Review

Gastrulation and pre-gastrulation morphogenesis, inductions, and gene expression: Similarities and dissimilarities between urodelean and anuran embryos

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A B S T R A C T

Studies of meso-endoderm and neural induction and subsequent body plan formation have been analyzed using mainly amphibians as the experimental model. *Xenopus* is currently the predominant model, because it best enables molecular analysis of these induction processes. However, much of the embryological information on these inductions (e.g., those of the Spemann–Mangold organizer), and on the morphogenetic movements of inductively interacting tissues, derives from research on non-model amphibians, especially urodeles. Although the final body pattern is strongly conserved in vertebrates, and although many of the same developmental genes are expressed, it has become evident that there are individually diverse modes of morphogenesis and timing of developmental events. Whether or not this diversity represents essential differences in the early induction processes remains unclear. The aim of this review is to compare the gastrulation process, induction processes, and gene expressions between a urodele, mainly *Cynops pyrrhogaster*, and an anura, *Xenopus laevis*, thereby to clarify conserved and diversified aspects.

Introduction

Gastrulation is a set of evolutionarily conserved morphogenetic movements in the early development of a wide variety of vertebrates. During gastrulation in amphibians, the cells of the dorsal marginal zone (DMZ) involute through the blastopore to form an archenteron roof (ARF) that underlies the future central nervous system (CNS). The three-dimensional germ layer structure of the embryo is established during gastrulation, and two major inductive interactions are required. One is meso-endoderm induction and establishment of the anteroposterior and dorsoventral regional characteristics of the induced mesoderm. The other is neural induction, in which induced and regionally specific mesoderm within the ARF or associated with it, spatially and temporally interacts with the presumptive neuroectoderm to form the CNS. Thus, the early patterning of the embryo involves a complex set of spatial and temporal inductions, morphogenetic movements and regional interactions.

Spemann and Mangold (1924) first discovered in urodeles the organizational activity of the early gastrula dorsal blastoporal lip
Xenopus become specified later than in For example, in frogs with slow development, the mesoderm may results compared with those for Urodele and Anura families have been performed and the Elinson, 2007; Venegas-Ferrín et al., 2010) of several members of et al., 2003; del Pino et al., 2007; Moya et al., 2007; Nath and Keller, 2008a, b), developmental profiles (Ninomiya et al., 2001; del Pino et al., 2007; Moya et al., 2007; Nath and Keller, 2008a, b), developmental profiles (Ninomiya et al., 2001; Hamburger, 1988). The heritage of embryological knowledge derived from urodelean experimental embryology is translated into the current amphibian model of the anuran, Xenopus laevis, which best enables analysis of the molecular nature of the meso-endoderm and neural induction processes. However, there may be some misinterpretation of basic embryological processes, because Xenopus and urodeles differ significantly in the structure of their respective DMZ, the state of commitment of the early gastrula DMZ, the mode of morphogenetic movement and the spatiotemporal interactions between the ARF and the overlying presumptive neuroectoderm (for reviews, see Nieuwkoop, 1996, 1997).

Comparative studies of gastrulation mechanisms (Shook and Keller, 2008a, b), developmental profiles (Ninomiya et al., 2001; Collazo and Keller, 2010) and gene expression patterns (Beckham et al., 2003; del Pino et al., 2007; Moya et al., 2007; Nath and Elinson, 2007; Venegas-Ferrín et al., 2010) of several members of the Urodele and Anura families have been performed and the results compared with those for Xenopus. From these it has become evident that the gastrulation mechanism and/or the timing of gene expression in the embryo differs according to the embryo's size, developmental speed and/or amount and omnipresence of yolk (for reviews, see Arendt and Nübler-Jung, 1999; Elinson and Beckham, 2002; Solnica-Krezel, 2005; Gallery, 2006). For example, in frogs with slow development, the mesoderm may become specified later than in Xenopus (del Pino et al., 2007; Moya et al., 2007). Recently, the gene regulatory network (GRN) for endoderm and mesoderm specification and differentiation, and its essential role in body plan formation, have been elucidated (e.g., Swiers et al., 2010; Rankin et al., 2011; for review, see Davidson and Erwin, 2006). These results suggest that there are generally conserved but individually diverse modes of morphogenesis even among the anuran species. However, whether or not this diversity represents essential differences in the early patterning of the embryo remains obscure. Although the final anterior-posterior and dorsoventral body patterns and maps of gene expression domains are strongly conserved in all vertebrates (Elinson and Kezmoh, 2010), it is necessary to analyze the similarities and dissimilarities of these basic processes to deepen our understanding of the fundamental principles of the early patterning of the embryos. In this review, we aim to verify the similarities and dissimilarities of the gastrulation process, and the spatially and temporally controlled mesoderm and neural induction processes, mainly from an embryological point of view, between a urodele, primarily the Japanese newt Cynops pyrrhogaster (formerly Triturus pyrrhogaster) and an anura, Xenopus laevis, both of which have extensive data and histories of investigation.

Structure, prospective fate, morphogenesis and functional gene expression domains of the urodele and anuran early gastrula DMZ

The spatial location or topological architecture of the DMZ varies among vertebrates but the essential feature is well conserved (Arendt and Nübler-Jung, 1999; Solnica-Krezel, 2005). In amphibians, the early gastrula DMZ occupies an arc-shaped region restricted to the dorso-vegetal part of the embryo. The DMZ is a mixture of multiple heterologous domains with differing prospective fates. However, the early gastrula DMZ of both Xenopus and Cynops shows considerable differences in its germ layer structure (Figs. 1 and 2 and Table 1).
Structure, prospective fate and morphogenesis of the early gastrula DMZ

*Xenopus laevis* and other anurans

At the late blastula and early gastrula stages (Fig. 1A, B), the *Xenopus* DMZ (the dorsal sector of the involuting marginal zone, Keller, 1975, 1976) is a multilayered structure in which at least two layers are recognized: a superficial epithelial layer (superficial layer) and a deep mesenchymal layer (deep layer). Although the degree of multilayering varies among anurans (Vogt, 1929; Keller, 1975, 1976; Dettlaff, 1983; Smith and Malacinski, 1983; Campos Casal and Manes, 1999), the multilayered DMZ structure is characteristic feature in the Anura family. The deep layer is divided into sub-classes of the leading edge meso-endoderm (LEM) for the future pharyngeal endoderm/prechordal plate and axial mesoderm for future notochord and somites (Keller, 1975, 1976; Smith and Slack, 1983; Shook et al., 2004; for reviews, see Gerhart, 2001; Keller and Shook, 2004; Shook and Keller, 2008a, b). The superficial layer comprises the archenteron floor. Anurans have relatively little superficial mesoderm compared with urodeles, and *Xenopus* has the least amount of superficial mesoderm (Minsuk and Keller, 1997; Keller and Shook, 2004). Fate mapping reveals that the dorsal mesoderm is derived mainly from the deep layer and partially from the superficial layer in both *Xenopus* (Shook et al., 2004) and *Rana pipiens* (Delarue et al, 1994). About 20% of presumptive notochord and about 5% of presumptive mid to posterior somites are distributed in the superficial layer in *Xenopus* (Shook et al., 2004).

*Xenopus* shows characteristic morphogenetic movements of the DMZ (Fig. 2A). At the late blastula to early gastrula stages, prior to the onset of gastrulation as defined by bottle cell formation (Fig. 1A), cells of the deep layer start to involute independently of bottle cell formation (Nieuwkoop and Florschütz, 1950; Keller, 1976) by “pre-gastrulation movements” such as the pre-gastrulation epiboly movement (Keller, 1980; Bauer et al., 1994; Papan et al., 2007) and vegetal rotation (Winklbauer and Schürfeld, 1999). At the early gastrula stage when bottle cells have been formed (Fig. 1B), the LEM has already started to involute and its anterior part has reached beneath the forebrain/midbrain part of the presumptive neuroectoderm (Poznanski and Keller, 1997), and the Cleft of Brachet is formed. Slightly later, the presumptive notochord of the deep layer also starts to involute and then bottle cells form in the most vegetal region of the superficial layer (Fig. 1B), and then the superficial layer starts to involute (Fig. 2A). During gastrulation, the archenteron is formed and the involuted superficial layer lines the ARF (see reviews, Keller and Shook, 2004). As will be discussed in detail later, these processes indicate that the onset of gastrulation in *Xenopus* has two steps: involution of the deep layer LEM by pre-gastrulation movements before bottle cell formation, and involution of the deep and superficial layers after bottle cell formation.

During gastrulation, bottle cells are located at the anterior end of the archenteron, and then spread bilaterally until finally they are located beneath the anterior region of the deep presumptive notochord at the late gastrula stage (Fig. 2A, b–d). During gastrulation, the ARF is composed mainly of endoderm and partially of presumptive mesoderm. Presumptive notochordal and pre-somitic cells originate from the superficial layer ingresses and move into the underlying deep presumptive notochord and somites that originated from the deep layer during neurulation (Shook et al., 2004), demonstrating that the dorsal mesodermal tissues of *Xenopus* have a dual origin with the same fate. In addition to these, LEM and presumptive notochord show different movements during gastrulation. Involuted LEM migrates anteriorly and spreads bilaterally. The presumptive notochord involutes and then extends anteroposteriorly by convergence and extension (CE) movements. After that, the presumptive notochord elongates posteriorly and bilaterally shears the pre-somatic mesoderm during the neurula stages (for reviews, see Keller and Shook, 2004; Shook and Keller, 2008a).

*Cytops pyrrhogaster* and other urodeles

Fig. 1C shows the DMZ structure of the *Cytops* stage 11 beginning gastrula, in which bottle cells has just formed at the blastopore site (*Cytops* stages are according to Okada and Ichikawa, 1947) and Fig. 2B shows the gastrulation process of *Cytops*.

The structure of the urodelean early gastrula shows significant differences when compared with that of *Xenopus*. Animal cap presumptive ectoderm consists of a layer of cells about 3–4 deep in the late blastula and early gastrula of *Ambystoma mexicanum* (Slack, 1984). In *Cytops*, the animal cap of the blastula to gastrula embryo is a single-cell-layered structure (Komazaki, 1992; Imoh, 1988). The *Cytops* early gastrula DMZ is defined as the area between the pigment line (bottle cells) and the limits of involution (Fig. 3A, angle of about 50° from the pigment line; Kaneda and Hama, 1979), and the *Cytops* early gastrula DMZ has a rather simple structure than that of *Xenopus* with two major differences (Table 1). One is that the *Cytops* early gastrula DMZ is a single-cell-layered structure except for the most vegetal region near the blastopore (Figs. 1C, 3A, 3B, Imoh, 1988; Suzuki et al., 1997). During gastrulation, the DMZ involutes through blastopore as a monolayered sheet (Fig. 3F), and forms the ARF (Hama et al., 1985; Imoh, 1988).

The other difference is the distribution of presumptive pharyngeal endoderm, prechordal plate and dorsal mesoderm. Urodela embryos have a larger presumptive mesoderm and presumptive pharyngeal endoderm/prechordal plate than *Xenopus*, and all these components are entirely located on the embryo’s surface (Fig. 4A). Presumptive pharyngeal endoderm/prechordal plate and notochord are located in the vegetal (future anterior) and animal (future posterior) halves of the DMZ, respectively (Figs. 1C, 3, 4). Fate mapping has been unable to define the exact location of the presumptive prechordal plate. Because it is hard to assign an exact location of the prechordal plate, or to distinguish precisely the limits between the prechordal plate and notochord on many urodelean fate maps (Vogt, 1929; Pasteels, 1942; Nakamura, 1942; Hama, 1978; see *Cytops* early gastrula fate map, Fig. 4A). Bottle cells form at the most vegetal part of the surface DMZ prior to the onset of involution (Fig. 1C). This tissue arrangement of the *Cytops* DMZ resembles that of the avian Koller’s sickle region, in which anterior definitive endoderm, presumptive notochord and presumptive neuroectoderm are planarly arranged (Arendt and Nübler-Jung, 1999).

In *Cytops*, and other urodeles (Vogt, 1929), the first sign of the onset of gastrulation is formation of bottle cells at the blastopore site. In *Xenopus*, the deep layer starts involuting by pre-gastrulation movements prior to bottle cell formation (Fig. 2A). However, in urodeles, bottle cells involute first and they are always located at the anterior end of the involuting archenteron during gastrulation. Following bottle cell involution, the presumptive pharyngeal endoderm/prechordal plate and presumptive notochord sequentially involute and form the single-cell-layered ARF in *Cytops*.
In *Xenopus*, the surface of the ARF is lined with ARF endoderm originating from the superficial layer, but in *Cynops* and other urodeles, such as *A. mexicanum* (Shook et al., 2002), the surface of the ARF is not covered with endoderm during gastrulation. The *Cynops* ARF is progressively covered with lateral endodermal crest derived from the lateral wall of the archenteron that rolls up during neurulation (Fig. 3G). Ignoring the multilayered structure of the DMZ, the spatial architecture of the late blastula/early gastrula DMZ of *Xenopus*, in which bottle cells have not yet formed, is roughly similar to that of the *Cynops* early gastrula DMZ (Fig. 1A, C). However, the structure of the *Xenopus* early gastrula DMZ, in which bottle cells have formed (Fig. 1B), is fundamentally different from that of *Cynops*, because of the pre-gastrulation morphogenesis of *Xenopus*.

It has been demonstrated that the vegetal (lower) half of the *Cynops* early gastrula DMZ (LDMZ) forms the anterior half of the ARF that self-differentiates only into endodermal tissues, such as pharyngeal endoderm (Kaneda et al., 2009). The animal (upper) half of the DMZ (UDMZ) forms the entire notochord after involution. The anterior half domain of the involuting ARF is, therefore, designated as the fore-notochordal endodermal roof (FNE). As shown later, the *Cynops* ortholog of *goosecoid* (*Cygsc*) is expressed in the LDMZ at the early gastrula stage (Figs. 4 and 5) but the expression is progressively restricted to the intermediate region between the FNE and notochord at the late gastrula to neurula stage (Figs. 5 and 6). Thus, the presumptive prechordal plate does not have a definite presumptive location in the early *Cynops* gastrula, and the LDMZ should be identified as a prechordal region by its gene expression pattern and prospective fate (Table 1, Fig. 1C), as proposed in avian embryos by Foley et al. (1997).

The LDMZ and UDMZ of *Cynops* show different morphogenetic movements during gastrulation (Fig. 2C). Like *Xenopus*, bottle cells
and presumptive pharyngeal endoderm/prechordal plate elongate anteriorly and spread bilaterally after involution. Dorsal CE movements in the axial mesoderm are relatively weak and occur later in *A. mexicanum* than in *Xenopus* (Keller and Jansa, 1992; Shook et al., 2002). *Cyprinids* presumptive notochord shows the same morphogenetic behavior; it elongates anteriorly after involution, and at the neurula to tail-bud stages it in turn starts to extend posteriorly and bilaterally shears the still involving presumptive tail somite (Hama, 1978, see Fig. 2C). These regional differences in the morphogenetic movements of the *Cyprinids* ARF may be controlled by the same morphogenetic mechanics as in *Xenopus*.

