

When does the anterior endomesoderm meet the anterior-most neuroectoderm during *Xenopus* gastrulation?

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ABSTRACT During amphibian gastrulation, the anterior endomesoderm is thought to move forward along the inner surface of the blastocoel roof toward the animal pole where it comes into physical contact with the anterior-most portion of the prospective head neuroectoderm (PHN), and it is also believed that this physical interaction occurs during the mid-gastrula stage. However, using *Xenopus* embryos we found that the interaction between the anterior endomesoderm and the PHN occurs as early as stage 10.25 and the blastocoel roof ectoderm at this stage contributed only to the epidermal tissue. We also found that once the interaction was established, these tissues continued to associate in register and ultimately became the head structures. From these findings, we propose a new model of *Xenopus* gastrulation. The anterior endomesoderm migrates only a short distance on the inner surface of the blastocoel roof during very early stages of gastrulation (by stage 10.25). Then, axial mesoderm formation occurs, beginning dorsally (anterior) and progressing ventrally (posterior) to complete gastrulation. This new view of *Xenopus* gastrulation makes it possible to directly compare vertebrate gastrulation movements.

KEY WORDS: *vertebrates, amphibian, gastrulation movement, leading edge mesoderm, Xenopus*

Introduction

In vertebrates, direct interaction between the prospective head neuroectoderm (PHN) and a specific region known as the head organizer (equivalent to the anterior endomesoderm in *Xenopus*, anterior visceral endoderm in mouse, primitive endoderm or anterior hypoblast in chick and dorsal yolk syncytial layer in zebrafish), is key to the head formation.

Amphibian embryogenesis studies have shown that the anterior endomesoderm lies near the dorsal lip, and the PHN is located in the blastocoel roof ectoderm in early gastrulae (Keller, 1976; Gerhart and Keller, 1986; Keller *et al.*, 1992). Thus, these two tissues are spatially distinct from one another in the early gastrula stages. In order to contact the PHN, the anterior endomesoderm moves along the inner surface of the blastocoel roof towards the prospective forebrain region during gastrulation. Previous fate mapping experiments have indicated that the anterior mesoderm does not progress anteriorly to underlie and contact the prospective forebrain ectoderm until stages 11-11.5 (Keller *et al.*, 1992). However, in *Xenopus*, it is still unclear when and where this physical interaction is established, because the prospective neuroectoderm is relatively short and wide, so that leading edge mesoderm tissue can physically contact the PHN in the earliest

phase of gastrulation (Nieuwkoop and Florshutz, 1950; Vodicka and Gerhart, 1995).

Although gastrulation seems to be a molecularly conserved process among vertebrate species, the morphogenetic movements of amphibian gastrulation are distinct. The leading edge mesoderm is closely opposed to the PHN at very early gastrula stages in several vertebrate species when these tissues are physically distinct from each other in early *Xenopus* gastrula (Beddington and Robertson, 1998). This apparent discrepancy between *Xenopus* and other vertebrates makes it difficult to propose a general model for the mechanisms regulating head specification, although several researchers have proposed such (Streit *et al.*, 1994; Holley and Ferguson, 1997; Beddington and Robertson, 1998; Arendt and Nubler-Jung, 1999).

In this report, we show that the blastocoel floor apposition (the anterior endomesoderm) already extends to the anterior-most portion of the prospective neuroectoderm as early as stage 10.25. Once this interaction is established, these two tissues are continuously associated in register throughout the gastrulation process. Based on these findings, we propose that at least some aspects of

Abbreviations used in this paper: HO, head organizer; Nb, Nileblue; PHN, prospective head neuroectoderm; WISH, whole-mount *in situ* hybridization.

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vertebrate gastrulation have a common evolutionary origin, and we discuss comparisons of gastrulation movements among vertebrate species.

Results

The Anterior Endomesodermal Tissue reaches the Anterior-most Portion of the Prospective Head Neuroectoderm by Stage 10.25

To determine when the physical interaction between the migrating leading edge tissue and the PHN initially occurs, we took *Xenopus* embryos at stages between 9 and 11, and labeled the inner surface of their blastocoel cavities with a Nileblue dye. The embryos were allowed to develop and the fates of the labeled cells were examined at the neurula stage. Our assumption was that once the neuroectoderm is in direct contact with the leading edge endoderm, the area of contact would be devoid of Nileblue staining. Thus, if the blastocoel cavities of gastrula stage embryos were injected with Nileblue after the physical interaction between the leading edge endoderm and the prospective neuroectoderm had occurred, the anterior portion of the neural tissue should not be labeled. This allows us to determine the extent of the physical contact between the leading edge endoderm and PHN (see Fig. 1 A-C and its legend).

