CONTROL OF CELL DIFFERENTIATION AND MORPHOGENESIS BY ACTIVIN A DURING EARLY AMPHIBIAN DEVELOPMENT

アクチビンAによる両生類の初期発生における細胞分化および形態形成の制御

有泉高史

Control of Cell Differentiation and Morphogenesis by Activin A during Early Amphibian Development

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Science

by

Takashi Ariizumi

University of Tokyo

December, 1995

Contents

General introduction

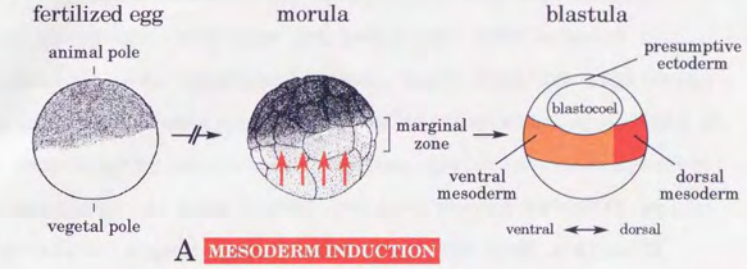
General introduction

Embryonic induction plays an important role in establishing the fundamental body plan during early amphibian development. About seventy years ago, Spemann and Mangold (1924) discovered the phenomenon of primary embryonic induction using a heteroplastic transplantation technique in newt embryos. Because the ability to induce a secondary embryo was restricted to the dorsal lip, this region was named "the organizer" in the sense of a morphogenetic center. Following the discovery of the organizer in amphibian embryos, many investigators tried to isolate factors which could act as the organizer, and to elucidate the source of the organizer, and to reveal the molecular mechanisms of the embryonic induction. As a result of these attempts, it was found that the mesodermal anlagen, including the organizer, develop from equatorial (marginal zone) cells under the influence of signals from vegetal hemisphere cells at an earlier stage of development (Nieuwkoop, 1969; Nakamura and Takasaki, 1970; Nakamura et al., 1971; Asashima, 1975). This is the first inductive phenomenon to be observed during amphibian development and is referred to as "mesoderm induction" (Fig. 1A). During gastrulation, the invaginating cells of the dorsal marginal zone (the organizer) induce central nervous system anlagen by secreting certain factors to the overlying layer of ectodermal cells. This inductive phenomenon is called

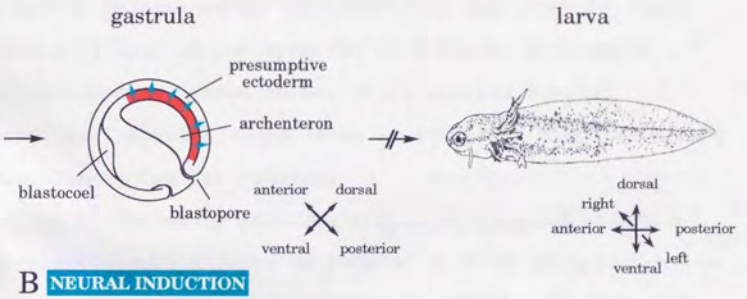
Figure 1

Model of embryonic induction during
early amphibian development

Mesoderm induction is the first embryonic induction, in which mesoderm anlagen including the organizer are induced by vegetal hemisphere cells (A). Dorsal mesoderm (the organizer) induces central nervous system anlagen in the overlying presumptive ectoderm during gastrulation. This inductive phenomenon is called neural induction (B).



A **MESODERM INDUCTION**



B **NEURAL INDUCTION**

"neural induction" (Fig. 1B). The fundamental body plan of the embryo is established as a result of these two major inductive interactions during the process of amphibian embryonic development.

Amphibians provide excellent material for experiments to study the mechanisms of embryonic induction. Urodela, such as *Cynops*, *Triturus* and *Ambystoma*, have several advantages over other amphibians. Their eggs are relatively large, and the embryos develop relatively slowly, so that it is very easy to perform surgical manipulation. Consequently, urodele embryos have proved the most useful material in traditional experimental embryology. The most available material used today is the anuran, *Xenopus laevis*. *Xenopus* is easily maintained in the laboratory and produces large numbers of eggs at one spawning in response to hormone injections every three month. Their embryos develop relatively rapidly, which is an advantage in biochemical and molecular biological studies.

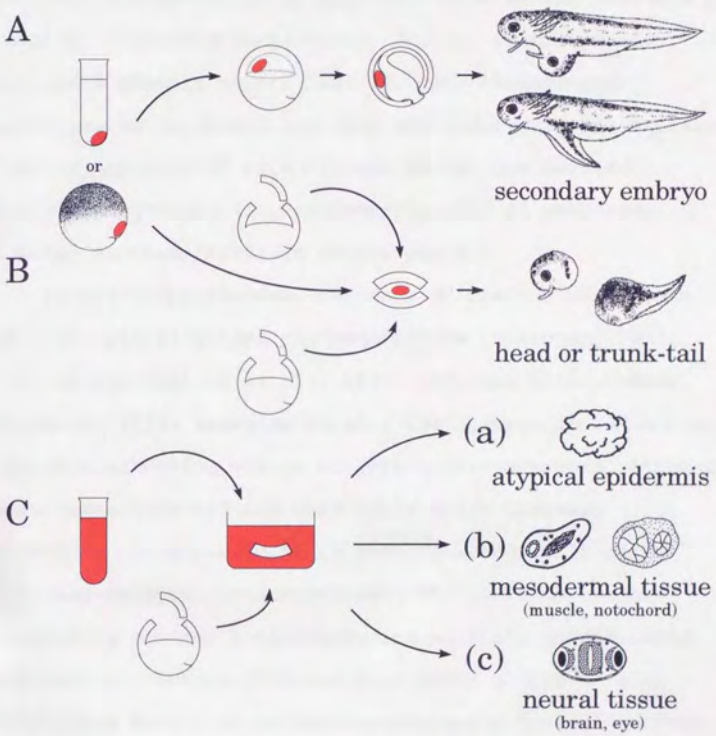
Up to the early gastrula stage, presumptive ectoderm has pluripotency and can be induced to form neural tissue or mesodermal and endodermal tissues by addition of inducers. Taking an advantage of the pluripotency of presumptive ectoderm, three main methods of testing the inducing activity of tissues and substances have been devised (Fig. 2). In the "implantation method" or "Einsteck method" (Mangold, 1923), a piece of tissue or coagulated inducing substance is pushed into the blastocoele of a late blastula or early gastrula through a slit at the animal pole of the

host embryo (Fig. 2A). As gastrulation proceeds, the implant becomes pressed against the ventral ectoderm of the host embryo some hours after implantation. If the organizer (the dorsal lip of the blastopore) is implanted, a secondary embryo is eventually formed on the ventral side of the host embryo. To eliminate any possible influence by the host embryo, two explantation ways have been improved. In the "sandwich culture method", conceived by Holtfreter (1933), the solid inducer is placed between two sheets of the presumptive ectoderm and interacts with them from the inside of a vesicle (Fig. 2B). When the inducer is soluble in saline, the "piece culture method" or "animal cap assay" (Becker et al., 1959; Yamada and Takata, 1961; Saxén and Toivonen, 1962) is generally used to test its inducing activity (Fig. 2C). A piece of presumptive ectoderm (animal cap) is excised from a late blastula or early gastrula and dipped in the saline containing the inducing substance for a certain period. This last method is easy to perform and has many advantages over other methods. Investigators can test large numbers of fractions of inducing substances in solution and estimate their inducing activity both qualitatively and quantitatively at the histological and molecular levels. It is also quite easy to determine the induction properties of the substance and the competence of the reacting tissues when presumptive ectoderm at different stages or of different sizes is treated with various concentrations of the inducer for a fixed period.

Figure 2

Schematic diagrams of three methods of testing the inducing activity of tissues and substances

(A) Implantation method. A piece of tissue (e.g., the organizer) or coagulated substance inserted into the blastocoele induces a secondary embryo on the ventral side of host embryo. (B) Sandwich culture method. Inducer is placed between two pieces of presumptive ectoderm. (C) Piece culture method. A piece of ectoderm is put in saline containing the inducing substance in solution. Ectoderm forms atypical epidermis without inducers (a). Differentiation of mesodermal tissues, such as notochord and muscle, indicates the presence of a mesoderm-inducing factor (b), whereas that of neural tissues, such as forebrain and eyes, indicates the presence of a neural inducing factor (c).


A
or
secondary embryo
B
head or trunk-tail
C
(a)
atypical epidermis
(b)
mesodermal tissue
(muscle, notochord)
(c)
neural tissue
(brain, eye)

In the piece culture method, the presumptive ectoderm forms irregular-shaped epidermis, referred to as atypical epidermis, in the absence of inducers (Fig. 2C, a), but can be induced to form neural tissue, or mesodermal and endodermal tissues by adding inducers. Differentiation of notochord and muscle in an explant indicates the presence of a mesoderm-inducing factor (Fig. 2C, b). If the saline contains a certain neural inducer, such archencephalic structures as forebrain and eyes are induced in the explants (Fig. 2C, c). Use of this culture method has enabled remarkable advances in the identification of mesoderm-inducing factors (MIFs) in recent years.

In the early studies, extracts of various vertebrate tissues, such as guinea pig bone marrow (Toivonen, 1953), chick embryo (Geithe et al., 1975) and carp swim bladder (Kawakami, 1976; Asashima et al., 1987), were found to have a mesoderm-inducing effect on presumptive ectoderm. Although these "heterogenous" inducers could mimic mesoderm induction, the molecule which induces mesoderm in normal amphibian embryos remains unknown. The first molecule reported to possess potent inducing activity was isolated from chicken embryos (Born et al., 1972; Geithe et al., 1981). This factor is called "vegetalizing factor" because of its mesoderm- and endoderm-inducing effect on the presumptive ectoderm (Kocher-Becker and Tiedemann, 1971; Grunz, 1979, 1983; Minuth and Grunz, 1980). More recently, a factor capable of inducing dorsal mesoderm was found in the culture medium of a *Xenopus* tadpole cell line (XTC cell) and

designated XTC-MIF (Smith, 1987). XTC-MIF is believed to be a natural mesoderm-inducing factor because of its origin.

The most remarkable achievement in MIF research has been the identification of several peptide growth factors belonging to the fibroblast growth factor (FGF) and transforming growth factor-ß (TGF-ß) families as MIF candidates (reviewed by Tiedemann, 1990; Dawid et al., 1992; Asashima, 1994). In 1987, Slack et al. (1987) reported that mammalian basic fibroblast growth factor (bFGF) has mesoderm-inducing activity. Presumptive ectoderm treated with bFGF differentiated solely into ventral mesoderm, such as blood cells and coelomic epithelium, and no dorsal mesoderm with notochord was detected. These results suggest that other factors are required for complete mesoderm formation (Grunz et al., 1988). In the same study, they showed that heparin could block the inducing activity of FGF, and suggested that an FGF-like substance which binds to heparin may be a natural inducer. Knöchel et al. (1987, 1989) and Rosa et al. (1988) found that TGF-ß1 and TGF-ß2 also have mesoderm-inducing activity.

Independent of these reports, several peptides having mesoderm-inducing activity were isolated from the conditioned medium of the human K-562 cell line (Nakano et al., 1990). These peptides were found to be closely related to activin A, which is identical with erythroid differentiation factor (EDF) (Asashima et al., 1990a). Activin A belongs to the TGF-ß superfamily and was originally identified as a gonadal hormone that promotes the

release of follicle stimulating hormone (FSH) from the anterior pituitary gland (Ling et al., 1986; Vale et al., 1986). After that, certain mesoderm-inducing factors derived from different sources have been shown to be identical to activin A or to be activin homologues: XTC-MIF from XTC cell conditioned medium (Smith et al., 1990; Eijnden-Van Raaij et al., 1990), WEHI-MIF from murine leukemia cells (Albano et al., 1990), vegetalizing factor from calf kidney and chicken embryo (Asashima et al., 1990b, 1991a, c; Tiedemann et al., 1992), and PIF from mouse macrophage cell line P388D1 (Sokol et al., 1990; Thomsen et al., 1990).

Activin A can induce a variety of mesodermal tissues in *Xenopus* presumptive ectoderm in the piece culture method, and thus it is the best candidate for the natural mesoderm-inducing factor (Asashima et al., 1990a). The tissues induced by activin are exactly the same at the histological and molecular levels as those found in normal embryos. Genes normally expressed during development are also expressed sequentially after exposure of presumptive ectoderm to activin (reviewed by Asashima, 1994; Ariizumi and Asashima, 1995b). The most characteristic property of activin is induction of organizer activity in presumptive ectoderm (Cooke, 1989; Ruiz i Altaba and Melton, 1989; Cho et al., 1991a). The isolated ectoderm of the *Xenopus* late blastula or early gastrula itself forms atypical epidermis in the absence of activin. After treatment of isolated ectoderm with activin, however, the same region induces a secondary embryo, as the organizer does when implanted into the

blastocoele of another embryo. These findings suggest that activin can not only induce various mesodermal tissues but elicit the organizer as a morphogenetic center.

Activin or a homologue actually exists in early *Xenopus* embryos. Asashima et al. (1991b) attempted to extract native activin protein directly from the early *Xenopus* embryos by reversed phase high performance liquid chromatography (HPLC). After HPLC fractionation using thousands of unfertilized eggs or blastulae, only certain fractions exhibited the same retention times as activin A and showed potent mesoderm-inducing activity. The peptide(s) in these fractions was identified as an activin homologue because its mesoderm-inducing activity was inhibited by follistatin, an activin-specific binding protein. These fractions also possessed erythroid differentiation factor (EDF) activity. Estimates of the EDF activity in these fractions revealed that about 1 pg (0.5 ng/ml) of the activin homologue is present at least in a *Xenopus* egg. Fukui et al. (1994) further succeeded in extracting three types of activin (A, AB, B) and follistatin from early *Xenopus* embryos. The activin and follistatin protein molecules were found to be localized uniformly in yolk platelets in early *Xenopus* oocytes, but not in other cytoplasmic organelles (Uchiyama et al., 1994). This suggests that a novel role of yolk platelets as a reservoir for inductive signals transported by vitellogenin, which is synthesized in the liver. These findings can be interpreted to mean that activin connects with vitellogenin forming the yolk platelets and the

polarity during oogenesis and that this activin has an important role in producing the embryonic body plan by influencing the mesoderm induction.

Thus, at present, activin is currently considered a strong candidate for the first inducing signal to establish body plan during early amphibian development. Using activin (as the inducer) and presumptive ectoderm (as the pluripotent reacting tissue), it is possible in theory to reproduce embryonic induction and design a fundamental embryonic form *in vitro*. In the present study, I have focused on activin as the first molecular signal in the chain of inductive events, and examined the extent to which cell differentiation and morphogenesis during early amphibian development can be controlled *in vitro* with activin. First, the induction property of activin A was examined by changing the concentration and treatment time of activin A on *Xenopus* presumptive ectoderm at different stages and of different regions (chapter 1; Ariizumi et al., 1991a, b). Activin A induced various mesodermal tissues at clear dose thresholds, such that low doses induced ventral mesoderm and high doses induced dorsal mesoderm. The dose of activin A required to induce mesodermal tissues was inversely proportional to the duration of exposure of the presumptive ectoderm. Furthermore, there existed stage- and regional differences in competence to the activin A treatment within the presumptive ectoderm, and these differences are considered to be important for the patterning of mesoderm in early *Xenopus* development. Second,

the induction property of activin A on presumptive ectoderm of the urodele, *Cynops pyrrhogaster*, was examined by changing the concentration of activin A and the size of presumptive ectoderm (chapter 2; Ariizumi and Asashima, 1995a; Ariizumi et al., 1996). Activin A behaved like the vegetalizing factor, and preferentially induced yolk-rich endodermal tissues on presumptive ectoderm. Mesodermal tissues, including beating heart rudiments, were also induced as the size of presumptive ectoderm increased. These results suggest that activin A is one of the substances involved in triggering endodermal differentiation, and that mesodermal tissues are induced by endodermally determined tissues as a secondary interaction in *Cynops* early development. Lastly, the regional induction-specificity of activin-treated ectoderm was examined employing the sandwich culture technique, after preculturing it for various periods in saline (chapter 3; Ariizumi and Asashima, 1994, 1995a). The activin-treated ectoderm displayed regional induction-specificity depending on the duration of preculture, such that trunk-tail structures were induced by shorter-term precultured ectoderm and head structures were induced by longer-term precultured ectoderm. These results are agreement with those of the previous experiment using the organizer as the inducer, and support the idea that activin acts as first inducing signal in the chain of inductive events in amphibian embryogenesis.

Chapter 1

Induction Property of Activin A on *Xenopus* Presumptive Ectoderm: Dose and Time-dependent Mesoderm-inducing Activity of Activin A on Presumptive Ectoderm of Different Developmental Stages and Regions

Introduction

In the early 1950's, many investigators grappled with the analysis of embryonic induction using vertebrate tissues as "heterogenous" inducers. As a result of these attempts, hypotheses about the establishment of fundamental body plan were proposed based on the gradients of inducers (reviewed by Nakamura and Toivonen, 1978; Hamburger, 1988). The liver of guinea pig induces only archencephalic structures, such as forebrain, eye and nose, whereas bone marrow induces mesodermal tissues (Toivonen, 1938, 1940, 1953). On the basis of experiments using these tissues, Toivonen and Saxén (1955a, b) proposed the "two-gradient hypothesis". They postulated the concentration gradients of two agents in the early amphibian embryos: The gradient of the neuralizing agent is highest at the dorsal midline and declines toward the lateral and ventral parts of the embryo. That of the mesodermalizing agent is highest at the posterior dorsal end and declines toward the anterior and lateral parts of the embryo. Each region of the embryo has a different concentration of the neuralizing and mesodermalizing agents. These gradient concepts are very important in understanding cell differentiation and morphogenesis during early amphibian development. In this chapter, presumptive ectoderm of *Xenopus laevis* was treated with various concentrations of activin A to examine whether mesoderm formation can be controlled by a concentration gradient of activin A. Activin

A could control mesoderm formation in *Xenopus* ectoderm in a gradient fashion, which agrees with classical gradient theories to some extent.

The competence of reacting tissues is also important in mesoderm formation during early *Xenopus* development (Sokol and Melton, 1991, 1992; Kinoshita et al., 1993, 1995; Kinoshita and Asashima, 1995). In normal development there seem to be close relationships between the mesoderm-inducing signal(s) and competence of presumptive mesodermal cells. It is, however, almost impossible to isolate the presumptive mesoderm, which has not yet received the mesoderm-inducing signal, from the early *Xenopus* embryos. Although findings observed on the presumptive ectoderm can not completely explain the competence of natural reacting tissues (presumptive mesoderm) in mesoderm induction, they will mimic this inductive event because presumptive ectoderm has a pluripotency to the inducing signals. In this chapter, competence of reacting tissues to the activin A treatment was also investigated using *Xenopus* presumptive ectoderm at different stages and of different regions. It was speculated that there exist stage- and regional differences in competence within the responding tissues, and that these differences are also important for the patterning of mesoderm during early *Xenopus* embryogenesis.

Materials and Methods

Eggs and Embryos

Eggs of *Xenopus laevis* were obtained by the injection of human chorionic gonadotropin (Gestron; Denka Seiyaku, Japan). Both males and females were injected with 800 IU of gonadotropin. Staging of embryos was according to Nieuwkoop and Faber (1956). The jelly coat was removed with Steinberg's solution containing 4.5% cysteine hydrochloride (pH 7.8). The vitelline membrane was manually removed with a pair of watchmaker's tweezers under a binocular microscope.

Activin A solution

Human recombinant activin A (EDF, erythroid differentiation factor; Murata et al., 1988) purified from the culture supernatant of CHO cells expressing the human inhibin ßA chain gene was used as the inducer in all experimental series. It yielded a single peak on reversed phase high performance liquid chromatography (HPLC) and a single band in silver-stained sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gel under both reducing and non-reducing conditions.

Activin A was dissolved in Steinberg's solution (SS; 58 mM NaCl, 0.7 mM KCl, 0.3 mM $Ca(NO_3)_2$, 0.8 mM $MgSO_4$, 4.6 mM TRIS at pH 7.4) with 0.1 g/l kanamycin sulfate (Banyu, Japan) at concentrations of 0-100 ng/ml. 0.1% bovine serum albumin (BSA, A-7888; Sigma, USA) was added to the solution

to avoid adsorption of activin A to the glass or plastic surfaces. Activin A solutions were put in non-surface-treated polystyrene wells (SUMILON 24-well plate, MS-80240; Sumitomo Bakelite, Japan).

Experimental series

All of the following procedures were carried out under sterile conditions. Schematic diagrams of the procedures and culture methods are shown in Fig. 3.

Series 1

Presumptive ectodermal sheets (0.4 x 0.4 mm) cut from late blastulae (stage 9, about 1.2 mm in diameter) were treated with activin A solutions (0, 0.1, 0.25, 0.5, 1, 10, 50 and 100 ng/ml) for various periods (5, 10, 30 min, 3 hr, 1 day and 3 days) with the inner blastocoelic surface facing up. After washing in two changes of SS with gentle pipetting, they were cultured in non-surface-treated polystyrene wells filled with SS at 20°C until control embryos reached stage 40 (for 3 days).

Series 2

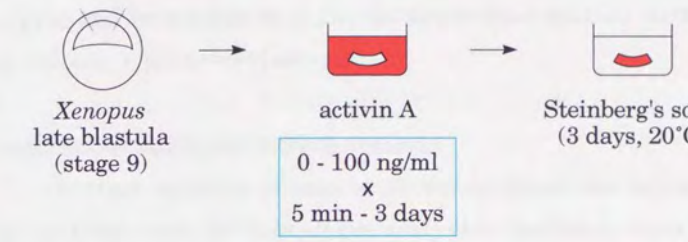
Presumptive ectodermal sheets of blastulae (stage 7-9) and early gastrulae (stage 10) were used as responding tissues. They were cut in following patterns:

1) prospective dorsal + ventral region (a circle with a radius of 0.4 mm round the animal pole)
2) prospective dorsal region
3) prospective ventral region

The prospective dorsal and ventral regions were determined by pigmentation. Prospective ventral region is more heavily pigmented than dorsal region.

They were treated with 0, 0.5, 1, 5 and 10 ng/ml of activin A solutions for 1 hr and cultured in SS as described in series 1.

Series 1



Series 2

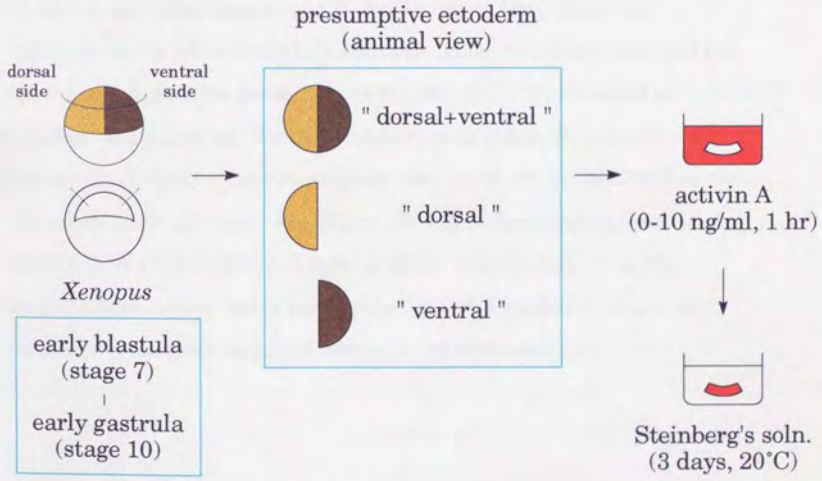


Figure 3

Schematic diagrams of the procedures and culture methods

Histological examination

Specimens were fixed in Bouin's solution for 3 hr. They were then dehydrated through a graded series of ethanols, cleared in xylene, embedded in paraffin (Histprep 568; Wako, Japan) and sectioned at 5 μm. Sections were stained with Delafield's hematoxylin/eosin.

Indirect immunofluorescence analysis

Differentiation of muscle in the explants was detected by indirect immunofluorescence analysis. Specimens were fixed in methanol for 10 min at 0°C, embedded in O.C.T. compound (Tissue-tek 4583; Miles, USA) and frozen in liquid nitrogen. Frozen sections were cut at 10 μm using a cryostat at -30°C and air-dried for 1 hr at room temperature. Sections were blocked with 1% skim milk in phosphate buffer (PB) for 10 min at room temperature, then reacted with 1/100 diluted solution of rabbit anti-myosin from chicken breast muscle (AB-1160; Didets, Japan) for 1 hr at room temperature and with 1/40 diluted solution of FITC-conjugated goat anti-rabbit IgG (1612-0081; Cappel, USA) for 30 min at room temperature. They were mounted with glycerol:PB (8:2) and observed under an epifluorescence microscope.

Results

Series 1: Dose and time-dependent mesoderm-inducing activity of activin A on *Xenopus* presumptive ectoderm

Morphological change of the presumptive ectoderm after the treatment with activin A

The activin-treated presumptive ectoderm rounded up over about 3 hr to form spheres with the original blastocoelic surface interior (Fig. 4A). They began to elongate at about 6 hr (Fig. 4B), when control embryos were middle gastrulae (stage 11-11.5). The degree of elongation depended on the concentration of activin A. At about 10 hr, when control embryos were early neurulae (stage 14-15), the explants treated with 1-10 ng/ml of activin A elongated dramatically (Fig. 4D) as compared with those of 0.5 ng/ml-treatment (Fig. 4C). The explants treated with a higher concentration of activin A (50 ng/ml) ceased elongating and began to push internal cells out of the explants (Fig. 4E). At 3 days, when control embryos reached stage 40, the explants showed four types of morphology depending on the concentration of activin A (Fig. 5). The control explants, which were cultured without activin A, rounded up and became smaller and wrinkled (A type). At 0.5 ng/ml of activin A, the explants swelled to become smooth and oval (B type). At 1-10 ng/ml, the explants elongated and were several times longer than the control explants (C type). At 50 ng/ml, the

explants did not elongate but remained spherical and became fragile. Cells often detached from the surface of the explants giving a ragged outline (D type).

In the next experiment, presumptive ectoderm was treated with various concentrations of activin A (0-100 ng/ml) for various periods (5 min-3 days). In each combination, 25 pieces of ectoderm were treated with activin A. More than 80% of the explants in each combination showed a similar morphology after 3 days of culture, which could be classified into four types as shown in Fig. 5. The concentration of activin A required to induce morphological change of the explants was inversely proportional to the duration of exposure of the presumptive ectoderm (Fig. 6). If the presumptive ectoderm was treated with activin A briefly (e.g., 5 min), a high concentration (10 ng/ml) was needed. Conversely, if treated for a long time (e.g., 3 hr), the concentration required was lower (0.25 ng/ml). Morphological change did not occur under 0.25 ng/ml, even if the treatment time was longer than 3 hr.

Histological examination of the activin-treated presumptive ectoderm

Sections of the four typical explants, which were treated with various concentrations of activin A for 3 days, are shown in Fig. 7. All of the control explants formed atypical epidermis, without any mesodermal derivatives. The ventral mesodermal tissues, such as blood-like cells, coelomic epithelium and mesenchyme, differentiated well at a

low concentration of activin A (0.5 ng/ml). At intermediate concentrations (1-10 ng/ml), muscle and neural tissue were induced as well. At a high concentration (50 ng/ml), notochord, the most dorsal mesodermal tissue was induced. Irregular shaped yolk-rich tissues, which are considered to be undifferentiated endoderm, were also formed around notochord in the explant.

The results of histological examination of the activin-treated explants are expressed diagrammatically in Fig. 8. Fig. 8A is the result of examination of the explants that were treated for 5 min, representing a brief treatment of activin A. Fig. 8B is that of 1 day-treatment, representing a long-term treatment. At 1 day-treatment, the minimum concentration of activin A needed for mesodermal differentiation was much lower than that of 5 min-treatment (0.25 ng/ml and 10 ng/ml, respectively). But in both cases, the explants differentiated into ventral to dorsal mesodermal tissues, depending on the concentration of activin A.

Indirect immunofluorescence analysis of muscle differentiation by anti-myosin antibody

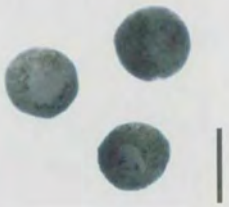
A polyclonal antibody specific for muscle myosin was used to detect muscle differentiation. Sections of the normal larva (stage 41) were examined as a positive control. The antibody clearly stained the muscle region specifically (data not shown). A large area of the explants treated with 10 ng/ml of activin A for 3 days was stained brightly (Fig.

9B) in contrast to the negative staining of the control explants (Fig. 9A). All explants which by histological inspection differentiated into muscle could be stained with anti-myosin antibody.

Figure 4

Morphological change of the presumptive ectoderm after the treatment with activin A

The activin-treated ectoderm rounded up during the first 3 hr (A). At about 6 hr, they began to elongate and showed a pear-shape (B). The degree of elongation depended on the concentration of activin A used. At about 10 hr, the explants treated with 10 ng/ml of activin A (D) elongated more lankily than those of 0.5 ng/ml-treatment (C). The explants treated with 50 ng/ml of activin A began to push internal cells out of the explants (E). Scale bar, 500 μm.



A 3 hr

B 6 hr

C 10 hr (0.5 ng/ml)

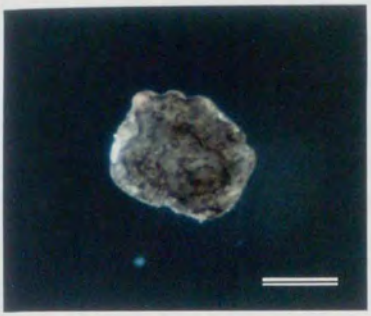
D 10 hr (10 ng/ml)

E 10 hr (50 ng/ml)

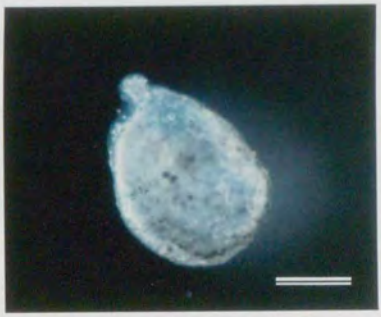
Figure 5

External views of the explants treated with various concentrations of activin A for 3 days

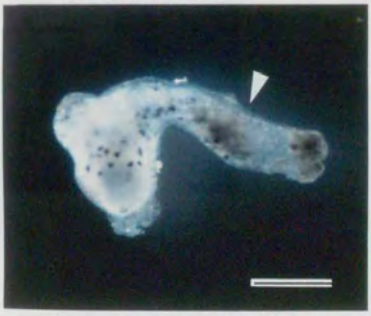
The activin-treated ectoderm underwent four types of morphological change depending on the concentration of activin A. Control explants cultured without activin A rounded up and became wrinkled (A type). At 0.5 ng/ml of activin A, explants showed vesicular forms (B type). At 1-10 ng/ml, explants elongated lankily (*arrow head*) (C type). At 50 ng/ml, explants became fragile giving ragged outline and cells often detached from the surface (*arrow head*) (D type). Scale bar, 200 μm.



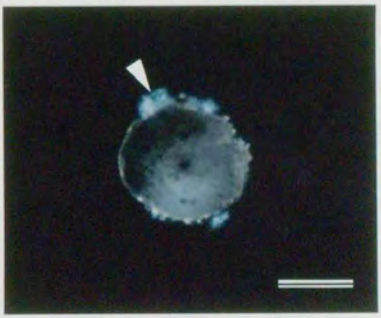
A type: 0 ng/ml



B type: 0.5 ng/ml



C type: 1-10 ng/ml



D type: 50 ng/ml

Figure 6

Correlation between the concentration and duration of activin A treatment on presumptive ectoderm

Twenty-five pieces of presumptive ectoderm were tested in each combination, and the type of explant (A-D type in Fig. 5) usually obtained in each case is shown. The concentration of activin A required to cause morphological change of the explants is inversely proportional to the duration of exposure of presumptive ectoderm.

treatment time of activin A	concentration of activin A (ng/ml)							
	0	0.1	0.25	0.5	1	10	50	100
5 min								
10 min								
30 min								
3 hr								
1 day								
3 days								

: A type, rounded and wrinkled

: B type, smooth and oval

: C type, elongated

: D type, fragile and ragged

Figure 7

Histological sections of the explants treated with various concentrations of activin A for 3 days

(A) Control explants cultured without activin A formed atypical epidermis. (B) Explants treated with 0.5 ng/ml of activin A differentiated into ventral mesodermal tissues, such as coelomic epithelium and blood-like cells. (C) At 10 ng/ml, a large block of muscle accompanied by nonspecific neural tissue was formed. (D) At 50 ng/ml, the most dorsal mesoderm, notochord, was induced surrounded by yolk-rich tissue. No epidermis was formed at the surface of the explants. *bl*, blood-like cells; *epi*, epidermis; *ce*, coelomic epithelium; *mus*, muscle; *not*, notochord; *nt*, nonspecific neural tissue; *yo*, yolk-rich tissue. Scale bar, 100 μm.

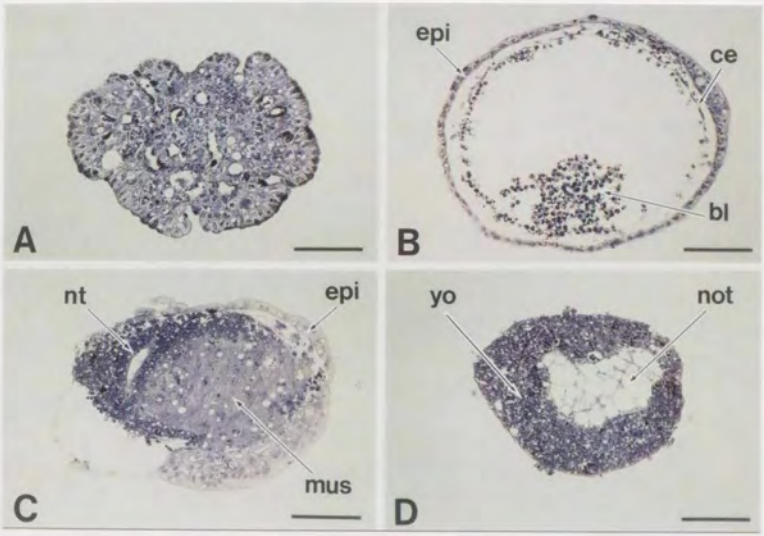

A
epi
ce
bl
B
nt
epi
mus
C
yo
not
D

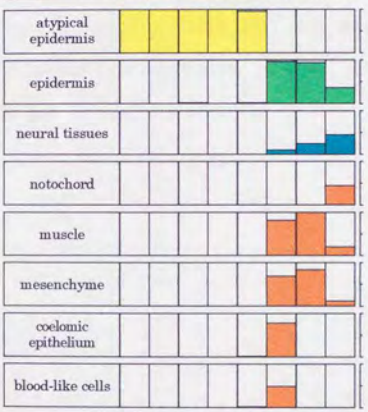
Figure 8

Dose and time-dependent mesoderm induction of activin A on presumptive ectoderm

Bar charts indicate the frequencies of inductions as a percentage of the total. The minimum concentration of activin A to cause mesoderm induction is higher in 5 min-treatment (A) than that in 1 day-treatment (B). Activin A induces various mesodermal tissues from ventral (situated on the bottom side) to dorsal (situated on the top side) in character in a dose-dependent manner.

A. 5 minutes-treatment

activin A (ng/ml)	0	0.1	0.25	0.5	1	10	50	100
no. of specimens	24	25	26	24	25	24	20	26



B. 1 day-treatment

activin A (ng/ml)	0	0.1	0.25	0.5	1	10	50	100
no. of specimens	28	25	26	25	22	26	28	28

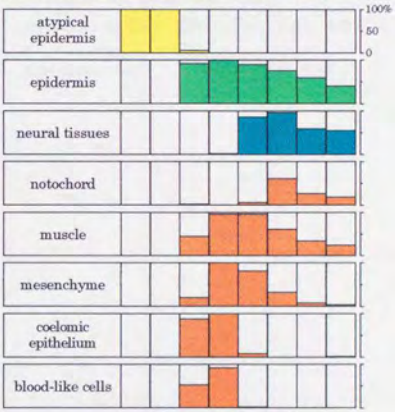
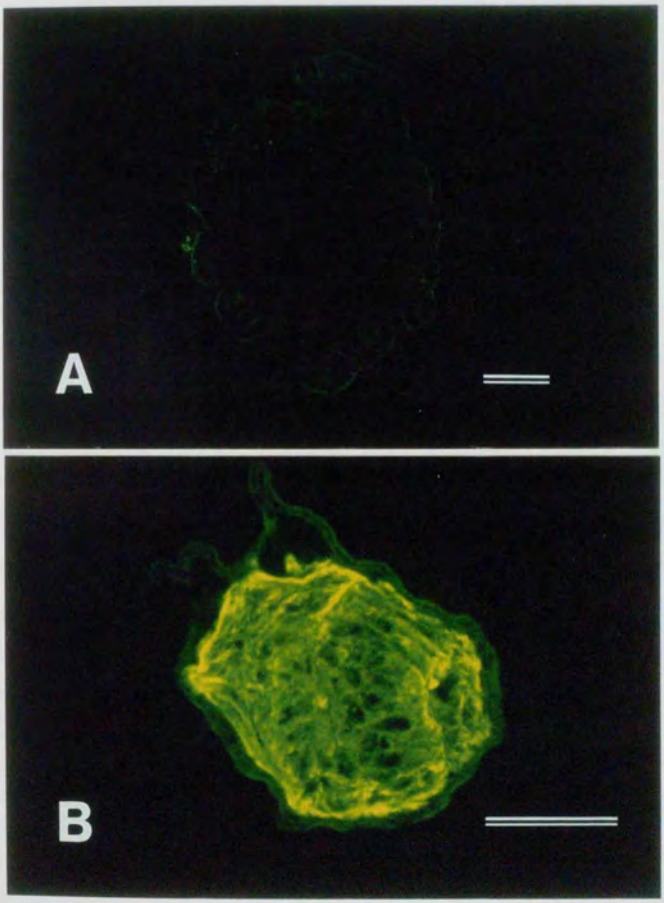


Figure 9

Detection of muscle differentiation by anti-myosin antibody

A large area of the explant treated with 10 ng/ml of activin A for 3 days (B) was stained brightly in contrast to the negative staining of the control explant (A). Scale bar, 100 μm.


A
B

Series 2: Mesoderm-inducing activity of activin A on *Xenopus* presumptive ectoderm of different developmental stages and regions

Typical sections of the explants are shown in Fig. 10. The results of histological examination are expressed diagrammatically in Fig. 11.

Mesoderm-inducing activity of activin A on presumptive ectoderm at different developmental stages

The whole presumptive ectoderm (prospective dorsal + ventral region) dissected at stage 7 (early blastula)-stage 10 (early gastrula) differentiated into various mesodermal tissues (Fig. 11A). The concentration effect of activin A as shown in series 1 was clearly observed in the explants dissected at stage 8 and stage 9. The type of mesodermal tissue induced at high frequencies, however, differed with developmental stages of the presumptive ectoderm. Notochord, most dorsal mesoderm, differentiated well at earlier stages (stage 7-8), whereas coelomic epithelium, one of the ventral mesodermal tissues, was induced well at later stages (stage 8-9) of the presumptive ectoderm.

Mesoderm-inducing activity of activin A on presumptive ectoderm of different regions

At stage 7, both prospective dorsal and ventral halves scarcely differentiated into any mesodermal tissues at 0.5-1 ng/ml of activin A, but they differentiated preferentially

into notochord at 10 ng/ml of activin A (Fig. 11B). Regional differences of the histological differentiation could be seen in the two halves dissected at stage 8-10. At 0.5 ng/ml of activin A, dorsal half of the stage 8 ectoderm differentiated into notochord at 60% of cases, whereas coelomic epithelium was formed at only 20%. In contrast, ventral half did not differentiate into notochord but differentiated into coelomic epithelium at a high frequency (82%) (Fig. 11B). Similar tendency was also observed in the two halves of stage 9 and stage 10 ectoderm when they were treated with 1 ng/ml and 10 ng/ml of activin A, respectively (Fig. 11C). However, these regional differences in the two halves became unclear as the concentration of activin A increased. For example, both halves of stage 8 and stage 9 ectoderm preferentially differentiated into notochord at 10 ng/ml of activin A.

Figure 10

Histological sections of the presumptive ectoderm of different regions treated with activin A

Three patterns of the presumptive ectoderm were dissected at middle blastula (stage 8) according to pigmentation. They were treated with 1 ng/ml of activin A for 1 hr and cultured for 3 days. (A) Control explants never exposed to activin A developed as atypical epidermis. (B) Section of the prospective dorsal + ventral explant. Various ectodermal tissues (cement gland, eye, ear vesicle, nonspecific neural tissue) and mesodermal tissues (mesenchyme, muscle, notochord) were formed. (C) Section of the prospective dorsal half explant. A large cement gland and dorsal mesodermal tissues (notochord and muscle) were differentiated very well. (D) Section of the prospective ventral half explant. Explant showed vesicular form and ventral mesodermal tissues (mesenchyme and coelomic epithelium) were formed. Small amount of muscle also existed between coelomic epithelium and epidermis. *ce*, coelomic epithelium; *cg*, cement gland; *ev*, ear vesicle; *mes*, mesenchyme; *mus*, muscle; *not*, notochord; *nt*, nonspecific neural tissue. Scale bar, 200 μm.

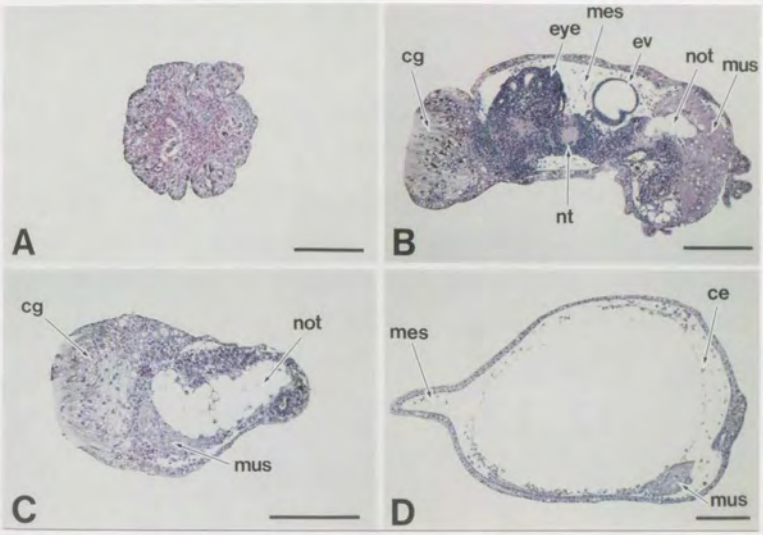

A
B
mes
eye
ev
cg
not
mus
nt
C
cg
not
mus
D
ce
mes
mus

Figure 11

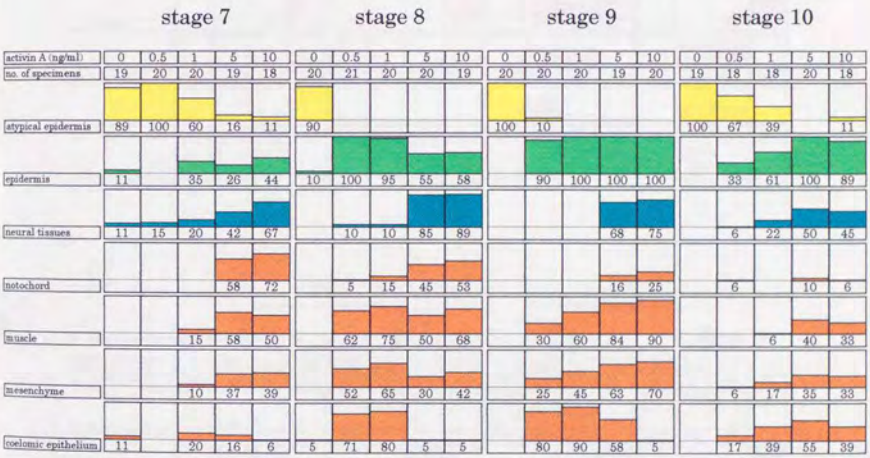
Differentiation of the presumptive ectoderm of different developmental stages and regions after the treatment with activin A

Bar charts and values indicate the frequencies of inductions as a percentage of the total. (A) Differentiation of the whole presumptive ectoderm (prospective dorsal + ventral region). (B),(C) Differentiation of the prospective dorsal and ventral halves of presumptive ectoderm.

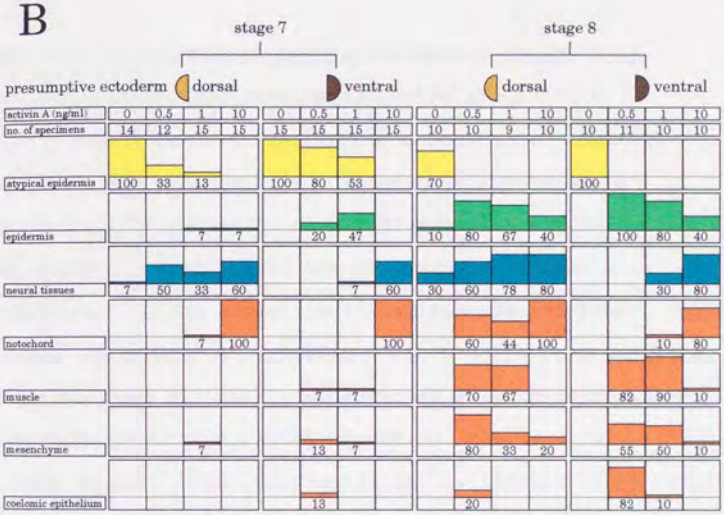
A

presumptive ectoderm

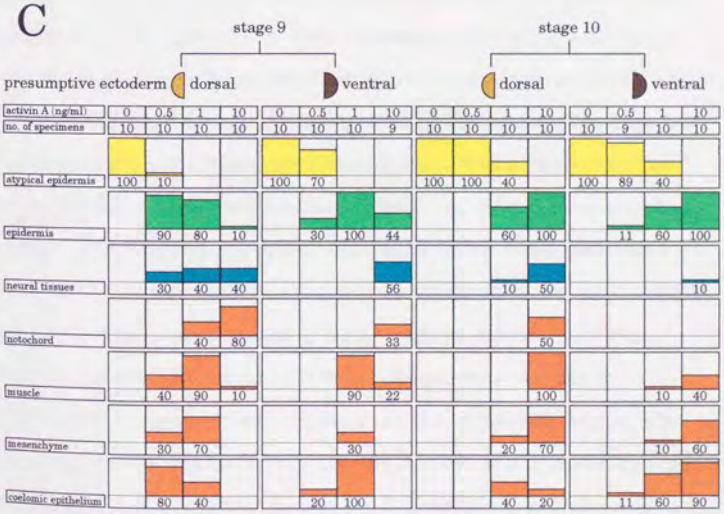
dorsal+ventral



B


stage 7
stage 8
presumptive ectoderm
dorsal
ventral
dorsal
ventral
activin A (ng/ml)
0 0.5 1 10 0 0.5 1 10 0 0.5 1 10 0 0.5 1 10
no. of specimens
14 12 15 15 15 15 15 15 10 10 9 10 10 11 10 10
atypical epidermis
100 33 13 100 80 53 70 100
epidermis
7 7 20 47 10 80 67 40 100 80 40
neural tissues
7 50 33 60 7 60 30 60 78 80 30 80
notochord
7 100 100 60 44 100 10 80
muscle
7 7 70 67 82 90 10
mesenchyme
7 13 7 80 33 20 55 50 10
coelomic epithelium
13 20 82 10

C


stage 9
stage 10
presumptive ectoderm
dorsal
ventral
dorsal
ventral
activin A (ng/ml)
0 0.5 1 10 0 0.5 1 10 0 0.5 1 10 0 0.5 1 10
no. of specimens
10 10 10 10 10 10 10 9 10 10 10 10 10 9 10 10
atypical epidermis
100 10 100 70 100 100 40 100 89 40
epidermis
90 80 10 30 100 44 60 100 11 60 100
neural tissues
30 40 40 56 10 50 10
notochord
40 80 33 50
muscle
40 90 10 90 22 100 10 40
mesenchyme
30 70 30 20 70 10 60
coelomic epithelium
80 40 20 100 40 20 11 60 90

Discussion

Dose and time-dependent mesoderm-inducing activity of activin A on *Xenopus* presumptive ectoderm

In the present study, it was demonstrated that the minimum concentration of activin A required to cause mesoderm differentiation of the presumptive ectoderm was inversely proportional to the duration of activin A treatment. Activin signal is transmitted to the target cells through the action of specific receptors (Mathews and Vale, 1991; Kondo et al., 1991; Attisano et al., 1992; Hemmati-Brivanlou and Melton, 1992; Hemmati-Brivanlou et al., 1992; Mathews et al., 1992; Nishimatsu et al., 1992). If we assume whether mesoderm induction occurs or not is determined by the number of activin molecules binding to its predicted receptor-site, the above result seems very plausible.

It was also shown that activin A induced several kinds of mesodermal tissues in the presumptive ectoderm in a gradient fashion. Similar results have been reported by Grunz (1983) using vegetalizing factor, and Green et al. (1990, 1992) using XTC-MIF. These factors have now been shown to be identical with activin (Asashima et al., 1990b, 1991a, c; Tiedemann et al., 1992; Smith et al., 1990; Eijnden-Van Raaij et al., 1990). Moreover, no clear difference in induction properties is observed among the three types of activin (A, AB, B). They all induce several different mesodermal tissues in a gradient fashion, as

described in the present study (Nakamura et al., 1992; Fukui et al., 1993). Follistatin, an activin-specific binding protein (Ueno et al., 1987; Nakamura et al., 1990), inhibits the mesoderm-inducing activity of activin A in a dose-dependent manner (Asashima et al., 1991a). At high concentrations of activin A, dorsal mesodermal tissues, such as muscle and notochord, are induced in presumptive ectoderm. When follistatin is added to activin A solution, the mesoderm-inducing activity of activin A is weakened and the type of tissue induced shifts from dorsal mesoderm to ventral mesoderm.

These results of *in vitro* studies suggest that the type of mesodermal tissue is determined by a concentration gradient of activin. Follistatin may also contribute to forming this gradient by its regulatory effect on the inducing activity of activin. The concentration gradient of the mesoderm-inducing factor (mesodermalizing agent) is assumed in the "two-gradient hypothesis" (Toivonen and Saxén, 1955a, b). In this theory, the mesodermalizing agent is concentrated in the posterior dorsal end of the embryo and induces various kinds of mesodermal tissues along the dorso-ventral axis in a gradient fashion. Although activin protein is present in early *Xenopus* embryos (Asashima et al., 1991b; Fukui et al., 1994), this putative concentration gradient, however, has not been confirmed. Another potent mesoderm-inducing factor, bFGF, preferentially induces ventral mesoderm *in vitro* (Slack et al., 1987). bFGF exists in early *Xenopus* embryos in the form of both mRNA and

protein (Kimelman et al., 1988; Slack and Isaacs, 1989), and bFGF protein is localized in the presumptive marginal zone (Shiurba et al., 1991). Moreover, evidence that FGF plays a very important role in *Xenopus* embryogenesis has been accumulated during the past few years (Musci et al., 1990; Amaya et al., 1991, 1993; Isaacs et al., 1992). Although several kinds of mesodermal tissues can be induced by the activin gradient *in vitro*, it may be impossible that the patterning of mesoderm during normal development is also established by this gradient alone. Further study will be needed to determine how many factors are required for complete mesoderm formation *in vivo*.

Competence of *Xenopus* presumptive ectoderm to respond to activin A

Presumptive ectoderm (prospective dorsal + ventral region) at each stage differentiated into various mesodermal tissues depending on the concentration of activin A. However, presumptive ectoderm at stage 7 tended to form notochord frequently even at 5 ng/ml of activin A (58%, Fig. 11A), whereas that at stage 10 scarcely formed it. As shown in series 1, higher concentrations of activin A are required to induce notochord. Conversely, the ability of presumptive ectoderm to form notochord at low concentrations seems to reflect its high competence to respond to activin A. Supposing that the mesoderm induction takes place at earlier cleavage stages (Nieuwkoop, 1969; Nakamura and Takasaki, 1970; Nakamura et al., 1971; Asashima, 1975), the above

result that competence of presumptive ectoderm to respond to activin A is highest at stage 7 (early blastula) is consistent with the early *Xenopus* embryogenesis.

It was also shown that prospective dorsal and ventral halves of presumptive ectoderm, except those at stage 7, responded differently to the same concentration of activin A. At stage 7, both halves differentiated into notochord at a high concentration of activin A (10 ng/ml, Fig. 11B). If activin A forms a gradient along dorso-ventral axis, the reacting tissue at the dorsal side, where activin A is concentrated, can form dorsal mesoderm (notochord). On the other hand, the reacting tissue at the ventral side never forms dorsal mesoderm because of lower concentrations of activin A. Thus, the above result seems plausible if we assume a concentration gradient of activin A. The two halves dissected at stage 8-10 responded differently to the same concentration of activin A. Prospective dorsal half tended to form dorsal mesoderm, whereas prospective ventral half formed ventral mesoderm. Again, if we assume a concentration gradient of activin A, these results can be explained as follows. The reacting tissue at the ventral side can not form any mesodermal derivatives at earlier stage (stage 7) because of lower concentrations of activin A. However, it becomes to obtain the competence to respond to lower concentrations of activin A as its developmental stage progresses (stage 8-10) and differentiates into ventral mesoderm. On the other hand, the reacting tissue at the dorsal side can form dorsal mesoderm because of its high

competence and higher concentrations of activin A. Therefore, differences in competence between prospective dorsal and ventral halves of presumptive ectoderm are considered to reflect the assistant role of the reacting tissues for the gradient of mesoderm-inducer.

Sokol and Melton (1991) reported that prospective dorsal and ventral halves of presumptive ectoderm of *Xenopus* blastulae (stage 6.5-9.5) responded differently to the same concentration of PIF, which is an activin A homologue derived from the mouse macrophage cell line (Sokol et al., 1990; Thomsen et al., 1990). Prospective dorsal half differentiated into dorsoanterior structures, such as eye, neural tissue and notochord, whereas the ventral half preferentially differentiated into ventrolateral structures, such as muscle and mesenchyme. In their report, these regional differences could be seen at all stages of presumptive ectoderm used and ventral half never formed dorsoanterior structures even at high concentrations. Moreover, they examined the interaction of *Wnt* and activin A in the dorsal mesoderm induction (Sokol and Melton, 1992). The ventral half of presumptive ectoderm injected with *Xwnt-8* mRNA did not differentiate into ventrolateral structures but into dorsoanterior structures when treated with PIF. These results suggest that "modifiers" such as *Wnt* gene products, *noggin* and lithium can change the response of the reacting tissues to the mesoderm-inducer. Although there are some differences between their results and the results shown in the present study, the patterning of mesoderm should be

discussed from dual aspects: the localization of the mesoderm-inducer(s) and the differential competence of the reacting tissues.

Neural and endodermal differentiation of the *Xenopus* activin-treated ectoderm

In the present study, considerable amounts of nonspecific neural tissues were observed in the activin-treated ectoderm at high concentrations of activin A. These tissues were never induced without mesodermal tissues, such as notochord and muscle. Therefore, it is impossible to assume that activin A acts as a neural inducing factor on presumptive ectoderm. These neural tissues are considered to be induced in the non-induced cells of the explants by the mesodermalized cells as a secondary interaction (Suzuki et al., 1986). This possibility is further supported by the results of sandwich experiments in which most of the neural tissues differentiated from the surrounding untreated ectoderm, not from the inner activin-treated ectoderm (chapter 3).

On the other hand, irregular shaped yolk-rich tissues were often induced around notochord when presumptive ectoderm was treated with high concentrations of activin A. Although these tissues can be considered undifferentiated endoderm from their histological features, they never differentiated into typical endodermal tissues, such as pancreas, liver and intestine. As shown in chapter 2, yolk-rich tissues are also induced in the *Cynops* activin-treated

ectoderm at high concentrations of activin A. Mesodermal tissues, such as notochord and muscle, are induced as well in the explants, however, the frequencies are relatively low. These yolk-rich tissues are identified as endoderm because they often exhibit a columnar appearance, a characteristic of endodermal epithelium in the alimentary canal of normal embryos. Thus, it is likely that activin A acts as a vegetalizing (endoderm-inducing) factor, not a mesoderm-inducing factor, on *Cynops* presumptive ectoderm, and that mesodermal tissues seem to be induced in the non-induced cells by the endodermalized cells as a secondary interaction (Grunz, 1979; Minuth and Grunz, 1980). Jones et al. (1993) have identified endodermal tissues in *Xenopus* ectoderm treated with XTC-MIF (*Xenopus* activin A) using a monoclonal endodermal marker. Although the existence of typical endodermal tissues was not confirmed at histological level in the present study, activin A may also have a vegetalizing effect on *Xenopus* ectoderm. Further study will be needed to confirm the vegetalizing effect of activin A on *Xenopus* early embryogenesis.

Summary

Human recombinant activin A, which is identical with erythroid differentiation factor (EDF), was tested for its mesoderm-inducing activity using presumptive ectoderm of *Xenopus* late blastula as the reacting tissue. The concentration of activin A required to induce mesodermal tissues was inversely proportional to the duration of exposure of the presumptive ectoderm. Depending on the concentration of activin A, several different mesodermal tissues from ventral to dorsal in character were induced in ectodermal explants at clear dose thresholds. At low concentrations of activin A, ventral mesodermal tissues, such as blood-like cells, coelomic epithelium and mesenchyme, were induced. At intermediate concentrations, muscle accompanied by nonspecific neural tissues was induced. At high concentrations, notochord, the most dorsal mesoderm was induced.

To examine the competence of reacting tissues to respond to the mesoderm-inducing factor, presumptive ectoderm of different developmental stages and regions were treated with activin A. The most dorsal mesoderm, notochord, tended to differentiate at high frequencies in earlier stages of ectoderm. Furthermore, prospective dorsal half of presumptive ectoderm tended to differentiate into notochord, whereas ventral half form ventral mesodermal tissues, such

as mesenchyme and coelomic epithelium, at the same concentration of activin A.

These results suggest that activin A acts as an intrinsic inducing molecule responsible for establishing the dorso-ventral axis in early *Xenopus* development. There are following two possibilities about the patterning of mesoderm. (1) Activin A forms a concentration gradient in the early *Xenopus* embryo. (2) There exist stage- and regional differences in competence to respond to activin A within the reacting tissues.

Chapter 2

Induction Property of Activin A on *Cynops* Presumptive Ectoderm: Vegetalization and Heart Formation of Presumptive Ectoderm by Activin A

Introduction

After the discovery that previously characterized peptide growth factors belonging to the FGF and TGF-ß families are capable of mesoderm induction, the study of embryonic induction has expanded rapidly (reviewed by Tiedemann, 1990; Dawid et al., 1992; Asashima, 1994; Ariizumi and Asashima, 1995b). Most of these recent studies have focused on the anuran, *Xenopus laevis*, embryos because of their advantages for biochemical and molecular biological studies. However, the embryonic induction was originally discovered on the urodele embryos (Spemann and Mangold, 1924) and significant theories about embryonic induction are based on the experiments using urodele embryos (reviewed by Nakamura and Toivonen, 1978; Hamburger, 1988). Although the urodele embryos have several shortcomings, such as the paucity of molecular markers, they have been good materials for the microsurgery and histological analysis *in vitro*. The pluripotent presumptive ectoderm of the urodele, *Cynops pyrrhogaster*, embryos is made up of a single cell layer, and is more homogeneous than that of *Xenopus laevis* (Asashima and Grunz, 1983). Thus, it appears that for *in vitro* studies the urodele might be a more amenable amphibian model system. There are, however, few reports about the effect of the peptide growth factors on the urodele embryos (Moriya and Asashima, 1992).

Urodele heart development also provides favorable features for studying embryonic induction. The induction phenomenon it exhibits has been well characterized (reviewed by Jacobson and Sater, 1988). Anterior pharyngeal endoderm contacts prospective cardiogenic mesoderm and stimulates morphogenesis and differentiation. This is a long process, which is initiated in urodeles certainly by the stage of neurulation, and likely much earlier. The tissue displacements and morphogenesis (e.g., folding) displayed during cardiogenesis are profound, and easily monitored. Differentiation can be easily assayed, since the cardiac muscle proteins are well characterized and cytodifferentiation is also relatively easy to recognize.

In this chapter, the induction property of activin A on *Cynops* ectoderm was investigated employing various concentrations of activin A and various sizes of presumptive ectoderm. The activin-treated ectoderm itself differentiated into endodermal tissues and induced mesodermal tissues, including beating heart rudiments, from non-induced ectoderm as a secondary interaction. The beating hearts exhibited a substantial array of the cytodifferentiated and functional properties of a naturally (*in vivo*) developing heart. These results suggest a novel role of activin A in the vegetalization and heart formation during early *Cynops* embryogenesis. It is likely that activin-treated *Cynops* explants will be a useful model for a variety of studies on the embryonic induction and heart development.

Materials and Methods

Eggs and Embryos

Eggs of *Cynops pyrrhogaster* were obtained by injecting females with two 100 IU doses of human chorionic gonadotropin (Gestron; Denka Seiyaku, Japan) every other day. Staging of embryos was according to Okada and Ichikawa (1947). Embryos were sterilized in 70% ethanol for 30 sec and then quickly washed with Holtfreter's solution (HS; 60 mM NaCl, 0.7 mM KCl, 0.9 mM $CaCl_2$, 4.6 mM HEPES at pH 7.6) with 0.1 g/l kanamycin sulfate (Banyu, Japan) at least five times. The jelly coat and vitelline membrane were manually removed with a pair of watchmaker's tweezers under a binocular microscope.

Activin A solution

Human recombinant activin A (EDF, erythroid differentiation factor; Murata et al., 1988) was used as the inducer in all experimental series. Activin A was dissolved in HS containing 0.1% bovine serum albumin (BSA, A-7888; Sigma, USA) at concentrations of 0-100 ng/ml. Activin A solutions were put in polystyrene dishes (Tissue Culture Dish 25000; Iwaki Glass, Japan).

Experimental series

All of the following procedures were carried out under sterile conditions. Schematic diagrams of the procedures and culture methods are shown in Fig. 12.

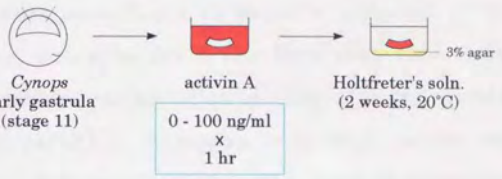
Series 1

Presumptive ectodermal sheets (1.0 x 1.0 mm) cut from early gastrulae (stage 11, about 2.3 mm in diameter) were treated with activin A solutions (0, 0.1, 0.5, 1, 5, 10, 50 and 100 ng/ml) for 1 hr with the inner blastocoelic surface facing up. After washing in two changes of HS with gentle pipetting, they were cultured for 2 weeks at 20°C in 3% agar-coated culture dishes (SUMILON 24-well plate, MS-80240; Sumitomo Bakelite, Japan) filled with HS.

Series 2

In the isolation-culture mode, presumptive ectodermal sheets of early gastrulae (st. 11) were cut out in size of 0.6 x 0.6 mm, 0.9 x 0.9 mm, 1.2 x 1.2 mm and 1.5 x 1.5 mm. They were then treated with 0 and 100 ng/ml of activin A solutions for 1 hr. In the combination-culture mode, the activin-treated ectoderm (0.6 x 0.6 mm) was combined with untreated ectoderm of early gastrulae in size of 0.6 x 0.6 mm, 0.9 x 0.9 mm, 1.2 x 1.2 mm and 1.5 x 1.5 mm. In the sandwich-culture mode, the activin-treated ectoderm (0.6 x 0.6 mm) was sandwiched between two sheets of untreated ectoderm of early gastrulae (1.2 x 1.2 mm). They were cultured in HS for 2-5 weeks as described in series 1.

Series 1



Series 2

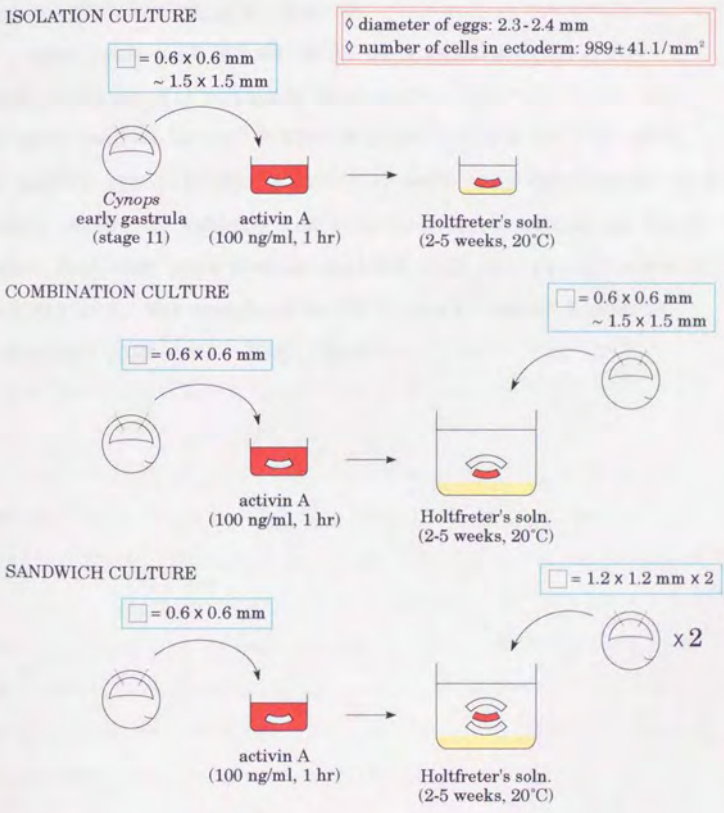


Figure 12

Schematic diagrams of the procedures and culture methods

Histological examination

Specimens were fixed in Bouin's solution or Susa (Heidenhain) solution for 3 hr. They were then dehydrated through a graded series of ethanols, cleared in xylene, embedded in paraffin (Histprep 568; Wako, Japan) and sectioned at 6-8 μm. Sections were stained with Delafield's hematoxylin/eosin.

Transmission electron microscopy

Specimens were fixed in 3% paraformaldehyde/2.5% glutaraldehyde/0.1 M cacodylate buffer (pH 7.4) for 1 day, and then washed in the buffer and post-fixed in 1.0% OsO_4 and buffer (pH 7.4) for 2 hr. They were then dehydrated in a graded series of ethanol and acetone, and embedded in epoxy resin. Sections were double-stained with uranyl acetate and lead citrate, and examined under a transmission electron microscope (JEM-100C; JEOL, Japan).

Results

Series 1: Inducing activity of activin A on *Cynops* presumptive ectoderm

Typical sections of the explants are shown in Fig. 13. The results of histological examination are expressed diagrammatically in Fig. 14.

All of the control explants cultured without activin A formed irregular-shaped epidermis referred to as atypical epidermis (Fig. 13A). Most explants treated with 0.1 ng/ml of activin A also formed atypical epidermis. As the concentration of activin A increased, the frequency of atypical epidermis decreased gradually.

At concentrations higher than 0.1 ng/ml, several kinds of ectodermal and mesodermal tissues were induced in the explants. However, the concentration effect of activin A shown on *Xenopus* ectoderm (chapter 1) was not clearly observed in *Cynops* ectoderm. There was not an optimum concentration of activin A for the differentiation of a certain tissue. Regardless of the concentration of activin A, epidermis and mesenchyme were induced constantly at about 20% and 15% of the cases, respectively. Nonspecific neural tissues and mesodermal tissues, such as notochord, muscle and blood cells, were also induced in the explants, but the percentages were very low (less than 5%).

The explants treated with higher concentrations of activin A (more than 5 ng/ml), on the other hand, exclusively formed a large block of yolk-rich endodermal cells (Fig. 13D). They were often surrounded by endodermal epithelium exhibiting a columnar appearance, a characteristic found in the alimentary canal of normal embryos (Fig. 13E). In the explants treated with lower concentrations of activin A (0.5-5 ng/ml), small amounts of yolk-rich endodermal tissues were also induced, and were usually accompanied by mesodermal tissues, such as blood cells and muscle (Fig. 13B, C).

Figure 13

Histological sections of the presumptive ectoderm treated with various concentrations of activin A

(A) Control explants cultured without activin A formed atypical epidermis with wrinkled surfaces. (B) At 0.5 ng/ml, activin A induced presumptive ectoderm to form smooth surfaces. Blood cells were induced in the explants, and were usually accompanied by yolk-rich endodermal tissues. (C) At 1 ng/ml of activin A, explants differentiated into dorsal mesodermal tissues, such as muscle and notochord, which were also accompanied by yolk-rich endodermal tissues. (D) The presumptive ectoderm treated with 100 ng/ml of activin A differentiated exclusively into a large block of endodermal cells. These cells were rich in yolk granules. (E) The activin-treated ectoderm, which differentiated into yolk-rich endodermal tissue, was often surrounded by columnar-shaped endodermal epithelium (*small arrows*). *bl*, blood cells; *ee*, endodermal epithelium; *epi*, epidermis; *mus*, muscle; *not*, notochord; *yo*, yolk-rich endodermal tissue. Scale bar, 100 μm.

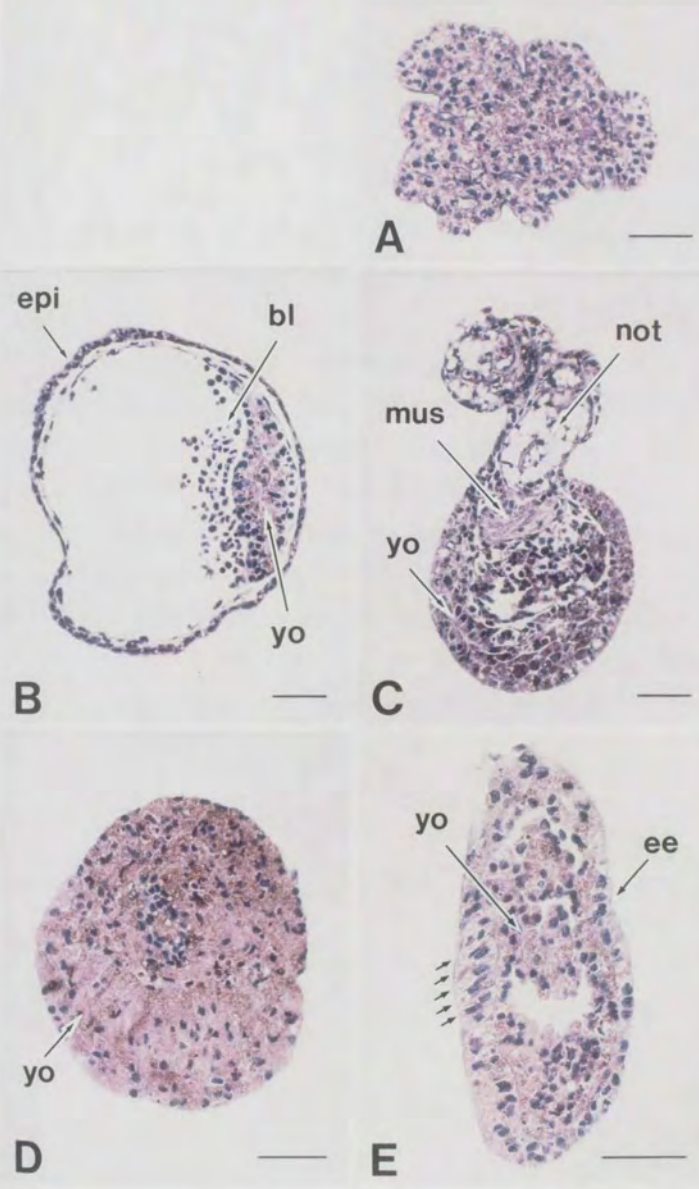
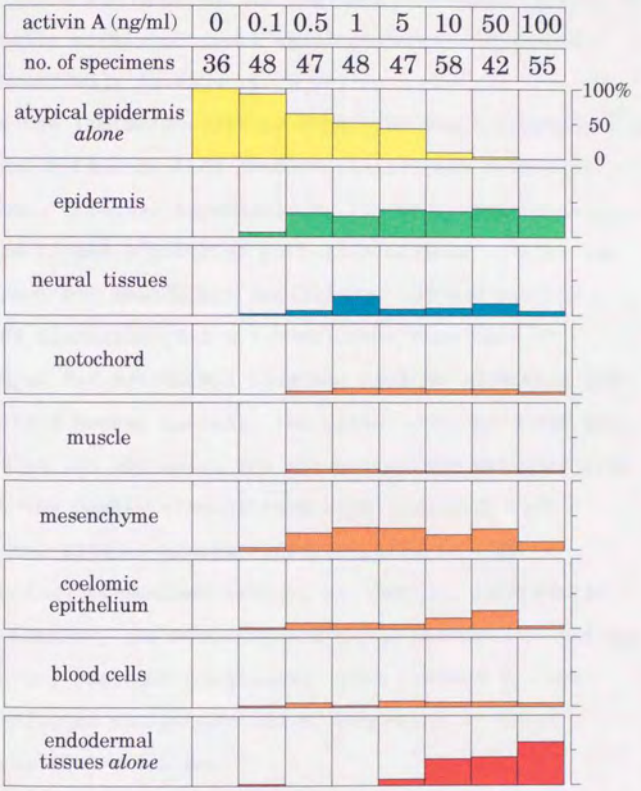

A
epi
bl
yo
B
not
mus
yo
C
yo
D
yo
ee
E

Figure 14

Inducing activity of activin A on *Cynops* presumptive ectoderm

Bar charts indicate the frequencies of inductions as a percentage of the total. Dose-dependent mesoderm induction by activin A shown on *Xenopus* ectoderm (Fig. 8) was not clearly observed in *Cynops* ectoderm. Several kinds of ectodermal and mesodermal tissues were induced in the explants at concentrations higher than 0.1 ng/ml, but the percentages were relatively low. At 10-100 ng/ml of activin A, the activin-treated ectoderm differentiated exclusively into a large block of yolk-rich endodermal cells.


activin A (ng/ml)
0
0.1
0.5
1
5
10
50
100
no. of specimens
36
48
47
48
47
58
42
55
atypical epidermis *alone*
epidermis
neural tissues
notochord
muscle
mesenchyme
coelomic epithelium
blood cells
endodermal tissues *alone*
100%
50
0

Series 2: Vegetalization and heart formation of presumptive ectoderm of different sizes by activin A

Vegetalization of presumptive ectoderm of different sizes by activin A

Typical sections of the explants are shown in Fig. 15. The results of histological examination are expressed diagrammatically in Fig. 16.

In the isolation-culture mode, the small explants (0.6 x 0.6 mm) differentiated preferentially into yolk-rich endodermal tissues. Approximately 50% of activin-treated ectoderm formed a block of yolk-rich tissues without any ectodermal and mesodermal derivatives. As the size of explants increased (1.2 x 1.2 mm), the frequency of mesenchyme and ectodermal tissues, such as epidermis and nonspecific neural tissues, increased. Notochord was also induced in the explants, but the percentage was very low.

In the combination-culture mode, the yolk-rich endodermal tissues always differentiated at high frequencies. Ectodermal tissues (epidermis, nonspecific neural tissues) and mesodermal tissues (notochord, muscle, mesenchyme, coelomic epithelium) were induced at high frequencies as the proportion of untreated ectoderm increased (1.2 x 1.2 mm).

In the sandwich-culture mode, explants often showed trunk-tail formation with axial structures, such as central nervous system, notochord and muscle. The yolk-rich endodermal tissues were also found in these explants and

differentiated into intestine at about 30% of the cases. Typical cytodifferentiation pattern expected of a liver and intestine was confirmed in the 5-week old sandwich cultures under the transmission electron microscopy (Fig. 17).

Heart formation of presumptive ectoderm by activin A

An external view of a 2-week old isolation culture (1.5 x 1.5 mm) that contains a beating heart-like rudiment inside is shown in Fig. 18A. The formation of heart rudiment was confirmed in a histological section (Fig. 18B). At 2-weeks, the sandwich cultures formed trunk-tails, with the beating-heart rudiment inside (Fig. 18C, D). In the isolation-culture mode, the frequency of heart formation depended on the size of presumptive ectoderm. Beating-heart rudiments were observed more frequently in the plants in size of 1.5 x 1.5 mm as compared with those of 0.9 x 0.9 mm (Table 1). In the combination-culture mode, beating-heart rudiments were formed at high frequencies as the proportion of untreated ectoderm increased. In the sandwich-culture mode, approximately 20% of activin-treated explants developed a beating heart (Table 1). In these three modes, control explants never exposed to activin A developed as atypical epidermis and never formed a beating heart (Fig. 18E, F).

The heartbeat in 4-week old isolation cultures was counted at various temperatures from 5°C to 30°C (Fig. 19). It was regular, and displayed the temperature-dependent frequency expected of a normal, beating *Cynops* embryonic heart (Taguchi et al., 1989; Uehara et al., 1989). These

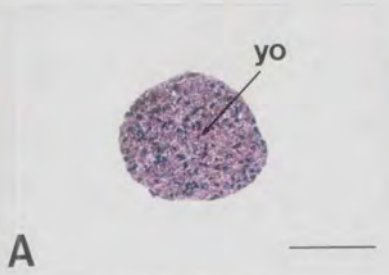
beating characteristics were observed in virtually all of the cases scored as "beating heart". That is, a beating heart was the sole criterion for scoring an explant culture as positive for heart development.

Five-week old sandwich cultures with a beating heart, examined with the transmission electron microscope, exhibited the cytodifferentiation pattern expected of a functioning heart, as shown in Fig. 20.

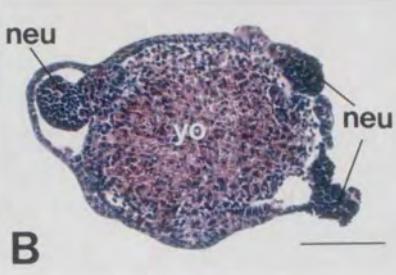
Figure 15

Histological sections of the presumptive ectoderm of different sizes treated with activin A

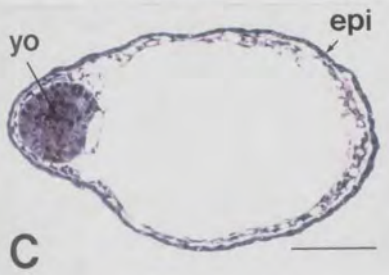
(A) The small isolation cultures (0.6 x 0.6 mm) differentiated preferentially into yolk-rich endodermal tissues. (B) Nonspecific neural tissues were also induced in the large isolation cultures (1.2 x 1.2 mm). (C) The yolk-rich endodermal tissues always differentiated at high frequencies in the combination-culture mode. (D) Mesodermal tissues, such as notochord, muscle and mesenchyme, were induced as the proportion of untreated ectoderm increased. (E) Sandwich cultures often showed trunk-tail formation with axial structures, such as notochord, muscle and central nervous system. Intestine consisted of yolk-rich columnar cells were also induced in these explants. (F) Control cultures never exposed to activin A became wrinkled, and developed as atypical epidermis. *AE*, activin-treated ectoderm; *UE*, untreated ectoderm; *br*, brain; *epi*, epidermis; *int*, intestine; *mes*, mesenchyme; *mus*, muscle; *neu*, nonspecific neural tissue; *not*, notochord; *yo*, yolk-rich endodermal tissue. Scale bar, 200 μm.



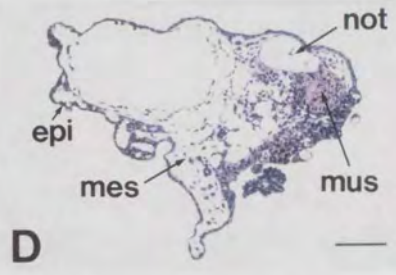
ISOLATION - small -
AE: 0.6 x 0.6 mm



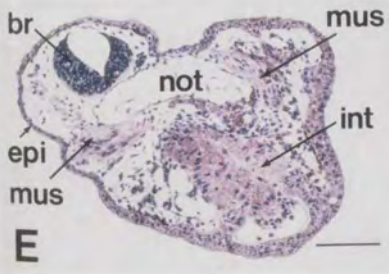
ISOLATION - large -
AE: 1.2 x 1.2 mm



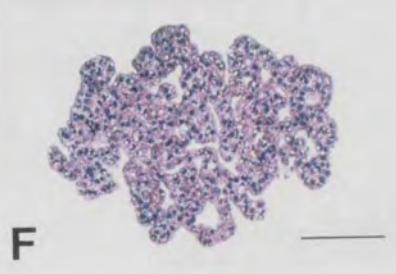
COMBINATION - small -
AE: 0.6 x 0.6 mm
UE: 0.6 x 0.6 mm



COMBINATION - large -
AE: 0.6 x 0.6 mm
UE: 1.2 x 1.2 mm



SANDWICH
AE: 0.6 x 0.6 mm
UE: 1.2 x 1.2 mm x 2



CONTROL
UE: 1.2 x 1.2 mm

Figure 16

Inducing activity of activin A on *Cynops* presumptive ectoderm of different sizes

Bar charts indicate the frequencies of inductions as a percentage of the total. The yolk-rich endodermal tissues differentiated at high frequencies in each culture mode. In the large isolation cultures (1.2 x 1.2 mm), several kinds of ectodermal and mesodermal tissues were induced in addition to endodermal tissues. These tissues were induced well in the combination-culture mode as the proportion of untreated ectoderm increased. In the sandwich-culture mode, most explants formed typical trunk-tails with axial structures (CNS, notochord, muscle) and intestine.

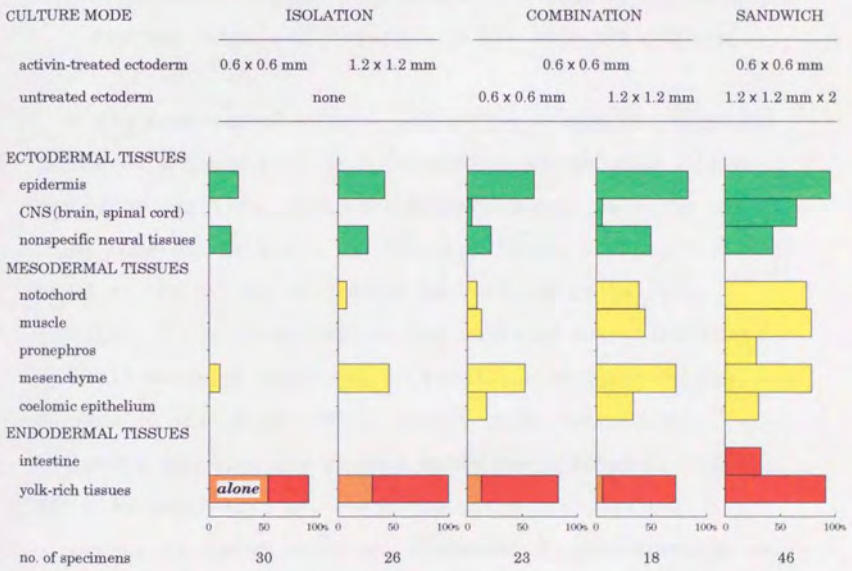

CULTURE MODE
ISOLATION
COMBINATION
SANDWICH
activin-treated ectoderm
0.6 x 0.6 mm
1.2 x 1.2 mm
0.6 x 0.6 mm
0.6 x 0.6 mm
untreated ectoderm
none
0.6 x 0.6 mm
1.2 x 1.2 mm
1.2 x 1.2 mm x 2
ECTODERMAL TISSUES
epidermis
CNS (brain, spinal cord)
nonspecific neural tissues
MESODERMAL TISSUES
notochord
muscle
pronephros
mesenchyme
coelomic epithelium
ENDODERMAL TISSUES
intestine
yolk-rich tissues
alone
0
50
100%
no. of specimens
30
26
23
18
46

Figure 17

Transmission electron microscopy of 5-week old activin-induced liver and intestine in the sandwich culture

(A) Portions of hepatic cells from 5-week old sandwich cultures. A large pool of glycogen has accumulated in the cytoplasm. Well-developed rough endoplasmic reticulum and Golgi body can be seen. The bile capillary, a characteristic found in the liver, is present between two cells. (B) Portions of absorptive epithelial cells of intestine from 5-week old sandwich cultures. Microvilli have lined on the surface of epithelial cells. Goblet cells containing secretory granules are present under the epithelial cells. *BC*, bile capillary; *ER*, rough endoplasmic reticulum; *G*, glycogen; *Go*, Golgi body; *Ly*, lysosome; *M*, mitochondria; *Mv*, microvilli; *PG*, pigment granule; *SG*, secretory granule. Scale bar, 1 μm.

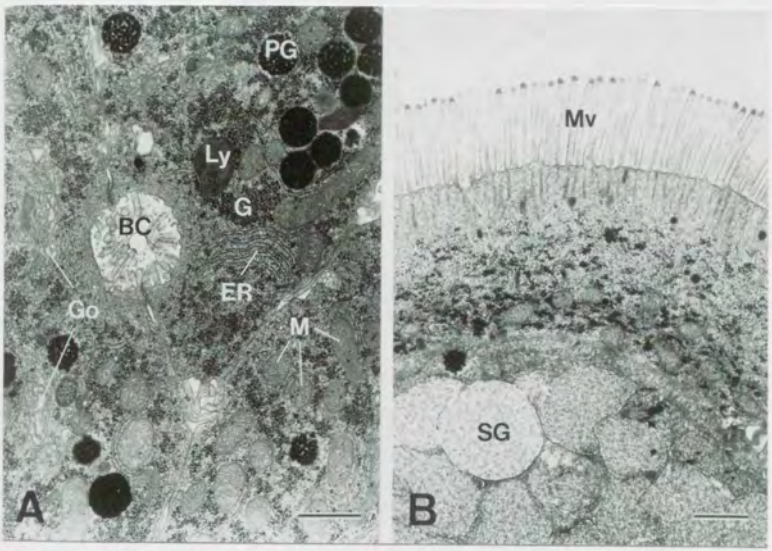

PG
Ly
G
BC
ER
Go
M
A
Mv
SG
B

Figure 18

External views and histological sections of 2-week old cultures with beating heart rudiments

(A) An isolation culture showing a beating heart-like rudiment in its interior (B). (C) A sandwich culture showing trunk-tail formation, with a beating heart rudiment inside (D). (E) Control cultures never exposed to activin A became wrinkled, and developed as atypical epidermis (F). *arrow head*, heart rudiment; *br*, brain; *epi*, epidermis; *ev*, ear vesicle; *mus*, muscle; *not*, notochord; *yo*, yolk-rich endodermal tissue. Scale bar, 200 μm.

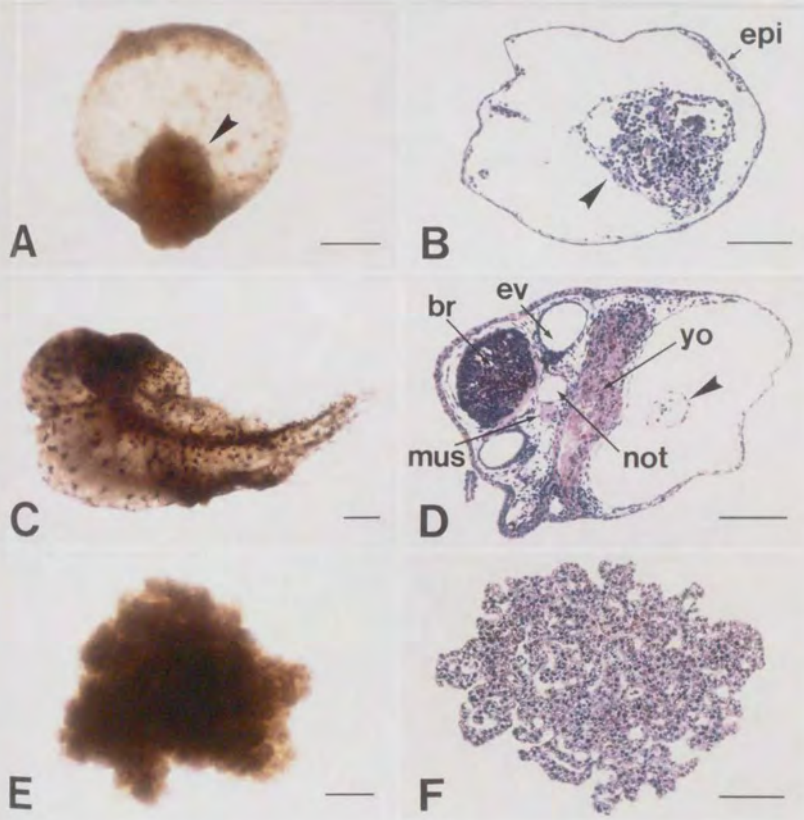

epi
br
ev
yo
mus
not
A
B
C
D
E
F

Table 1. Heart formation of *Cynops* presumptive ectoderm by activin A

culture mode	isolation		combination		sandwich
AE* (mm x mm)	0.9 x 0.9	1.5 x 1.5	0.6		0.6
UE** (mm x mm)	none		0.9 x 0.9	1.5 x 1.5	1.2 x 1.2 x 2
no. of specimens	33	33	19	20	24
% of heart formation	3.0	12.1	5.3	10.0	20.8

* activin-treated ectoderm
** untreated ectoderm

Figure 19

Measurement of pulsation of heart rudiment developed from activin-treated ectoderm after 4-week culture period

The beat frequency was measured at various temperatures from 5°C to 30°C. Control larvae (stage 47) and explants were incubated for 30 min at each temperature before heart rate was measured. Each point represents 12 larvae and 12 explants with a beating heart. Bars show the S.E.

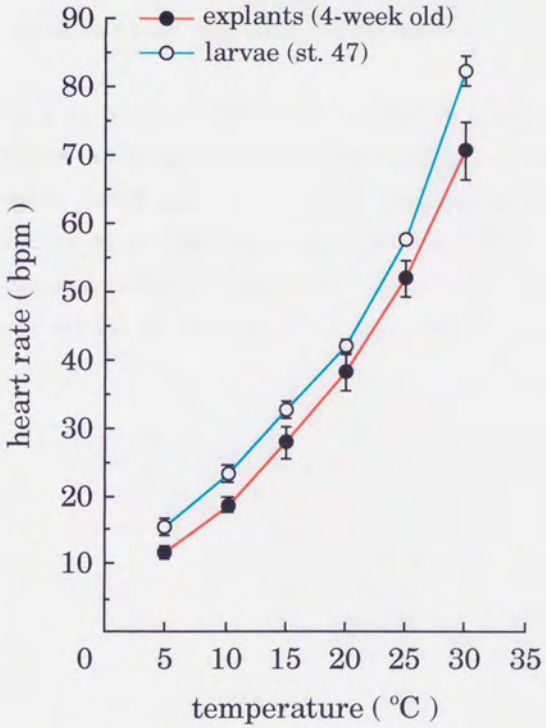
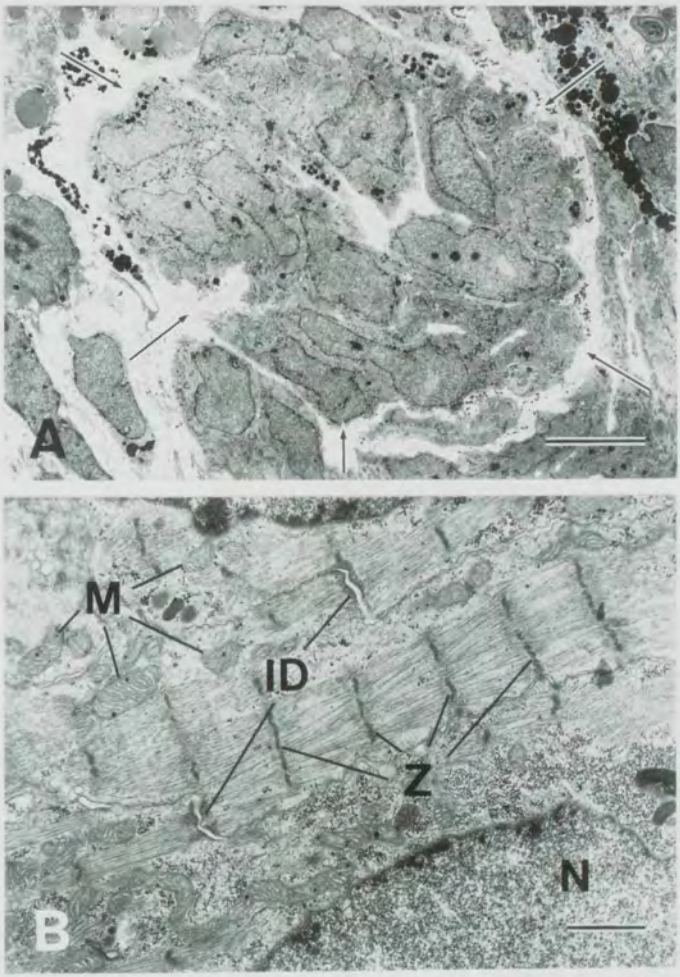

explants (4-week old)
larvae (st. 47)
heart rate (bpm)
90
80
70
60
50
40
30
20
10
0
5
10
15
20
25
30
35
temperature (°C)

Figure 20

Transmission electron microscopy of 5-week old activin-induced heart rudiments in the sandwich culture

(A) A large block of myocardial cells (*arrows*) can be seen. These cells are well developed because yolk platelets are absent. Scale bar, 10 μm. (B) Higher magnification view of a portion of a myocardial cell. The myofibrils are well organized and several intercalated discs (*ID*) have formed. *M*, mitochondria; *N*, nucleus; *Z*, Z band. Scale bar, 1 μm.


A
M
ID
Z
N
B

Discussion

Vegetalizing activity of activin A on *Cynops* presumptive ectoderm

The induction property of activin A on *Cynops* presumptive ectoderm was different from that on *Xenopus* ectoderm in some points. The dose-dependent mesoderm induction by activin A shown on *Xenopus* ectoderm (chapter 1) was not clearly observed in *Cynops* ectoderm. Furthermore, *Cynops* ectoderm treated with high concentrations of activin A differentiated preferentially into yolk-rich tissues without any other tissues. These tissues often exhibited a columnar appearance, a characteristic of endodermal epithelium in the alimentary canal. From these histological features, they were identified as endoderm-derived tissues in the present study. Other irregular-shaped yolk-rich tissues are considered to be undifferentiated endoderm (Grunz, 1979, 1983). Small amounts of mesodermal tissues and nonspecific neural tissues were also induced in the ectodermal explants at low frequencies. Using vegetalizing factor as an inducer, Grunz (1979) and Minuth and Grunz (1980) showed that mesodermal tissues are formed by secondary interactions between endodermalized and non-induced ectodermal cells. The vegetalizing factor has now been identified as activin A based on its chemical properties and biological activities (Asashima et al., 1990b, 1991a, c; Tiedemann et al., 1992). In the present

study, the endodermally determined cells, because of activin A treatment, may have induced mesodermal tissues from the adjacent non-induced cells as a secondary interaction. This possibility is also supported by the results of combination-culture mode in which mesodermal tissues were induced frequently as the proportion of the untreated (non-induced) ectoderm increased. Induction of neural tissue could be explained as a further interaction between mesodermalized and non-induced ectoderm (Suzuki et al., 1986).

In the sandwich-culture mode, the yolk-rich endodermal tissues were found in most of the explants. These tissues were better organized than those induced in the isolation and combination-culture modes. They often formed typical endoderm-derived organs, such as intestine and liver. A large amount of axial mesoderm and central nervous system also differentiated in the explants at relatively high rates. The increased number of non-induced ectoderm may be a major cause of mesodermal and neural differentiation at high rates. Okada (1954a, b, 1960) showed that the presence of mesoderm is a prerequisite for the formation of well-organized endodermal organs. Thus, it would seem that the existence of a large amount of axial mesoderm also affects endodermal differentiation in the sandwich explants.

Heart formation of *Cynops* presumptive ectoderm by activin A

Since the beating of explant was employed as the criterion for successful heart formation, there can be little doubt or uncertainty about the extent to which *Cynops*

ectoderm responds to activin A treatment. Approximately 20% of the sandwich explants developed into beating hearts. It is likely that the other 80% display heart formation to one extent or another, but never develop to a fully functional state. That is, if less rigorous criteria, such as the presence of one of the myosin, actin or other muscle-specific proteins, were employed, the scoring result would likely show more "positives" than the 20% observed in the present study.

Beating heart rudiments have never been detected in any of the *Xenopus* ectodermal explants (chapter 1, 3). As already mentioned above, the induction properties of activin A on *Cynops* ectoderm are different from those on *Xenopus* ectoderm. The activin-treated *Cynops* ectoderm itself differentiates solely into yolk-rich endodermal tissues and the frequency of mesoderm differentiation is relatively low. Differentiation of the heart rudiment during normal development is known to depend on the influence of anterior pharyngeal endoderm (Jacobson and Sater, 1988). Although it has not been confirmed that the yolk-rich endodermal tissues are identical to anterior pharyngeal endoderm, the presence of these tissues seems to be requisite for the formation of beating hearts.

The role of activin as a regulatory molecule in early amphibian embryogenesis has been well documented (reviewed by Asashima, 1994; Ariizumi and Asashima, 1995b). Recent results reveal, however, that activin might act in combination with other regulatory molecules (e.g., other

peptide growth factors) during cardiogenesis (Sugi and Lough, 1995; Muslin and Williams, 1991). A culture system such as the one described in this chapter should provide a good system for analyzing phenomena such as the role of multiple signal molecules in promoting cardiac induction. Not only could positive signal molecules be searched for, but as well signal molecules which act to inhibit the action of peptide growth factors such as activin could be identified with this system. It is highly unlikely that only "positive" signal molecules act during the course of an embryological induction. "Negative" signals which act to down-regulate the action of opposing forces likely play a role in the complex circuitry of cardiogenesis. This activin/*Cynops* ectoderm system should provide a useful bio-assay for identifying such additional regulatory molecules which comprise the mechanism(s) of cardiac induction.

Shortcomings are, however, associated with this experimental system. First, is of course the paucity of molecular markers for urodele cardiogenesis. Most amphibian studies have focused on *Xenopus* (Logan and Mohun, 1993; Cox and Neff, 1995). Perhaps it would be possible to use the available *Xenopus* cDNA markers (or even those from zebra fish [Stanier and Fishman, 1994] or *Drosophila* [Bodmer, 1995]) to screen appropriate urodele cDNA libraries. As well, it should be possible to perform differential display analyses to detect relatively low abundance gene products in early stages of cardiogenesis. Second is the observation that only 20% of the sandwich explants display a beating

heart. Future experiments will be designed to improve that frequency. But as mentioned above, since a beating heart represents a highly completed differentiation program, it is likely that the other explants exhibit heart development to one extent or another. Considering both positive and negative features, it is likely that activin-treated *Cynops* explants will be a useful model for a variety of studies on early heart development.

Summary

Induction property of activin A was examined using *Cynops* gastrula ectoderm. Dose-dependent mesoderm induction of activin A shown on *Xenopus* ectoderm was not clearly observed in *Cynops* ectoderm. The frequency of mesodermal differentiation was relatively low. On the other hand, ectoderm treated with high concentrations of activin A differentiated solely into yolk-rich tissues. These tissues were considered endoderm from their histological features.

As the size of activin-treated ectoderm increased, various kinds of ectodermal and mesodermal tissues, including beating heart rudiments, were induced in addition to yolk-rich endodermal tissues. Furthermore, these tissues were induced at a high frequency when the activin-treated ectoderm was combined with a large size of untreated ectoderm. In the sandwich explants with a larger amount of untreated ectoderm, well-developed axial mesoderm and central nervous system accompanied by endodermal tissues, such as liver and intestine, were induced frequently.

These results suggest that activin A is one of the substances involved in triggering endodermal differentiation of *Cynops* gastrula ectoderm, and the endodermally determined ectoderm may have induced ectodermal and mesodermal tissues, including beating heart rudiments, from the adjacent non-induced ectoderm as a secondary interaction.

Chapter 3

In Vitro Control of the Embryonic Body Plan by Activin A: Regional Induction-specificity of Activin-treated Ectoderm

Introduction

Mangold and Spemann (1927) demonstrated by the implantation method that the dorsal lip of the *early* salamander gastrula induces a secondary head, while that of the *late* gastrula induces a secondary trunk and tail. Spemann (1931) defined the former as the "head organizer" and the latter as the "trunk organizer". Mangold (1933) further examined the inducing abilities of the dorsal lip after invagination. The anterior region of the archenteron roof, corresponding to the invaginated head organizer, induced a head, and the posterior region, the trunk organizer, induced a trunk and tail. These results indicate that at least two distinct regionalities are present in the organizer.

The dorsal lip of the early gastrula (defined as the head organizer by Spemann, 1931), however, induces trunk-tail structures in the sandwich culture or affixed transplantation. The same region induces the formation of heads when it is precultured in saline for a definite period (Okada and Takaya, 1942a, b; Okada and Hama, 1943; Hama, 1949). This means that the regional induction-specificity of the organizer changes autonomously during gastrulation in normal development. In the experiment performed by Mangold and Spemann (1927), the dorsal lip of the early gastrula was implanted into the blastocoele. As gastrulation proceeds, its induction-specificity may transform from trunk-tail to

head and eventually induce a secondary head on the ventral ectoderm of the host embryo. Although these findings seem to be very important in establishing the fundamental body plan, further investigation has long been awaited.

Presumptive ectoderm treated with XTC-MIF (*Xenopus* activin A) induces a well-organized secondary embryo when implanted into the blastocoele or ventral marginal zone of early *Xenopus* gastrula (Cooke, 1989; Ruiz i Altaba and Melton, 1989; Cho et al., 1991a). As is well known, a similar embryo is also obtained by transplanting the organizer (Spemann and Mangold, 1924). If activin induces organizer-activity in presumptive ectoderm, it may well be that activin-treated ectoderm also exhibits regional induction-specificity, as shown in the classical organizer experiments (Okada and Takaya, 1942a, b; Okada and Hama, 1943; Hama, 1949). In this chapter, it was investigated whether the activin-treated ectoderm of *Xenopus laevis* and *Cynops pyrrhogaster* acts as the head organizer and/or trunk-tail organizer, employing the implantation and sandwich culture techniques. The activin-treated ectoderm showed a clear regional induction-specificity depending on the concentration of activin A and the time after activin A treatment. These results are also in agreement with those of the previous experiment using the organizer as the inducer (Hama et al., 1985), and suggest that activin A can not only induce various mesodermal and endodermal tissues but can also facilitate formation of the organizer as a morphogenetic center.

Materials and Methods

Eggs and Embryos

Xenopus laevis embryos were obtained from hormone-stimulated males and females as described in chapter 1 and staged according to Nieuwkoop and Faber (1956). *Cynops pyrrhogaster* embryos were obtained from hormone-stimulated females as described in chapter 2 and staged according to Okada and Ichikawa (1947). Removal of the jelly coat and vitelline membrane was the same procedure as described in chapter 1 and 2.

Activin A solution

Human recombinant activin A was dissolved in Steinberg's solution (SS; pH 7.4) at concentrations of 0-100 ng/ml for *Xenopus* explants and in Holtfreter's solution (HS; pH 7.6) at concentrations of 0 and 100 ng/ml for *Cynops* explants. 0.1% bovine serum albumin (BSA, A-7888; Sigma, USA) was added to the solution to avoid adsorption of activin A to the glass or plastic surfaces. Activin A solutions were put in non-surface-treated polystyrene wells (SUMILON 24-well plate MS-80240; Sumitomo Bakelite, Japan) or polystyrene dishes (Tissue Culture Dish 25000; Iwaki Glass, Japan).

Experimental series

All of the following procedures were carried out under sterile conditions. Schematic diagrams of the procedures and culture methods are shown in Fig. 21.

Series 1·1

Presumptive ectodermal sheets (0.4 x 0.4 mm) cut from late blastulae of *Xenopus laevis* (stage 9, about 1.2 mm in diameter) were treated with 10, 50 and 100 ng/ml of activin A solutions for 10 min. As a control, they were cultured in SS containing 0.1% BSA for the same period. After washing in two changes of SS with gentle pipetting, they were precultured for 0, 1, 3, 5 and 7 hr in SS at 20°C. They were then sandwiched between two sheets of presumptive ectoderm (0.8 x 0.8 mm) from other late blastulae of *Xenopus laevis*. Explants were cultured for 4 days at 20°C in 3% agar-coated culture dishes (SUMILON 24-well plate, MS-80240, Sumitomo Bakelite, Japan) filled with SS.

Series 1·2

Embryos of *Xenopus laevis* at the 2-cell stage were injected with a total volume of 10 nl of 1% fluorescein-dextran-amine (FDA, D-1820; Molecular Probes, USA). After they were raised until late blastula stage in SS containing 5% Ficoll (F-4375; Sigma, USA), presumptive ectodermal sheets (0.4 x 0.4 mm) were cut and treated with 100 ng/ml of activin A solution for 10 min. After having been precultured for 1 or 5 hr in SS at 20°C, they were sandwiched between

two sheets of presumptive ectoderm (0.8 x 0.8 mm) from non-labeled late blastulae of *Xenopus laevis*. The sandwich explants were cultured as described in series 1·1.

Specimens were fixed in 4% paraformaldehyde, buffered at pH 7.4 with 0.1 M sodium cacodylate, for 12 hr at 4°C. After they had been washed in 0.1 M NaCl, 10 mM sodium phosphate buffer (pH 7.4) for 4 hr, specimens were dehydrated through an ethanol series, embedded in paraffin and sectioned at 8 μm. After deparaffinization, sections were observed under an epifluorescence microscope.

Series 2·1

Presumptive ectodermal sheets (1.0 x 1.0 mm) cut from early gastrulae of *Cynops pyrrhogaster* (stage 11, about 2.3 mm in diameter) were treated with 100 ng/ml of activin A solution for 1 hr. As a control, they were cultured in HS containing 0.1% BSA for the same period. After washing in two changes of HS with gentle pipetting, they were precultured for 0, 12, 24 hr in HS at 20°C. They were then implanted into the blastocoele of other early gastrulae of *Cynops pyrrhogaster*. Embryos were raised on the filter paper in culture dishes (SUMILON 12-well plate, MS-80120, Sumitomo Bakelite, Japan) filled with 1/10 HS.

Series 2·2

After the same procedure as described in series 2·1, the activin-treated ectodermal sheets were precultured for 0, 6, 12, 18 and 24 hr in HS at 20°C. They were then

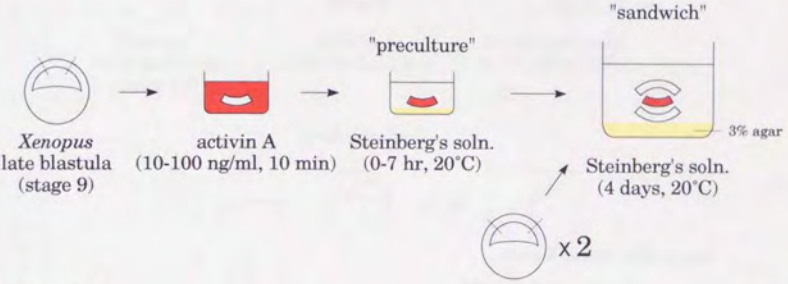
sandwiched between two sheets of presumptive ectoderm (1.5 x 1.5 mm) from other early gastrulae of *Cynops pyrrhogaster* and cultured for 2 weeks at 20°C in 3% agar-coated culture dishes (SUMILON 24-well plate, MS-80240, Sumitomo Bakelite, Japan) filled with HS.

Series 2·3

Embryos of *Cynops pyrrhogaster* at the 2- or 4-cell stage were injected with a total volume of 50 nl of 1% Texas Red-dextran-amine (TRDA, D-1863; Molecular Probes, USA). After they were raised until early gastrula stage in HS, presumptive ectodermal sheets (1.0 x 1.0 mm) were cut and treated with 100 ng/ml of activin A solution for 1 hr. After having been precultured for 0 or 24 hr in HS at 20°C, they were sandwiched between two sheets of presumptive ectoderm (1.5 x 1.5 mm) from other early gastrulae of *Cynops pyrrhogaster*, which were injected with a total volume of 50 nl of 1% fluorescein-dextran-amine (FDA, D-1820; Molecular Probes, USA) at the 2- or 4-cell stage. The sandwich explants were cultured as described in series 2·2.

Specimens were fixed in half-strength Karnovsky's fixative for 15 min and phosphate-buffered 10% commercial formalin for 12 hr (Tandler, 1990). They were then dehydrated through an ethanol series, embedded in paraffin and sectioned at 10 μm. After deparaffinization, sections were mounted in PermaFluor Aqueous Mounting Medium (434990; Immunon, USA) and examined with a confocal laser scanning microscope (TCS 4D; Leica).

Series 1.1



Series 1.2

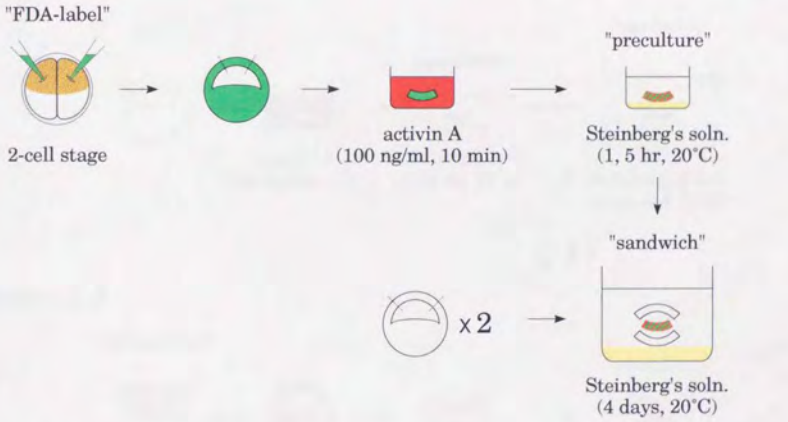
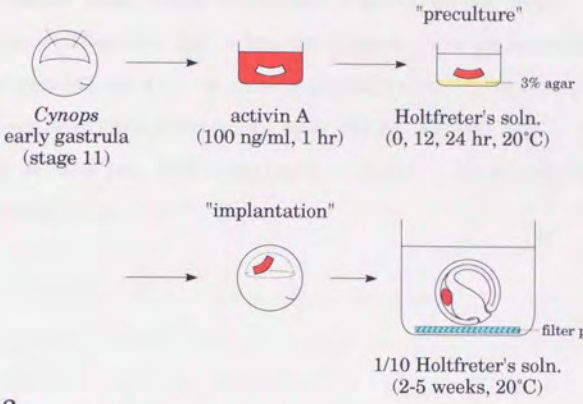


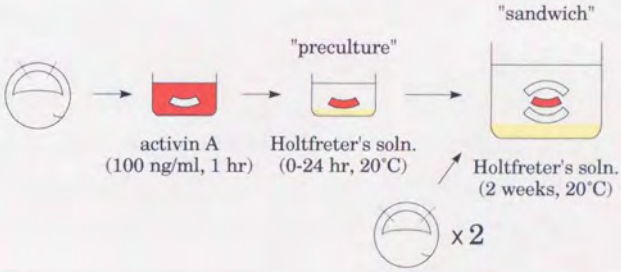
Figure 21

Schematic diagrams of the procedures and culture methods

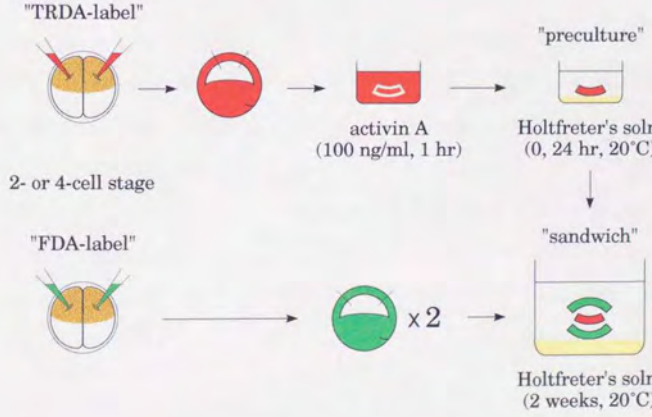
Series 2.1



Series 2.2



Series 2.3



Histological examination

Specimens were fixed in Bouin's solution or Susa (Heidenhain) solution for 3 hr. They were then dehydrated through a graded series of ethanols, cleared in xylene, embedded in paraffin (Histprep 568; Wako, Japan) and sectioned at 6-8 μm. Sections were stained with Delafield's hematoxylin/eosin.

Results

Series 1: Regional induction-specificity of activin-treated ectoderm in *Xenopus laevis*

Dose and time-dependent regional induction-specificity of activin-treated ectoderm

Regardless of the duration of preculture, untreated ectoderm never induced head or trunk-tail structures in the sandwich culture. All of the control explants, sandwiching untreated ectoderm, formed atypical epidermis. Typical explants and their histological sections obtained from the following experiments are shown in Fig. 22.

Ectoderm treated with 10 ng/ml of activin A itself differentiated into ventral mesodermal tissues, such as mesenchyme, coelomic epithelium and blood cells. When they were sandwiched with untreated ectoderm, sandwich explants showed vesicular or fin-like forms and differentiated into ventral mesodermal tissues, regardless of the duration of preculture (Fig. 23A). These tissues could be considered to differentiate from the inner activin-treated ectoderm, or as a result of homoiogenetic induction by activin-treated ectoderm to the surrounding untreated ectoderm.

At 50 ng/ml of activin A, ectoderm differentiated into more dorsal mesodermal tissues (pronephros and muscle). When they were sandwiched immediately (0 hr), sandwich explants formed only the same tissues as the explants treated with 10

ng/ml of activin A. When the activin-treated ectoderm was precultured for more than 1 hr, trunk-tail structures such as muscle, pronephros and notochord were also differentiated. Furthermore, when they were precultured for more than 5 hr, the sandwich explants formed head structures (cement gland, brain, ear vesicle) in addition to trunk-tail structures (Fig. 23B). These head structures were presumed to be induced by the inner activin-treated ectoderm because activin-treated ectoderm itself differentiated exclusively into mesodermal tissues at 50 ng/ml of activin A.

At 100 ng/ml of activin A, the activin-treated ectoderm differentiated mainly into notochord and nonspecific neural tissue. In sandwich culture, sandwich explants formed trunk-tail structures when the activin-treated ectoderm was precultured briefly (0-1 hr). Besides these structures, head structures were induced by long-term preculture of the ectoderm (3-7 hr). As compared with the results of treatment with 50 ng/ml of activin A, the acquisition of head-inducing ability by the activin-treated ectoderm progressed more rapidly under this condition (Fig. 23C).

Cell-lineage analysis of the Xenopus sandwich explants

To confirm whether inner activin-treated ectoderm acted as the organizer and induced head structures from the surrounding ectoderm, FDA-labeled ectoderm was sandwiched between non-labeled ectoderm, and their cell lineage was traced. In this experiment, FDA-labeled ectoderm was treated with 100 ng/ml of activin A for 10 min. After 1 hr of

preculture (representing a short-term of preculture) or 5 hr of preculture (representing a long-term of preculture), FDA-labeled ectoderm was sandwiched with non-labeled ectoderm.

Explants sandwiching 1 hr-precultured ectoderm differentiated into trunk-tail structures including various mesodermal tissues, such as muscle, pronephros, mesenchyme and coelomic epithelium. Head structures (cement gland and ear vesicle) were also induced at very low frequencies. The FDA-labeled descendants formed a large part of trunk-tail structures and were not found in these head structures at all (Table 2). Explants sandwiching 5 hr-precultured ectoderm showed head formation. In histological sections, differentiation of head structures (brain, cement gland and ear vesicle) was seen in addition to mesodermal derivatives (Fig. 24). Fluorescence of FDA-labeled descendants was not found in head structures, but was observed in mesodermal tissues (Table 2, Fig. 24). It was confirmed that the inner activin-treated ectoderm itself differentiated exclusively into mesodermal tissues and induced head structures from the surrounding ectoderm as the organizer does in normal *Xenopus* development.

Figure 22

External views and histological sections
of the *Xenopus* sandwich explants

(A) Explant with head structures. After 5 hr of preculture, ectoderm treated with 100 ng/ml of activin A induced typical head structures, such as eye and cement gland. (B) Explant with trunk-tail structures. After 1 hr of preculture, ectoderm treated with 10 ng/ml of activin A induced trunk-tail structures equipped with caudal fin. (C) Histological section of the explant with head structures as shown in (A). A large mass of neural cells formed brain ventricles with an eye surrounded by pigment epithelium. No mesodermal tissue, except a small amount of mesenchyme, differentiated in this explant. (D) Histological section of the explant with trunk-tail structures as shown in (B). Muscle and pronephros were formed in the explant. *br*, brain; *cg*, cement gland; *mus*, muscle; *pr*, pronephros. Scale bar, 500 μm in A and B, 200 μm in C and D.

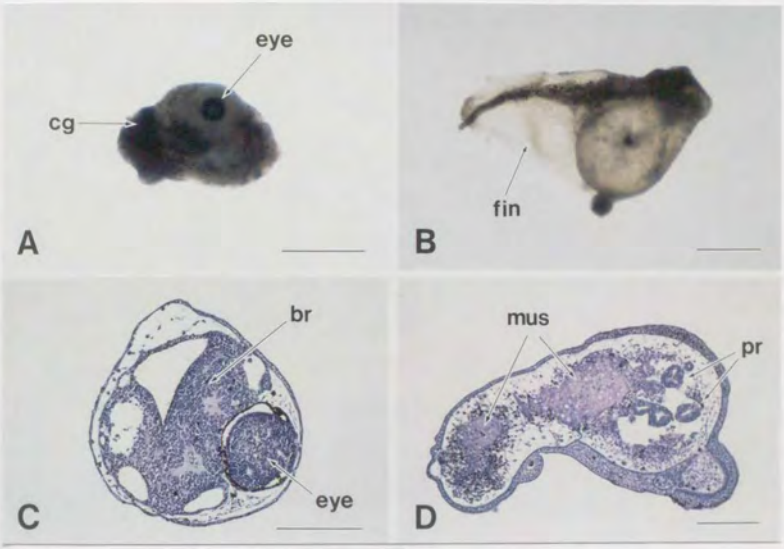

eye
cg
A
fin
B
br
eye
C
mus
pr
D

Figure 23

Differentiation of the *Xenopus* sandwich explants

Bar charts indicate the frequencies of induction as a percentage of the total. (A) Differentiation of explants sandwiching the 10 ng/ml activin-treated ectoderm. Explants differentiated into ventral mesodermal tissues, such as mesenchyme, coelomic epithelium and blood cells, regardless of the duration of preculture. (B) Differentiation of the explants sandwiching the 50 ng/ml activin-treated ectoderm. When the activin-treated ectoderm was precultured for more than 1 hr, trunk-tail structures, such as muscle, pronephros and notochord, differentiated well. In addition to these structures, head structures, such as cement gland, brain and ear vesicle, were induced when the activin-treated ectoderm was precultured for over 5 hr. (C) Differentiation of explants sandwiching the 100 ng/ml activin-treated ectoderm. Explants formed trunk-tail structures regardless of the duration of preculture. In addition to these structures, head structures were induced by the long term-precultured ectoderm (3-7 hr). The acquisition of head-inducing ability of activin-treated ectoderm progressed more rapidly in (B).

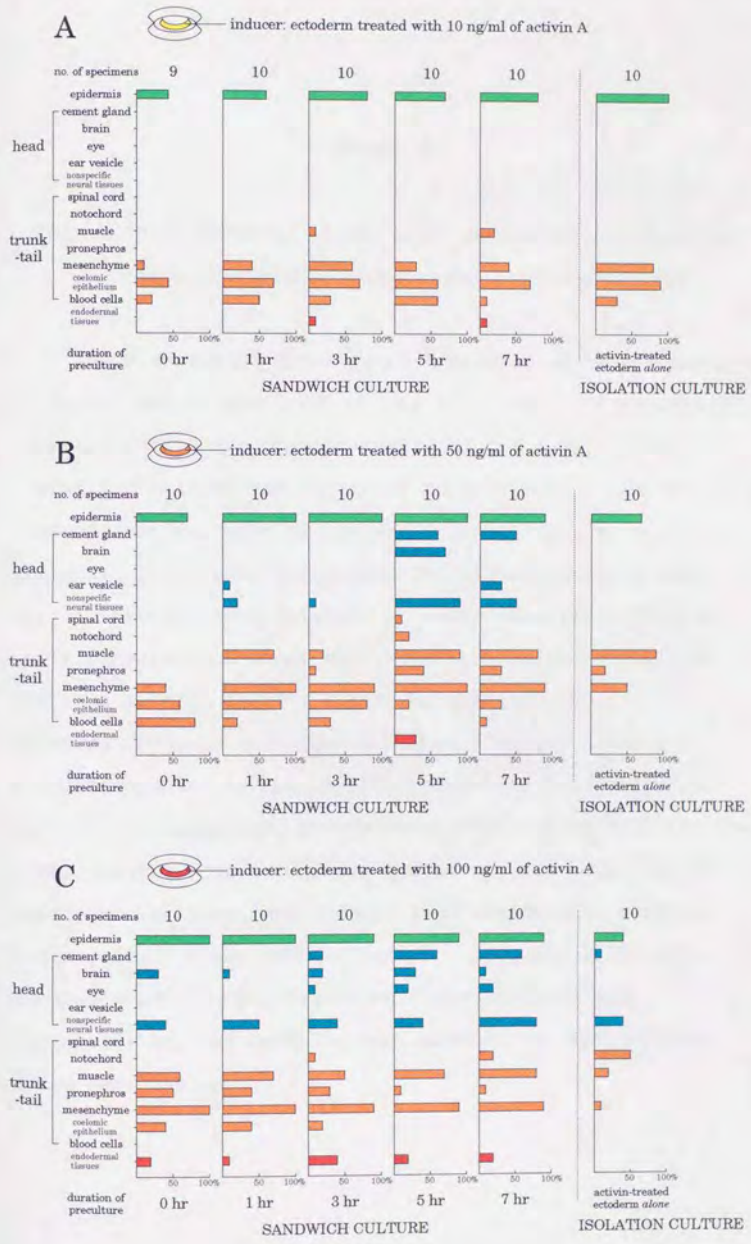

A
inducer: ectoderm treated with 10 ng/ml of activin A
no. of specimens
9
10
10
10
10
10
epidermis
cement gland
brain
eye
ear vesicle
nonspecific neural tissues
spinal cord
notochord
muscle
pronephros
mesenchyme
coelomic epithelium
blood cells
endodermal tissues
head
trunk -tail
50
100%
duration of preculture
0 hr
1 hr
3 hr
5 hr
7 hr
activin-treated ectoderm alone
SANDWICH CULTURE
ISOLATION CULTURE
B
inducer: ectoderm treated with 50 ng/ml of activin A
no. of specimens
10
10
10
10
10
10
epidermis
cement gland
brain
eye
ear vesicle
nonspecific neural tissues
spinal cord
notochord
muscle
pronephros
mesenchyme
coelomic epithelium
blood cells
endodermal tissues
head
trunk -tail
50
100%
duration of preculture
0 hr
1 hr
3 hr
5 hr
7 hr
activin-treated ectoderm alone
SANDWICH CULTURE
ISOLATION CULTURE
C
inducer: ectoderm treated with 100 ng/ml of activin A
no. of specimens
10
10
10
10
10
10
epidermis
cement gland
brain
eye
ear vesicle
nonspecific neural tissues
spinal cord
notochord
muscle
pronephros
mesenchyme
coelomic epithelium
blood cells
endodermal tissues
head
trunk -tail
50
100%
duration of preculture
0 hr
1 hr
3 hr
5 hr
7 hr
activin-treated ectoderm alone
SANDWICH CULTURE
ISOLATION CULTURE

Figure 24

Cell-lineage analysis of the activin-treated ectoderm and untreated ectoderm in the *Xenopus* sandwich explants

(A) Presumptive ectoderm of FDA-injected embryo was cut at late blastula and treated with 100 ng/ml of activin A for 10 min. After having been precultured for 5 hr, it was sandwiched between two sheets of untreated ectoderm. After 4 days of culture, most of the sandwich explants showed head formation under this condition. The tissues derived from activin-treated (FDA-labeled) ectoderm were identified by their fluorescence under epifluorescent illumination. (B) The same section as (A), which was stained with hematoxylin/eosin and observed under a bright field. Fluorescence in (A) corresponds to muscle, mesenchyme and part of the epidermis. It was shown that the activin-treated (FDA-labeled) ectoderm itself differentiated mainly into mesodermal tissues, and induced head structures, such as brain, cement gland and ear vesicle, from the outer non-labeled ectoderm. *br*, brain; *cg*, cement gland; *epi*, epidermis; *ev*, ear vesicle; *mes*, mesenchyme; *mus*, muscle. Scale bar, 200 μm.

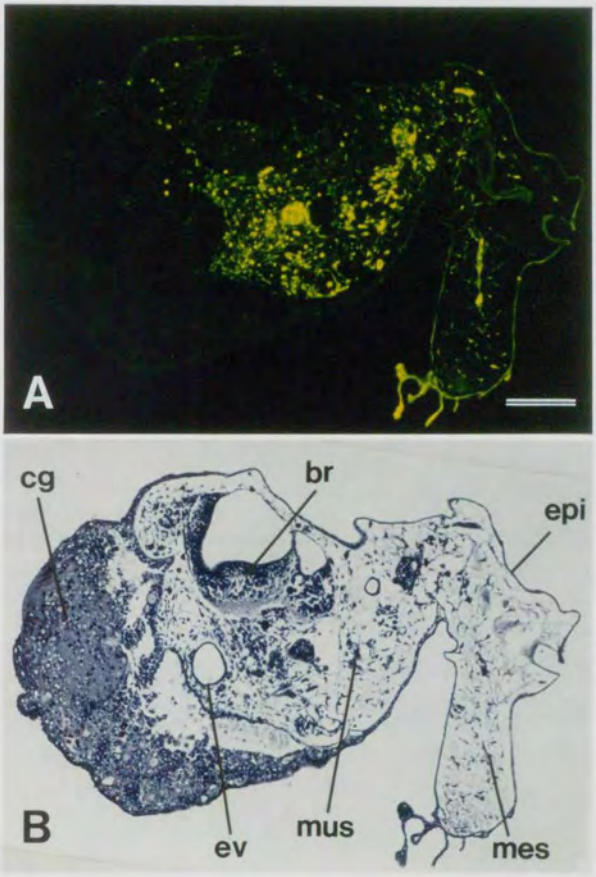

A
cg
br
epi
B
ev
mus
mes

Table 2. Cell-lineage analysis of the *Xenopus* sandwich explants

duration of preculture	short term (1 hr)		long term (5 hr)	
no. of specimens	10		12	
tissues	differentiated*	FDA-labeled**	differentiated*	FDA-labeled**
epidermis	10	10	12	9
cement gland	2	0	10	0
brain	0	0	4	0
eye	0	0	3	0
ear vesicle	3	0	2	0
nonspecific neural tissues	3	2	10	5
notochord	1	1	5	4
muscle	9	8	10	10
pronephros	3	3	2	2
mesenchyme	9	8	11	10
coelomic epithelium	4	3	0	0

* number of differentiated explants

** number of explants having FDA-labeled tissues

Series 2: Regional induction-specificity of activin-treated ectoderm in *Cynops pyrrhogaster*

Head and trunk-tail organizing effects of activin-treated ectoderm in the implantation method

Presumptive ectoderm of *Cynops* early gastrula was treated with 100 ng/ml of activin A for 1 hr. As described in chapter 2, the activin-treated ectoderm itself differentiates preferentially into yolk-rich endodermal tissue under this condition. After having been precultured in Holtfreter's solution for 0, 12, 24 hr, it was implanted into the blastocoele of other early gastrulae. The activin-treated ectoderm induced secondary embryos with head or trunk-tail structures as the organizer does (Fig. 25). The type of secondary embryo induced depended on the duration of preculture of activin-treated ectoderm (Fig. 26). Short-term (0 hr) precultured ectoderm mainly induced trunk-tail structures with a secondary axis (spinal cold, notochord, somite), fin and fore-limb. As the duration of preculture was prolonged, the proportion of secondary trunk and tails were decreased. On the other hand, secondary heads with eye and balancer were induced by longer-term (12 and 24 hr) precultured ectoderm.

Time-dependent regional induction-specificity of activin-treated ectoderm

Regardless of the duration of preculture, untreated ectoderm never induced head or trunk-tail structures. All of

the control explants sandwiching untreated ectoderm formed atypical epidermis (Fig. 27A). In the experimental series, presumptive ectoderm was treated with 100 ng/ml of activin A for 1 hr and sandwiched after precultured in Holtfreter's solution for various periods (0-24 hr). Explants sandwiching short-term precultured ectoderm often formed a complete body axis composed of deuterencephalic and spinocaudal structures, such as hindbrain, spinal cord, notochord and muscle (Fig. 28A, C). They closely resembled normal larvae not only in shape but in tissue and organ differentiation. These explants quivered convulsively when stimulated with a needle and swam a short distance. They were also equipped with a rhythmically beating heart. The formation of heart structure was confirmed in histological sections (Fig. 27B). However, archencephalic structures were either absent or poorly developed in these explants. In contrast, the explants sandwiching long-term precultured ectoderm developed into archencephalic structures, such as forebrain and eye (Fig. 28B, D). The eye induced consisted of a lens, neural retina and pigment epithelium.

There was a tendency that the activin-treated ectoderm precultured for short periods (0-6 hr) induced trunk and tails characterized by deuterencephalic and spinocaudal structures, whereas when precultured for long periods (12-24 hr) it induced heads with archencephalic structures (Fig. 29). The yolk-rich endodermal tissues, which are considered to differentiate from the activin-treated ectoderm, were

found in the sandwich explants at high frequencies, regardless of the duration of preculture.

Cell-lineage analysis of the Cynops sandwich explants

Fluorescent dyes (TRDA and FDA) were employed to trace the lineage of activin-treated ectoderm and untreated ectoderm in the sandwich explants. A typical section is shown in Fig. 30 and the results of histological examination are summarized in Table 3.

Activin-treated ectoderm labeled with TRDA had mainly differentiated into endodermal cells. The FDA-labeled untreated ectoderm also participated in the formation of endoderm in 7 out of 26 specimens. All of the portions of the central nervous system (forebrain, hindbrain and spinal cord), sensory organs (eye and ear vesicle) and fins were derived from untreated ectoderm alone. Mesodermal derivatives, such as notochord, muscle and pronephros, consisted of untreated ectoderm in about two thirds of the specimens (15 out of 24, 16 out of 24, and 6 out of 8, respectively). However, the remainder were made up of both activin-treated ectoderm and untreated ectoderm. Notochord and muscle consisted of activin-treated ectoderm alone in only one case. It was confirmed that the activin-treated ectoderm differentiated mainly into endodermal cells itself and then induced mesodermal tissues and central nervous system in the untreated ectoderm.

Figure 25

Secondary embryos induced by the *Cynops* activin-treated ectoderm

Activin-treated ectoderm precultured in Holtfreter's solution for a long term (12 hr) induced a secondary head (A), whereas that precultured for a short term (0 hr) induced a secondary trunk-tail (B). A well-developed eye, consisted of a lens, neural retina and pigment epithelium, was seen in the secondary head (C). A well-organized secondary axis, consisted of a spinal cord, notochord and somite, was formed in the secondary trunk-tail (D). *arrow*, secondary embryo; *fl*, fore-limb; *not*, notochord; *sc*, spinal cord; *so*, somite. Scale bar, 2 mm in A and B, 200 μm in C and D.

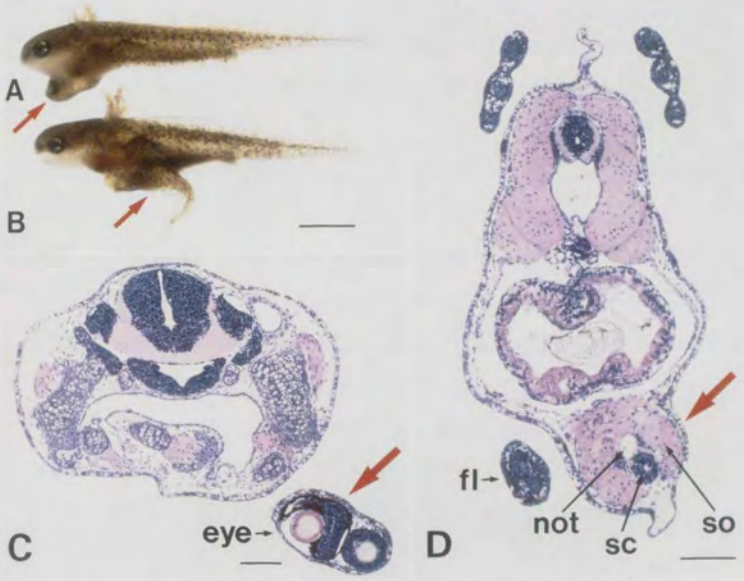

A
B
C
eye
D
fl
not
sc
so

Figure 26

Head and trunk-tail organizing effects of the activin-treated ectoderm in the implantation method

The type of secondary embryos depended on the duration of preculture of activin-treated ectoderm. Secondary trunk-tail structures, characterized by secondary axis, fin and fore-limb, were induced by 0 hr-precultured ectoderm at a high frequency. On the other hand, secondary head structures with eye and balancer were induced well by longer-term (12-24 hr) precultured ectoderm. The head and trunk-tail organizing activities of the activin-treated ectoderm decreased as the duration of preculture prolonged.

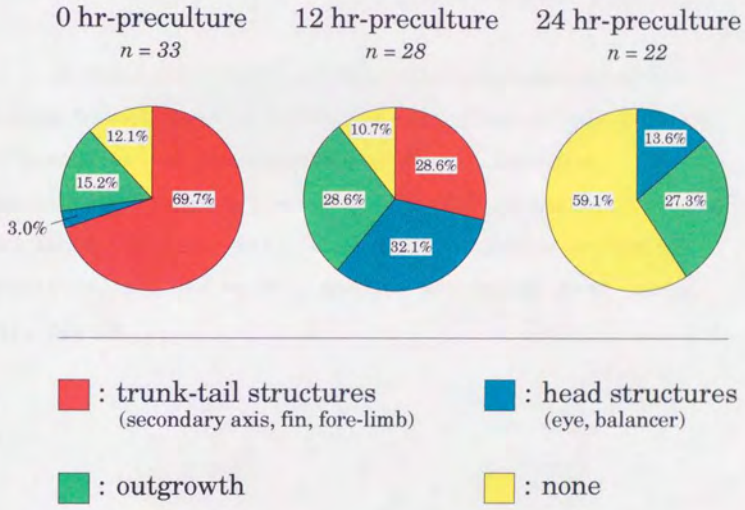

0 hr-preculture
n = 33
12 hr-preculture
n = 28
24 hr-preculture
n = 22
12.1%
15.2%
69.7%
3.0%
10.7%
28.6%
28.6%
32.1%
13.6%
59.1%
27.3%
: trunk-tail structures
(secondary axis, fin, fore-limb)
: head structures
(eye, balancer)
: outgrowth
: none

Figure 27

Heart differentiation in the *Cynops* sandwich explants

(A) Control explants sandwiching untreated ectoderm always formed atypical epidermis regardless of the duration of preculture of untreated ectoderm. (B) Explants, sandwiching the activin-treated ectoderm precultured for 0-6 hr, exhibited trunk-tail formation accompanied by heart structure. *ht*, heart; *mus*, muscle; *sc*, spinal cord. Scale bar, 200 μm.

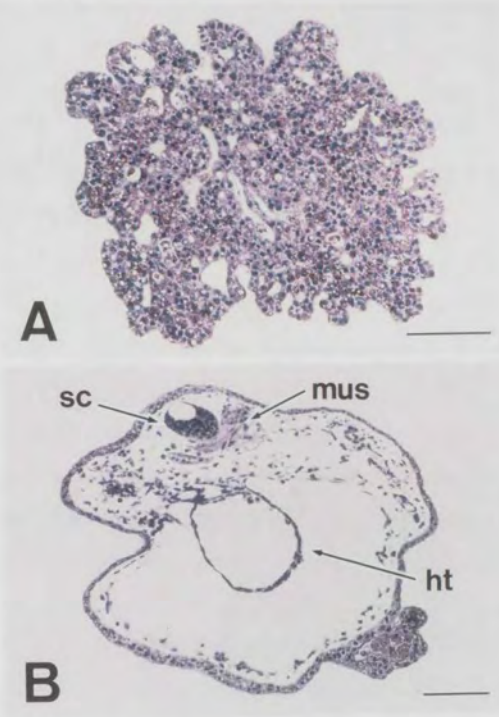

A
sc
mus
ht
B

Figure 28

External views and histological sections
of the *Cynops* sandwich explants

Presumptive ectoderm of early *Cynops* gastrula was treated with 100 ng/ml of activin A for 1 hr. After precultured in Holtfreter's solution for various periods (0-24 hr), its regional induction-specificity was examined by the sandwich culture method. (A) Explant with trunk-tail structures induced by the activin-treated ectoderm without preculture. (B) Explant with head structures induced by the activin-treated ectoderm that was precultured for 12 hr in Holtfreter's solution. (C) Histological section of (A). Spinal cord, axial mesoderm and gut differentiate in the same arrangement as in normal larvae. (D) Histological section of (B). Forebrain is accompanied by a well-differentiated eye. *CNS*, central nervous system; *end*, endodermal tissue; *fb*, forebrain; *mus*, muscle; *not*, notochord; *pr*, pronephros; *sc*, spinal cord. Scale bar, 1 mm in A and B, 100 μm in C and D.

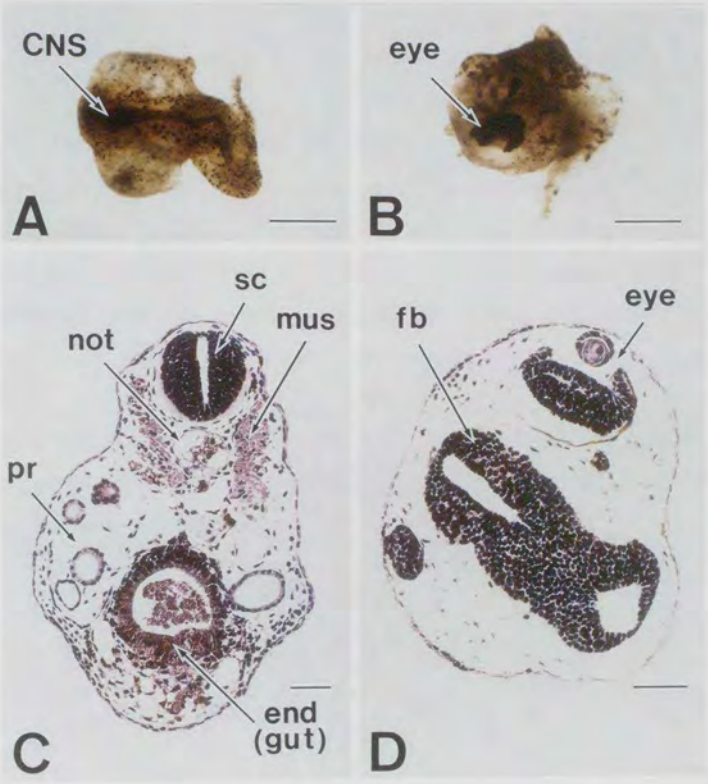

CNS
A
eye
B
sc
not
mus
pr
end
(gut)
C
fb
eye
D

Figure 29

Differentiation of the *Cynops* sandwich explants

Bar charts indicate the frequency of induction as a percentage of the total. Heads with archencephalic structures, such as forebrain and eye, were formed frequently in explants sandwiching over 12 hr-precultured ectoderm, whereas trunk and tails characterized by deuterencephalic and spinocaudal structures, such as spinal cord, notochord and muscle, were often induced by shorter-term precultured ectoderm. Yolk-rich endodermal tissues differentiated at high frequencies, regardless of the duration of preculture.

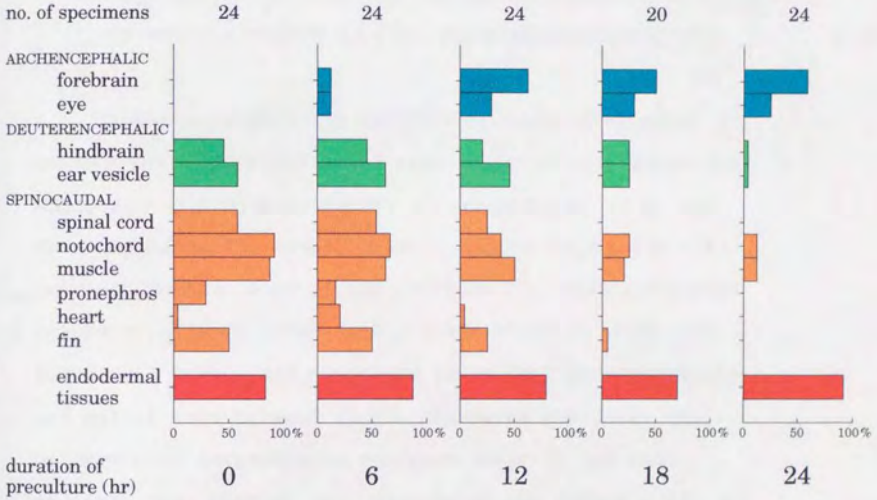

no. of specimens
24
24
24
20
24
ARCHENCEPHALIC
forebrain
eye
DEUTERENCEPHALIC
hindbrain
ear vesicle
SPINOCAUDAL
spinal cord
notochord
muscle
pronephros
heart
fin
endodermal tissues
0
50
100%
duration of preculture (hr)
0
6
12
18
24

Figure 30

Cell-lineage analysis of the activin-treated ectoderm and untreated ectoderm in the *Cynops* sandwich explants

Explants sandwiching the activin-treated ectoderm without preculture exhibited typical trunk-tail formation. These explants were sectioned transversely at 10 μm and observed under a confocal laser scanning microscope. (A) Epifluorescence image of the section. The activin-treated ectoderm differentiated into a large block of yolk-rich endodermal tissue and notochord (*red*), and induced muscle and spinal cord (*green*) in the untreated ectoderm. (B) Differential interference contrast image of the same section. *mus*, muscle; *not*, notochord; *sc*, spinal cord; *yo*, yolk-rich endodermal tissue. Scale bar, 200 μm.

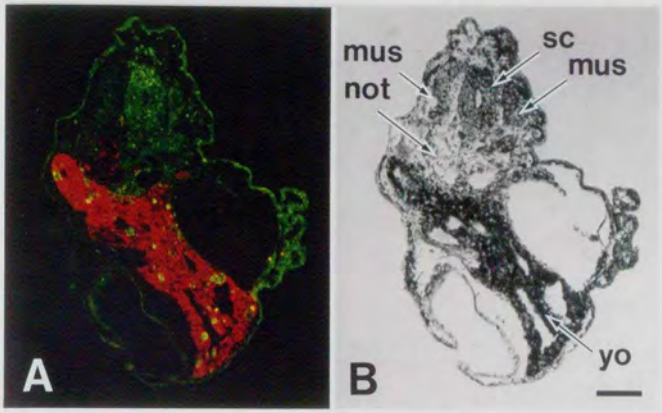

A
mus
not
sc
mus
yo
B

Table 3. Cell-lineage analysis of the *Cynops* sandwich explants

	differentiation		label		
	$n = 26$	(%)*	TRDA**	FDA***	both
ARCHENCEPHALIC					
forebrain	2	(8)	0	2	0
eye	1	(4)	0	1	0
DEUTERENCEPHALIC					
hindbrain	22	(85)	0	22	0
ear vesicle	10	(38)	0	10	0
SPINOCAUDAL					
spinal cord	14	(54)	0	14	0
notochord	24	(92)	1	15	8
muscle	24	(92)	1	16	7
pronephros	8	(31)	0	6	2
fin	18	(69)	0	18	0
endodermal tissues	26	(100)	19	0	7

* figures in parentheses indicate the percentage of the number of specimens

** number of explants having TRDA-labeled tissues

*** number of explants having FDA-labeled tissues

Discussion

Activin-treated ectoderm as an initiator in establishing the fundamental body plan

The concentrations of activin A (10-100 ng/ml) used in the present study are relatively high and will be much above the concentration of the corresponding molecule in the *Xenopus* embryo (Asashima et al., 1991b). As already mentioned in chapter 1, the minimum concentration of activin A required to induce presumptive ectoderm of *Xenopus laevis* is inversely proportional to the duration of treatment. It was necessary to shorten the duration of activin A treatment, because changes in the regional induction-specificity of presumptive ectoderm after the treatment were focused in the present study. Therefore, relatively high concentrations of activin A were employed in all experimental series in this chapter.

As shown in previous chapters, tissues induced by activin A in ectodermal pieces are poorly organized, and explants never display a clear embryonic axis and form. It has been shown that PIF, an activin A homologue derived from a mouse macrophage cell line, induces miniature embryos called "embryoids" from *Xenopus* blastula ectoderm (Sokol et al., 1990; Thomsen et al., 1990). They are equipped with a rudimentary anterior-posterior axis, brain and eyes. However, embryoids are never formed when a small piece of ectoderm, excluding cells close to the marginal zone

(presumptive mesoderm), is used as a reacting tissue (Dawid et al., 1992). In the present study, on the other hand, ectoderm treated with activin induced a well-organized secondary embryo as the organizer does when implanted into the blastocoele of early gastrula. These findings suggest that activin A induces organizer activity in the ectoderm and the activin-treated ectoderm acts as an initiator in establishing the fundamental body plan.

Regional induction-specificity of activin-treated ectoderm in *Xenopus laevis*

In the experiments using *Xenopus* ectoderm, it was shown that activin-treated ectoderm not only differentiated into mesodermal tissues but also behaved like the organizer. Ectoderm treated with a low concentration of activin A (10 ng/ml) never formed head structures even if the duration of preculture was prolonged. On the other hand, ectoderm treated with high concentrations of activin A (50 and 100 ng/ml) induced head structures in addition to trunk-tail structures as the duration was prolonged. These results suggest that ectoderm treated with higher concentrations of activin A obtains head organizer activity for head formation, depending on the duration of preculture. When activin-treated ectoderm is immediately sandwiched with untreated ectoderm, the sandwich explant is determined to form trunk-tail structures because the activin-treated ectoderm does not have head organizer activity at this time. After being precultured for a definite period (more than 3

hr), activin-treated ectoderm obtains head organizer activity and induces head structures from the surrounding untreated ectoderm.

In the classical organizer experiments using *Cynops* early gastrulae, the dorsal lip region obtained head organizer activity in return for losing trunk-tail organizer activity during the process of preculture (Okada and Takaya, 1942a, b; Okada and Hama, 1943; Hama, 1949). The regional induction-specificity of dorsal lip is considered to have transformed from trunk-tail to head autonomously. On the other hand, in the present study, the activin-treated ectoderm also obtained head organizer activity but mesoderm differentiation for trunk-tail formation scarcely decreased after long-term preculture. Although the activin-treated ectoderm did not show conspicuous transformation of regional induction-specificity, it will be made more clear by making experimental conditions, *i.e.*, concentration of activin A and duration of preculture, closer to the normal *Xenopus* development.

Presumptive ectoderm treated with XTC-MIF, which is identical to *Xenopus* activin A (Smith et al., 1990; Eijnden-Van Raaij et al., 1990), induces a well-organized secondary embryo when implanted into the blastocoele or ventral marginal zone of early *Xenopus* gastrula (Cooke, 1989; Ruiz i Altaba and Melton, 1989; Cho et al., 1991a). The type of secondary embryo was dependent on the concentration of XTC-MIF. Head structures were often induced at high concentrations, whereas trunk-tail structures were induced

at low concentrations (Ruiz i Altaba and Melton, 1989). The XTC-MIF-treated ectoderm is thrust against ventral ectoderm some hours after implantation as a result of the host's gastrulation. Therefore, the above results may be equivalent to the differentiation of the long term-precultured sandwich explants in the present study. The activin-treated ectoderm precultured for long periods (more than 3 hr) induced structures ranging from trunk-tail to head structures in a dose-dependent manner.

Hama et al. (1985) divided the dorsal lip of *Cynops* early gastrula into three regions, and compared their inducing abilities on presumptive ectoderm using the sandwich culture method. Both the region nearest to the blastopore and the adjacent region changed their inducing abilities from trunk-tail to head, depending on the duration of preculture. These changes in inducing abilities progressed more rapidly in the former than in the latter region. The third region, nearest to the boundary of invagination, induced only trunk-tail structures, regardless of the duration of preculture. There is a parallel between the results using the organizer and those using activin-treated ectoderm. In the present study, ectoderm treated with the highest concentration of activin A (100 ng/ml) showed the same inducing ability as the organizer nearest to the blastopore. On the other hand, ectoderm treated with the lowest concentration of activin A (10 ng/ml) showed similar ability to the organizer farthest from the blastopore. However, the tissue differentiation of these sandwich

explants is not always identical with each other. For example, the frequency of the differentiation of spinal cord is very low in the present study. Spinal cord is characteristic of the trunk-tail region and induced by the trunk-tail organizer at very high frequency. Takaya (1956a, b, 1977) reported that the regionalization of the central nervous system is affected by the quality and quantity of surrounding mesodermal tissues. Forebrain differentiates in the presence of a few mesenchyme, whereas more posterior central nervous systems (hindbrain and spinal cord) differentiate surrounded by the axial mesodermal tissues, such as notochord and muscle. Although the explants sandwiching the shorter term-precultured ectoderm showed trunk-tail formation in appearance, the axial mesoderm, especially notochord, did not differentiate in these explants sufficiently. For the reason above, the difference in the tissue differentiation seems to be caused between organizer experiments and present study.

Regional induction-specificity of activin-treated ectoderm in *Cynops pyrrhogaster*

In the experiments using *Cynops* ectoderm, the sandwich explants differentiated into typical trunk-tail or head structures depending on the duration of preculture of the activin-treated ectoderm. The arrangement of tissues and organs in the explants was similar to that of normal embryos. The explants sandwiching short-term precultured ectoderm often formed a complete embryonic axis accompanied

by a beating heart. Differentiation of the heart anlage is known to depend on the influence of endoderm during normal development (Mangold, 1956; Orts-Llorca and Gil, 1965; Jacobson and Sater, 1988). Considering that the activin-treated ectoderm itself differentiated into yolk-rich endodermal tissues, the presence of endoderm seems to be important to the formation of heart structures in the sandwich explants. On the other hand, the explants sandwiching long-term precultured ectoderm formed archencephalic structures, such as forebrain and eye, at high rates. Although these changes in the regional induction-specificity will occur autonomously during preculture, the mechanism is not at all certain at present. Motility of the inducing tissue and cell interactions between the inducing and reacting tissues will need to be taken into account to resolve this question (Hama et al., 1985).

The inducing ability of the dorsal lip of the early gastrula of *Cynops pyrrhogaster* has been examined by certain Japanese groups in great detail (Okada and Takaya 1942a, b; Okada and Hama 1943; Hama et al., 1985). The dorsal lip region next to the blastopore (presumptive pharyngeal endoderm) itself differentiated into yolk-rich endodermal tissues. This region induced trunk-tail structures when immediately sandwiched between ectodermal sheets. However, the same region induced head structures after being precultured for a definite period in Holtfreter's solution (Hama et al., 1985). Thus, the activin-treated ectoderm

seems to have exactly the same induction properties as the dorsal lip region next to the blastopore and to display "trunk-tail organizer" and "head organizer" effects (Spemann, 1931) depending on the duration of preculture.

Based on the results in the present study and those reported in other papers, it can be postulated that the process of establishment of the body plan in the *Cynops* sandwich explants is as follows. The activin-treated ectoderm differentiates mainly into endodermal tissues in the first step. Next, the endodermalized ectoderm acts as an initiator of a chain of induction events, like the dorsal lip region next to the blastopore (Hama et al., 1985), and induce axial mesoderm, such as notochord and somites, from the adjacent non-induced ectoderm. The induced axial mesoderm further induces central nervous system from the remaining non-induced ectoderm (Suzuki et al., 1986). Lastly, the anterior-posterior pattern of the central nervous system is determined by the quality and quantity of surrounding mesodermal tissues (Takaya, 1956a, b, 1977). The existence and function of activin in early *Cynops* embryos are not at all certain at present. However, the present study will serve as a suitable test system to analyze the establishment of the fundamental body plan of amphibian embryos *in vitro*.

Summary

Regional induction-specificity of the presumptive ectoderm of *Xenopus laevis* and *Cynops pyrrhogaster* was examined after the treatment with activin A. In the sandwich experiments using the *Xenopus* blastula ectoderm, the activin-treated ectoderm precultured for a short period induced trunk-tail structures, whereas that precultured for a long period induced head structures in addition to trunk-tail structures. These time-dependent changes in regional induction-specificity occurred more rapidly when the concentration of activin A was higher. Lineage analysis of the sandwich explants, which formed typical heads, revealed that the activin-treated ectoderm differentiated mainly into mesodermal tissues and induced head structures with brain and eye in the untreated ectoderm. These results suggest that the activin-treated ectoderm functioned as a "trunk-tail organizer" or "head organizer" depending on the concentration of activin A and the duration of preculture.

In the experiments using the *Cynops* gastrula ectoderm, ectoderm treated with a high concentration of activin A (100 ng/ml) also displayed time-dependent changes in induction-specificity both in the implantation and sandwich culture methods. However, the *Cynops* sandwich explants showed some differences in histological differentiation as compared with the *Xenopus* explants. The *Cynops* activin-treated ectoderm itself differentiated preferentially into yolk-rich

endodermal tissues under this condition. Furthermore, lineage analysis of the sandwich explants revealed that the activin-treated ectoderm differentiated mainly into endodermal tissues and induced axial mesoderm and central nervous system in the untreated ectoderm. These results indicate that activin A is one of the substances involved in triggering endodermal differentiation, and that the presumptive ectoderm induced to form endoderm displays regional induction-specificity as shown in the classical organizer experiments.

General discussion

General discussion

In the present study, I have focused on activin A, a strong candidate for the mesoderm-inducing factor, and examined its induction properties on pluripotent presumptive ectoderm through a series of experiments. Activin A could control mesoderm formation in *Xenopus* ectoderm in a gradient fashion, which agrees with classical gradient theories. The vegetalizing (endoderm-inducing) effect of activin A was also revealed by the experiment using *Cynops* ectoderm as a reacting tissue. Furthermore, ectoderm treated with activin A behaved as the organizer and exhibited clear regional induction-specificity for the formation of complete embryonic body plan. Thus, using the activin/ectoderm experimental system, it has been possible to control cell differentiation and morphogenesis during early amphibian development, and design a fundamental embryonic form *in vitro*. However, there are many problems still to be solved to understand the establishment of embryonic body plan more precisely.

Concentration gradient of activin and competence of reacting tissue

The most characteristic property of activin on *Xenopus* ectoderm is induction of a variety of mesodermal tissues in a gradient fashion. Mesodermal tissues along the dorso-ventral axis were induced in the presumptive ectoderm with

clear dose thresholds (chapter 1, series 1). A number of genes induced by activin on *Xenopus* ectoderm have recently been reported (reviewed by Dawid et al., 1992; Asashima, 1994). For example, *Xgoosecoid* (*Xgscd*), a *Xenopus* homeobox gene cloned from the cDNA library of the dorsal lip region of early gastrulae (Cho and De Robertis, 1990; Cho et al., 1991b; Blumberg et al., 1991), is expressed within 30 min after the activin treatment. *Xgscd* seems to be one of the early response genes directly induced by activin, because its expression is not inhibited in the presence of cycloheximide, a specific inhibitor of new protein synthesis. *Xgscd* is expressed in only the dorsal lip region of early gastrulae. Another early response gene, *Xbrachyury* (*Xbra*), is expressed in the mesodermal cells of marginal zone at early gastrula stage (Smith et al., 1991). *Xbra* is immediately induced by both activin and FGF on the isolated ectoderm. Green et al. (1992, 1994) exposed dissociated ectodermal cells to different concentration of activin, and found that the type of genes expressed in these cells is related to the concentration of activin used. The dorsal mesodermal marker, *Xgscd*, is expressed at high concentrations of activin, whereas the pan-mesodermal marker, *Xbra*, is expressed at lower concentrations. These results support the concentration effects of activin for the mesodermal differentiation of *Xenopus* ectoderm at the molecular level. Furthermore, Gurdon et al. (1994) showed that the expression of *Xbra* and *Xgscd* is spatially related to the source of activin signal. *Xbra* is expressed at a

distance from the activin source and *Xgscd* is expressed in the intervening space between the activin source and cells expressing *Xbra*. These results suggest that activin acts as a morphogen, and that the type of gene activation and cell differentiation undergone by reacting tissues is related to the concentration of activin that they receive. However, the putative concentration gradient of activin has not actually been seen, although activin protein exists in the early *Xenopus* embryos (Asashima et al., 1991b; Fukui et al., 1994). Activin proteins are found in yolk platelets in the oocytes (Uchiyama et al., 1994). How these proteins are concerned in the mesoderm formation during early *Xenopus* development should be examined. If activin forms a concentration gradient in the embryos as expected from *in vitro* studies, the mechanism of gene activation and cell differentiation caused by this gradient is not at all certain at present. Several kinds of *Xenopus* activin type II receptors have been cloned (Kondo et al., 1991; Hemmati-Brivanlou et al., 1992; Mathews et al., 1992; Nishimatsu et al., 1992), and there are slight differences in the affinity of activin depending on the type of receptor. These differences seem to affect the differentiation of target cells. On the other hand, recent studies have revealed that activin might act in combination with other regulatory molecules. Combination of activin A and retinoic acid can induce pronephric tubules at high rates (Moriya et al., 1993). The synergic effect of activin and other molecules also seems to be important for the mesoderm formation.

Evidence that another potent mesoderm-inducing factor, FGF, plays a very important role in *Xenopus* embryogenesis has also been accumulated during the past few years (Slack et al., 1987; Kimelman et al., 1988; Slack and Isaacs, 1989; Musci et al., 1990; Shiurba et al., 1991; Amaya et al., 1991, 1993; Isaacs et al., 1992). Further studies will be needed to know how these molecules are related with each other and participate in the mesoderm formation during the early *Xenopus* development.

It was also shown that there exist stage- and regional differences in competence to respond to activin within the reacting tissues. The assistant role of the reacting tissues for the concentration gradient of activin was discussed (chapter 1, series 2). Sokol and Melton (1991) showed the different competence to respond to PIF (an activin homologue) between the prospective dorsal and ventral regions of presumptive ectoderm. They concluded that activin does not endow ectodermal cells with polarity but rather reveals their pre-existent pattern. Although the competence prepattern of response to activin exists as early as the 8-cell stage (Kinoshita et al., 1993), the process of prepatterning is not understood sufficiently. The prepatterning is not likely to depend directly on the number activin receptors, because mRNA encoding receptors is uniformly distributed in the early embryos (Kondo et al., 1991; Mathews et al., 1992). Recent studies revealed that the modifiers, such as *Xwnt-8* (Sokol and Melton, 1992) and lithium (Kinoshita and Asashima, 1995), alter the competence

prepattern of animal hemisphere cells to respond to activin. We should investigate how the competence prepattern is established and participates in the pattern formation of mesoderm during early *Xenopus* development. The patterning of mesoderm should be discussed, at present, from dual aspects: the localization (gradient) of the mesoderm-inducing factor(s) and the differential competence prepattern of the reacting tissues.

Mesoderm-inducing activity and vegetalizing activity of activin

The activin-treated ectoderm of *Cynops pyrrhogaster* itself differentiated into yolk-rich endodermal tissues, which suggests a novel role of activin for the vegetalization during early *Cynops* development (chapter 2). Transmission electron microscopy of the sandwich explants revealed that these yolk-rich tissues exhibited the cytodifferentiation pattern expected of lever and intestine. Activin is not likely to induce directly these well-developed endodermal tissues, because the mesodermal tissues induced in the sandwich explants seem to affect the differentiation of the initially endodermalized cells (Okada, 1954a, b, 1960). As shown in the combination and sandwich experiments, it is also clear that the mesodermal tissues are induced as a secondary interaction between the endodermalized cells and non-induced cells (Grunz, 1979; Minuth and Grunz, 1980). Thus, it is likely that activin

acts as a vegetalizing (endoderm-inducing) factor, not a mesoderm-inducing factor, on *Cynops* presumptive ectoderm.

On the other hand, such the well-developed endodermal tissues as liver and intestine were never found in the *Xenopus* activin-treated explants (chapter 1 and chapter 3, series 1). However, nonspecific yolk-rich tissues, which could be considered undifferentiated endoderm (Grunz, 1983), were induced around notochord at high concentrations of activin A. Jones et al. (1993) identified endoderm in the activin-treated ectoderm using a monoclonal endodermal marker. A *Xenopus* homeobox gene, *Mix. 1*, is immediately expressed after the activin treatment on presumptive ectoderm (Rosa, 1989). In normal development, *Mix. 1* mRNA is localized in the presumptive endoderm and mesoderm region in the vegetal hemisphere. *XlHbox 8*, another *Xenopus* homeobox gene expressed in the anterior endodermal cells fated to develop into pancreas and duodenum (Wright et al., 1988), is induced in the presumptive ectoderm treated with activin (Gamer and Wright, 1995). Although I was unable to confirm the endodermal differentiation of *Xenopus* presumptive ectoderm at the histological level in the present study, the above results based on the molecular studies suggest that activin may also have a vegetalizing effect on *Xenopus* ectoderm. The *Cynops* presumptive ectoderm is made up of a homogeneous cell layer. On the other hand, *Xenopus* ectoderm consists of heterogeneous cells, such as inner layer and outer layer (Asashima and Grunz, 1983). If activin induces mesoderm as a secondary induction *via* vegetalized cells, the

heterogeneity of reacting tissue may be a major cause of mesodermal differentiation at high rates in the *Xenopus* activin-treated ectoderm. There is another possibility, however, that activin directly induces mesoderm and endoderm depending on its concentration. As mentioned above, the expression of mesodermal marker genes, *Xbra* and *Xgscd*, is directly induced by activin without new protein synthesis. *Mix. 1*, normally expressed in the presumptive endoderm and mesoderm region of the embryos, is also directly induced by high concentrations of activin. Furthermore, the expression of a endodermal marker gene, *XlHbox 8*, only occurs at activin doses well above those that induce mesodermal tissues (Gamer and Wright, 1995). Further study will be needed to confirm whether mesodermal tissues are directly induced by activin or formed as a result of secondary interactions between endodermalized and non-induced cells in *Xenopus laevis*.

The formation of beating hearts is characteristic of the *Cynops* activin-treated ectoderm (chapter 2, series 2), which also supports the vegetalizing activity of activin A on *Cynops* ectoderm. Differentiation of the heart rudiment during normal development is known to depend on the influence of anterior pharyngeal endoderm (reviewed by Jacobson and Sater, 1988). Anterior pharyngeal endoderm contacts prospective cardiogenic mesoderm and stimulates morphogenesis and differentiation. This indicates that at least two distinct tissues act as anterior pharyngeal endoderm and presumptive cardiogenic mesoderm are present in

the activin-treated ectoderm. Although it has not been confirmed that the yolk-rich endodermal tissues induced by activin in *Cynops* ectoderm are identical to anterior pharyngeal endoderm, the presence of these tissues seems to be requisite for the formation of beating hearts.

Regional induction-specificity of the activin-treated ectoderm

Activin A induced head and trunk-tail organizing activities in the presumptive ectoderm (chapter 3). The activin-treated ectoderm behaved as the organizer and exhibited clear regional induction-specificity from trunk-tails to heads depending on the concentration of activin A and the time after the activin treatment. These findings suggest that activin acts as the first molecular signal in the chain of inductive events in amphibian embryogenesis.

The regional induction-specificity of the organizer is closely related to the establishment of fundamental body plan. In normal development, the organizer induces central nervous system anlagen to the overlying layer of presumptive ectodermal cells. The anterior-posterior pattern of the central nervous system is known to be determined by the quality and quantity of surrounding mesodermal tissues (Takaya, 1956a, b, 1977). Forebrain (characterizes heads) differentiates in the presence of a few mesenchyme, whereas hindbrain and spinal cord (characterize trunk and tails) differentiate surrounded by the axial mesodermal tissues, such as notochord and muscle. In the present study using

Cynops ectoderm (chapter 3, series 2), the axial mesoderm was induced by shorter-term precultured activin-treated ectoderm at high rates. The explants sandwiching the shorter-term precultured activin-treated ectoderm formed trunk and tails. Thus, there seems to be a close relationship between the regional induction-specificity and the axial mesoderm-inducing ability of the activin-treated ectoderm. Although the mechanism of the regional induction-specificity of the organizer is not yet known at the molecular level, this activin/ectoderm experiment will serve as a useful system to analyze this problem.

The *Cynops* activin-treated ectoderm differentiated into yolk-rich endodermal tissues and induced axial mesoderm and central nervous system in the untreated ectoderm. These results are in agreement with those of the previous experiment using presumptive pharyngeal endoderm as the inducer. From their extensive sandwich experiments on the dorsal lip of the early *Cynops* gastrula, Hama et al. (1985) concluded that presumptive pharyngeal endoderm is the core of the organizer activity. Recently, *Siamois*, a *Xenopus* homeobox gene expressed in the dorsal endoderm including presumptive pharyngeal endoderm of early gastrulae has been cloned (Lemaire et al., 1995). *Siamois* generates a complete secondary embryo when injected ventrally at the 4-cell stage. The injected *Siamois* mRNA is inherited by anterior endodermal cells that do not contribute to the secondary axial structure. These results are consistent with the hypothesis that presumptive pharyngeal endoderm acts as the

initiator of the organization center (Hama et al., 1985). Although it has not been confirmed that the yolk-rich endodermal tissues induced by activin A in *Cynops* ectoderm is identical to the presumptive pharyngeal endoderm, these results support the idea that activin acts as the first molecular signal in the chain of inductive events in amphibian embryogenesis.

Conclusion

Conclusion

The present study was undertaken to control cell differentiation and morphogenesis during early amphibian development employing the microsurgery and *in vitro* culture techniques. Activin A, a member of the transforming growth factor-ß (TGF-ß) superfamily, was used as the inducer and presumptive ectoderm of *Xenopus laevis* (Anura) and *Cynops pyrrhogaster* (Urodela) early embryos were used as the pluripotent reacting tissues throughout the experimental series.

Activin A could control mesodermal differentiation of *Xenopus* presumptive ectoderm in a gradient fashion, such that low doses induced ventral mesoderm and high doses induced dorsal mesoderm, which agrees with classical gradient theories using vertebrate tissues as the inducers to some extent. It was also shown that presumptive ectoderm at different developmental stages responded differently to the activin A treatment, and that prospective dorsal and ventral regions of the presumptive ectoderm responded differently to the same concentration of activin A. These differences in competence to the inducer within the reacting tissue may also be important for the patterning of mesoderm in early *Xenopus* development.

The induction property of activin A on *Cynops* presumptive ectoderm was different from that on *Xenopus* ectoderm. The activin-treated *Cynops* ectoderm differentiated

preferentially into yolk-rich tissues that were identified as endodermal derivatives from their histological characteristics. Mesodermal tissues including beating heart rudiments were formed at high frequencies when larger sizes of presumptive ectoderm were used as the reacting tissues or untreated (non-induced) ectoderm was combined with the activin-treated ectoderm. These results suggest that activin A has a vegetalizing effect on *Cynops* presumptive ectoderm, and that mesoderm is induced as a result of secondary interactions between vegetalized (endodermalized) and non-induced tissues.

The activin-treated ectoderm of *Xenopus laevis* and *Cynops pyrrhogaster* behaved as the organizer on the non-induced ectoderm and changed their regional induction-specificity from trunk-tails to heads depending on the time after the activin A treatment. The arrangement of tissues and organs induced in the explants was similar to that of normal embryos. The regional induction-specificity of the organizer is closely related to the establishment of fundamental body plan during early amphibian development. Although the mechanism of the regional induction-specificity of the organizer remains to be solved, these results observed *in vitro* should provide a useful experimental system to analyze this problem.

In conclusion, the present study has shown that cell differentiation and morphogenesis during early amphibian development can be essentially reproduced and controlled *in vitro* using activin and pluripotent presumptive ectoderm.

These facts thus suggest that activin is one of the first induction signals responsible for establishing the embryonic body plan in early amphibian development. Although the results of the present study performed *in vitro* can not completely explain the role of activin *in vivo*, they will serve as an excellent model system for further analysis of cell differentiation, morphogenesis and organogenesis at the histological and molecular levels.

Acknowledgements

Acknowledgements

I wish to express my deepest indebtedness to Prof. Dr. Makoto Asashima and Dr. Akimasa Fukui, Department of Biology, College of Arts and Science, University of Tokyo, for their constant guidance, encouragement, and valuable discussion during the course of my study.

Thanks are also due to members of Prof. Asashima's laboratory, for their valuable discussion, friendship and help during the course of my study.

I am indebted to Prof. Dr. George M. Malacinski, Department of Biology, Indiana University, Dr. Shinji Komazaki, the 2nd Department of Anatomy, Saitama Medical School, and Mr. Hideho Uchiyama, Department of Biology, Yokohama City University, for their excellent suggestion and warm encouragement on my study.

I am also grateful to Dr. Yuzuru Eto, the Central Research Laboratories of Ajinomoto Co. Inc., for supplying human recombinant activin A.

References

References

Albano RM, Godsave SF, Huylebroeck D, Nimmen K van, Isaacs HV, Slack JMW, Smith JC (1990) A mesoderm-inducing factor produced by WEHI-3 murine myelomonocytic leukemia cells is activin A. Development 110: 435-443

Amaya E, Musci TJ, Kirschner MW (1991) Expression of a dominant negative mutant of the FGF receptor disrupts mesoderm formation in *Xenopus* embryos. Cell 66: 257-270

Amaya E, Stein PA, Musci TJ, Kirschner MW (1993) FGF signaling in the early specification of mesoderm in *Xenopus*. Development 118: 477-487

Ariizumi T, Asashima M (1994) *In vitro* control of the embryonic form of *Xenopus laevis* by activin A: Time and dose-dependent inducing properties of activin-treated ectoderm. Dev Growth Differ 36: 499-507

Ariizumi T, Asashima M (1995a) Head and trunk-tail organizing effects of the gastrula ectoderm of *Cynops pyrrhogaster* after treatment with activin A. Roux's Arch Dev Biol 204: 427-435

Ariizumi T, Asashima M (1995b) Control of the embryonic body plan by activin during amphibian development. Zool Sci 12: 509-521

Ariizumi T, Moriya N, Uchiyama H, Asashima M (1991a) Concentration-dependent inducing activity of activin A. Roux's Arch Dev Biol 200: 230-233

Ariizumi T, Sawamura K, Uchiyama H, Asashima M (1991b) Dose and time-dependent mesoderm induction and outgrowth formation by activin A in *Xenopus laevis*. Int J Dev Biol 35: 407-414

Ariizumi T, Komazaki S, Asashima M, Malacinski GM (1996) Activin treated urodele ectoderm: A model experimental system for cardiogenesis. Int J Dev Biol (in press)

Asashima M (1975) Inducing effects of the presumptive endoderm of successive stages in *Triturus alpestris*. Wilhelm Roux' Arch Entwicklungsmech Org 177: 301-308

Asashima M (1994) Mesoderm induction during early amphibian development. Dev Growth Differ 36: 343-355

Asashima M, Grunz H (1983) Effects of inducers on inner and outer gastrula ectoderm layers of *Xenopus laevis*. Differentiation 23: 206-212

Asashima M, Nakano H, Matsunaga T, Sugimoto M, Takano H (1987) Purification of mesoderm-inducing substances from carp swim bladders. I. Extraction and isoelectric focusing. Dev Growth Differ 29: 221-227

Asashima M, Nakano H, Shimada K, Kinoshita K, Ishii K, Shibai H, Ueno N (1990a) Mesodermal induction in early amphibian embryos by activin A (erythroid differentiation factor). Roux's Arch Dev Biol 198: 330-335

Asashima M, Nakano H, Uchiyama H, Davids M, Plessow S, Loppnow-Blinde B, Hoppe P, Dau H, Tiedemann H (1990b) The vegetalizing factor belongs to a family of mesoderm-inducing proteins related to erythroid differentiation factor. Naturwissenschaften 77: 389-391

Asashima M, Nakano H, Uchiyama H, Sugino H, Nakamura T, Eto Y, Ejima D, Davids M, Plessow S, Cichocka I, Kinoshita K (1991a) Follistatin inhibits the mesoderm-inducing activity of activin A and the vegetalizing factor from chicken embryo. Roux's Arch Dev Biol 200: 4-7

Asashima M, Nakano H, Uchiyama H, Sugino H, Nakamura T, Eto Y, Ejima D, Nishimatsu S, Ueno N, Kinoshita K (1991b) Presence of activin (erythroid differentiation factor) in unfertilized eggs and blastulae of *Xenopus laevis*. Proc Natl Acad Sci USA 88: 6511-6514

Asashima M, Uchiyama H, Nakano H, Eto Y, Ejima D, Sugino H, Davids M, Plessow S, Born J, Hoppe P, Tiedemann H, Tiedemann H (1991c) The vegetalizing factor from chicken embryos: its EDF(activin A)-like activity. Mech Dev 34: 135-141

Attisano L, Wrana JL, Cheifetz S, Massagué J (1992) Novel activin receptors: Distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. Cell 68: 97-108

Becker U, Tiedemann H, Tiedemann H (1959) Versuche zur Determination von embryonalem Amphibiengewebe durch Induktionsstoffe in Lösung. Z Naturforsch 14b: 608-609

Blumberg B, Wright CVE, De Robertis EM, Cho KWY (1991) Organizer-specific homeobox genes in *Xenopus laevis* embryos. Science 253: 194-196

Bodmer R (1995) Heart development in *Drosophila* and its relationship to vertebrates. TCM 5: 21-28

Born J, Geithe H-P, Tiedemann H, Tiedemann H, Kocher-Becker U (1972) Isolation of a vegetalizing inducing factor. Z Physiol Chem 353: 1075-1084

Cho KWY, De Robertis EM (1990) Differential activation of *Xenopus* homeobox genes by mesoderm inducing growth factors and retinoic acid. Genes Dev 4: 1910-1916

Cho KWY, Morita EA, Wright CVE, De Robertis EM (1991a) Overexpression of a homeodomain protein confers axis-forming activity to uncommitted *Xenopus* embryonic cells. Cell 65: 55-64

Cho KWY, Blumberg B, Steinbeisser H, De Robertis EM (1991b) Molecular nature of Spemann's organizer: The role of the *Xenopus* homeobox gene goosecoid. Cell 67: 1111-1120

Cooke J (1989) Mesoderm-inducing factors and Spemann's organizer phenomenon in amphibian development. Development 107: 229-241

Cox WG, Neff AW (1995) Cardiac myosin heavy chain expression during heart development in *Xenopus laevis*. Differentiation 58: 269-280

Dawid IB, Taira M, Good PJ, Rebagliati MR (1992) The role of growth factors in embryonic induction in *Xenopus laevis*. Mol Rep Dev 32: 136-144

Eijnden-Van Raaij AJM van den, Zoelent EJJ van, Nimmen K van, Koster CH, Snoek GT, Durston AJ, Huylebroeck D (1990) Activin-like factor from a *Xenopus laevis* cell line responsible for mesoderm induction. Nature 345: 732-734

Fukui A, Nakamura T, Sugino K, Takio K, Uchiyama H, Asashima M, Sugino H (1993) Isolation and characterization of *Xenopus* follistatin and activin. Dev Biol 158: 131-139

Fukui A, Nakamura T, Uchiyama H, Sugino K, Sugino H, Asashima M (1994) Identification of activins A, AB and B and follistatin proteins in the *Xenopus* embryos. Dev Biol 163: 279-281

Gamer LW, Wright CVE (1995) Autonomous endodermal determination in *Xenopus*: Regulation of expression of the pancreatic gene *XlHbox 8*. Dev Biol 171: 240-251

Geithe H-P, Asashima M, Born J, Tiedemann H, Tiedemann H (1975) Isolation of a homogeneous morphogenetic factor, inducing mesoderm and endoderm derived tissues in *Triturus* ectoderm. Exp Cell Res 94: 447-449

Geithe H-P, Asashima M, Asahi K, Born J, Tiedemann H, Tiedemann H (1981) A vegetalizing inducing factor. Isolation and chemical properties. Biochem Biophys Acta 676: 350-356

Green JBA, Smith JC (1990) Graded changes in dose of a *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. Nature 347: 391-394

Green JBA, New HV, Smith JC (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: 731-739

Green JBA, Smith JC, Gerhart JC (1994) Slow emergence of a multithreshold response to activin requires cell-contact-dependent sharpening but not prepattern. Development 120: 2271-2278

Grunz H (1979) Change of the differentiation pattern of amphibian ectoderm after the increase of the initial cell mass. Wilhelm Roux' Arch Entwicklungsmech Org 187: 49-57

Grunz H (1983) Change in the differentiation pattern of *Xenopus laevis* ectoderm by variation of the incubation time and concentration of vegetalizing factor. Roux's Arch Dev Biol 192: 130-137

Grunz H, McKeehan WL, Knöchel W, Born J, Tiedemann H, Tiedemann H (1988) Induction of mesodermal tissues by acidic and basic heparin binding growth factors. Cell Differ 22: 183-190

Gurdon JB, Harger P, Mitchell A, Lemaire P (1994) Activin signalling and response to a morphogen gradient. Nature 371: 487-492

Hama T (1949) Explantation of the urodelen organizer and the process of morphological differentiation attendant upon invagination. Proc Imp Acad Jpn 25: 4-11

Hama T, Tsujimura H, Kanéda T, Takata K, Ohara A (1985) Inductive capacities of the dorsal mesoderm of the dorsal marginal zone and pharyngeal endoderm in the very early gastrula of the newt, and presumptive pharyngeal endoderm as an initiator of the organization center. Dev Growth Differ 27: 419-433

Hamburger V (1988) The heritage of experimental embryology Hans Spemann and the organizer. Oxford Univesity Press, New York

Hemmati-Brivanlou A, Melton DA (1992) A truncated activin receptor inhibits mesoderm induction and formation of axial structures in *Xenopus* embryos. Nature 359: 609-614

Hemmati-Brivanlou A, Wright DA, Melton DA (1992) Embryonic expression and functional analysis of a *Xenopus* activin receptor. Dev Dynam 194: 1-11

Holtfreter J (1933) Nachweis der Induktionsfähigkeit abgetöteter Keimteile. Isolations- und Transplantationsversuche. Wilhelm Roux' Arch Entwicklungsmech Org 128: 584-633

Isaacs HV, Tannahill D, Slack JMW (1992) Expression of a novel FGF in the *Xenopus* embryo. A new candidate inducing factor for mesoderm formation and anteroposterior specification. Development 114: 711-720

Jacobson A, Sater AK (1988) Features of embryonic induction. Development 104: 341-359

Jones EA, Abel MH, Woodland HR (1993) The possible role of mesodermal growth factors in the formation of endoderm in *Xenopus laevis*. Roux's Arch Dev Biol 202: 233-239

Kawakami I (1976) Fish swimbladder: an excellent mesodermal inductor in primary embryonic induction. J Embryol exp Morph 36: 315-320

Kimelman D, Abraham JA, Haaparanta T, Palisi TM, Kirschner MW (1988) The presence of fibroblast growth factor in the frog egg: its role as a natural mesoderm inducer. Science 242: 1053-1056

Kinoshita K, Asashima M (1995) Effect of activin and lithium on isolated *Xenopus* animal blastomeres and response alteration at the midblastula transition. Development 121: 1581-1589

Kinoshita K, Bessho T, Asashima M (1993) Competence prepattern in the animal hemisphere of the 8-cell-stage *Xenopus* embryo. Dev Biol 160: 276-284

Kinoshita K, Bessho T, Asashima M (1995) Onset of competence to respond to activin A in isolated eight-cell stage *Xenopus* animal blastomeres. Dev Growth Differ 37: 303-309

Knöchel W, Born J, Hoppe P, Loppnow-Blinde B, Tiedemann H, Tiedemann H (1987) Mesoderm-inducing factors: their possible relationship to heparin-binding growth factors and transforming growth factor-ß. Naturwissenschaften 74: 604-606

Knöchel W, Tiedemann H, Tiedemann H (1989) Mesoderm induction by transforming growth factor: medium conditioned by TGF-ß treated ectoderm enhances the inducing activity. Naturwissenschaften 76: 270-272

Kocher-Becker U, Tiedemann H (1971) Induction of mesodermal and endodermal structures and primordial germ cells in *Triturus* ectoderm by a vegetalizing factor from chick embryos. Nature 233: 65-66

Kondo M, Tashiro K, Fujii G, Asano M, Miyoshi R, Yamada, R, Muramatsu M, Shiokawa K (1991) Activin receptor mRNA is expressed early in *Xenopus* embryogenesis and the level of the expression affects the body axis formation. Biochem Biophy Res Commun 181: 684-690

Lemaire P, Garrett N, Gurdon JB (1995) Expression cloning of *Siamois*, a *Xenopus* homeobox gene expressed in dorsal-vegetal cells of blastulae and able to induce a complete secondary axis. Cell 81: 85-94

Ling N, Ying SY, Ueno N, Shimasaki S, Esch F, Hotta M, Guillemin R (1986) A homodimer of the ß-subunits of inhibin A stimulates the secretion of pituitary follicle-stimulating hormone. Biochem Biophys Res Commun 138: 1129-1137

Logan M, Mohun T (1993) Induction of cardiac muscle differentiation in isolated animal pole explants of *Xenopus laevis* embryos. Development 118: 865-875

Mangold O (1923) Transplantationsversuche zur Frage der Spezifität und der Bildung der Keimblätter. Arch Mikrosk Anat Entwicklungsmech 100: 193-301

Mangold O (1933) Über die induktionsfähigkeit der verschiedenen Bezirke der Neurula von Urodelen. Naturwissenschaften 21: 761-766

Mangold O (1956) Experimente zur Analyse der Herzentwicklung bei Triton. Naturwissenschaften 43: 287

Mangold O, Spemann H (1927) Über Induktion von Medullarplatte durch Medullarplatte im Jüngeren Keim. Wilhelm Roux' Arch Entwicklungsmech Org 111: 593-665

Mathews LS, Vale WW (1991) Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell 65: 973-982

Mathews LS, Vale WW, Kintner CR (1992) Cloning of a second type of activin receptor and functional characterization in *Xenopus* embryos. Science 255: 1702-1705

Minuth M, Grunz H (1980) The formation of mesodermal derivatives after induction with vegetalizing factor depends on secondary cell interactions. Cell Differ 9: 229-238

Moriya N, Asashima M (1992) Mesoderm and neural inductions on newt ectoderm by activin A. Dev Growth Differ 34: 589-594

Moriya N, Uchiyama H, Asashima M (1993) Induction of pronephric tubules by activin and retinoic acid in presumptive ectoderm of *Xenopus laevis*. Dev Growth Differ 35: 123-128

Murata M, Eto Y, Shibai H, Sakai M, Muramatsu M (1988) Erythroid differentiation factor is encoded by the same mRNA as that of the inhibin ßA chain. Proc Natl Acad Sci USA 85: 2434-2438

Musci TJ, Amaya E, Kirschner MW (1990) Regulation of the fibroblast growth factor receptor in early *Xenopus* embryos. Proc Natl Acad Sci USA 87: 8365-8369

Muslin AJ, Williams LT (1991) Well-defined growth factors promote cardiac development in axolotl mesodermal explants. Development 112: 1095-1101

Nakamura O, Takasaki H (1970) Further studies on the differentiation capacity of the dorsal marginal zone in the morula of *Triturus pyrrphogaster*. Proc Jpn Acad 46: 546-551

Nakamura O, Toivonen S (1978) Organizer -A milestone of a half century from Spemann. Elsevier/North-Holland Biomedical Press, Amsterdam

Nakamura O, Takasaki H, Ishihara M (1971) Formation of the organizer from combinations of presumptive ectoderm and endoderm. Proc Jpn Acad 47: 313-318

Nakamura T, Takio K, Eto Y, Shibai H, Titani K, Sugino H (1990) Activin binding protein from rat ovary is follistatin. Science 247: 836-838

Nakamura T, Asashima M, Eto Y, Takio K, Uchiyama H, Moriya N, Ariizumi T, Yashiro T, Sugino K, Titani K, Sugino H (1992) Isolation and characterization of native activin B. J Biol Chem 267: 16385-16389

Nakano H, Kinoshita K, Ishii K, Shibai H, Asashima M (1990) Activities of mesoderm-inducing factors secreted by mammalian cells in culture. Dev Growth Differ 32: 165-170

Nieuwkoop PD (1969) The formation of mesoderm in urodelan amphibians, Part 1 induction by the endoderm. Wilhelm Roux' Arch Entwicklungsmech Org 162: 341-373

Nieuwkoop PD, Faber J (1956) Normal table of *Xenopus laevis* (Daudin). North-Holland publishing company, