, 214–222 doi: 10.1111/dgd.12122

Original Article

Tail structure is formed when blastocoel roof contacts blastocoel floor in *Xenopus laevis*

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The tail organizer has been assessed by such transplantation methods as the Einsteck procedure. However, we found that simple wounding of blastocoel roof (BCR) made it possible to form secondary tails without any transplantation in *Xenopus laevis*. We revealed that the ectopic expression of *Xbra* was blocked by inhibiting the contact between BCR and blastocoel floor (BCF), and wounding per se seemed to be not directly related to the secondary tail formation. Therefore, the secondary tail might be induced by the contact between BCR and BCF due to the leak of blastocoel fluid from the wound. This secondary tail was similar to the original tail in the expression pattern of tail genes, and in the fact that the inhibition of fibroblast growth factor signaling prevented the secondary tail induction. Our results imply that the secondary tail formation reflects the developmental processes of the original tail, indicating that simple wounding of BCR is useful for the analysis of tail formation in normal development.

Key words: blastocoel floor, blastocoel roof, ectopic tail, tail formation, *Xenopus laevis*.

Introduction

In 1924, Spemann and Mangold revealed that the dorsal blastopore lip of amphibians can induce a new body axis (Spemann & Mangold 1924), and today, this region is called the Spemann–Mangold organizer. Thereafter, Spemann showed that the organizer activity changes by developmental stage: the dorsal lip of early gastrulae has head-to-tail inducing activity, whereas that of late gastrulae can induce only the tail structure (Spemann 1931). These important findings were derived from transplantation assays. There are two main transplantation methods to assess organizer activity. One involves graft transplantation into the ventral side of a host gastrula, and the other is the Einsteck procedure, which involves graft insertion into the host gastrula blastocoel. Because of ease of use, the Einsteck procedure is applied to various grafts, such as injected animal caps (ACs) (Ruiz i Altaba & Melton 1989). However, it is reported that the results obtained by those two methods differ (Nakamura & Kawakami 1977) and hence, we should consider the results carefully.

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Received 14 December 2013; revised 10 January 2014; accepted 11 January 2014.

The tail is a continuation of the structures of the main body axis posterior to the anus and contains the neural tube, the notochord, and somites. After the discovery of the Spemann–Mangold organizer, many studies were performed to identify the head-inducing factor (Nakamura & Kawakami 1977), whereas few focused on tail induction. The molecular mechanism of tail formation is gradually being revealed by recent loss-of-function experiments. Blocking of fibroblast growth factor (FGF) signaling in *Xenopus* embryo by the expression of a dominant negative mutant form of the FGF type I receptor (dnFGFR1) can give rise to embryos with normal heads and anterior trunk tissues, but not tails (Amaya *et al.* 1991, 1993). Conversely, the injection of flrt3, which promotes FGF signaling, can generate ectopic tails (Bottcher *et al.* 2004). In addition, Tucker and Slack presented a model for tail formation in *Xenopus* that a tail bud will be determined when the junction of mesodermal and neural regions of the posterior neural plate directly overlies the underlying dorsal mesoderm (Tucker & Slack 1995a). Whereas the mechanism of tail bud outgrowth has begun to be revealed (Beck & Slack 1999; Beck *et al.* 2001), the mechanism of early tail formation is still only partially understood.

In many studies, tail organizer activity has been assessed by the Einsteck procedure (Gont *et al.* 1993; Slack & Isaacs 1994). However, Kornikova *et al.* (2009) reported that a mechanically relaxed embryo made by orthotopic transplantation of a suprablastoporal area at

gastrula stage often possesses a tail-like protrusion and wounding activates ERK (LaBonne & Whitman 1997; Christen & Slack 1999), which is downstream of FGF signaling. Hence, those reports led us to hypothesize that a secondary tail is formed when blastocoel roof (BCR) is simply wounded during normal development. In the present study, we found that an ectopic tail is formed when a slit is made on the BCR at the early gastrula stage in *Xenopus.* Analyses of marker gene expression revealed that this process of ectopic tail formation reflects that of original tail formation. We showed that this ectopic tail formation requires physical contact between BCR and blastocoel floor (BCF), and wound per se seems not needed. Our result implies that, in transplantation assay, embryos have potential to form ectopic tail without graft. Therefore, at least in *Xenopus*, the mechanisms of tail formation, including the concept of the tail organizer, should be re-evaluated. Moreover, this simple wounding of BCR may be useful for studies of early tail formation.

