



The gastrocoel roof plate in embryos of different frogs

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ABSTRACT

The morphology of the gastrocoel roof plate and the presence of cilia in this structure were examined in embryos of four species of frogs. Embryos of *Ceratophrys stolzmanni* (Ceratophryidae) and *Engystomops randi* (Leiuperidae) develop rapidly, provide comparison for the analysis of gastrocoel roof plate development in the slow-developing embryos of *Epipedobates machalilla* (Dendrobatidae) and *Gastrotheca riobambae* (Hemiphractidae). Embryos of the analyzed frogs develop from eggs of different sizes, and display different reproductive and developmental strategies. In particular, dorsal convergence and extension and archenteron elongation begin during gastrulation in embryos of rapidly developing frogs, as in *Xenopus laevis*. In contrast, cells that involute during gastrulation are stored in the large circumblastoporal collar that develops around the closed blastopore in embryos of slow-developing frogs. Dorsal convergence and extension only start after blastopore closure in slow-developing frog embryos. However, in the neurulae, a gastrocoel roof plate develops, despite the accumulation of superficial mesodermal cells in the circumblastoporal collar. Embryos of all four species develop a ciliated gastrocoel roof plate at the beginning of neurulation. Accordingly, fluid-flow across the gastrocoel roof plate is likely the mechanism of left-right asymmetry patterning in these frogs, as in *X. laevis* and other vertebrates. A ciliated gastrocoel roof plate, with a likely origin as superficial mesoderm, is conserved in frogs belonging to four different families and with different modes of gastrulation.

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0. Introduction

The surface presumptive mesoderm involutes around the dorsal blastopore lip during gastrulation in *Xenopus laevis*, *Xenopus tropicalis*, other frogs, and urodeles (Delarue et al., 1994; Purcell and Keller, 1993; Shook et al., 2002). The involuted surface presumptive mesoderm transiently coats the posterior gastrocoel roof of the neurula and forms the gastrocoel roof plate (grp). The grp consists of an epithelium with monociliated cells of the superficial presumptive notochord, presumptive hypochord, and superficial presumptive somitic mesoderm (Shook et al., 2004). The grp is flanked on both sides by large endodermal cells of the lateral endodermal crests (lec) (Shook et al., 2004). By the end of neurulation, the grp is internalized into the notochord and somites by bottle cell ingression in *X. laevis* and relamination in *X. tropicalis* (Shook et al., 2004). The lec cover the somites and notochord, whereas ingression of hypochordal cells occurs after neurulation (Shook et al., 2004). The grp triggers the left-right asymmetry of *X. laevis* embryos by fluid-flow toward the left side, generated by the movement of the grp cilia (Blum et al., 2007, 2009a, b; Schweickert et al., 2007, 2010;

Vick et al., 2009). The term gastrocoel refers to the gut cavity that contains presumptive mesoderm in its roof. The archenteron is the gut cavity completely lined by endoderm (Shook et al., 2004).

Given the diversity of gastrulation modes that occurs in frogs (del Pino et al., 2007), it was of interest to determine whether a typical ciliated grp develops in the early neurula of frogs with deviant modes of gastrulation. Analysis of the grp was done in the following frog species: *Ceratophrys stolzmanni* (Ceratophryidae), the túngara frog *Engystomops randi* (Leiuperidae), *Epipedobates machalilla* (Dendrobatidae, previously known as *Colostethus machalilla*), and the marsupial frog *Gastrotheca riobambae* (Hemiphractidae). We choose *C. stolzmanni* for this comparison because the surface presumptive mesoderm is large in the related species, *Ceratophrys ornata* (Purcell and Keller, 1993). The analyzed frogs differ in egg size, developmental rate, and onset of dorsal convergence and extension (dce) (Table 1) (del Pino et al., 2007).

Embryos of *C. stolzmanni* and *E. randi* share with *X. laevis* a rapid developmental rate (Table 1). Eggs of *C. stolzmanni* have aquatic development, whereas the eggs of *E. randi* are deposited in foam nests that float in the water (Romero-Carvajal et al., 2009). The white embryos of *E. randi* resemble *X. laevis* albino embryos due to their small size and white appearance. In contrast, eggs of *C. stolzmanni* have a darkly pigmented animal hemisphere. Onset of dce starts in the midgastrula of *E. randi* embryos, as in *X. laevis*, and the cells that involute move away from the blastopore lip during gastrulation (Moya et al., 2007;

Abbreviations: cbc, Circumblastoporal collar; dce, Dorsal convergence and extension; grp, Gastrocoel roof plate; lec, lateral endodermal crests.

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Table 1
Developmental characteristics of different frogs.

Species	Reproductive mode	Egg diameter (mm)	Developmental time ^a	Stage at onset of dce ^b
Rapid Development				
<i>Xenopus laevis</i>	Aquatic	1.2	14 h	11
<i>Ceratophrys stolzmanni</i>	Aquatic	2.2	10 h	?
<i>Engystomops randi</i>	Foam nest	1.1	24 h	11
Slow Development				
<i>Epipedobates machalilla</i>	Terrestrial nest	1.6	4 day	13
<i>Gastrotheca riobambae</i>	Maternal pouch	3.0	14 day	13

^a Time from fertilization to the end of gastrulation, reviewed in del Pino et al. (2007).

^b Detected by the elongation of the notochord, reviewed in del Pino et al. (2007).

del Pino et al., 2007; Romero-Carvajal et al., 2009; Venegas-Ferrín et al., 2010) (Table 1). Similarly, dce may begin during gastrula stages in *C. stolzmanni*, as the gastrocoel elongated and the notochord became visible during gastrula stages.