**Gene expression domains of the early gastrula DMZ**

The gene expression profiles in *Xenopus* have been extensively analyzed, but only limited observations are available for urodele. In *Xenopus*, although some gene expressions overlap in the early gastrula DMZ, *goosecoid* (*gsc*, Cho et al., 1991), *chordin* (Sasai et al., 1994), *Xlim-1* (Taira et al., 1997), *noggin* (Smith and Harland, 1992) and *Cerberus* (Bouwmeester et al., 1996) are expressed mostly in the lower half of the late blastula/early gastrula DMZ (Fig. 5A). The upper half of the DMZ expresses *Xnot* and *Xbra* (Smith et al., 1991). Based on these results, the *Xenopus* early gastrula DMZ has been divided into two domains according to the gene expressions: the lower *gsc*-expressing domain of LEM and upper *Xbra/Xnot*-expressing domain of presumptive notochord (Vodicka and Gerhart, 1995; Zoltewicz and Gerhart, 1997; Winklbauer and Schürfeld, 1999). The *gsc*-expressing domain in *Xenopus*, and also in chick and fish embryos, remains only in cells that involute early, and later reach the prechordal plate region (e.g., Cho et al., 1991; Sasai et al., 1994; Izzpisúa-Belmonte et al., 1993; De Robertis, 2009). *Xlim-1* expression follows similarly (Taira et al., 1997). At the late gastrula to neurula stage, strong *gsc* expression is observed in the prechordal plate. *Chordin* and *Xlim-1* expressions overlap with that of *gsc* and their expressions extend to the anterior notochord (Sasai et al., 1994; Taira et al., 1997). On the other hand, at the late blastula/early gastrula stage, the *Xbra*-expressing domain is located entirely on the deep marginal zone and its expression is progressively restricted to the entire notochord after involution (Smith et al., 1991, Zoltewicz and Gerhart, 1997; Conlon and Smith, 1999). These gene expression patterns in *Xenopus* are in good agreement with the self-differentiation and organizing activity of each domain of the early gastrula DMZ (for reviews, see Gerhart, 2001; Bouwmeester, 2001; De Robertis, 2009).

In *Cyprinids*, *goosecoid* (*Cygsc*), *noggin* (*Cynog*), *chordin* (*Cychd*), *Lim-1* (*CyLim-1*) and *VegT* (*CyVegT*) expressions are preferentially restricted to the LDMZ; their expression does not extend to the UDMZ or the sub-blastoporal region (Sone et al., 1997; Yokota et al., 1998; Doi et al., 2000; Kaneda et al., 2009; Motoki et al., unpublished; see Figs. 4B, 5B and Table 1). All these gene expressions appear on the surface of the LDMZ. As shown later, the *Cygsc*-expressing region coincides with the area that has secondary axis-inducing activity. *Cygsc* expression starts in the late blastula, and reaches its maximum in the early gastrula (Sone et al., 1997). *Cygsc*-expressing LDMZ completely involutes by the mid-gastrula (stage 12b/c) and then the *Cygsc* expression is progressively restricted and confined to the intermediate region between the FNE and notochord beneath the anterior tip of the neural groove in the late gastrula to mid-neurula (Figs. 5B, 6A). Although the germ layer structures are considerably different, the *Cyprinids* LDMZ is roughly homologous to the *Xenopus* prechordal plate/pharyngeal endoderm domain of the deep DMZ and in part to the endodermal portion of the superficial layer of the DMZ (Table 1, Figs. 1 and 2).

In *Xenopus*, *chordin* is expressed in the anterior part of the ARF during gastrulation, and at the neurula stages there is broad but strong expression in the whole prechordal plate region and the anterior part of the notochord (Sasai et al., 1994). Our preliminary observations show that, in the early gastrula stages, *Cychd* is expressed in the LDMZ. Unlike *Xenopus*, however, *Cychd* expression progressively expands to the notochord, and at the neurula stage the expression is restricted to the entire notochord. The expression is negligible or absent in the FNE that underlies the forebrain. At this stage, the *Cychd* expression pattern is nearly the same as that of *Cybra* (Fig. 6B, Motoki et al. unpublished). In *Cyprinids*, *Wnt-8* and *BMP-2/4* are expressed in the ventro-vegetal part of the embryo from the mid-blastula transition (MBT) and they cooperatively pattern the mesoderm (e.g., Hoppler and Moon, 1998). In the early gastrula of *A. mexicanum*, *Wnt-8* is expressed in the ventro-vegetal region of the early gastrula (Bachvavora et al., 2001). Our observations (Motoki et al. unpublished) show that *Cyprinids* *Wnt-8* (Cywnt-8) is expressed by vegetal endoderm around the vegetal pole and the expression extends to the sub-blastoporal endoderm (Fig. 4B) at the early gastrula stage.

**Fig. 2.** Gastrulation process of *Cyprinids* and *Xenopus*, and morphogenetic movement of the dorsal lip of each stage of the *Cyprinids* gastrula. (A) Gastrulation process of *Xenopus* larvae. Prior to the onset of traditionally defined gastrulation, as marked by blastopore formation, *Xenopus* undergoes pre-gastrulation movements such as the pre-gastrulation epiboly movement (red arrow in a; Keller, 1980; Bayer et al., 1994; Papan et al., 2007), and vegetal rotation (blue arrow in a; Winklbauer and Schürfeld, 1999). During pre-gastrulation morphogenesis, the germ layer structure of the DMZ is drastically changed, and leading edge meso-endoderm (*LEM, light green*) and presumptive notochord (*NT, light magenta*) start to involute independently of bottle cells (*dark green*) formation. At the early gastrula stage (b) when the bottle cells are formed, the LEM and vegetal (future anterior) part of the notochord have already started to involute, and the Cleft of Brachet (CB) is formed. Vertical contact between the LEM and future neuroectoderm has thus begun at this stage. As involution proceeds (c, d), the LEM and the deep notochord elongate anteriorly. Slightly after LEM involution, bottle cells are formed at the blastopore site (Blip, b). Following bottle cell involution, the surface layer (*dark yellow*) starts to involute to form the roof of the archenteron (c, d). Bottle cells are located beneath the anterior end region of the involuted notochord throughout gastrulation (b, c), and they spread bilaterally and forms respreading bottle cells (d). Presumptive mesoderm originating from superficial layer ingress and move into the underlying deep mesoderm layer during neurulation. (Modified from Shook et al., 2004, Keller and Shook, 2004,).; (B) Gastrulation process of *Cyprinids* pyrroghaster. At 20 C, involution of the DMZ starts about 3 h after the first appearance of bottle cells at stage 11 (b), and gastrulation is almost completely finished about 30 h from the onset of involution (detailed staging criteria for *Cyprinids* gastrulae are shown in Kaneda and Hama, 1979). It takes approximately 12 h from stage 11 (b) to the mid-gastrula (stage 12b/c) and 24 h to stage 13b/c (d). At the late blastula/early gastrula stages (a, b), the *Cyprinids* DMZ is subdivided into UDMZ (presumptive notochord, Pre-NT, light magenta) and LDMZ (presumptive pharyngeal endoderm/prechordal plate, Pre-Phe + Ptc, light green). Prior to the onset of gastrulation, bottle cells (dark green) are formed at the most vegetal region of the DMZ (b). Following bottle cell involution, the LDMZ and then UDMZ sequentially involute and form a single-cell-layered ARF (b-d). Thus, bottle cells are always located at the anterior end of the involuting archenteron during gastrulation. At the mid-gastrula stage (c), involuted LDMZ underlies the surface UDMZ. The UDMZ is induced to notochord (NT) during the early to mid-gastrula stages. Involuting notochord starts to vertically interact with surface presumptive neuroectoderm (Pre-Neu; *dark blue*) and induces neuroectoderm (*Neu*). From the late gastrula onward, the LDMZ is segregated into fore-notochordal endodermal roof (FNE) and *Cygsc*-expressing prechordal plate (Ptc, d). C Morphogenetic movement of dorsal lip (DLP) material of *Cyprinids* during gastrulation. The DLP of each gastrula stage was vital stained at the same size (0.4 x 0.4 mm) and the movement of the dye mark was traced during gastrulation (Redrawn from Hama, 1978). After involution, the LDMZ-equivalent DLP (DLP of stages 11 and 12a: green light and light yellow) elongates anteriorly and spreads bilaterally, occupying the anterior half of the ARF at the mid-gastrula to early neurula stages (a-d). It then spreads bilaterally to form the FNE (c, d and e). The UDMZ-equivalent DLP (*light magenta* and gray at stage 12b and 12c) involutes and elongates anteriorly and then anteroposteriorly by a convergent–extension movement (c, d). Finally, it occupies the posterior half of the ARF (d-e) and forms notochord. During the late gastrula to neurula stages (d-e), notochord elongates posteriorly and bilaterally shears the still involuting tail somites (e, gray and brown).
Table 1
Prospective fate, self-differentiation and organizing specificity of each domain of the dorsal marginal zone (DMZ) of Xenopus and Cynops early gastrulae.