Materials and methods

Animals

Adults of *Xenopus laevis* purchased from Watanabe Zoshoku (Hyogo, Japan) were maintained in our laboratory. All animals were maintained and used in accordance with the guidelines established by JT Biohistory Research Hall for the use and care of experimental animals. The eggs were obtained by injecting female frogs with 500 IU human chorionic gonadotropin (Sigma-Aldrich, St. Louis, MO, USA) prior to *in vitro* fertilization. The jelly coat was removed by treatment with 1.5% cysteinium chloride (pH 8.0). The embryos were raised in 0.1 × Barth solution (88 mmol/L NaCl, 1 mmol/L KCl, 2.4 mmol/L $NaHCO_3$, 0.82 mmol/L $MgSO_4$, 0.33 mmol/L $Ca(NO_3)_2$, 0.41 mmol/L $CaCl_2$, 10 mmol/L HEPES, pH 7.6) until the indicated stage according to Nieuwkoop and Faber (Nieuwkoop & Faber 1956) at 13–16°C.

Wounding

The vitelline membrane was removed mechanically with round-ended forceps without wounding. A slit (approximately 300 μm) was made on BCR at the animal pole or the lateral side with an eyebrow needle in 0.5 × Steinberg solution (58 mmol/L NaCl, 0.67 mmol/L KCl, 0.44 mmol/L $Ca(NO_3)_2$, 1.3 mmol/L $MgSO_4$, 4.6 mmol/L Tris, pH 7.6) containing 100 mg/L kanamycin. As the need arises, a small piece (approximately 300 μm × 300 μm) of plastic wrap (Asahi Kasei Home Products, Tokyo, Japan) was inserted under the slit. For fate mapping of the wound, the slit was stained immediately after wounding with 3 mg/mL DiI in dimethylformamide (DMF) and Nile-blue-soaked agar. Unless otherwise specified, the wounded embryos were kept wounded side down on 1.2% agarose-coated dish at 17.5°C.

Histology

Thirty-micrometer cryosections were prepared as described previously (Kawasaki-Nishihara *et al.* 2011). After cleaning of the compound, the sections were stained with hematoxylin and eosin.

In situ *hybridization*

Whole-mount *in situ* hybridization was performed as described previously (Harland 1991) with minor modifications. Plasmids for antisense RNA probe template containing *Xbra*, *bmp4*, *chd*, and *fgf8* were a kind gift from Dr K. Cho. *Xnot*-containing plasmid was described previously (Yamaguti *et al.* 2005) and pCS2AT+ plasmids of other genes were constructed with the following primers: *cdx4*-F (5′-cgGAATTCcaccATGGACATCACATGTGGGAGAC), *cdx4*-R (5′-AggcgcgccTCATTGGGACAGAGTGACATGC); *flrt3*-F (5′-cgGAATTCcaccATGACTACGGACACTTGGAA), *flrt3*-R (5′-AggcgcgccGCATCATGAATGTGAATGAT); *tbx6*-F (5′-cgGAATTCcaccATGTACCACTCTGAGCTCTTCCAGC) and *tbx6*-R (5′-AggcgcgccAATCAATAGTCTCACATCCAG). Digoxigenin-labeled antisense RNAs were generated by *in vitro* transcription with a MAXIscript Kit (Ambion, Life Technologies, Carlsbad, CA, USA) and a DIG RNA Labeling Kit (Roche GmbH, Mannheim, Germany).

Inhibition of FGF signaling

For the inhibition of FGF signaling at BCR, capped mRNA of *dnFGFR1* was synthesized with SP6 RNA polymerase (mMESSAGE mMACHINE Kit, Ambion) from the Not1-linearized template of *dnFGFR1*/pCS2AT+ constructed with *dnFGFR1*-F (5′-cgcGAATTCcaccATGTTCTCCGGAATGTCCCTC) and *dnFGFR1*-R (5′-AggcgcgccTCACGGGTGCTTCATTTTAAAGATAATG). To minimize the effect of normal development, *dnFGFR1* mRNA was microinjected at the 8-cell stage in four animal blastomeres (1 ng/cell) near the animal pole. The injected embryos were incubated and wounded at stage 10.

AC assay

Animal caps were dissected from the wounded embryos with fine forceps at the indicated times and incubated until 7 h after wounding. Then, ACs were fixed with MEMFA (0.1 mol/L MOPS, pH 7.4, 2 mmol/

L EGTA, 1 mmol/L $MgSO_4$, and 4% formaldehyde) and *in situ* hybridization was performed with *Xbra* or *flrt3* digoxigenin-labeled RNA probe.

Results

Secondary tail formation

We hypothesized that a secondary tail is formed when BCR is simply wounded during normal development. To confirm this, we made a slit on the BCR of early gastrulae at the animal pole and incubated the embryos by putting the wounded side down (Fig. 1A). As expected, the wounded embryos had tail-like protrusions (Fig. 1B). Some anterior protrusions were fused with the primary axis in the head region, and the embryos lacked eyes on the fused side (data not shown). The tail-like protrusions had well-formed dorsal and ventral fins, pigment cells, muscles (the protrusions wiggled), and neural-tube-like tissues (Fig. 1C). Some wounded embryos had also notochord-like tissues (Fig. 1C). Note that the tail structure is independent of the original axis, because we checked it did not branch from the original axis by serial sections (data not shown).