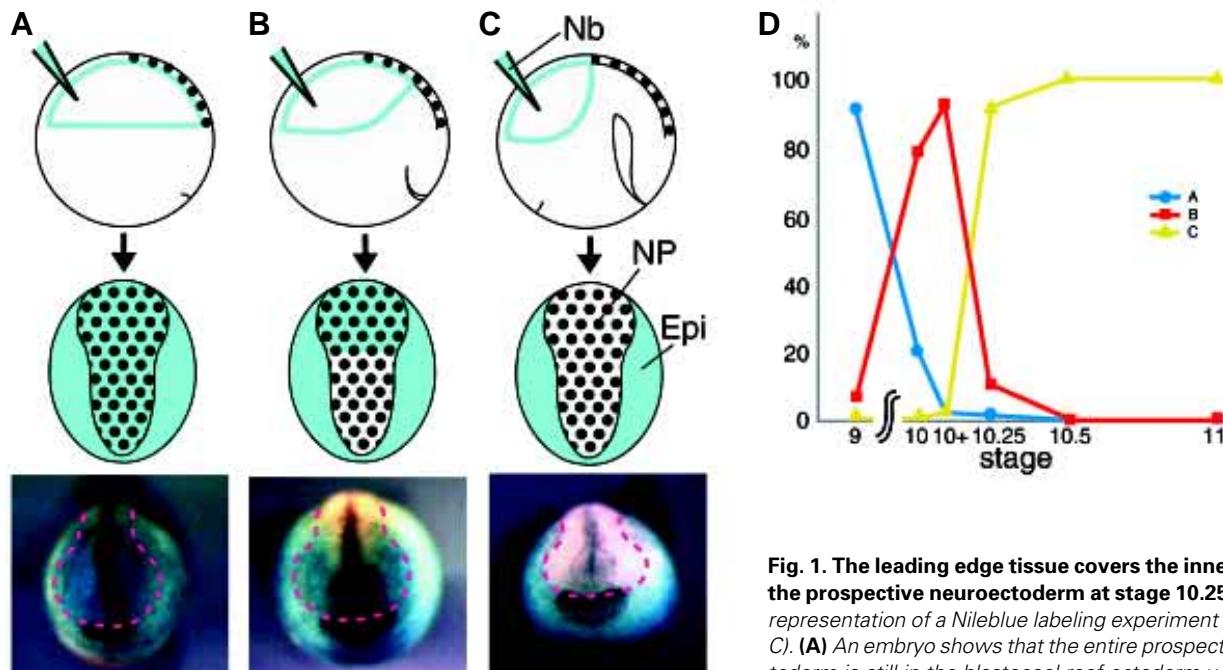
Descendents of the cells from the blastocoel roof ectoderm contribute to all neural and epidermal ectodermal lineages when embryos were labeled prior to stage 10 (Fig. 1 A,D). At stages

between 10 and 10+, no contribution of the labeled cells to posterior neural tissue was observed, although Nileblue positive cells were still found in the neural tissues anterior from the hindbrain region (Fig. 1 B,D, and other data not shown). Between stages 10+ and 10.25, the number of embryos devoid of the anterior neural plate staining increased rapidly, implying that the presumptive forebrain is in contact with the leading edge endoderm (Fig. 1 C,D).

The Fate of Regions of the Ectodermal Layer in Stage 10.25 Embryos

To find the precise location of the prospective forebrain ectoderm in early gastrula at stage 10.25, we took advantage of Keller sandwich experiments (Keller and Danilchik, 1988; Keller, 1991; Doniach *et al.*, 1992; Blitz and Cho, 1995). In a Keller sandwich explant, mesoderm lies posterior to and in the same plane as the ectoderm; thus vertical contact between ectoderm and endo- and/or mesoderm does not occur, unlike in the embryo where anterior endoderm moves underneath the ectoderm and is in contact with it along its entire length. In these explants, the mesoderm and ectoderm differentiate into axial mesodermal and neural tissues, respectively. Genes such as *Xotx2*, *En2* and *Krox20*, which normally exhibit region-specific patterns within neural tissue, are expressed with correct anterior-posterior orientations in the explant (Doniach *et al.*, 1992; Blitz and Cho, 1995).

