

Comparison of induction during development between *Xenopus tropicalis* and *Xenopus laevis*

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ABSTRACT Several *in vitro* systems exist for the induction of animal caps using growth factors such as activin. In this paper, we compared the competence of activin-treated animal cap cells dissected from the late blastulae of *Xenopus tropicalis* and *Xenopus laevis*. The resultant tissue explants from both species differentiated into mesodermal and endodermal tissues in a dose-dependent manner. In addition, RT-PCR analysis revealed that organizer and mesoderm markers were expressed in a similar temporal and dose-dependent manner in tissues from both organisms. These results indicate that animal cap cells from *Xenopus tropicalis* have the same competence in response to activin as those from *Xenopus laevis*.

KEY WORDS: *activin, chd, cer, Xbra, tissue differentiation, Xenopus laevis, Xenopus tropicalis*

Introduction

Xenopus laevis has been widely used as an amphibian model in studies of early embryonic development (Ariizumi *et al.*, 2000, Sive *et al.*, 1998). The presumptive ectodermal region of the late blastulae of *Xenopus laevis* is composed of undifferentiated cells, known as the animal cap. These cells show competency in response to certain growth factors to differentiate into several different cell lineages. Many *in vitro* inductive systems using animal cap cells have been developed and various cell and tissue types have been induced using animal caps from *Xenopus laevis*, including heart, pronephros, pancreas, cartilage and eye (Ariizumi *et al.*, 2003, Asashima *et al.*, 2000a, Chan *et al.*, 1999, Furue *et al.*, 2002, Moriya *et al.*, 2000, Myoishi *et al.*, 2004, Osafune *et al.*, 2002, Sedohara *et al.*, 2003). In addition, animal cap assays have been used to investigate mechanisms of cell differentiation by treating the cells with various growth factors and to analyze gene function by injecting with various mRNAs.

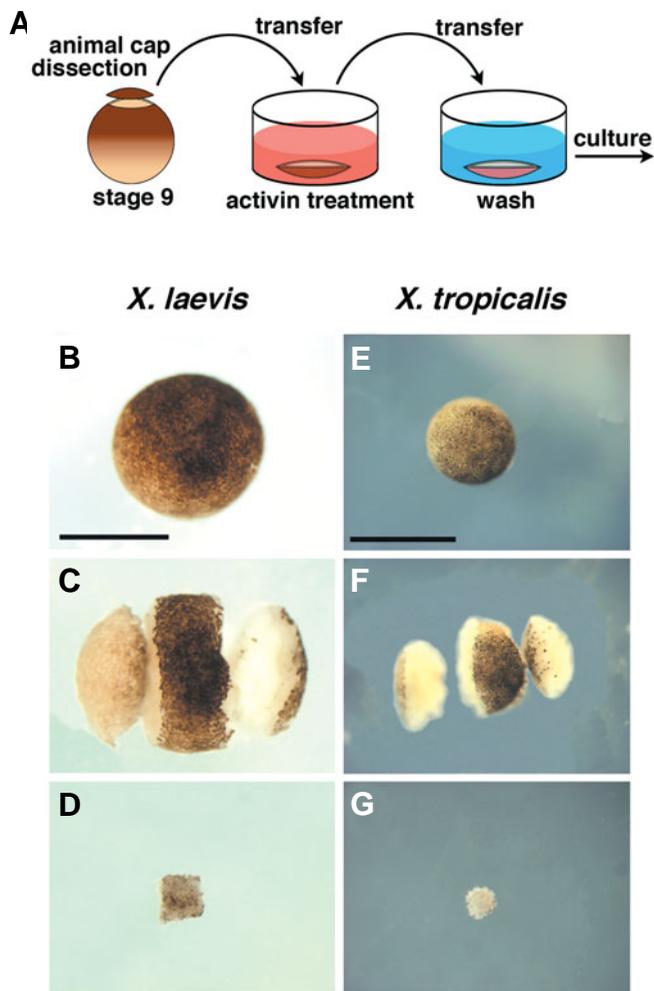
Activin A is a member of the transforming growth factor β (TGF- β) superfamily (Asashima *et al.*, 1990, Smith *et al.*, 1990) and is a strong inducer of mesoderm in animal cap cells. Activin is expressed at the protein level in developing *Xenopus laevis* embryos (Ariizumi *et al.*, 1991, Fukui *et al.*, 1993, Fukui *et al.*, 1994, Smith *et al.*, 1991). Activin can interact with specific receptors to activate the expression of genes, including *Xbrachyury* (*Xbra*), *chordin* (*chd*) and *cerberus* (*cer*) through the smad2/4 intracellular signaling pathway (Attisano and Wrana, 1998, Bourillot

et al., 2002, Bouwmeester *et al.*, 1996, Hill, 2001, Miyazono *et al.*, 2000, Sasai *et al.*, 1994, Smith, 1993, Smith *et al.*, 1991). Treatment with activin induces animal cap cells to differentiate into specific tissues via the expression of tissue-specific genes in a tightly controlled temporal and dose-dependent manner (Ariizumi *et al.*, 1991, Green *et al.*, 1992, Gurdon *et al.*, 1999, Smith, 1993, Smith *et al.*, 1991).

There have been many developmental studies of morphogen gradients and axes patterning (Bourillot *et al.*, 2002, Dubrulle *et al.*, 2001, Dyson and Gurdon, 1998, Fujii *et al.*, 2002, Gamse and Sive, 2000, Gamse and Sive, 2001, Green *et al.*, 1997, Green *et al.*, 1992, Gurdon *et al.*, 1996, Gurdon *et al.*, 1999, Ninomiya *et al.*, 2004, Tabata and Takei, 2004). Morphogen gradients were thought to regulate the positioning of cells in a developing embryo, as well as controlling gene expression and tissue-specific differentiation. Traditionally, activin A (human recombinant activin A) treatment of animal caps from amphibian embryos such as *Xenopus laevis* were used in this research (Ariizumi and Asashima, 1995, Ariizumi *et al.*, 1991, Asashima *et al.*, 2000b, Green *et al.*, 1997, Green *et al.*, 1992, Gurdon *et al.*, 1996, Gurdon *et al.*, 1999, McDowell *et al.*, 1997, Ryan *et al.*, 2000). At low concentrations of activin, ventral mesoderm tissues such as coelomic epidermis and blood cells were induced in the animal cap explants. At moderate doses of activin, the animal cap cells differentiated into

Abbreviations used in this paper: RT-PCR, reverse transcriptase polymerase chain reaction; SS, Steinberg's solution; WE, whole embryo.

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dorsal mesoderm such as muscle. At high doses of activin, axial mesoderm tissues, such as notochord and endodermal cells were induced in the animal cap explants (Ariizumi *et al.*, 1991). In addition, the animal cap cells treated with a high dose of activin could be induced to form head structures in conjugation and transplantation experiments, indicating that the animal cap cells treated with high doses of activin act as the head organizer (Ariizumi and Asashima, 1995; Ninomiya *et al.*, 1999; Sedohara *et al.*, 2002).