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<th>Subdomain</th>
<th>Xenopus</th>
<th>Cynops</th>
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| **Pharyngeal endoderm** | ■ Location: Deep layer of the DMZ
Leading edge endoderm that starts involution prior to bottle cell formation |
■ Fate: Anterior part of the involuting deep layer
Pharyngeal endoderm and foregut endoderm |
■ Gene expression: *gsc, Lim-1, cerberus, chordin* |
■ Organizing activity: Induces head structure (forebrain, midbrain) |
| **Prechordal plate**   | ■ Location: Vegetal to the presumptive notochord in the deep DMZ
Leading edge meso-endoderm that starts involution prior to bottle cell formation |
■ Fate: Prechordal endomesoderm
Intermediate region between pharyngeal endoderm and notochord |
■ Gene expression: *gsc, Lim-1, cerberus, chordin, noggin* (same as pharyngeal endoderm) |
■ Organizing activity: Induces head structure (forebrain, midbrain) |
| **Notochord**          | ■ Location: Animal half in the deep DMZ |
■ Fate: Notochord |
■ Gene expression: *Chordin, noggin, Xbra, Xnot etc.* |
■ Organizing activity: Trunk–tail organizer activity
Induces trunk–tail structure |
| **Superficial epithelial layer** | ■ Location: Surface, suprablastoporal region of the DMZ, |
■ Fate: Bottle cells (most vegetal region)
ARF endoderm
Part of notochord and somite mesoderm that ingress to deep mesoderm during neurulation |
■ Gene expression: *Gsc, Lim-1, cerberus, noggin* |
■ Organizing activity: Notochord-inducing activity
Trunk–tail organizer activity |
| **Nonexistent**        | ■ Location: Nonexistent |


Cywnt-8 expression does not extend beyond the pigment line, thus Cygsc-expressing LDMZ and Cywnt-8-expressing endoderm are clearly separated by the blastopore. Cywnt-8-expressing endoderm involutes through the ventral lip and forms the floor endoderm of the archenteron and the yolk plug during gastrulation (Fig. 5B).

The most critical difference is the *brachyury* expression pattern. In *Xenopus*, ring-shaped *Xbra* expression is clearly detected before the onset of gastrulation. However, *Cybra* expression is never detected in any part of the *Cynops* beginning gastrula, but is first detectable from the mid-gastrula stage onward in the surface presumptive notochord region of the upper blastopore (Fig. 9C).

At the yolk-plug stage, *Cybra* expression is restricted to involuted and elongated notochord and surface presumptive tail mesoderm around the blastopore (*Sone et al., 1997; Doi et al., 2000; Kaneda et al., 2009*). At the neurula stage, *Cybra* expression is detected in the entire notochord (Figs. 5B, 6C). *Brachyury* expression is clearly shown to couple with dorsal mesoderm (notochord) induction in vertebrates (*Smith et al., 1991*). These results indicate critical differences in the state of commitment of the early DMZ of *Cynops* and *Xenopus*. The *Xenopus* early gastrula DMZ is already well determined according to the prospective fate at the early gastrula stage; however, dorsal mesoderm induction and regional specification of the mesoderm occurs during gastrulation in *Cynops*. 
(Kaneda et al., 2002), as indicated by the self-differentiation and inducing activities of the DMZ (Figs. 7 and 8). The situation should be the same as in \textit{A. mexicanum} in which \textit{Axbra} expression starts at the mid-gastrula stage onward (Swiers et al., 2010).

Summarizing these results, at the early gastrula stage, the \textit{Xenopus} DMZ is divided into two gene expression domains of a \textit{Xbra/Xnot}-expressing notochord and a \textit{gsc}-expressing LEM, whereas the \textit{Cynops} early gastrula has only \textit{Cygsc}-expressing notochord.
LDMZ and a Cybra-expressing domain is not detected (Table 1, Figs. 4 and 5).

The expressions of Bra and Lim-1 proteins were analyzed in several anuran families and compared with *Xenopus* (del Pino, 1996; del Pino et al., 2007; Moya et al., 2007; Venegas-Ferrin et al., 2010). Lim-1 was simultaneously detected in the prechordal plate and anterior notochord at the mid-gastrula stage of the rapidly developing embryos of *Xenopus* and *Engystomops randi*. In contrast, only the prechordal plate expressed Lim-1 in the slowly developing embryos of *Colostethus machalilla*. The notochord became Lim-1-positive after blastopore closure in *C. machalilla* and *Gastrotheca riobambae* embryos. Like *Xenopus*, a wide ring of Bra expression is detected in the deep and surface layers of the marginal zone at the blastula stages of *C. machalilla* or in the early gastrula of *G. riobambae* (del Pino, 1996). Bra distribution shows a considerably diverse pattern in relation to notochord elongation (del Pino et al., 2007). These observations indicate that the DMZ has a different schedule of gene expression even among frog families. However, the structure, fate, morphogenesis and organizing activities of each DMZ domain of these frogs are not yet clear (Venegas-Ferrin et al., 2010). It is therefore difficult, at present, to compare these results with those of urodeles. However, Bra expression in the early gastrula of these frogs indicates that there are essential differences in the mesoderm induction process and the mode of DMZ formation between anurans and urodeles.

Self-differentiation and organizing activity of the early gastrula DMZ

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organizing capacities vary among studies according to the defining criteria used, the self-differentiation and organizing activities of the early gastrula DMZ have been extensively studied in many urodeles (e.g., Spemann and Mangold, 1924; Holtfreter, 1938a; Holtfreter-Ban, 1965; Kaneda and Hama, 1979; Kaneda, 1980, 1981; Slack, 1984; Cleine and Slack, 1985; Hama et al., 1985; Delarue et al., 1992; Yamamoto and Suzuki, 1994; Imoh et al., 1998; Kaneda et al., 2002, 2009) and anuran species (e.g., Holtfreter, 1938b; Smith and Slack, 1983; Stewart and Gerhart, 1990, 1991; Shih and Keller, 1992; Domingo and Keller, 1995; Lane and Keller, 1997; Manes and Campos Casal, 1997; Fujii et al., 2002). By inserting the DLP into the blastocoel, earlier experiments revealed that the DLP of the early gastrula has secondary axis organizing activity in which the inserted DLP differentiates into notochord, somites and endoderm, while the secondary neural axis originates from the host embryo (e.g., Spemann and Mangold, 1924). In some cases, notochord and/or somites are also derived from host cells. Nevertheless, the early gastrula DLP is defined as an organizer (the Spemann–Mangold organizer), which is "a cell population capable of releasing inducers to adjacent cells and self-differentiating into dorsal mesoderm such as notochord and somites". This definition has led to the concept that the organizer has been determined to self-differentiate into notochord at the early gastrula stage. The self-differentiation of the Xenopus organizer, defined as an arc of about 60° in the early gastrula DMZ (Stewart and Gerhart, 1990, 1991), has been analyzed by grafting experiments (e.g., Smith and Slack, 1983). In these experiments, the DLMZ itself differentiates into a variety of tissues such as notochord, head mesenchyme and endoderm. However, these studies used rather large isolates of DMZ, which included almost the entire DMZ. Fujii et al. (2002) isolated the early gastrula DMZ of a stage 10 Xenopus, which is much smaller (about 0.3 × 0.3 mm) than the DMZ studied in previous works, and found that the central region (core region) of the DMZ only induced anterior structures, while it differentiated into notochord and endoderm. Furthermore, they demonstrated that the sub-blastoporal region, which has endodermal differentiation, has no inducing activity. The prospective fate of the "core region" is not exactly defined; however, these results demonstrate that the inducing activity of the Xenopus stage 10 early gastrula is restricted to the DMZ, and that the DMZ has already been specified to the regions that induce head and trunk–tail, as indicated by the gene expression patterns.