Our result shown in Fig. 1 suggests that the prospective anterior neuroectoderm has already made physical contact with the leading



resulting in the staining of the entire neural plate. (B) An embryo is labeled when the leading edge tissue is on the way to the anterior end of the prospective neural region. The posterior portion of the central nervous system is not labeled in neurulae. (C) An embryo, whose entire prospective neuroectoderm including head territories was underlain by the inner tissue, was injected with Nileblue. The overall area of the central nervous system was thus prevented from Nileblue staining. The stippled area represents the neuroectoderm or neural plate. Nb, Nileblue; NP, neural plate; Epi, epidermis. The dashed line indicates the actually labeled area of the central nervous system of the embryo. (D) Percentages of Nileblue injected embryos of type-A (blue), type-B (red) and type-C (yellow) are shown. Fifty embryos were injected with Nileblue at each stages indicated. It is obvious that the inner tissue moves relatively gradually on the inner surface of the prospective neuroectoderm toward the anterior boundary from stage 9 through 10.25, and that the physical interaction of the leading edge of the inner tissue with the anterior border of the prospective neuroectoderm is established at stage 10.25.

Fig. 1. The leading edge tissue covers the inner surface of the prospective neuroectoderm at stage 10.25. Schematic representation of a Nileblue labeling experiment is shown (A-C). (A) An embryo shows that the entire prospective neuroectoderm is still in the blastocoel roof ectoderm when labeled,

edge tissue at stage 10.25. To check this point more precisely, we constructed Keller sandwich explants from embryos that had been injected with Nileblue in their blastocoel cavities at stage 10.25 (Fig. 2A). These explants were fixed and processed for whole mount *in situ* hybridization (WISH) analysis using epidermal keratin and *Xotx2* probes at the equivalent of stage 18 (Fig. 2B-E). As shown in Fig. 2B,C, the Nileblue staining in the explant completely overlaps with the region of epidermal keratin staining. The expression of *Xotx2*, a marker for the most anterior neural region (forebrain and midbrain), was found in tissues adjacent to and clearly distinct from the region of Nileblue staining in the explants (Fig. 2D,E), indicating that the prospective neuroectoderm has been in physical contact with inner tissues, such as the anterior endoderm and axial mesoderm.

Therefore it is concluded that prospective neuroectoderm, which is at first located in the blastocoel floor region during blastula stages, has contacted vertically with the leading edge tissue by stage 10.25.

The Leading Edge Tissue at Stage 10.25 corresponds to the Head Organizer

To find out whether the leading edge tissue, which contacts the PHN at stage 10.25, corresponds to the anterior endomesoderm (the head organizer), we examined the expression patterns of *Xdkk1* and *Xcer* by WISH. In other vertebrate species, related genes of *Xdkk1* and *Xcer* are expressed in the head organizer (Schneider and Mercola, 1999; Glinka *et al.* 1999; Jones *et al.*, 1999). As shown in Fig. 3, the leading edge endoderm of a stage 10.25 embryo expressed both *Xdkk1* and *Xcer* (Fig. 3 A,B), but not *Xchd* (Fig. 3C). From these findings, we conclude that the leading edge tissue at the stage 10.25 is the *Xenopus* head organizer.