Xenopus laevis is an allotetraploid-derived species. For this reason, it has been hard to investigate the function of genes in

Fig. 1. Schematic diagrams of animal cap assay using *Xenopus tropicalis* and *Xenopus laevis*. (A) Schematic diagrams of animal cap assay. Animal caps were dissected from late blastulae (stage 9). The animal cap explants were treated with various doses of activin for 1 hour at 20°C. The activin-treated animal caps were washed three times and cultured in Steinberg solution (SS) containing 0.1% BSA. (B-G) Method of animal cap dissection from late blastulae of *Xenopus laevis* (B-D) and *Xenopus tropicalis* (E-F). (B,E) External views of late blastulae embryo of *Xenopus laevis* (B) and *Xenopus tropicalis* (E). (C,F) Removal of bilateral side of embryo. (D,G) Animal cap cells were dissected from late blastulae. The animal cap explant contained 445 ± 14.0 (*Xenopus laevis*) or 448 ± 18.5 (*Xenopus tropicalis*) cells. ac; animal cap. Scale bar in B and E indicates 1 mm.

early development using a conventional genetic approach, such as gene-knockout experiments. *Xenopus tropicalis*, a related species of *Xenopus laevis* is now also used as a model species in developmental biology studies (Amaya *et al.*, 1998; Song *et al.*, 2003), because *Xenopus tropicalis* is the diploid species and therefore more amenable to genetic studies (Smith *et al.*, 1991). The genome of *Xenopus tropicalis* was sequenced recently to construct a genetic linkage map (Kochan *et al.*, 2003). Previous studies have compared the developmental processes in *Xenopus laevis* and *Xenopus tropicalis* (Khokha *et al.*, 2002; Sargent and Mohun, 2005; Shook *et al.*, 2004). Fortunately, the same methods established to investigate early development in *Xenopus laevis*, including mRNA injection, whole-mount *in situ* hybridization and transplantation can also be used in the study of *Xenopus tropicalis* development (Khokha *et al.*, 2002; Offield *et al.*, 2000). In addition, many of the genes involved in early development in *Xenopus laevis* are expressed in the same regions and have the same functions in *Xenopus tropicalis* (D'Souza *et al.*, 2003; Fisher *et al.*, 2003; Fletcher *et al.*, 2004; Haramoto *et al.*, 2004; Knochel *et al.*, 2001; Sekizaki *et al.*, 2004). However, there is little known about the competency of animal caps from *Xenopus tropicalis* in response to activin treatment. In this study, we compared competence in activin-treated animal cap cells dissected from late blastulae of *Xenopus tropicalis* and *Xenopus laevis*.

Results

Animal cap assay using late blastulae of *Xenopus tropicalis*

Schematic diagrams illustrating the animal cap assays performed in this study are shown in Figure 1A. Animal caps were dissected from late blastulae (stage 9). These animal cap ex-

TABLE 1

PCR PRIMER PAIRS

<i>Xenopus laevis</i>				<i>Xenopus tropicalis</i>			
PCR primer pair	Reference	Sequence	Annealing temp. (°C)	PCR primer pair	Reference	Sequence	Annealing temp. (°C)
Xbra	Sun <i>et al.</i> , 1999	F: ttctgaagggtgagcatgtcg R: gtttgactttgctaaaagagacagg	55	Xbra	New	F: ccagatgatgcgtcacagcac R: gtcagaccactcacacctg	55
cerberus	Heasman <i>et al.</i> , 2000	F: gcttgcaaaaccttgcctt R: ctgatggaacagagatcttg	60	cerberus	New	F: tggcgcaagatgttctgga R: atgagcttcacatgcacatt	55
chordin	XMMR	F: aactgccaggactggatggt R: ggcaggatttagagttgcttc	55	chordin	New	F: gctcagcaggtcacgcatgg R: gttaggtatgtgacctgttc	55
ODC	Heasman <i>et al.</i> , 2000	F: gccattgtgaagactctctccattc R: ttccgggtgactcttgccac	55	ODC	Haramoto <i>et al.</i> , 2004	F: gcacatgtcaagccagttct R: tgcgctcagttctggtactt	55

plants were treated with various doses of activin (0-100 ng/ml) for 1 hour at 20°C. The activin-treated animal caps were washed two times to remove activin completely. For analysis of gene expression, the resultant explants were sampled immediately until sibling embryos reach stage 10.5 (early gastrulae) or cultured for 5 hours until sibling embryos reach stage 12 (late gastrulae) after activin treatment at 20°C. We did activin treatment and culturing animal caps for PCR analysis at same temperature in *Xenopus laevis* and *Xenopus tropicalis* (20°C), because temperature is very critical for signaling pathway. The gastrulation proceeds almost equally between *Xenopus tropicalis* and *Xenopus laevis* at 20°C (Khokha *et al.*, 2002). For histological analysis, the resultant explants were cultured for 2 days at 25°C (*Xenopus tropicalis*) or 3 days at 20°C (*Xenopus laevis*) until sibling embryos reached stage 40 (Fig. 1A). The method of animal cap dissection is shown in Figure 1B-D (*Xenopus laevis*) and Figure 1E-G (*Xenopus tropicalis*). The diameter of late blastula of *Xenopus laevis* is 1.3-1.4 mm (Fig. 1B), compared to 0.6-0.8 mm for the late blastula of

Xenopus tropicalis (Fig. 1E). A chorion was removed from the blastulae of both species by fine forceps under a stereomicroscope. The animal pole of the blastulae was directed towards the top and then a bilateral side of the embryo was removed by tungsten needle (Fig. 1C, 1F). Yolk-rich cells at the vegetal pole of the late blastula were omitted from these embryos. The remainder of the animal cap explant was trimmed down to 0.5 mm x 0.5 mm (*Xenopus laevis*) or 0.25 x 0.25 mm (*Xenopus tropicalis*) (Fig. 1D, G). The animal cap explant was composed of 445 ± 14.0 or 448 ± 18.5 cells in *Xenopus laevis* or *Xenopus tropicalis*, respectively. These trimmed animal cap explants were used for all subsequent experiments.