Holtfreter (1938a, b) comprehensively investigated the self-differentiation of the urodelean (Triton alpestris, T. taeniatus and A. mexicanum) and anuran (Rana esculenta and Bombinator pachypus) early gastrula DMZ. Holtfreter-Ban (1965) further analyzed the self-differentiation of the Cynops and Ambystoma tigrinum early gastrula DMZ. She demonstrated that the upper animal part of the early gastrula DMZ of both Cynops and A. tigrinum preferentially differentiates into epidermis. The lower vegetal part tends to differentiate into endodermal derivatives. Mesodermal tissues are obtained from the middle and lower portions, but the frequency of notochord and somite differentiation is unexpectedly low. In addition, neural tissues often differentiate from all parts of the DMZ.

"Self-differentiation" can mean "differnetiation of a given embryonic part according to its normal fate" or "differnetiation according to the state of determination of the embryonic part at a given stage and place" (Hamburger, 1988). The results of Holtfreter-Ban (1965) are incompatible with the definition of the "organizer". All endodermal, dorsal mesodermal and neural tissues are differentiated when the DMZ is isolated in a larger piece that includes all parts of the DMZ. A relatively large DLP was isolated and used in earlier experiments, in which dorsal mesodermal tissues should have been induced by induction within the grafted DLP. Holtfreter-Ban (1965) thus proposed that the "self-differentiation capacity of the Cynops and Ambystoma organizer is not yet determined, even at early gastrula stage, and still stays at the labile state". Her proposal was later confirmed in Cynops. The Cynops DMZ was divided into three parts and the respective self-differentiation and organizing activities analyzed (Fig. 7). The most vegetal part (presumptive pharyngeal endoderm) exclusively self-differentiates into an endodermal cell mass, but induces the trunk–tail structure. The most animal part (future trunk–tail notochord) preferentially differentiates into atypical epidermis and has almost no inducing activity. The intermediate part that is fated to the posterior pharyngeal endoderm, putative prechordal plate and anterior part of the notochord differentiates into an endodermal cell mass and/or notochord, and induces the trunk–tail axis (Fig. 7, see Hama et al., 1985; Kaneda and Hama, 1979). Much later, dividing the Cynops early gastrula DMZ into the UDMZ and LDMZ further confirmed that the LDMZ self-differentiates into an endodermal cell mass, but has potent notochord-inducing activity (Fig. 8), and that the UDMZ has epidermal self-differentiation capacity (Kaneda, 1981; Kaneda and Suzuki, 1983; Suzuki et al., 1984; Kaneda et al., 2002, 2009; Sakaguchi et al., 2002).

Grafting experiments (Yamamoto and Suzuki, 1994) confirm the secondary axis organizing activity of the LDMZ, in which grafted LDMZ differentiates into endoderm but induces a complete secondary axis, and that the secondary axis-inducing activity...
activity of Cynops DMZ is restricted to the suprablastoporal region of an area 30° from the blastopore along the animal–vegetal axis and 60° laterally from the dorsal–midline, an area identical to the gsc-expressing LDMZ. Moreover, grafting the Cynops LDMZ and Xenopus early gastrula DMZ into the ventral marginal zone, Imoh et al. (1998) compared the secondary axis organizing activity. As shown in Fig. 2E, they found that the grafted Cynops LDMZ itself forms the FNE and differentiates into pharyngeal endoderm and anterior gut endoderm, but could also organize a complete secondary axis, whereas the DMZ of Xenopus organizes a rather poor or site-restricted secondary axis. Thus, Imoh et al. (1998) proposed that the early gastrula DMZ of both Xenopus and Cynops has critical differences in its specifications; the Xenopus DMZ is committed much earlier than that of Cynops.

As summarized above, these results and the absence of Cybra expression show that, in Cynops, the mesoderm is not yet induced even at the early gastrula stage. At that stage, the early gastrula DMZ is simply divided into two domains of UDMZ, which is fated to notochord and a Cygsc-expressing LDMZ, which has notochord-inducing activity.

Mesoderm induction before and after the onset of gastrulation

Is bottle cell formation coupled with mesoderm induction?

In general, parts of the embryo that are initially located at a remote distance can interact with each other by means of gastrulation movements. During gastrulation, internalization of the marginal zone begins with blastopore formation in all chordates (Arendt and Nübler-Jung, 1999; Solnica-Krezel, 2005; Shook and Keller, 2008a). In Xenopus, bottle cells are progressively formed by cells of the lower half of the suprablastoporal endoderm layer around the entire circumference of the marginal zone, but neither bottle cells nor the blastopore forms on the ventral side in Cynops (Doi et al., 2000). Cell lineage analyses have suggested that blastopore (bottle cell)-forming activity is not always defined in a particular blastomere at the cleavage stage in either urodeles or Xenopus (Holtfreter, 1944; Nakamura and Kishiyama, 1971; Doucet-de Bruïne, 1973; Smith and Slack, 1983; Gimlich, 1985; Takasaki, 1987; Hardin and Keller, 1988; Bauer et al., 1994; Suzuki et al., 2002), indicating that the bottle cells are induced before the onset of gastrulation. It can therefore be assumed that bottle cell induction and mesoderm induction occur in a temporally synchronized manner.

Many studies have evaluated the synchronicity between bottle cell formation and mesoderm induction in the DMZ (Hardin and Keller, 1988; Black, 1989; Kurth and Hausen, 2000). In Xenopus, it appears that dorsal extension movement and axis formation are inhibited in an antimorphic gsc-injected embryo, but bottle cell formation is not (Ferreiro et al., 1998). On the other hand, Kurth and Hausen (2000) found that ectopic bottle cells are induced in Activin- or Xnr1-injected ectoderm, and they proposed that the processes of both bottle cell and mesoderm formation are closely interlinked in Xenopus. These findings indicate that simultaneous mesoderm induction and bottle cell induction in the DMZ is necessary to initiate gastrulation and normal axis formation of the embryo. These induction processes may depend on a separate set of maternal molecules, as suggested for Xenopus (Sakai, 1996, 2008; Nagano et al., 2000) and for Cynops (Doi et al. 2000).

In Cynops, ultraviolet-irradiated eggs form an abnormal blastopore and thus dorsal axis formation is arrested (Doi et al., 2000;
Suzuki et al., 2002), while the notochord-inducing activity of the LDMZ remains active (Suzuki et al., 2002). Suzuki et al. (2002) treated Cynops embryos with anti-morphogenesis reagents such as suramin (aspecific FGF-inhibitor, Gerhart et al., 1991; Grunz, 1992; Oschwald et al., 1993; Cardellini et al., 1994; Wallingford et al., 1997; Kaneda et al., 2002; Sakaguchi et al., 2002) or nocodazole (inhibitor for microtubule polymerization, Lane and Keller, 1997). Suramin injection and nocodazole treatment inhibited involution of the DMZ, but the blastopore formed normally. Cybra expression was activated in the nocodazole-treated embryos but not in the suramin-injected embryos. The notochord-inducing activity of the LDMZ in the nocodazole-treated gastrulae remained active, but the LDMZ of the suramin-injected embryos completely lost its notochord-inducing activity. These results strongly indicate that blastopore formation and dorsal mesoderm induction in the DMZ occur independently. Cynops gastrulation indispensably requires bottle cells (Suzuki et al., 2002), but in Xenopus, involution of the deep layer occurs independently to bottle cell formation (Fig. 2A). Bottle cells appear to make a significant but not the major contribution to gastrulation in Xenopus (Keller, 1976; Hardin and Keller, 1988). These observations indicate that Cynops and Xenopus bottle cells have different functions during gastrulation movements.

**Pre-gastrulation movements and the onset of gastrulation**

The movements of embryonic cells that occur before the onset of gastrulation, as defined by bottle cell formation, have been investigated in many amphibians. In Xenopus, these comprise epiboly (Keller, 1980; Bauer et al., 1994; Papan et al., 2007), uninodal progression (Züst and Dixon, 1975), vegetal rotation (Winklbauer and Schürfeld, 1999) and the movements of pre-chordal plate migration and notochord CM morphogenesis (Nieuwkoop and Florschütz, 1950; Keller, 1975, 1976). Pre-gastrulation movements mostly start from the mid-blastula stage onward. By these movements, the internal tissue arrangements are drastically changed and the deep layer involutes independently of blastopore formation in Xenopus.

In contrast, urodeles undergo these pre-gastrulation movements only moderately or not at all (Schechterman, 1934; Nieuwkoop and Florschütz, 1950; Harris, 1964; for reviews, see Nieuwkoop, 1969a, 1996). Komazaki (1992) demonstrated that the animal cap presumptive ectoderm of the Cynops blastula and gastrula undergoes epiboly movement. By epibolyic extension, the single-cell-layered animal cap is formed, but there are no structural changes of the embryo. These movements may not be so critical for DMZ configuration in the Cynops embryo, and the simple planar (animal–vegetal) juxtaposition of the DMZ is retained before and after the onset of gastrulation (Fig. 1C). Consequently, retaining its initial structure the surface DMZ starts to internalize through the blastopore as a monolayer sheet (Figs. 2B, 3F).

The significance of the “pre-gastrula epiboly movement” on the formation of the multilayered Xenopus DMZ has been described. Using cell lineage tracing (Bauer et al., 1994) or time-lapse microscopic magnetic resonance imaging (Papan et al., 2007), it has been demonstrated that the tissue movements leading to the formation of the three-dimensional multilayered DMZ begin by stage 8 or at least by stage 9 in Xenopus. Pre-gastrula epiboly drastically moves animal cap tissue into the DMZ, but not into the ventral marginal zone (Papan et al., 2007). Following this, the deep layer involutes independently of blastopore formation by a series of pre-gastrulation movements (Fig. 5). Vertical interaction between the presumptive neuroectoderm and involuting deep layer is thus established prior to formation of the blastopore. Traditionally defined gastrulation movement starts after blastopore/bottle cell formation at stage 10/10+ in Xenopus. Archenteron formation and the vertical interaction between neuroectoderm and the involuted ARF is finished by stage 10.25 to 10.5.

In Xenopus, the pre-gastrulation movements are much more extensive than commonly thought (Bauer et al., 1994; De Robertis et al., 1994; Papan et al., 2007), and substantially constitute the onset of gastrulation. Embryological evidence indicates that Xenopus gastrulation should be revised to beginning at stage 8 as a two-step process: “pre-blastoporal gastrulation”, which starts at stage 8 when the pre-gastrulation movements start, and the traditionally defined gastrulation (“traditional gastrulation”) in which marginal zone cells involute through the blastopore. During pre-blastoporal gastrulation, dorsal mesoderm induction in the DMZ should occur, because functional domains characterized by their specific gene expression profile, self-differentiation, inducing activities and capacity for dorsal CE movements are established before the onset of traditional gastrulation.

On the other hand, urodeles synchronously undergo bottle cell (blastopore) formation and traditional gastrulation. In addition to the pre-gastrulation movements, significant differences between anuran and urodelean gastrulation mechanics are also identified: no autonomous dorsal CE movements of the axial mesoderm occur in Pleurodeles (Shi et al., 1989), and the dorsal CE mechanisms in the axial mesoderm are relatively weak but occur later in urodeles (Keller and Jansa, 1992; Shook et al., 2002). The planar cell polarity (PCP) pathway occurs in the trunk mesoderm (notochord), but not in the head mesoderm. As the dorsal CE movements induced by the Wnt/PCP pathway are a specific character of notochord (mesoderm) cells (Keller and Danilich, 1988; Keller and Shook, 2004), it is evident that urodeles sequentially undergo, first, bottle cell/blastopore formation and the onset of traditional gastrulation, and second, dorsal mesoderm induction, much later than in anura.

**Meso-endoderm induction in the marginal zone**

Nieuwkoop (1969a, b) and Nakamura et al. (1970) found in A. mexicanum and Xenopus that the vegetal endodermal hemisphere induces dorsal mesoderm in the presumptive ectoderm. Thus it was proposed that the Spemann–Mangold organizer is induced in the DMZ by this “meso–endoendoderm induction”. Further investigation revealed that meso-endoderm inducing activity of the vegetal hemisphere is activated after MBT at stage 8 in Xenopus (Wylie et al., 1996) and thus meso-endoderm induction starts after MBT as proposed (Nagano et al., 2000; Vonica and Gumbiner, 2007; reviewed by Sakai, 2008). Furthermore, Nakamura et al. (1971) demonstrated that the inducing activity of the Xenopus DMZ first appears at stage 9 and that the organizing activities of the Xenopus DMZ are fixed until stage 10. As discussed earlier, pre-blastoporal gastrulation in Xenopus starts at stage 8 and the results indicate that meso–endoendoderm induction of the Xenopus DMZ occurs during pre-blastoporal gastrulation, although the notochord–inducing activity of the superficial epithelium is maintained during traditional gastrulation (Shih and Keller, 1992).

Several maternal molecules are found to be key molecules for meso–endoendoderm induction in Xenopus. Two models are currently proposed to explain this process. One is the three-signals model (Smith and Slack, 1983; Dale and Slack, 1987), in which localized distribution of maternal VegT (Zhang et al., 1998) and Wnt/β-catenin in the oocyte cortex (Wylie et al., 1996; Moon and Kimelman, 1998) activate the GRN for meso–endoendoderm induction, and finally, signals emanating from dorso–vegetal cells induce and specify the DMZ. The other proposed model in Xenopus and Bufo is that the organizer is cell-autonomously specified by synergic action of maternal cytoplasmic determinants without inducing signals from dorso–vegetal cells (Sakai, 1996; Manes and Campos Casal, 2002; Fujii et al., 2002;
Notochord induction before and after the onset of gastrulation

In Xenopus, although the size and spatial location varies according to the criteria used, the stage 10 early gastrula DMZ (Smith and Slack, 1983) or the core region of the DMZ (Fuji et al., 2002) shows notochord and endoderm differentiation. Gsc is expressed after MBT and reaches its maximum level just before the onset of traditional gastrulation. Xbra expression starts after MBT and is clearly detected at stage 9, but not at stage 8/8.5 (e.g., Smith et al., 1991; Eimon and Harland, 2002; Fukuda et al., 2010, see Fig. 5A). Thus, it is possible that in Xenopus the gsc-expressing tissues such as the deep layer LEM and superficial endoderm are induced earlier than the Xbra-expressing tissues, rather than both gsc- and Xbra-expressing tissues being induced at the same time.

In Cynops, the entire prospective notochord is not yet induced to become notochord until the mid-gastrula (Figs. 5, 7, 8). To clarify when and how notochord is induced, the spatial location of the presumptive notochord can be traced at each stage of the gastrulae and the temporal changes in self-differentiation and neural-inducing activity analyzed. It is clear that notochord/somite self-differentiation capacity and trunk–tail neural-inducing activity of the presumptive notochord is evoked from the mid-gastrula stage onward (Kaneda and Hama, 1979). The notochord- and Cybra-inducing activity of the LDMZ disappears soon after involution or culture in vitro (Kaneda, 1980, 1981; Suzuki et al., 1984; Kaneda et al., 2002; see Fig. 10). At the mid-gastrula when the involuted LDMZ underlies the surface presumptive notochord (Fig. 9B, C), it has already been induced to become notochord. Using a Keller sandwich of the early gastrula DMZ confirmed that the Cygsc-expressing LDMZ planarly induces Cybra expression in the UDMZ (Fig. 8F). Thus, it is proposed that notochord is induced by planar induction signals from the LDMZ during the early to mid-gastrula stages (Kaneda et al., 2009), which indicates that formation of the LDMZ is the direct target of meso-endoderm induction, and that the LDMZ in turn acts as the source of notochord-inducing signals.

Taking all these findings into consideration, the differences in the state of determination of the early Cynops and Xenopus gastrula DMZ and the process for notochord induction can be summarized as follows.

(1) At the onset of traditional gastrulation, dorsal mesoderm is not yet induced in any part of the Cynops embryo. Only a domain that has notochord-inducing activity exists at the suprablastoporal endodermal prechordal region (i.e., LDMZ). The activity is restricted to this region and does not extend to the sub-blastoporal endoderm region (Fig. 2B). Similar activity is distributed over a rather broad region of the suprablastoporal region in Xenopus, including superficial endoderm and deep layer LEM, all of them having notochord-inducing activity (Stewart and Gerhart, 1990, 1991; Shih and Keller, 1992).

(2) In Xenopus, the gsc-expressing deep layer LEM and the superficial endoderm will be induced and established earlier than Xbra/Xnot-expressing notochord (Fig. 5A). These gsc-expressing tissues play an essential role in inducing notochord during pre-blastoporal gastrulation. Thus, notochord induction in Xenopus is completed prior to the onset of traditional gastrulation. Cynops undergoes these processes after the onset of traditional gastrulation (Figs. 2, 5B).

(3) LDMZ planarly induces notochord in the neighboring cells during the early phase of traditional gastrulation in Cynops (Figs. 8 and 9). On grafting the LDMZ, a complete secondary axis is organized (Yamamoto and Suzuki, 1994; Imoh et al., 1998), indicating that grafted LDMZ can induce an anteroposterior regional difference in inducing activity of the secondary induced notochord (Fig. 3E). Secondary axis-forming gastrulation is thus induced by both the grafted LDMZ and induced notochord. This demonstrates that the initial action of the LDMZ is necessary and sufficient to trigger the sequential morphological network for the body plan formation (Sakaguchi et al., 2002).

(4) Although the spatial and temporal locations vary, notochord or dorsal mesoderm-inducing activity of endoderm around the blastopore may be conserved in vertebrates. Cynops, and perhaps other urodeles, evolved this as the LDMZ.

As summarized above, Xenopus undergoes notochord (dorsal mesoderm) induction prior to the onset of involution by a series of pre-gastrulation movements. During this process, planar and vertical intra-DMZ interactions facilitate establishment of the regional specification of the multilayered DMZ.

Although differences of the early gastrula DMZs are classified as above, there should be common mechanisms among them. To unify these differences, at least two problems need to be considered. One is the rearrangement of the organizing activity of the late blastula to early gastrula DMZ. In addition to the traditional concept of head and trunk–tail organizers, Xenopus embryology defines several organizers, centers and/or regions that have specific organizing activities on the blastula and gastrula embryo. For example, the blastula organizer, gastrula organizer, Nieuwkoop center, surface endodermal epithelium and deep endoderm are classified as leading tissues in Xenopus embryogenesis (for review, see Gerhart, 2001). The equivalent tissue has been investigated in other vertebrates (e.g., for reviews, see Arendt and Nübler-Jung, 1999; Stern, 2001; Solnica-Krezel, 2005); however, because these tissues are located in a restricted area of a rather small embryo and are spatially continuous, and because they change their spatial location and function with time, it is difficult to introduce unifying criteria even in the Anura family. The Cynops LDMZ equivalent tissue in Xenopus may be superficial endodermal epithelium and deep endoderm, however, re-evaluation and rearrangement of the multiple organizers and centers in
Xenopus is required, especially around stage 8 at the onset of preblastoporal gastrulation. The other problem is the criterion of staging the embryo. Traditionally, the stage at which the visible blastopore or pigment line has just formed is defined as the first stage of the gastrula. Xenopus stage 10/10 and Cynops stage 11 are thus defined as the first stage of the gastrula (Fig. 1). Although the final body pattern is strongly conserved, and although many of the same developmental genes are expressed, there are essential differences between Xenopus and Cynops in self-differentiation, gene expression patterns and organizing activity of each part of the embryo. It is worthwhile identifying the equivalent stages between different species using a combination of the gene expression profiles in the area of definite prospective fate and the morphological characters. Using gsc and Bra expressions as a marker, De Robertis et al. (1994) have proposed that the stages of maximal gsc expression are equivalent in many organisms. Furthermore, the tissue with maximal gsc expression can be identified as homologous, such as the DMZ in Xenopus and the early Hensen’s node in chicks. In addition to these definitions, it may be most important to define the criteria for the substantive onset of gastrulation.

According to these definitions, it is reasonable to conclude that Xenopus stage 10/10 is equivalent to the Cynops mid-gastrula (stage 12b/c), the stage at which notochord has been induced and neural induction between the induced notochord and future neuroectoderm begins (Kaneda et al., 2009). As discussed again later, Xenopus traditional gastrulation should be specified as a process to realize neural patterning (“neural induction phase”) by inductive interactions between the already patterned ARF and the overlaying ectoderm, as suggested by Koide et al. (2002). Of course, other important events occur in this period, such as internalization of all the endoderm leading to archenteron formation, rearrangement of much of the mesoderm, and in Xenopus perhaps further induction of the somatic mesoderm by BMP antagonists such as Noggin and Chordin occurs. On the other hand, the Cynops stage 11 beginning gastrula is equivalent to stage 8/9 of Xenopus, because pre-blastoporal gastrulation starts at this stage. Sequential events for notochord (dorsal mesoderm) induction and patterning of the induced notochord that occur during the early to mid-gastrula stages (“notochord induction phase”) in Cynops should occur at stage 8 to 10 in Xenopus. It is thus necessary to rearrange the criteria for the real onset of
gastrulation to elucidate and verify the similarities and dissimilarities of the basic morphogenetic process.

**What is the true nature of the Spemann–Mangold organizer?**

Dividing the urodelean neurula ARF into several parts, the secondary axis-inducing activity (Mangold, 1933) or neural-inducing activity (Sala, 1955; Johnen, 1956a, 1956b) of each part of the neurula ARF was analyzed. The results show that most anterior quarter (anterior endoderm and prechordal plate) has little inducing activity, but the head and trunk–tail-inducing activity is broadly distributed from the second quarter (anterior notochord) to the posterior end of the ARF. These results support the existence of separate head and trunk–tail organizers, as first proposed by Spemann (1931). However, these experiments were done at the neurula stage, in which almost all of the basic morphogenesis has been completed. More recently, the role of the Spemann–Mangold organizer in the anteroposterior patterning of the trunk–tail in Xenopus has been analyzed (Jansen et al., 2007; Durston et al., 2010) and it has been proposed that interaction between the Spemann–Mangold organizer and non-organizer mesoderm during gastrulation establishes an anteroposterior axis in Xenopus. However, it remains unclear when and how the final regional difference in the inducing activities of the ARF is established, when neural induction is initiated, and the cellular source of signals for anteroposterior CNS patterning.

A crucial embryological observation was that the inducing activity of the Cynops DMZ changes on invagination (Okada and Takaya, 1942a). The Cynops early gastrula DMZ originally induces trunk–tail structures, whereas the same tissue, if isolated after involution, induces the head. This change in the organizing activity of the early gastrula DMZ has been confirmed and it has been revealed that the change is mimicked when the DMZ is isolated and cultured in vitro for a period of time (Okada and Takaya, 1942b; Okada and Hama, 1943, 1944; Suzuki et al., 1984; for review, see Takaya, 1978; Slack and Tannahill, 1992; Okada, 1994). Importantly, a similar experiment was performed using *Rana nigromaculata* and its early gastrula DMZ induced the head (Okada and Takaya, 1942a), which demonstrated that the inducing activity of the early gastrula DMZ is determined considerably earlier in *Rana* than in *Cynops*. These phenomena were fully confirmed later in *A. mexicanum* (Hoessels, 1957; for review, see Nieuwkoop, 1997). The change in the inducing activity of the early gastrula DMZ has been further confirmed in *Cynops* (Suzuki et al., 1984; Yokota et al., 1998; Kaneda et al., 2002). Furthermore, Ariizumi and Asashima (1994, 1995) and Ninomiya et al. (1998, 1999) treated *Cynops* and *Xenopus* animal cap ectoderm with a high dose of activin A and then cultured it *in vitro* for a period of time. There was a change in its neural-inducing activity from trunk–tail to head. Although the change in the inducing activity of the *Xenopus* DMZ has not been investigated, these results indicate that *Xenopus* DMZ shows the same change in its organizing activity *in vivo*.

The LDMZ of the early *Cynops* gastrula is confirmed to have notochord- and *Cybra*-inducing activities (Fig. 8). The notochord- or *Cybra*-inducing activity of the LDMZ is lost during normal involution, cultivation *in vitro* or with suramin treatment, but transiently retains weak but definite head-organizing activity (Suzuki et al., 1984; Fig. 10A). It is confirmed that Cysgc, Cychd, Cynog and Cylim-1 expressions of the LDMZ are retained during cultivation *in vitro* (Yokota et al., 1998; Kaneda et al., 2002). These results show that the LDMZ induces notochord first, and the induced notochord in turn organizes the trunk–tail structure (Fig. 10B). Thus, the uninvoluted LDMZ apparently shows trunk–tail organizing activity. On the other hand, after involution or culture *in vitro*, the notochord- and *Cybra*-inducing activities of the LDMZ disappear, and it changes its organizing activity. When the late gastrula DLP of *Cynops*, which has been committed to notochord, is isolated and cultured *in vitro*, a change in inducing activity is scarcely observed (unpublished). These observations suggest that the change in organizing activity of the LDMZ is
explained by the loss of notochord- and Cybra-inducing activity (Fig. 10B). Thus, the change in the organizing activity of the organizer is a specific feature of the LDMZ. Furthermore, the true form of the Spemann–Mangold organizer is indicated to be suprablastoporal endoderm that has notochord-inducing activity, at least in Cynops, and perhaps also in other urodeles.

The other questions are when neural induction is initiated, and the cellular source of signals for anteroposterior CNS patterning. Although the stage criteria and positioning of the ARF differ among studies, the anteroposterior regional inducing activities of the involuting and extending ARF have been investigated in Cynops (Okada and Hama, 1945; Suzuki et al., 1975; Kaneda et al., 2009), and in A. mexicanum and P. waltlhi (Eyal-Giladi, 1954). Dividing the Cynops gastrula ARF into several parts, the anterior one-third (Suzuki et al., 1975) or anterior half (Okada and Hama, 1945; Kaneda et al., 2009) of the mid- to late gastrula ARF is shown to have no neural-inducing activity. These results demonstrate that the neural-inducing activity of the involuting and extending ARF of the mid- to late gastrula of Cynops is restricted to the involuting presumptive notochord (posterior half of the ARF), which has been induced to become notochord by planar signals from the LDMZ. Vertical contact between the induced notochord and the overlying presumptive neuroectoderm occurs from the mid-gastrula stage onward (Fig. 9B), indicating that vertical neural induction starts at the mid-gastrula stage in Cynops. On the other hand, Poznanski and Keller (1997) demonstrated that vertical neural induction could begin within the first half hour of traditional gastrulation (between stages 10 and 10+) in Xenopus.

In general, the prechordal plate is thought to be the source of head-organizing signals in amphibians (e.g., Niehrs, 2004). However, it has been shown that the preshordal region does not have neural-inducing activity in the avian embryo (Foley et al., 1997). In Cynops, the Cycg-expressing prechordal region forms the FNE after involution (Figs. 2, 5, 8) but the FNE has no inducing activities. These results suggest that not the prechordal plate (region) but the notochord plays an essential role in neural induction. These observations in Cynops, and also in other urodeles, show that traditional gastrulation has two phases: the early phase of notochord induction in the early to mid-gastrula stage and the later phase of neural induction at the mid- to late gastrula stage (Kaneda et al., 2009). This suggests that neural induction in Cynops is initiated at the mid-gastrula stage, and the final CNS pattern is determined from the neurula stage onward as indicated in Xenopus (Saha and Grainger, 1992). On the other hand, Xenopus undergoes the notochord induction phase during pre-blastoporal gastrulation and the neural induction phase during traditional gastrulation.

In addition, the developing notochord induces neural tissue of a somewhat broader regional character than would be expected from its final position in the embryo in Cynops. This indistinct regional difference in the neural-inducing activity of the presumptive notochord indicates that forebrain-inducing activity is not restricted to a specific region, but is distributed over a broad region of the entire presumptive notochord during the gastrula stages (Kaneda et al., 2009). Therefore, the traditional concept of fixed tissues of the head and trunk–tail organizers needs to be re-evaluated, especially for the head organizer. Changing their position and function with time, and interacting with each other, the inducing activities of the ARF are progressively established during gastrulation.

Conclusion

Amphibian embryology has developed at least three general concepts of body plan formation. One is the significance of spatially and temporally controlled sequential inductive interactions. Uneven distribution of maternal molecules in the fertilized egg activates the GRN for dorsoventral and animal–vegetal regional specification in the embryo. The early gastrula DMZ is thus specified and in turn triggers the subsequent process that forms the anteroposterior CNS pattern. The second is the significance of morphogenetic movements. Cells that are committed to differentiate into their own fate acquire the capacity for cell-autonomous movements, such as CE movements. By these acquired capacities, cells initially located remotely can interact with each other by topological changes in the embryonic germ layers. The third is the synergy of sequential inductions and cell movements. Synchronicity of the morphogenetic movements and the spatiotemporally controlled induction process are necessary to establish the correct body pattern. Experiments analyzing these processes have been extensively performed using many amphibian species as models, and earlier works paid special attention to the third concept. Compiling the results from individual divergent species, the unifying features of the organizer and the processes for early body pattern formation have been theorized. Undoubtedly, Xenopus has advantages for analyzing the cellular and molecular nature of morphogenetic movements, and mesendoderm and neural induction processes. However, Xenopus shows a rather extreme mode of internal and external gastrulation, germ layer structure, origin of internal mesoderm and the timing of dorsal mesoderm induction. It is therefore difficult but useful to compare the experimental results from Xenopus with those from other animals such as urodeles, and it is also important to note that comparisons among amphibians provide insights for extending these results to the other vertebrates.

The current understanding of Xenopus development provides a comparative background for the analysis of amphibian development. Recent works on the comparative gene expression patterns provide evidence that the molecular mechanisms underlining early embryogenesis are conserved to a large extent. However, it has also become evident that timing and/or spatial gene expression patterns and the onset of morphogenetic processes are unexpectedly diverse among individual species. Despite the growing evidence, it is not yet clear whether or not this diversity represents essential embryological differences. This situation may arise largely because of the difficulties in identifying and comparing the equivalent stage or homologous tissues in each organism that has distinct germ layer construction, timing of mesoderm and neural induction, and initial signs of the onset of gastrulation. Combining the morphological characteristics and the gene expression patterns, we propose that amphibian gastrulation is divided into two phases: a notochord induction phase and a neural induction phase. Urodeles sequentially undergo these phases after the onset of traditional gastrulation, whereas Xenopus undergoes the notochord induction phase during pre-blastoporal gastrulation. In Xenopus, traditional gastrulation is specifically a process to realize neural induction as well as to internalize endoderm, rearrange mesoderm and reorganize the germ layer. Thus, the molecular and cellular characteristics of the Xenopus early gastrula would be equivalent to the mid-gastrula of urodeles. On the other hand, the event that occurs during the early to mid-gastrula stage of urodeles represents the events that occur during the pre-gastrula stage of Xenopus (Fig. 5). Furthermore, comparison of the structure, self-differentiation, fate and function of each domain of the early gastrula DMZ indicates that the true form of the Spemann–Mangold organizer is suprablastoporal gsc-expressing endoderm that has notochord-inducing activity. There is clearly still much to learn from the embryo.
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