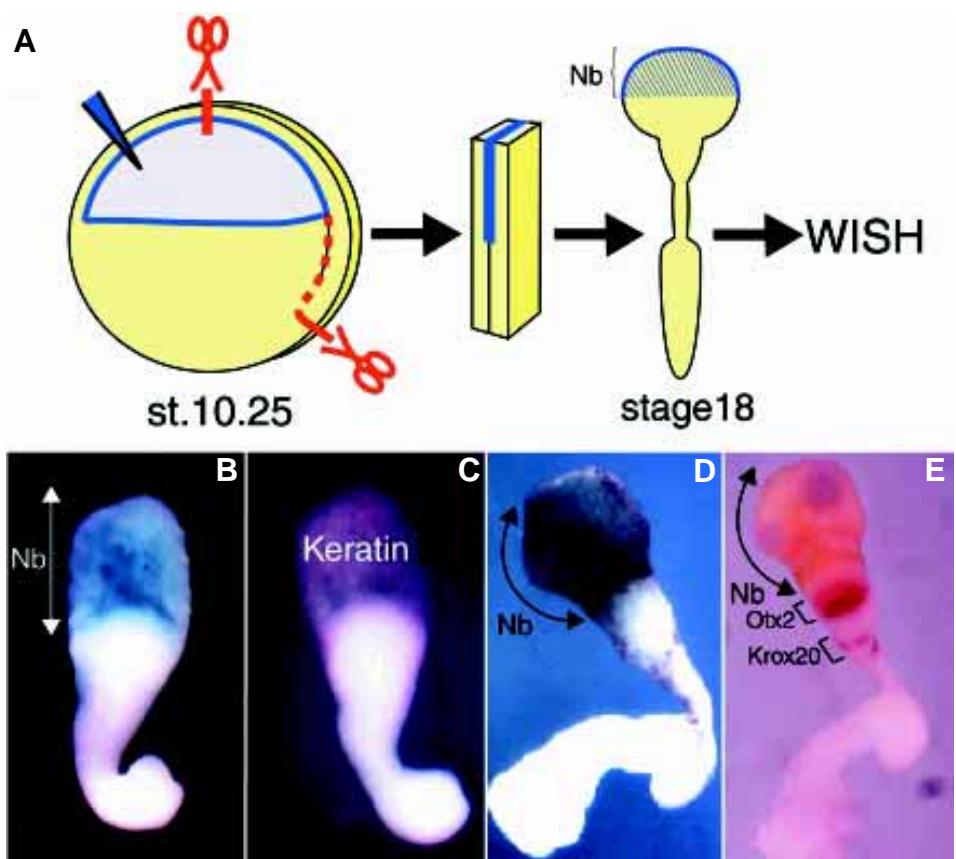
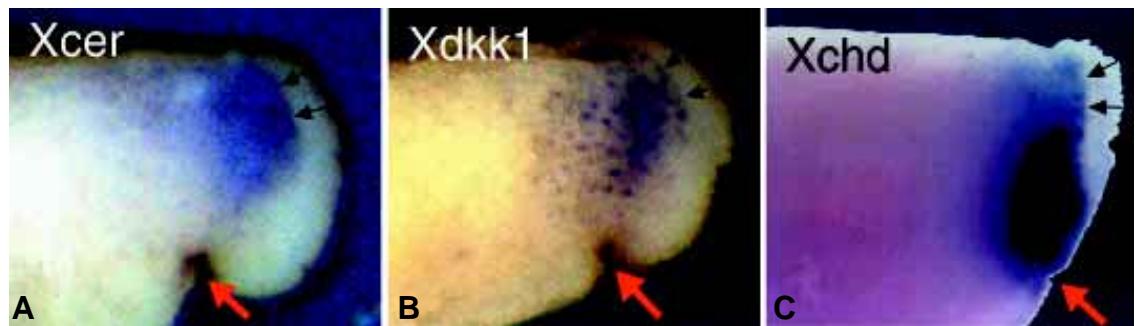


Fig. 2. Blastocoel roof ectoderm at stage 10.25 does not take neural, but an epidermal fate in the Keller sandwich explant. A schematic illustration shows the experimental procedure of making a Keller sandwich explant (A). Keller sandwiches were prepared from stage 10.25 embryo immediately after Nileblue (Nb) injection. The explants were developed to stage 18 and fixed for whole-mount *in situ* hybridization (WISH). Keller sandwich explants made from Nileblue injected stage 10.25 embryos (B,D), epidermal keratin expression (C) and *Xotx2* and *Krox20* expression (E) are shown. Because Nileblue signal disappears during the procedure of WHIS, pre-*in situ* explants are shown in B and D. All the sandwich explants ($n=8$) showed basically the same results.

The PHN and the Leading Edge Tissue are continuously associated with each other from Stage 10.25 until the End of Gastrulation

It is known that the head neuroectoderm of neurula embryo is directly underlain by head organizer tissue (de Souza and Niehrs, 2000; Niehrs *et al.*, 2001), and our observation showed that the head organizer tissue has already made a physical contact with the

Fig. 3. Expression patterns of *Xcer*, *Xdkk1* and *Xchd* in stage 10.25 embryos. Sagittally dissected embryos stained with *Xcer* (A), *Xdkk1* (B) and *Xchd* (C) probes are shown. Dorsal side is to the right. Dorsal lip is indicated by a red arrow. By this stage, Brachet's cleft has already been formed (black arrows).



It is obvious that the anterior endomesoderm (indicated by the *Xcer* or *Xdkk1* expression) is attached to the dorso-marginal ectodermal layer (A and B), but the trunk organizer (indicated by *Xchd* expression) is not physically associated with the ectoderm.