The expression of early marker genes was induced by activin treatment in a dose-dependent manner in animal cap explants of *Xenopus tropicalis*

We investigated whether the dose-dependent activin effects observed for *Xenopus laevis* in previous studies were also

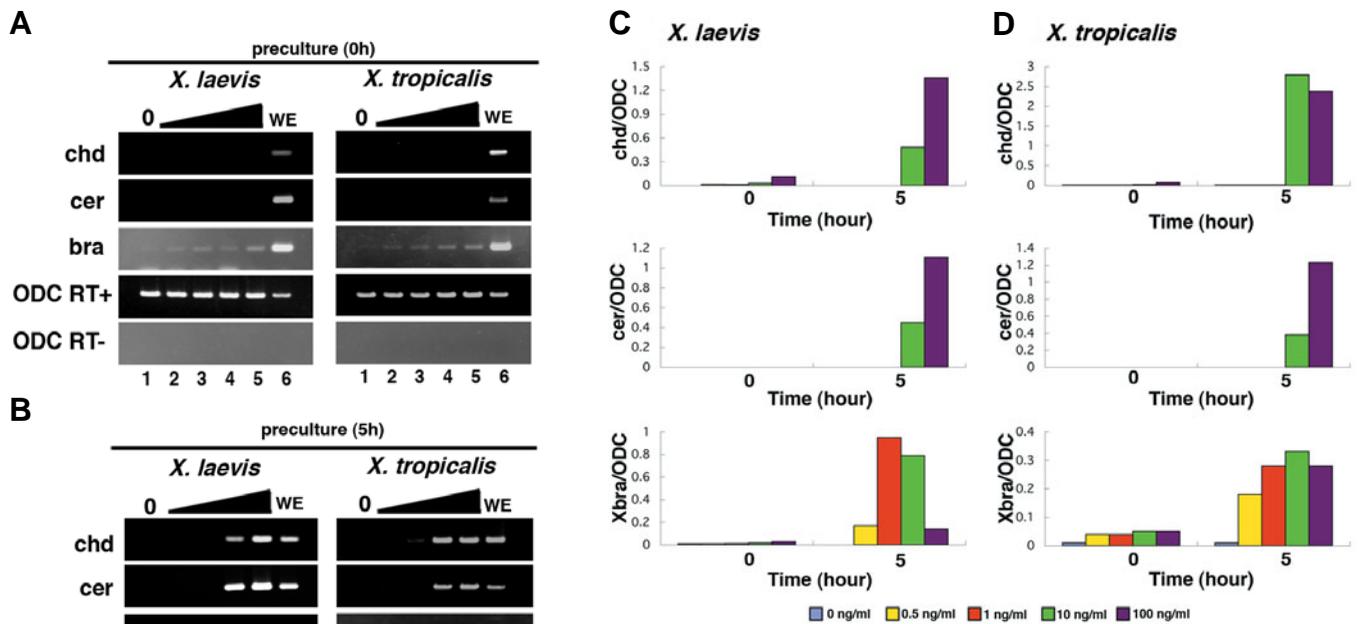


Fig. 2. Analysis of gene expression in activin-treated animal cap explants dissected from *Xenopus laevis* and *Xenopus tropicalis*. (A,B) RT-PCR analyses of activin-treated animal cap explants. Left panels indicate the results for *Xenopus laevis*. Right panels indicate the results for *Xenopus tropicalis*. For cDNA synthesis, total RNA was extracted from 10 or 15 animal caps in *Xenopus laevis*

or *Xenopus tropicalis*, respectively. This experiment was carried out three times with the same result. (A) RT-PCR analyses of animal cap explants treated with various doses of activin (0, 0.5, 1, 10, 100 ng/ml) for 1 hour at 20°C and sampled immediately when sibling embryos reached stage 10.5. In this condition, the expression of organizer marker genes, such as chd and cer, could not be detected at any doses. The expression of the mesodermal marker gene Xbra was detected at both low and high doses of activin. (B) RT-PCR analyses of animal cap explants treated with various doses of activin (0, 0.5, 1, 10, 100 ng/ml) for 1 hour at 20°C and cultured for 5 hours until sibling embryos reached stage 12 at 20°C. The expression of the organizer genes chd and cer was induced at the high dose of activin treatment (lanes 4,5). The high level of Xbra expression could be detected consecutively at every dose of activin treatment. (C,D) Quantitative analysis of gene expression for chordin, cerberus and Xbrachyury in activin-treated animal caps of *Xenopus laevis* (C) and *Xenopus tropicalis* (D) by real-time RT-PCR, relative to ODC expression. RNA samples were derived from animal caps treated with 0, 0.5, 1, 10, 100 ng/ml of activin for 1 hour at 20°C and then sampled immediately or 5 hours later at 20°C. The amount of cer expression was almost the same for the two species at high doses of activin for 5 hours. The expression levels of chd at 10 ng/ml of activin in *Xenopus tropicalis* was higher than that observed in *Xenopus laevis*, although the dose of activin for induction of gene expression was the same. The amount of Xbra expressed in *Xenopus laevis* was higher than that observed in *Xenopus tropicalis*, although Xbra expression was observed in 0.5 ng/ml activin in both species. chd, chordin; cer, cerberus; Xbra, Xbrachyury; WE, whole embryos.

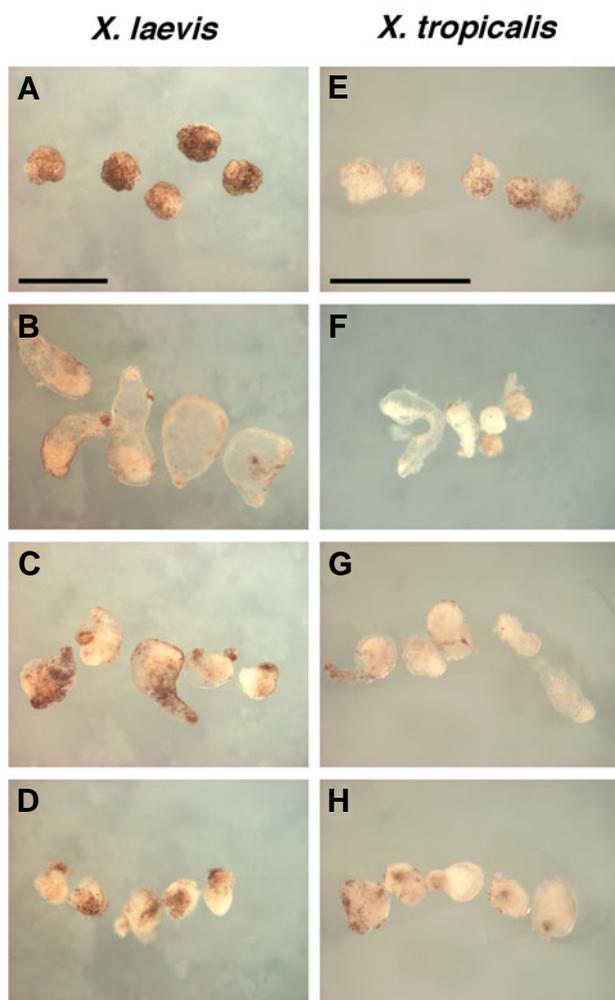


Fig. 3. External views of activin-treated animal cap explants cultured for 3 days at 20°C (*Xenopus laevis*, left panels) or 2 days at 25°C (*Xenopus tropicalis*, right panels) until sibling embryos reached stage 40. (A,E) Non-treated animal cap explants. (B,F) The animal cap explants were treated with 1 ng/ml activin. The explants had the appearance of transparent balloons. (C,G) 10 ng/ml and (D,H) 100 ng/ml activin. At this concentration, the explants became white and round. Note the similarity between explants from blastulae of *Xenopus tropicalis* treated with graded doses of activin and those from *Xenopus laevis* treated similarly. Scale bars in (A,E) indicate 1 mm.

apparent in animal cap cells dissected from *Xenopus tropicalis*. High doses of activin induced the expression of organizer marker genes, such as *chordin* (*chd*) and *cerberus* (*cer*), whereas lower doses of activin induced the mesodermal marker gene, *Xbrachyury* (*Xbra*) in animal cap explants from *Xenopus laevis* (Bouwmeester et al., 1996, Sasai et al., 1994, Smith, 1993, Smith et al., 1991). To do this, we analysed the expression of region-specific marker genes in activin-treated animal cap explants by RT-PCR and compared the gene expression in *Xenopus tropicalis* quantitatively with that in *Xenopus laevis*. The animal cap cells were dissected from the late blastulae of *Xenopus tropicalis* and *Xenopus laevis* and treated with various doses of activin (0, 0.5, 1, 10, 100 ng/ml) for 1 hour at 20°C. Non-treated animal cap explants were used as negative

controls. The activin-treated animal cap explants were sampled immediately or cultured for 5 hours at 20°C until sibling embryos reached stage 12. Total RNA was extracted from the animal caps of both species, as described. ODC was used as an internal control for all experiments. The expression of *chd* and *cer* (organizer marker genes) could not be detected at any dose of activin in the treated explants sampled immediately; this was the case for both species (Fig. 2A, left and panels indicates results of *Xenopus laevis* and *Xenopus tropicalis*, respectively). The mesodermal marker gene, *Xbra* was expressed slightly at low and high doses (0.5-100 ng/ml) of activin in explants from *Xenopus laevis* and *Xenopus tropicalis*, respectively (Fig. 2A, lanes 2-5). In contrast, in the explants sampled after culturing for 5 hours, the expression of *chd* and *cer* was induced only with the high-dose (10 ng/ml, 100 ng/ml) activin treatments (Fig. 2B, lanes 4-5). In contrast, strong expression of *Xbra* could be detected at every dose (0.5-100 ng/ml) of activin treatment for both embryo species (Fig. 2B, lanes 2-5). This result indicates that the animal cap cells dissected from the blastulae of *Xenopus tropicalis* have competency following activin treatment and that the expression of organizer and mesodermal marker genes was induced by activin in a time- and dose-dependent manner.

We next analyzed the gene expression induced by activin treatment quantitatively using real-time PCR analysis. We measured the expression of a range of marker genes, including *chd*, *cer* and *Xbra* in activin-treated animal caps of

TABLE 2

DIFFERENTIATION OF ACTIVIN TREATED ANIMAL CAPS IN *XENOPUS LAEVIS*

activin (ng/ml, 1 h)	0	1	10	100
number of experiments	17	14	14	15
notochord	0 (0)	0 (0)	2 (14)	3 (20)
muscle	0 (0)	10 (71)	14 (100)	15 (100)
pronephros	0 (0)	0 (0)	3 (21)	0 (0)
mesenchyme	0 (0)	14 (100)	13 (93)	10 (67)
neural tissues	0 (0)	0 (0)	10 (71)	6 (40)
epidermis	0 (0)	14 (100)	14 (100)	14 (93)
endodermal cells	0 (0)	0 (0)	1 (7)	4 (27)
atypical epidermis	17 (100)	0 (0)	0 (0)	0 (0)

Numbers in brackets indicate the percentage of tissue differentiation respectively.

TABLE 3

DIFFERENTIATION OF ACTIVIN TREATED ANIMAL CAPS IN *XENOPUS TROPICALIS*

activin (ng/ml, 1 h)	0	1	10	100
number of experiments	16	26	25	23
notochord	0 (0)	0 (0)	8 (32)	7 (30)
muscle	0 (0)	7 (27)	24 (96)	5 (22)
pronephros	0 (0)	0 (0)	0 (0)	0 (0)
mesenchyme	0 (0)	17 (65)	8 (32)	0 (0)
neural tissues	0 (0)	5 (19)	17 (68)	18 (78)
epidermis	0 (0)	19 (73)	24 (96)	4 (17)
endodermal cells	0 (0)	0 (0)	3 (12)	21 (91)
atypical epidermis	16 (100)	14 (54)	0 (0)	0 (0)

Numbers in brackets indicate the percentage of tissue differentiation respectively.

Xenopus laevis (Fig. 2C) and *Xenopus tropicalis* (Fig. 2D), relative to ODC expression. RNA samples were derived from animal caps treated with 0, 0.5, 1, 10, 100 ng/ml of activin for 1 hour and then sampled either immediately or 5 hours later at 20°C. In animal cap explants sampled immediately after activin treatment, no gene expression was detected in either species. After 5 hours of culturing, the expression of *chd* and *cer* was detected at 10 and 100 ng/ml of activin and *Xbra* expression was induced at 0.5-100 ng/ml of activin as described above (Fig. 2A, B). The amount of *cer* expression was similar at high doses of activin (10, 100 ng/ml) between the two species. The expression levels of *chd* at 10 ng/ml of activin in *Xenopus tropicalis* was higher than that observed in *Xenopus laevis*, although the dose of activin that induces the genes expression is the same. In particular, the expression of *chd* was 5 times higher at 10 ng/ml of activin in *Xenopus tropicalis* than at the same dose in *Xenopus laevis*. In contrast, the expression level of *Xbra* at 1-10 ng/ml of activin in *Xenopus laevis* was higher than that observed in *Xenopus tropicalis* and peaked at 1-10 ng/ml of activin in *Xenopus laevis* (there was no peak level of *Xbra* expression in *Xenopus tropicalis*). The quantitative

analysis by real-time PCR therefore highlighted a difference in the gene expression levels activated by activin treatment between the two species, although the activin needed to induce the gene expression was the same.

Activin-treated animal cap explants from *Xenopus tropicalis* showed the same morphology as the explants from *Xenopus laevis*

To further investigate the competency of animal cap explants from *Xenopus tropicalis* and *Xenopus laevis* following activin treatment (0, 1, 10, 100 ng/ml) for 1 hour, we compared the morphology of respective explants cultured for 3 days at 20°C (*Xenopus laevis*, left panels) or 2 days at 25°C (*Xenopus tropicalis*, right panels) until sibling embryos reached stage 40 as described above. The surface of non-treated explants from both *Xenopus laevis* and *Xenopus tropicalis* was quite rough (Fig. 3A, E). The explants treated with 1 ng/ml of activin had an elongated shape and the appearance of a transparent balloon (Fig. 3B, F). In contrast, treatment with 100 ng/ml of activin induced the explants to become white, round blocks (Fig. 3D, H), while those treated with 10 ng/ml of activin assumed a shape that

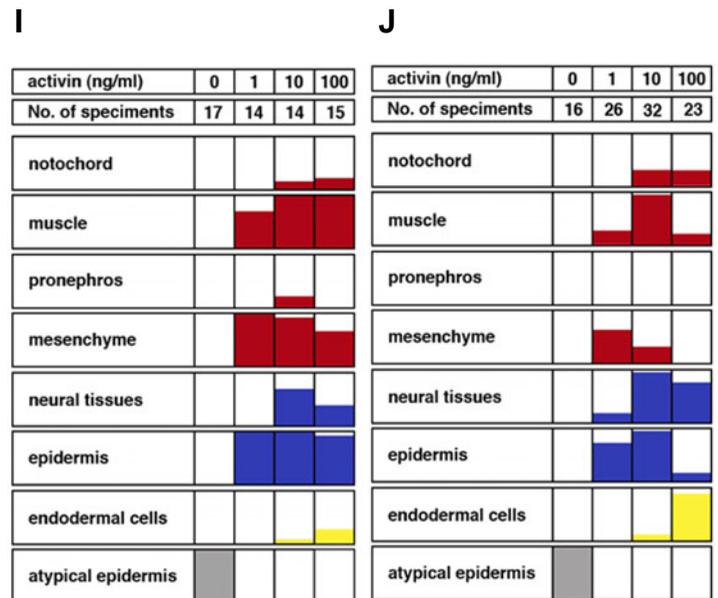
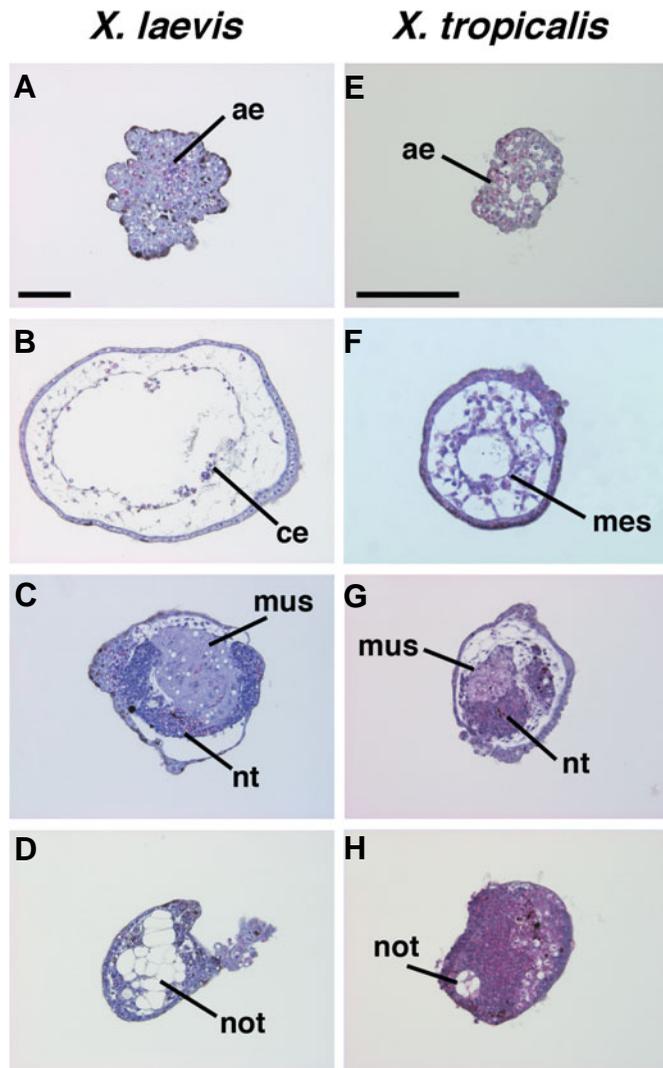


Fig. 4. Histological analysis of animal cap explants treated with various doses of activin (A-I, *Xenopus laevis*; E-J, *Xenopus tropicalis*). (A,E) Non-treated animal cap explants. The explants differentiated into atypical epidermis. Mesodermal or endodermal tissues were not observed. (B,F) Animal cap explants were treated with 1 ng/ml activin. The explants contained mesenchyme. (C,G) 10 ng/ml activin. Muscle and neural tissues were observed in the explants. (D,H) 100 ng/ml activin. The axial mesoderm, notochord could be observed at high frequency. In addition, many explants contained an endodermal cell mass. ae, atypical epidermis; ce, coelomic epidermis; mes, mesenchyme; mus, muscle; not, notochord; nt, neural tissues. Scale bars in (A,E) indicate 100 μm. (I,J) Histogram of the tissue differentiation in the activin-treated animal cap cells of *Xenopus tropicalis* and *Xenopus laevis* from Tables 2 and 3. Red, mesodermal tissues; Blue, ectodermal tissues; Yellow, endodermal tissue and Gray, atypical epidermis.

was somewhere between that observed with low and high doses of activin (Fig. 3C, G). This result indicates that the *Xenopus tropicalis* explants treated with a graded dose of activin had the same gross morphology as those dissected from *Xenopus laevis* and similarly treated.

Activin-treated animal cap explants from *Xenopus tropicalis* differentiated into mesodermal and endodermal tissues in a dose-dependent manner

To investigate what tissue types were differentiated from the treated explants of *Xenopus tropicalis*, we performed histological analyses of the cultured explants described above. Non-treated animal cap explants from both *Xenopus laevis* and *Xenopus tropicalis* differentiated into atypical epidermis (Fig. 4A, E). Mesodermal or endodermal tissues could not be observed in the histological sections. On the other hand, mesenchyme and well-defined epidermis could be observed in the animal cap explants treated with 1 ng/ml of activin (Fig. 4B, F). Following treatment with 10 ng/ml of activin, the animal cap explants contained muscle and neural tissues (Fig. 4C, G). The axial mesoderm and notochord could be observed at high frequency in the explants treated with 100 ng/ml of activin (Fig. 4D, H). In addition, many explants contained an endodermal cell mass (Fig. 4D, H). Tables 2 and 3 summarize the results from the observation of tissue differentiation in the activin-treated animal cap cells of *Xenopus tropicalis* and *Xenopus laevis*. Histogram representations of the results from these tables is shown in Figure 4I and J. It is clear from these more quantitative comparisons that there are many differences in the ratios of tissue differentiation between *Xenopus laevis* and *Xenopus tropicalis*. The *Xenopus tropicalis* explants differentiated into muscle much less frequently at the high and low doses of activin compared to *Xenopus laevis* and differentiated into neural tissues more frequently at all doses. The explants treated with 100 ng/ml of activin differentiated predominantly into endodermal tissues (91%) and neural tissues (78%) in *Xenopus tropicalis*, while endodermal tissues (93%), mesenchyme (67%) and muscle (100%) were predominantly induced in the explants from *Xenopus laevis* (Fig. 4I, J).

Discussion

Here, we aimed to perform animal cap assays from *Xenopus tropicalis* for histological analysis of *in vitro* inductive processes as reported in *Xenopus laevis*. This study demonstrated that animal cap cells dissected from late blastulae of *Xenopus tropicalis* have the same competence in response to activin as *Xenopus laevis* animal caps studied previously (Ariizumi et al., 1991, Green et al., 1997, Green et al., 1992). Organizer and mesoderm marker genes were expressed in the same temporal and dose-dependent pattern. Histological analysis also revealed that activin-treated animal cap explants of *Xenopus tropicalis* could differentiate into mesodermal and endodermal tissues in a dose-dependent manner. The animal cap cells from *Xenopus tropicalis* have responsiveness for various inductive signals such as activin and differentiate into various types of tissue *in vitro*, suggesting that *in vitro* systems of tissue induction using animal cap assays, as established in *Xenopus laevis*, may also be used to study *Xenopus tropicalis*.

This study also compared the responsiveness for activin treat-

ment in animal cap cells from *Xenopus laevis* and *Xenopus tropicalis*. The qualitative analysis using RT-PCR presented here revealed no difference in the dose of activin required to induce gene expression between *Xenopus tropicalis* and *Xenopus laevis*, suggesting that the rate and speed of signal transduction may be regulated by a common system in *Xenopus laevis* and *Xenopus tropicalis*. Interestingly, the quantitative analysis using real-time RT-PCR revealed several differences in the levels of individual marker gene expression activated by activin treatment between the two species, indicating that transcriptional regulation might differ between these two species. The expression levels of the organizer marker, *cer* was the same in the two species, indicating that the induction of these genes might be conserved during development. Differences in the amounts and peaks of expression of other marker genes (*chd*, *cer* and *Xbra*) between the two species suggested that there are different mechanisms to define expression levels in a germ layer- (tissue or position) or gene-specific manner between *Xenopus laevis* and *Xenopus tropicalis*.

Our experiments also revealed that activin-treated animal cap explants of *Xenopus tropicalis* can differentiate into mesodermal and endodermal tissues in a dose-dependent manner, as reported previously for *Xenopus laevis* (Ariizumi et al., 1991, Green et al., 1997, Green et al., 1992). This result indicates that there are common mechanisms of tissue differentiation, which are conserved among species. However, our results also indicated many differences in tissue differentiation between *Xenopus laevis* and *Xenopus tropicalis*. This may indicate that the threshold of responsiveness for activin treatment may be more highly defined in animal cap cells from *Xenopus tropicalis* than in those from *Xenopus laevis*. The main responsiveness for the activin dose did not differ significantly between the two species, although differences were observed in the ratios of tissues differentiated. It is thought that the cell differentiation is determined by which kind of signal the cell receives during normal development.

Our results support previous developmental comparisons of these two amphibians in demonstrating close similarities (Khokha et al., 2002, Sargent and Mohun, 2005, Shook et al., 2004). For example, many of the genes identified in *Xenopus laevis* are also expressed in the same regions and elicit homologous functions in *Xenopus tropicalis* (D'Souza et al., 2003, Fisher et al., 2003, Fletcher et al., 2004, Haramoto et al., 2004, Knochel et al., 2001, Sekizaki et al., 2004). However, many differences also exist in the developmental processes of these two closely related species. The embryos of *Xenopus tropicalis* develop more rapidly than those of *Xenopus laevis* and the embryos of *Xenopus tropicalis* are smaller than those of *Xenopus laevis* (Amaya et al., 1998, Khokha et al., 2002). In addition, the animal cap explants cultured from *Xenopus tropicalis* in this study were only half the size of those from *Xenopus laevis*. Further research is required to clarify whether similar signal transduction mechanisms exist in *Xenopus tropicalis* to those already defined in *Xenopus laevis* (Bourillot et al., 2002, Gurdon et al., 1999).

In summary, we described that animal cap cells of *Xenopus tropicalis* and *Xenopus laevis* display comparable competence for activin induction. This indicates the likelihood that the same method reported previously in *Xenopus laevis* can be used for tissue induction in *Xenopus tropicalis* (Ariizumi and Asashima, 1995, Ariizumi et al., 2003, Asashima et al., 2000a, Chan et al., 1999, Furue et al., 2002, Moriya et al., 2000, Myoishi et al., 2004,

Osafune *et al.*, 2002, Sedohara *et al.*, 2003). This new information will further advance both tissue induction research and our understanding of the molecular mechanisms of organ differentiation through genetic approaches that are possible by using *Xenopus tropicalis*.

Materials and Methods

Xenopus laevis and *Xenopus tropicalis* embryos

Xenopus tropicalis embryos were obtained by natural mating, where males and females were primed with 10 units of human chorionic gonadotropin (HCG; Gestron, Denka Seiyaku CO. Kawasaki Japan) at least 12 hours before a booster and incubated at 25°C. Both males and females were boosted with 100 units of HCG 4 hours before the frogs laid eggs. *Xenopus tropicalis* fertilized eggs were dejellied with 5% cysteine hydrochloride (Wako, Japan) (pH 7.8) in Steinberg's solution (SS). *Xenopus laevis* embryos were obtained as described previously (Sedohara *et al.*, 2002).

Embryo manipulation and conditioning of activin

The vitelline membrane was manually removed with fine forceps under a stereomicroscope. All operations were carried out under sterile conditions. Staging of the embryos of *Xenopus tropicalis* and *Xenopus laevis* was performed according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1956). Human recombinant activin was dissolved at various concentrations (0, 0.5, 1, 10, 100 ng/ml) in SS containing 0.1% bovine serum albumin (BSA; Sigma Chemical CO., St Louis, MO, USA) to avoid adsorption of activin to the plastic surfaces.

Analysis of gene expression using RT-PCR and real-time RT-PCR

Total RNA preparation and RT-PCR were performed as described previously (Sedohara *et al.*, 2002). Total RNA was extracted from 10 or 15 animal cap pieces for each dose of activin for *Xenopus laevis* and *Xenopus tropicalis*. Real-time RT-PCR was performed on an ABI PRISM 7700 (Applied Biosystems) using SYBR Green PCR Master Mix (QIAGEN) according to the QuantiTect SYBR Green Kit instructions, as described previously (Abe *et al.*, 2004). The PCR primer pairs are listed in Table 1 (Abe *et al.*, 2004, Haramoto *et al.*, 2004, Heasman *et al.*, 2000, Sun *et al.*, 1999). To ensure that the PCR was in the quantitative linear range, a sample dilution series was performed for each primer pair. Ornithine decarboxylase (ODC) was used as a loading control (Osborne *et al.*, 1991). The relative expression amounts were normalized to ODC.

Histology

For hematoxylin and eosin staining, the explants were fixed in Bouin's solution (75 ml saturated picric acid, 25 ml formalin and 5 ml glacial acetic acid) for 3 hours at room temperature. The samples were then dehydrated through a graded ethanol series (70%, 90%, 99.5% and 100%) for 15 minutes each, infiltrated in xylene for 20 minutes before embedding in paraffin (Histoprep 548). The samples were sectioned serially at 6 µm for hematoxylin and eosin staining.

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References

- ABE, T., FURUE, M., MYOISHI, Y., OKAMOTO, T., KONDOW, A. and ASASHIMA, M. (2004). Activin-like signaling activates notch signaling during mesodermal induction. *Int J Dev Biol* 48: 327-32.
- AMAYA, E., OFFIELD, M.F. and GRAINGER, R.M. (1998). Frog genetics: *Xenopus tropicalis* jumps into the future. *Trends Genet* 14: 253-5.
- ARIIZUMI, T. and ASASHIMA, M. (1995). Control of the embryonic body plan by activin during amphibian development. *Zool Sci* 12: 509-21.
- ARIIZUMI, T., KINOSHITA, M., YOKOTA, C., TAKANO, K., FUKUDA, K., MORIYAMA, N., MALACINSKI, G.M. and ASASHIMA, M. (2003). Amphibian in vitro heart induction: A simple and reliable model for the study of vertebrate cardiac development. *Int J Dev Biol* 47: 405-10.
- ARIIZUMI, T., SAWAMURA, K., UCHIYAMA, H. and ASASHIMA, M. (1991). Dose and time-dependent mesoderm induction and outgrowth formation by activin a in *Xenopus laevis*. *Int J Dev Biol* 35: 407-14.
- ARIIZUMI, T., TAKANO, K., ASASHIMA, M. and MALACINSKI, G.M. (2000). Bioassays of inductive interactions in amphibian development. *Methods Mol Biol* 135: 89-112.
- ASASHIMA, M., ARIIZUMI, T. and MALACINSKI, G.M. (2000a). In vitro control of organogenesis and body patterning by activin during early amphibian development. *Comp Biochem Physiol B Biochem Mol Biol* 126: 169-78.
- ASASHIMA, M., ARIIZUMI, T., TAKAHASHI, S. and MALACINSKI, G.M. (2000b). Bioassays for studying the role of the peptide growth factor activin in early amphibian embryogenesis. *Methods Mol Biol* 136: 15-26.
- ASASHIMA, M., NAKANO, H., SIMADA, K., KINOSHITA, K., ISHII, K., SHIBAI, H. and UENO, N. (1990). Mesodermal induction during early amphibian development. *Roux's Arch. Dev. Biol.* 198: 330-35.
- ATTISANO, L. and WRANA, J.L. (1998). Mads and smads in TGF beta signalling. *Curr Opin Cell Biol* 10: 188-94.
- BOURILLOT, P.Y., GARRETT, N. and GURDON, J.B. (2002). A changing morphogen gradient is interpreted by continuous transduction flow. *Development* 129: 2167-80.
- BOUWMEESTER, T., KIM, S., SASAI, Y., LU, B. and DE ROBERTIS, E.M. (1996). Cerberus is a head-inducing secreted factor expressed in the anterior endoderm of spemann's organizer. *Nature* 382: 595-601.
- CHAN, T.C., ARIIZUMI, T. and ASASHIMA, M. (1999). A model system for organ engineering: Transplantation of in vitro induced embryonic kidney. *Naturwissenschaften* 86: 224-7.
- D'SOUZA, A., LEE, M., TAVERNER, N., MASON, J., CARRUTHERS, S., SMITH, J.C., AMAYA, E., PAPALOPULU, N. and ZORN, A.M. (2003). Molecular components of the endoderm specification pathway in *Xenopus tropicalis*. *Dev Dyn* 226: 118-27.
- DUBRULLE, J., MCGREW, M.J. and POURQUIE, O. (2001). Fgf signaling controls somite boundary position and regulates segmentation clock control of spatiotemporal hox gene activation. *Cell* 106: 219-32.
- DYSON, S. and GURDON, J.B. (1998). The interpretation of position in a morphogen gradient as revealed by occupancy of activin receptors. *Cell* 93: 557-68.
- FISHER, M.E., PECK, W., BRANNEY, P.A. and POWNALL, M.E. (2003). Cloning and characterisation of myf5 and myod orthologues in *Xenopus tropicalis*. *Biol Cell* 95: 555-61.
- FLETCHER, R.B., WATSON, A.L. and HARLAND, R.M. (2004). Expression of *Xenopus tropicalis* *noggin1* and *noggin2* in early development: Two *noggin* genes in a tetrapod. *Gene Expr Patterns* 5: 225-30.
- FUJII, H., NAGAI, T., SHIRASAWA, H., DOI, J.-Y., YASUI, K., NISHIMATSU, S.-I., TAKEDA, H. and SAKAI, M. (2002). Anteroposterior patterning in *Xenopus* embryos: Egg fragment assay system reveals a synergy of dorsalizing and posteriorizing embryonic domains. *Dev. Biol.* 252: 15-30.
- FUKUI, A., NAKAMURA, T., SUGINO, K., TAKIO, K., UCHIYAMA, H., ASASHIMA, M. and SUGINO, H. (1993). Isolation and characterization of *Xenopus* follistatin and activins. *Dev Biol* 159: 131-9.
- FUKUI, A., NAKAMURA, T., UCHIYAMA, H., SUGINO, K., SUGINO, H. and ASASHIMA, M. (1994). Identification of activins a, ab and b and follistatin proteins in *Xenopus* embryos. *Dev Biol* 163: 279-81.
- FURUE, M., MYOISHI, Y., FUKUI, Y., ARIIZUMI, T., OKAMOTO, T. and ASASHIMA, M. (2002). Activin a induces craniofacial cartilage from undifferentiated *Xenopus* ectoderm in vitro. *Proc Natl Acad Sci USA* 99: 15474-9.
- GAMSE, J.T. and SIVE, H. (2000). Vertebrate anteroposterior patterning: The *Xenopus* neurectoderm as a paradigm. *BioEssays* 22: 976-986.
- GAMSE, J.T. and SIVE, H. (2001). Early anteroposterior division of the presumptive neurectoderm in *Xenopus*. *Mech. Dev.* 104: 21-36.
- GREEN, J.B., COOK, T.L., SMITH, J.C. and GRAINGER, R.M. (1997). Anteropos-

- terior neural tissue specification by activin-induced mesoderm. *Proc Natl Acad Sci USA* 94: 8596-601.
- GREEN, J.B., NEW, H.V. and SMITH, J.C. (1992). Responses of embryonic xenopus cells to activin and fgf are separated by multiple dose thresholds and correspond to distinct axes of the mesoderm. *Cell* 71: 731-9.
- GURDON, J.B., MITCHELL, A. and RYAN, K. (1996). An experimental system for analyzing response to a morphogen gradient. *Proc Natl Acad Sci USA* 93: 9334-8.
- GURDON, J.B., STANDLEY, H., DYSON, S., BUTLER, K., LANGON, T., RYAN, K., STENNARD, F., SHIMIZU, K. and ZORN, A. (1999). Single cells can sense their position in a morphogen gradient. *Development* 126: 5309-17.
- HARAMOTO, Y., TANEGASHIMA, K., ONUMA, Y., TAKAHASHI, S., SEKIZAKI, H. and ASASHIMA, M. (2004). Xenopus tropicalis nodal-related gene 3 regulates bmp signaling: An essential role for the pro-region. *Dev Biol* 265: 155-68.
- HEASMAN, J., KOFRON, M. and WYLIE, C. (2000). Beta-catenin signaling activity dissected in the early xenopus embryo: A novel antisense approach. *Dev Biol* 222: 124-34.
- HILL, C.S. (2001). Tgf-beta signalling pathways in early xenopus development. *Curr Opin Genet Dev* 11: 533-40.
- KHOKHA, M.K., CHUNG, C., BUSTAMANTE, E.L., GAW, L.W., TROTT, K.A., YE, J., LIM, N., LIN, J.C., TAVERNER, N., AMAYA, E. et al. (2002). Techniques and probes for the study of xenopus tropicalis development. *Dev Dyn* 225: 499-510.
- KNOCHEL, S., DILLINGER, K., KOSTER, M. and KNOCHEL, W. (2001). Structure and expression of xenopus tropicalis bmp-2 and bmp-4 genes. *Mech Dev* 109: 79-82.
- KOCHAN, K.J., WRIGHT, D.A., SCHROEDER, L.J., SHEN, J. and MORIZOT, D.C. (2003). Genetic linkage maps of the west african clawed frog xenopus tropicalis. *Dev Dyn* 226: 99-102.
- MCDOWELL, N., ZORN, A.M., CREASE, D.J. and GURDON, J.B. (1997). Activin has direct long-range signalling activity and can form a concentration gradient by diffusion. *Curr Biol* 7: 671-81.
- MIYAZONO, K., TEN DIJKE, P. and HELDIN, C.H. (2000). TGF-beta signaling by smad proteins. *Adv Immunol* 75: 115-57.
- MORIYA, N., KOMAZAKI, S. and ASASHIMA, M. (2000). In vitro organogenesis of pancreas in xenopus laevis dorsal lips treated with retinoic acid. *Dev Growth Differ* 42: 175-85.
- MYOISHI, Y., FURUE, M., FUKUI, Y., OKAMOTO, T. and ASASHIMA, M. (2004). Induction of tooth and eye by transplantation of activin a-treated, undifferentiated presumptive ectodermal xenopus cells into the abdomen. *Int J Dev Biol* 48: 1105-12.
- NIEUWKOOP, P.D. and FABER, J. (1956). *Normal table of xenopus laevis (daudin)*. North-Holland publishing company, Amsterdam.
- NINOMIYA, H., ELINSON, R.P. and WINKLBAUER, R. (2004). Antero-posterior tissue polarity links mesoderm convergent extension to axial patterning. *Nature* 430: 364-7.
- NINOMIYA, H., TAKAHASHI, S., TANEGASHIMA, K., YOKOTA, C. and ASASHIMA, M. (1999). Endoderm differentiation and inductive effect of activin-treated ectoderm in xenopus. *Dev Growth Differ* 41: 391-400.
- OFFIELD, M.F., HIRSCH, N. and GRAINGER, R.M. (2000). The development of xenopus tropicalis transgenic lines and their use in studying lens developmental timing in living embryos. *Development* 127: 1789-97.
- OSAFUNE, K., NISHINAKAMURA, R., KOMAZAKI, S. and ASASHIMA, M. (2002). In vitro induction of the pronephric duct in xenopus explants. *Dev Growth Differ* 44: 161-7.
- OSBORNE, H.B., DUVAL, C., GHODA, L., OMILLI, F., BASSEZ, T. and COFFINO, P. (1991). Expression and post-transcriptional regulation of ornithine decarboxylase during early xenopus development. *Eur J Biochem* 202: 575-81.
- RYAN, K., GARRETT, N., BOURILLOT, P., STENNARD, F. and GURDON, J.B. (2000). The xenopus eomesodermin promoter and its concentration-dependent response to activin. *Mech Dev* 94: 133-46.
- SARGENT, M.G. and MOHUN, T.J. (2005). Cryopreservation of sperm of xenopus laevis and xenopus tropicalis. *Genesis* 41: 41-6.
- SASAI, Y., LU, B., STEINBEISSER, H., GEISSERT, D., GONT, L.K. and DE ROBERTIS, E.M. (1994). Xenopus chordin: A novel dorsalizing factor activated by organizer-specific homeobox genes. *Cell* 79: 779-90.
- SEDOHARA, A., FUKUI, A., MICHIEU, T. and ASASHIMA, M. (2002). Role of bmp-4 in the inducing ability of the head organizer in xenopus laevis. *Zool Sci* 19: 67-80.
- SEDOHARA, A., KOMAZAKI, S. and ASASHIMA, M. (2003). In vitro induction and transplantation of eye during early xenopus development. *Dev Growth Differ* 45: 463-71.
- SEKIZAKI, H., TAKAHASHI, S., TANEGASHIMA, K., ONUMA, Y., HARAMOTO, Y. and ASASHIMA, M. (2004). Tracing of xenopus tropicalis germ plasm and presumptive primordial germ cells with the xenopus tropicalis daz-like gene. *Dev Dyn* 229: 367-72.
- SHOOK, D.R., MAJER, C. and KELLER, R. (2004). Pattern and morphogenesis of presumptive superficial mesoderm in two closely related species, xenopus laevis and xenopus tropicalis. *Dev Biol* 270: 163-85.
- SIVE, H.L., GRAINGER, R.M. and HARLAND, R.M. (1998). *Early development of xenopus laevis: A laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- SMITH, J.C. (1993). Mesoderm-inducing factors in early vertebrate development. *EMBO J* 12: 4463-70.
- SMITH, J.C., PRICE, B.M., GREEN, J.B., WEIGEL, D. and HERRMANN, B.G. (1991). Expression of a xenopus homolog of brachyury (t) is an immediate-early response to mesoderm induction. *Cell* 67: 79-87.
- SMITH, J.C., PRICE, B.M., VAN NIMMEN, K. and HUYLEBROECK, D. (1990). Identification of a potent xenopus mesoderm-inducing factor as a homologue of activin a. *Nature* 345: 729-31.
- SONG, M.O., FORT, D.J., MCLAUGHLIN, D.L., ROGERS, R.L., THOMAS, J.H., BUZZARD, B.O., NOLL, A.M. and MYERS, N.K. (2003). Evaluation of xenopus tropicalis as an alternative test organism for frog embryo teratogenesis assay—xenopus (fetax). *Drug Chem Toxicol* 26: 177-89.
- SUN, B.I., BUSH, S.M., COLLINS-RACIE, L.A., LAVALLIE, E.R., DIBLASIO-SMITH, E.A., WOLFMAN, N.M., MCCOY, J.M. and SIVE, H.L. (1999). Derriere: A tgf-beta family member required for posterior development in xenopus. *Development* 126: 1467-82.
- TABATA, T. and TAKEI, Y. (2004). Morphogens, their identification and regulation. *Development* 131: 703-12.

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