To confirm that the tail-like protrusions are secondary tails, we compared the expression of various genes in the protrusions with those in the original tail (Fig. 2). At the tadpole stage, *Xbra* (Fig. 2A), *cdx4* (Fig. 2B), *chd* (Fig. 2C), *fgf8* (Fig. 2D), and *Xnot* (Fig. 2E) were expressed at the tips of the tail-like protrusions as well as in the original tail. From the *myoD* expression pattern, we recognized segmented muscles in the protrusions (Fig. 2F). Also at the tail-bud stage, the transcripts of those genes were found in the protrusions (Fig. 2G–J). Some embryos also had anus-like structures in which *bmp4* was expressed similar to the original anus (Fig. 2K). From these observations, we concluded that the protrusion was the secondary tail.

If the secondary tail were organized by the same mechanism as the original tail, inhibition of FGF signaling would interfere with the secondary tail formation (Amaya *et al.* 1991, 1993). To verify this point, we made a slit on the BCR of *dnFGFR1*-injected gastrula embryos. As shown in Figure 2L, there is a distended epidermis in the ventral side of the embryos, but external tail buds are never formed. This result suggests that the process of secondary tail formation reflects that of original tail formation.

Early gene expression of secondary tail region

When does BCR have the competence for secondary tail formation? We made a slit on the BCR of embryos at stages from blastula to late gastrula stages and examined the rate of secondary tail formation (Fig. 3). More than 80% of embryos whose BCR was slit at blastula (stage 9) or early gastrula (stage 10.25) stage generated secondary tails, although the embryos whose BCR was slit at stage 9 had high malformation frequency. In contrast, the rate of secondary tail formation was significantly reduced after the mid-gastrula stage (stage 11). Therefore, BCR may possess the ability to form a secondary tail from the blastula stage through to early gastrula stage, and the ability disappears at mid-gastrula or later stages.

Next, we analyzed the early phase of secondary tail formation using embryos whose BCR was wounded at stage 10 (Fig. 4). At 3.5 h after wounding (stage 11), we recognized the weak expression of *Xbra* (Fig. 4A) and *flrt3* (Fig. 4B). At 7 h after wounding (stage 12),

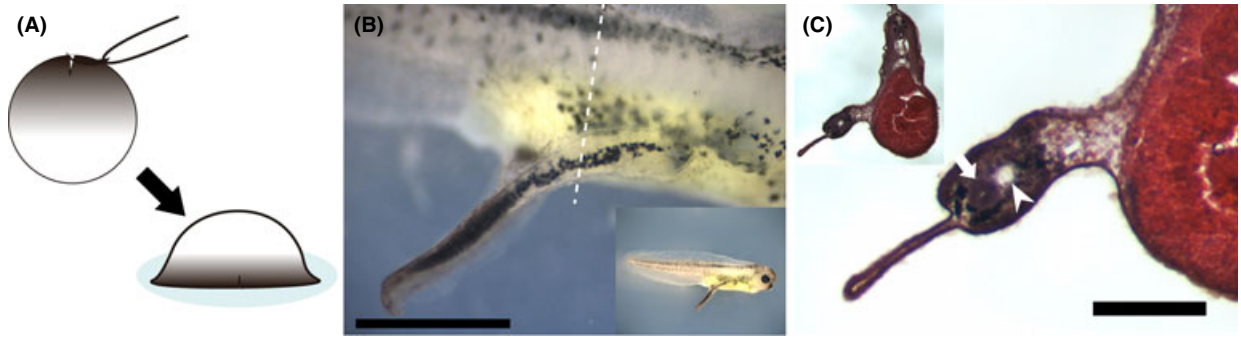

Fig. 1. Tail-like protrusion in embryo whose blastocoel roof (BCR) was wounded. (A) Schematic representation of wounding experiment. After removal of vitelline membrane, BCR of an embryo was wounded with an eyebrow needle and incubation was carried out animal side down. (B) Tail-like protrusion of wounded embryo at the tadpole stage. The protrusion had melanophores and fin, and was not continuous from the original axis. Inset shows whole image of the embryo. (C) Magnified image of the protrusion in the transverse cryosection at the broken line in B. Inset shows whole image of the section. Arrowhead indicates notochord-like structure and arrow indicates neural tube-like structure. Scale bars represent 500 μm in B and 200 μm in C.

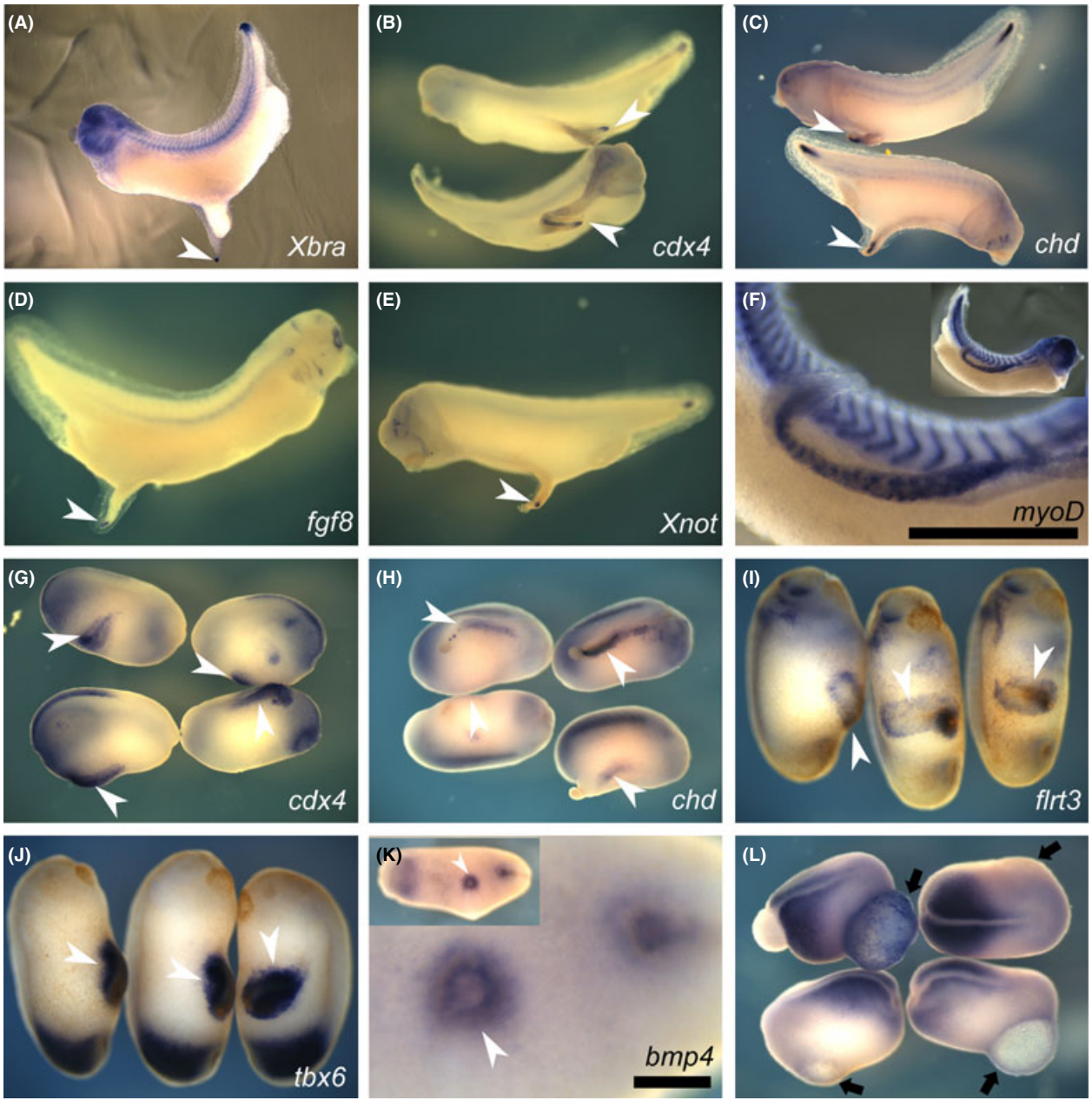

Fig. 2. Expression of tail marker genes in wounded embryos. (A–F) Wounded embryos at stage 33 stained with antisense RNA probes of *Xbra* (A), *cdx4* (B), *chd* (C), *fgf8* (D), *Xnot* (E), and *myoD* (F). Arrowheads in A–E indicate expression at tips of ectopic protrusions. (G–K) Wounded embryos at early tail-bud stage stained with antisense RNA probes of *cdx4* (G), *chd* (H), *flrt3* (I), *tbx6* (J), and *bmp4* (E). Arrowheads in G–J indicate expression in ectopic protrusions, and those in K indicate expression in ectopic anus-like structure. Scale bars represent 1 mm in F and 200 μm in K. (L) *dnFGFR1*-injected and wounded embryos stained with antisense RNA probe of *tbx6*. Arrows indicate distended epidermis.

the expressions became clear (Fig. 4C,D). The expression region showed thickening and there were interstices with internal tissue (Fig. 4E). The expression of *Xnot*, *cdx4*, and *chd* became recognizable at 7 h after wounding (Fig. 4F–I). As the transcription of *Xbra* and *flrt3* started within 3.5 h after wounding, those genes may be the first genes induced by wounding.

Conditions for secondary tail formation

To evaluate the conditions for ectopic tail formation, we examined other conditions using embryos whose BCR was wounded at stage 10. When the wounded embryos were incubated vegetal side down, the efficiency of secondary tail formation was half as much as

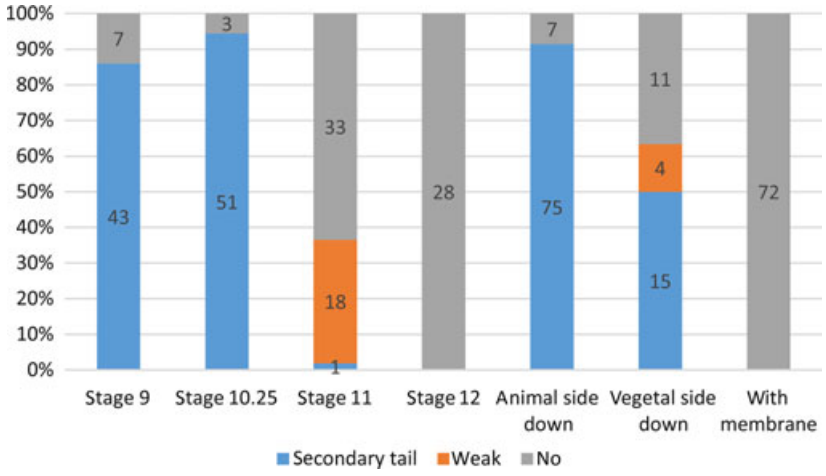

Fig. 3. Graph of percentages of secondary tail formation in various conditions. Numbers in each column indicate the number of wounded embryos. Blue columns indicate the percentage of secondary-tail-forming embryos. Orange columns indicate the percentage of small-lump-forming embryos. Gray columns indicate the percentage of wounded embryos without ectopic protrusions. Starting from the leftmost column, percentages of embryos wounded at stage 9, stage 10.25, stage 11, stage 12, stage 10 with animal side down, stage 10 with vegetal side down, and stage 10 with vitelline membrane intact, are shown.

when the wounded embryos were incubated animal side down (Fig. 3). Wounded embryos with vitelline membrane did not form a secondary tail (Fig. 3). As shown in Figure 5A, ACs of the wounded region explanted immediately after a slit was made on the BCR did not express *Xbra* and *flrt3* at 7 h after wounding (each gene; $n = 4$). Those genes were expressed in three out of seven ACs explanted from the wounded embryos incubated animal side down for 1.5 h (data not shown) and in all the ACs explanted at 3.5 h after wounding (*Xbra*; $n = 8$ in Fig. 5B, *flrt3*; $n = 7$). The results suggest that BCR wounding alone does not induce a secondary tail and incubating the embryo animal side down promotes ectopic tail induction.

From these observations, we hypothesized that the contact between BCR and BCF induces secondary tail formation. To ascertain this hypothesis, we analyzed ectopic *Xbra* expression using BCR-wounded embryos in which plastic wrap was inserted between BCR and BCF. As shown in Figure 5C and D, ectopic *Xbra* expression was not detected in the region where the plastic wrap inhibited the contact, whereas the expression was observed in the surrounding area ($n = 15$). Therefore, this result supported our hypothesis.

Relationship of wounding with secondary tail formation

The fact that the direct contact of BCR with BCF seems to be essential for the secondary tail induction raises the question of whether wounding is needed for the secondary tail induction. To answer this, we wounded the lateral side of BCR to establish physical contact with BCF while leaving AC intact, and explanted ACs at 3.5 h after wounding. The ACs expressed *Xbra* (22/23, Fig. 6B) as well as ACs with wound (Fig. 5B). As ACs were wounded by dissection from the embryos, there is a possibility that wounding is needed to induce the *Xbra* expression. For further verification, we labeled the wounded region with Nile blue and the fluorescent carbocyanine dye DiI, and analyzed the positional relationship between labeled cells and the secondary tail region (Fig. 6C–F, Movie S1). When we made a wound at the animal pole, the labeled cells were found in the secondary tail region (13/14, Fig. 6C,D, Movie S1). On the other hand, the labeled cells were found far from the secondary tail region in embryos whose lateral side of BCR was wounded (26/29, Fig. 6E,F). Together, the results suggest that wounding per se does not influence secondary tail formation directly.

Discussion

In this study, we found that wounding of BCR induced secondary tail formation without any transplantation or genetic manipulation (Fig. 1). The secondary tail induction started from the contact between BCR and BCF due to blastocoel fluid leakage from the wound (Fig. 5). In addition, we revealed that the process of secondary tail formation seems to mimic that of original tail formation (Fig. 2).

Wounding per se is not the key to tail formation

How does wounding induce secondary tail formation? Wounding induces the transient activation of ERK (Christen & Slack 1999; data not shown), and the ectopic activation of FGF signaling by wounding

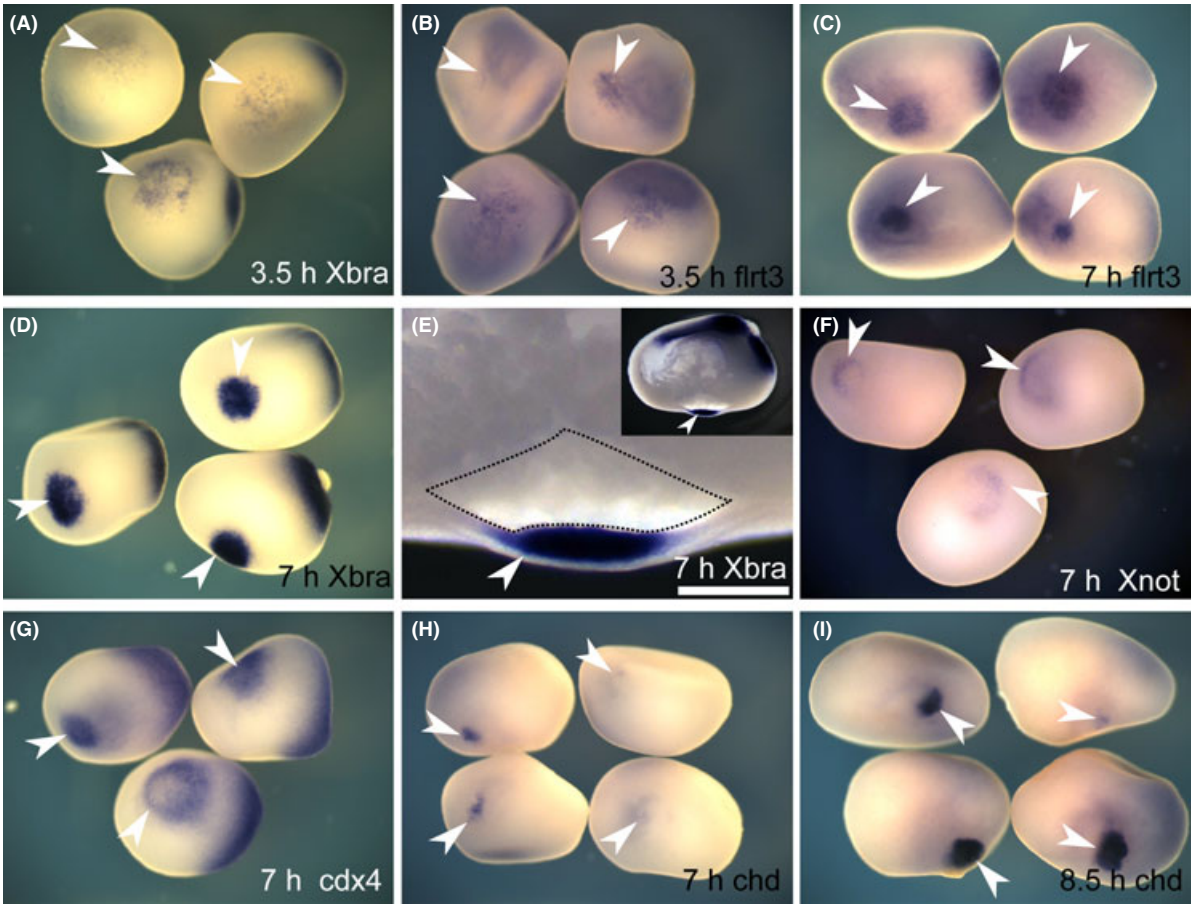

Fig. 4. Ectopic expression of tail marker genes in wounded gastrula embryos. (A–D, F–I) Ventral view of wounded embryos stained with antisense RNA probes of *Xbra* (A, D), *flrt3* (B, C), *Xnot* (F), *cdx4* (G), and *chd* (H, I) at 3.5 h (A, B), 7 h (C–H), and 8.5 h (I) after wounding. (E) Sagittal section of wounded embryo stained with *Xbra* probe. Scale bar represents 200 μm. Broken line indicates a hole. Arrowheads indicate ectopic staining. Part of the ectopic region showed very weak staining, but all the embryos showed strong staining in the normal expression region.

seems to induce the tail formation. However, the activation was not detected after the wound healed and the same activation was observed in wounded embryos with vitelline membrane intact (data not shown), which did not show any ectopic tail, indicating that the FGF signaling activation by wounding does not cause the ectopic tail formation. In addition, we also showed that the wounded site does not specify the position of the secondary tail (Fig. 6). Taken together, wounding per se seems not to directly contribute to any processes of secondary tail formation, but to indirectly permit BCR to contact BCF.

BCF (endoderm) directly converts BCR (ectoderm) into mesoderm (possible tail organizer)

Our results indicate that the secondary tail is formed simply by the physical contact between BCR and BCF (Fig. 5). It has been known from conjugation experiments that the mesoderm is induced via inductive signals emanating from the endoderm (Nieuwkoop 1969; Slack 1991). The same result was confirmed by the observation of the induction of mesodermal markers, such as *Xbra* and *chd* (Wylie *et al.* 1996; Agius *et al.* 2000). The tail induction ability was obviously lost from stage 11 (Fig. 3), and this result coincides with the report of Jones and Woodland that the ability of vegetal yolky cells to induce mesoderm formation disappears between stages 10.5 and 11 (Jones & Woodland 1987). In addition, embryos form a tail-shaped outgrowth when mesoderm inducing factor or activin A is injected into the blastocoel (Cooke *et al.* 1987; Ariizumi *et al.* 1991). From these findings, it seems that BCR (ectoderm) is converted into mesoderm by inducing factor(s) emanating from BCF (endoderm), and then the induced mesoderm, in turn, acts as a tail organizer.

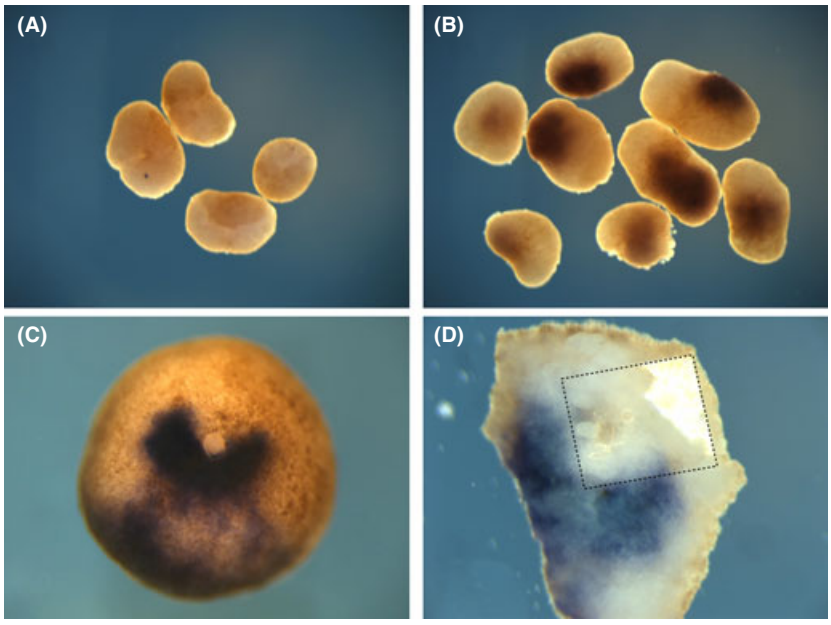

Fig. 5. Contact between blastocoel roof (BCR) and blastocoel floor (BCF) is essential for induction of ectopic *Xbra* expression. (A, B) *Xbra*-stained animal caps (ACs) of wounded regions explanted from wounded embryos at 0 h (A) and 3.5 h (B) after wounding. *Xbra* expression was not detected in A, but all the ACs in B were stained. (C, D) Animal view of an *Xbra*-stained embryo with plastic wrap inserted under the wound (C) and vegetal view of embryo BCR (D). *Xbra* expression was lacking in part of the ectopic region (C), and the lacking region was covered by plastic wrap (D). Broken line indicates a piece of plastic wrap.

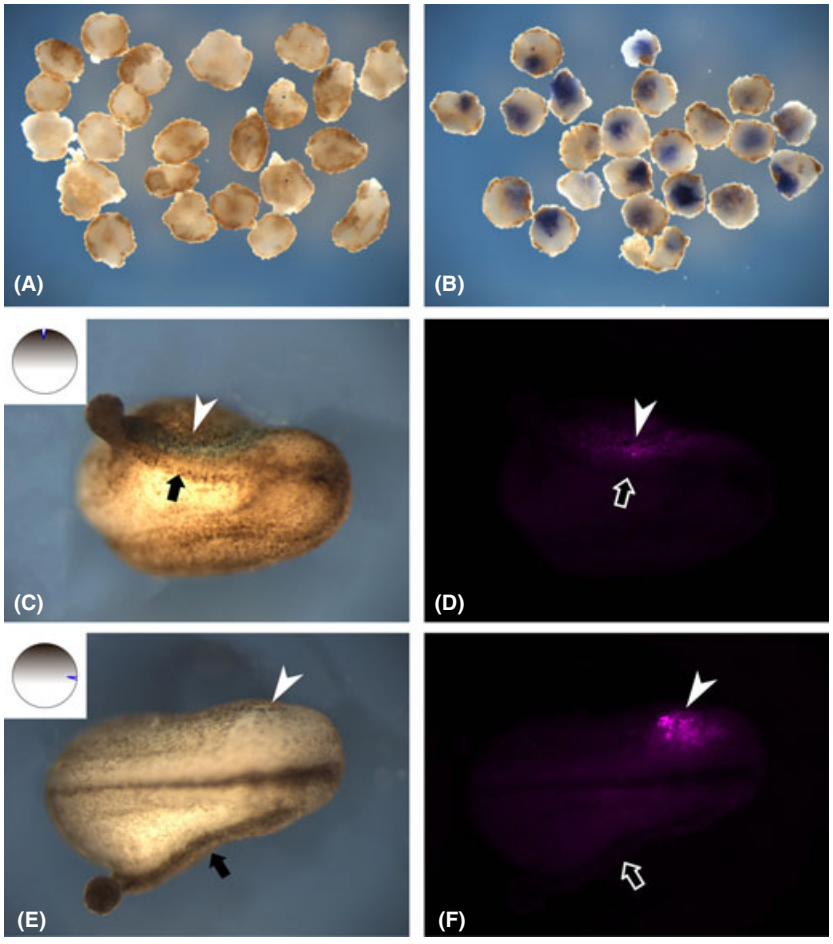

Fig. 6. Wounding is not related to secondary tail formation. (A, B) *Xbra*-stained animal caps (ACs) of non-wounded region explanted from wounded embryos at 3.5 h after wounding (B) and from intact siblings in B (A). *Xbra* expression was not detected in A, whereas most ACs in B showed staining. (C–F) Dorsal view of wounded embryos whose wound was stained with Nile blue (arrowheads in C, E) and DiI (arrowheads in D, F). Arrows indicate secondary axis. Insets show schematic representation of wounded region at stage 10. The animal pole of the embryo in C, D was wounded and the stained region overlapped with the secondary axis. On the other hand, the lateral side of blastocoel floor (BCF) of the embryo in E, F was wounded and the stained region was located opposite to the secondary axis.

How is original tail formed?

Two controversial views regarding tail formation have persisted for decades (reviewed by Handrigan 2003). One is that the tail is formed from a homogeneous mass of pluripotent mesenchymal cells by a separate and distinct process from that of the trunk, i.e. secondary neurulation (Holmdahl 1925). The other view is that the tail is formed as a continuation of the gastrulation process shaping the head and the trunk (Pasteels 1939). In *Xenopus*, in particular, there is little consensus (Gont *et al.* 1993; Tucker & Slack 1995b; Beck & Slack 1999). As shown in Figure 1, a set of tail structures was formed ectopically in the region where no neural or axial mesoderm should be present. This leads us to surmise that the tail arises directly, in agreement with the view of secondary neurulation. However, the secondary tails were very small at the tadpole stage (Fig. 1), indicating that complete tail structure could not be formed in the ectopic condition. It is said that most of the tail tissues in tadpole are derived from the trunk region of the early embryo (Tucker & Slack 1995b). Therefore, the tail itself may be basically formed by secondary neurulation and cells specified at primary neurulation take part in the process to establish complete tail structure.

The intrinsic tail-forming region of the embryo consists of multiple tissues, such as neuroectoderm, axial mesoderm, and posterior endoderm, and the normal developmental process establishes this complex. It is said that proper morphology leads to proper gene expression in the subsequent developmental process. In this study, we found that the tail is formed by physical contact between BCR and BCF, although those tissues never contact each other in normal development, and the physical contact may ectopically create the conditions for the tail formation in normal development. The simple association of those tissues may be sufficient for the tail formation, and the same process may take place in the original tail formation. Thus, it is interesting to think that tail formation may be explained without considering the existence of the tail organizer.

As the secondary tail induction shown in this study is very simple, we can easily observe the process of tail formation without any effects of complex morphogenetic movement. Therefore, this procedure may be useful for the detailed analysis of tail formation mechanisms.

Acknowledgments

We thank Dr Ken Cho for the gifts of plasmids. We also thank all the laboratory members for valuable discussions, animal maintenance, and assistance in experiments.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Movie S1. Process of secondary tail formation.