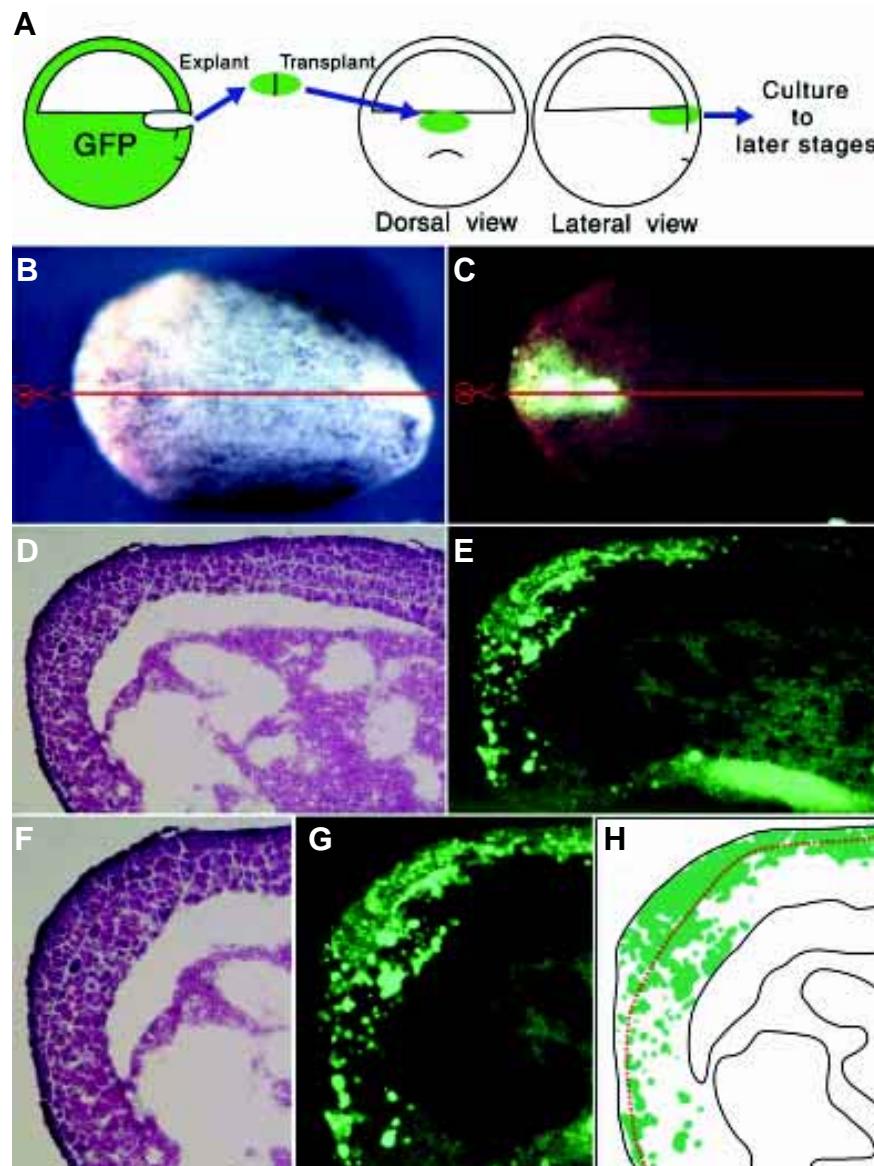


Fig. 4. Both the prospective head neuroectoderm and the head organizer endoderm are continuously associated with each other during gastrulation. (A) Schematic representation of the construction of the GFP-labeled embryos. Prospective head rudiment tissue is excised from a GFP-labeled stage 10.25 embryo and grafted into the homogeneous region of an unlabeled stage 10.25 embryo. The average size of the excised tissue is about a dorsal lip wide, 8-cells deep and 5-cells high. Light (B) and fluorescence (C) micrographs of GFP-labeled stage 14 embryo are shown. All operated embryos ($n=20$) developed normally. Grafted tissue located in the most anterior portion of neurula embryo. Light (D) and fluorescence (E) micrographs of midsagittal sections through the GFP-labeled stage 14 embryo shown in B and C. Higher magnification view of D and E are shown in (F,G) respectively. An illustration of the distribution of GFP labeled cells in the embryo is shown in (H). The boundary between outer (ectodermal) and inner (endo/mesodermal) tissue is indicated by a dashed line. GFP labeled cells of both ectodermal and endo/mesodermal tissues are arranged in register at the presumptive head rudiment.

PHN by stage 10.25, indicating that these tissues continuously associate with each other during entire gastrulation processes. To check whether the PHN and the leading edge mesoderm continue to be associated with each other in register throughout the gastrulation processes, we examined the fate of both the leading edge tissue and the ectodermal layer by grafting a small GFP-labeled tissue into unlabeled embryos. The presumptive head rudiment consisting of both tissues in the dorso-anterior deep layer and its overlying ectoderm were labeled by transplantation of a GFP labeled graft (about 200 μm wide, 200 μm high and 200 μm deep) at stage 10.25 (Fig. 4A). All the operated embryos ($n=20$), which survived surgery, developed normally, indicating that the labeling and transplantation techniques did not perturb normal processes. Labeled tissue was found in the anterior region at stage 14 (Fig. 4B,C). A sagittal section of a stage 14 embryo showed that both the ectoderm and endo-mesoderm of GFP-labeled tissues derived from the graft were approximately in register (Fig. 4E,G,H). The ectodermal thickening marked a region of the presumptive

anterior-most neuroectoderm (Fig. 4H), and suggested that the GFP labeled tissue contributed to the most anterior portion of the embryos.

Taken all together, it is reasonable to conclude that both the PHN and leading edge mesoderm were continuously and physically attached with each other throughout the gastrulation processes from stage 10.25, although we cannot eliminate the possibility that these tissues are physically associated but still sliding a little against each other after stage 10.25.

Discussion

It has been thought that the leading edge mesoderm does not progress anteriorly to underlie and contact the prospective forebrain ectoderm until a mid-gastrula stage (Keller *et al.*, 1992). However, we have shown that the direct interaction between PHN and the leading edge mesoderm as early as stage 10.25 in *Xenopus*. Consistent with our finding, it is reported that at stage

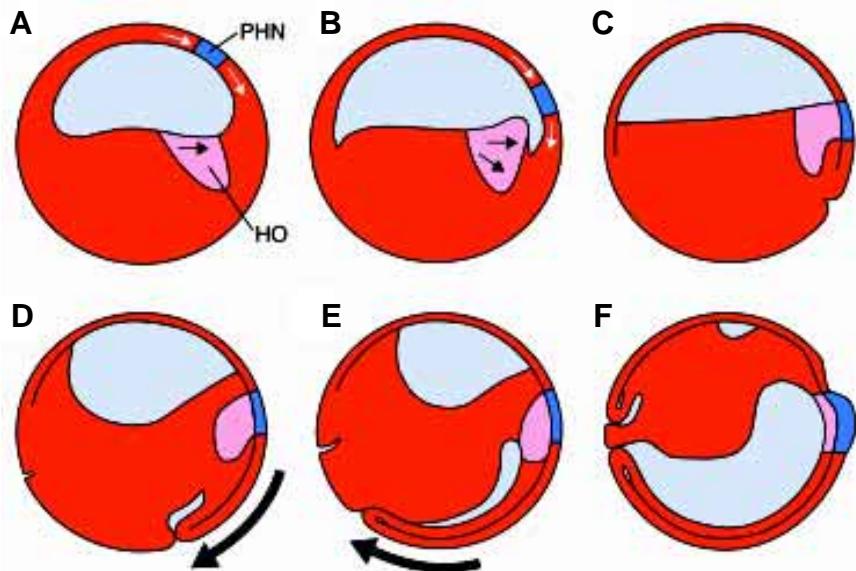


Fig. 5. Model of *Xenopus* gastrulation movements. A model of the presumptive head tissue movement during *Xenopus* gastrulation is shown. At stage 10, the prospective head neuroectoderm (PHN) is still in the blastocoel roof (**A**). At stages between 10 and 10.25 (**A**) and (**B**), the head organizer (HO) moves toward the dorsal marginal end (thin black arrows) and the PHN moves downward toward the dorsal marginal zone (white arrow). During this period, these tissues move relatively nearer to each other. At stage 10.25, vertical contact is established (**C**). After physical interaction has been established, the PHN is associated with the HO tissue during overall gastrulation processes, and thus the axial structure is formed from the anterior (dorsal) to the posterior (ventral) (thick black arrow) (**D-F**). The central nervous system is presumed to be found at the vegetal side of the embryo when free rotation of the developing embryo is inhibited.

10+, blastocoel floor apposition already extends to the prospective hindbrain (Poznanski and Keller, 1997). Although they mentioned that the physical interaction between the leading edge mesoderm and the PHN can possibly occur much earlier phase of gastrulation, they didn't indicate any particular stages in which it occurs. Therefore this is the first report indicating that the physical interaction of these two embryonic tissues appears to take place in the earliest phase of gastrulation in *Xenopus*, contrary to the previous held belief.