Embryos of *E. machalilla*, and *G. riobambae* develop slowly in comparison with *X. laevis* (Table 1) (del Pino et al., 2004, 2007). Early embryos of *E. machalilla* develop in terrestrial nests, whereas the eggs of the marsupial frog, *G. riobambae*, develop in a pouch on the back of the mother (Table 1) (del Pino et al., 2004). In the gastrula of these frogs, cells that involute during gastrulation remain in the blastopore lip, and contribute to form a large cbc at the time of blastopore closure. Elongation of the notochord only starts after blastopore closure in embryos of these frogs, indicating that dce is dissociated from gastrulation (Table 1) (del Pino, 1996; Benítez and del Pino, 2002; Moya et al., 2007; del Pino et al., 2007; Venegas Ferrín et al., 2010). Embryos of *G. riobambae* display the most divergent mode of frog gastrulation with the formation of a large cbc and an embryonic disk (del Pino and Elinson, 1983; del Pino, 1989; del Pino et al., 2007; Moya et al., 2007).

The presence of presumptive mesoderm on the surface of the gastrocoel roof is a common developmental feature in vertebrates (Shook et al., 2004; Blum et al., 2009a,b). Homologous structures with the *X. laevis* grp are the posterior notochord of the mouse embryo, also known as the node, and the Kupffer's vesicle of the zebrafish tailbud embryo (Shook et al., 2004; Blum et al., 2007, 2009b). All of these transient structures consist of epithelia with mono-ciliated cells that coat the posterior gastrocoel roof and display planar cell polarity (Blum et al., 2009b; Antic et al., 2010). Each epithelial cell bears a single cilium located in the posterior apical region of the cell. The combined clockwise beating of these cilia results in fluid-flow towards the left side. Fluid-flow is the most common mechanism for the onset of left-right asymmetry in vertebrates (Blum et al., 2009b; Schweickert et al., 2010). The exception is the chick that lacks both surface presumptive mesoderm and mono-ciliated presumptive mesodermal cells in the epithelium that coats its archenteron roof (Blum et al., 2009b; Shook et al., 2004; Schlueter and Brand, 2007).

The different modes of frog gastrulation allowed us to inquire whether formation of the grp and its origin as superficial mesoderm are conserved features of development in the analyzed frogs. We analyze whether a grp develops in frog embryos that develop slowly and store presumptive surface notochord in a cbc in comparison with rapidly developing frogs that start notochord elongation during gastrulation.

1. Materials and methods

1.1. Frogs embryos and staging

Embryos of *C. stolzmanni* and *E. randi* were donated by the Herpetology Laboratory of the Pontificia Universidad Católica del

Ecuador and their frog breeding program “Balsa de los sapos”. Embryos of *G. riobambae* and *E. machalilla* were obtained from frog colonies maintained in captivity. Collection sites and the methods for frog maintenance were previously described (Elinson et al., 1990; del Pino et al., 2004; Moya et al., 2007). Collection of frogs was done with authorization, 016-IC-FAU-DNBAP-MA, from the Ministry of the Environment, Ecuador.

Embryos of all frogs were staged according to the *X. laevis* normal table of stages until stage 14 (Nieuwkoop and Faber, 1994). After stage 14, the morphology of embryos differs from *X. laevis*. Neurulae of *E. machalilla* and *E. randi* were staged according to the tables of stages of these frogs (del Pino et al., 2004; Romero-Carvajal et al., 2009). The stages of *C. stolzmanni* were determined according to *C. ornata* (Purcell and Keller, 1993), and neurulae of *G. riobambae* were staged according to a general staging table for anurans (Gosner, 1960).

1.2. Processing of embryos and whole mount immunohistochemistry

Embryos were fixed in Smith's fixative, as previously described (Moya et al., 2007). The dorsal side of embryos at stages 12.5 to 16 was excised for the morphological analysis of the grp. The remaining portions of the embryos were discarded. Sections of 50–100 µm of neurulae were produced with a Vibratome 100 (Technical Products International, Inc. St Louis, MO, USA).

For the immunohistochemistry of cilia, embryos were fixed in Memfa buffer (Harland, 1991) for 3 h. Embryos were incubated with a 1:700 dilution of a mouse anti-acetylated-tubulin monoclonal antibody (Sigma, St. Louis, MO, USA). The secondary antibody was Cy3-conjugated rabbit anti-mouse IgG (Sigma, St. Louis, MO, USA) diluted 1:250, according to Vick et al., (2009). Embryos were analyzed and photographed with a Stemi SV6 and an AxioObserver.Z1 (Carl Zeiss, Oberkochen, Germany). Each microscope was attached to an AxioCam camera running the image acquisition program Axiovision 4.6.3 (Carl Zeiss, Oberkochen, Germany).

2. Results and discussion

2.1. Development of the grp in frogs with dissimilar modes of gastrulation

We investigated whether a ciliated grp develops in the neurula of the slow-developing frogs *E. machalilla* and *G. riobambae* despite the dissociation of gastrulation from dce that occurs in the embryos of these frogs. Analysis of the grp in the rapidly developing frogs, *C. stolzmanni* and *E. randi*, provided a comparative baseline. The dissimilar morphology of the gastrula in the analyzed frogs is diagrammed in Fig. 1A, B. We excised the gastrocoel roof, and photographed the likely grp region of

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