It has been said that it is difficult to determine internal morphology from external morphology because it does not always precisely

reflect the internal morphology. Brachet's cleft is formed at stage 10.25 in the embryos shown in this report, but Winklbauer and Schurfeld regard stage 10+ as when the cleft forms (Winklbauer and Schurfeld, 1999), suggesting that our 10.25 may correspond to their stage 10+. Thus, the physical interaction could possibly be established a little earlier than stage 10.25 in other batches of fertilization.

Because there seems to be no vertical induction present in the explant, the well-patterned expression of regional specific neural markers in the Keller sandwich has made investigators assume the existence of planar signal emanating from the organizer (Keller and Danilchik, 1988; Keller, 1991; Doniach *et al.*, 1992). However, as

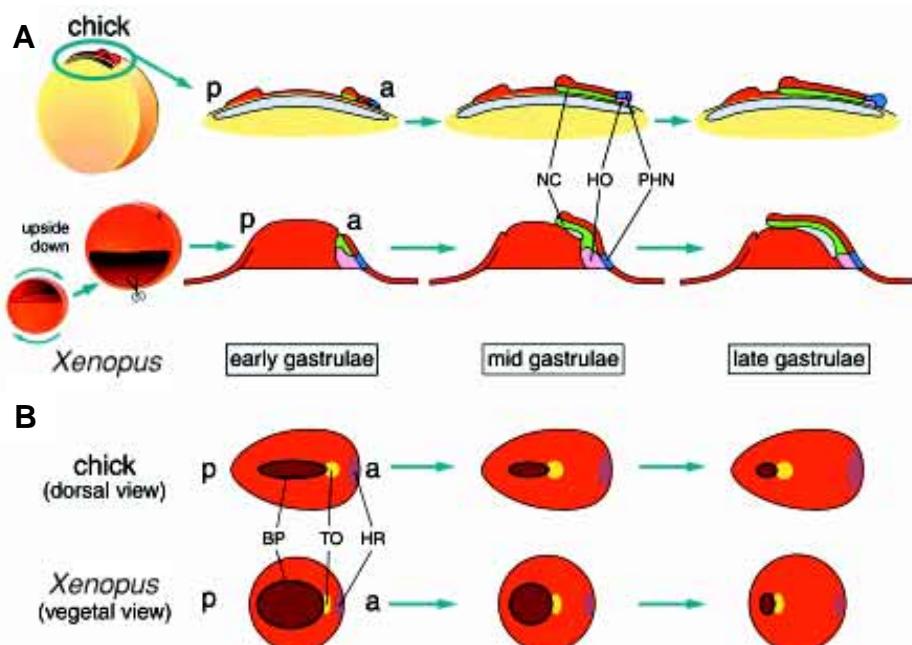


Fig. 6. Comparison of gastrulation movements between chick and *Xenopus*. Schematic sagittal (**A**) and external (**B**) views of predicted gastrulation movements of chick and *Xenopus* are shown. As seen in **A**, the *Xenopus* early gastrula is shown upside down. The blastocoel roof ectoderm was cut and opened in order to make it easy to compare *Xenopus* gastrulation movements directly with those in chick. The trunk organizer (Hensen's node in chick) is formed at the area posterior to the presumptive head rudiment in early gastrulae. As gastrulation proceeds from early through late gastrula stages, the trunk organizer moves in a posterior direction with the closing of the blastopore (primitive streak in chick), and axial mesoderm (notochord) develops in the area anterior to the trunk organizer. The relative arrangements of the homologous tissues (blastopore, trunk organizer and the presumptive head rudiment) are conserved between chick and *Xenopus* during the overall gastrulation period. (**B**) In this figure, the full-length primitive streak stage

embryo in chick is represented as an early gastrula, and an embryo, whose primitive streak closure has been completed, is shown as a chick late gastrula. The early gastrula is representing the stage 10.25 *Xenopus* embryo. To simplify the movement of the trunk organizer, other tissue such as a head folds are not shown here, though these tissue are formed during this period. Abbreviations: A, anterior; P, posterior; NC, notochord; HO, head organizer; PHN, presumptive head neuroectoderm; BP, blastopore; TO, trunk organizer; HR, the presumptive head rudiment.

shown in this paper, vertical contact has already been established in the sandwich explants generated at the earliest phase of gastrulation. Therefore the significance of such planar signals on *Xenopus* neural induction and patterning should be carefully reevaluated, especially for the induction of head neural tissue (Nieuwkoop and Koster, 1995; Nieuwkoop, 1997), although we cannot exclude a role for planar induction.

Taking these results together, we propose a new perspective for *Xenopus* gastrulation (Fig. 5). According to this model, *Xenopus* gastrulation can be divided into at least two steps: formation of the head rudiment by a short ingression movement of the leading edge endomesoderm and the formation of the trunk by a long regression movement of the chordamesoderm. At the onset of gastrulation, the leading edge endomesoderm is spatially separated from the PHN (Fig. 5A). Subsequently, the leading edge endomesoderm moves to make a physical contact with the PHN at the dorso-marginal region (Fig. 5 A,B). By stage 10.25, physical contact between the PHN and the leading edge endomesoderm is established, so that the presumptive head rudiment is specified (Fig. 5C). This means that the animalward migration of the axial mesoderm on the inner surface of blastocoel cavity, in principle, can never take place after this stage. Based on this viewpoint, the chordamesoderm and the neural plate are expected to be formed ventrally (Fig. 5 D-F). It is known, however, that the neural plate is naturally formed at the "dorsal" side of the embryo at the end of gastrulation in normal *Xenopus* development. The source of this discrepancy might be as follows. For a long time, we may have been led to believe that the embryo itself does not rotate during gastrulation, and that the whole embryo can be regarded as a reference system for location tissue position. However, as a matter of fact, the developing embryo rotates freely on its own axis due to the shift of its embryonic center of gravity, which is generated by extensive tissue movement during gastrulation. Therefore, it may be probable that the simple rotation of the embryo brings the head rudiment to the top and the neural plate to the "dorsal" side. This interpretation is supported by the fact that the neural plate is formed downward, when the free rotation of the embryo is blocked artificially (data not shown, Black *et al.*, 1996).

In addition, our model also indicates that the physical interaction between the mesoderm (and/or endoderm) and the blastocoel roof ectoderm is no longer required for the completion of the rest of gastrulation process. This is supported by the fact that anterior specification could not be suppressed even when the interaction between the migrating mesoderm and the blastocoel roof ectoderm was chemically prevented at the gastrulation stage (Brickman and Gerhart, 1994; Mitani, 1989; Kao and Danilchik, 1991) and that the gastrulation was not prevented by perturbation of fibronectin at the gastrulation stage in *Xenopus*, although the migration of mesodermal tissue on the blastocoel roof was inhibited (Winklbauer and Keller, 1996).

After comparing our new model with those of other vertebrates, we found that gastrulation movements, which outwardly appear to be different among vertebrate species, are indeed much more unified processes. For example, since primitive streak in the mouse and also in chick, is equivalent to the blastopore in *Xenopus* (Beddington and Robertson, 1998; Tam and Behringer, 1997), the movement of the leading edge mesoderm can be considered to be similar to that of AVE (Schneider and Mercola, 1999). The subsequent posteriorward progression of the notochord correlates well with the movements in chick (see Fig. 6 and its legend). According

to our model, it is obvious that the *Xenopus* blastocoel cavity is not necessary for the formation of the axial mesoderm, although the gastrulation movements progress posteriorly in the blastocoel cavity in chick. Therefore, we propose that the blastocoel of *Xenopus* can no longer be regarded as a structure homologous with that of chick. Instead, we surmise that the chick blastocoel floor tissue is homologous with the vegetal surface tissue of *Xenopus* gastrulae, the region that is encompassed by the blastopore.

Since mouse and chick embryos expand in size during gastrulation, it is difficult to study the actual morphogenetic movements. Therefore, further studies on *Xenopus* development should aid in a greater understanding of a general model for head formation in vertebrates.

Materials and Methods

Embryonic Manipulations

Embryos were *in vitro* fertilized, dejellied and cultured (Hawley *et al.*, 1995). For GFP labeling, 4 nl (0.1 mg/ml) of mRNA encoding a modified GFP (Ogawa *et al.*, 1995) was synthesized using mMessage mMACHINE (AMBION), and was injected into all four blastomeres at the four-cell stage. Embryos were fixed with MEMFA at stages indicated and sagittally dissected with a razor blade. For labeling, 20 nl of saturated Nileblue solution in saline was injected into the blastocoel cavity at stages indicated.

Whole-mount *In Situ* Hybridization

Whole-mount *in situ* hybridization was performed, essentially, as described previously (Harland, 1991).

Preparation of Keller Sandwich Explants

Keller sandwiches were prepared as described (Blitz and Cho, 1995) using gastrula embryos injected with Nileblue dye into their blastocoel cavities at stage 10.25. Sandwiches were allowed to develop until stage 18, and then fixed for whole-mount *in situ* hybridization.

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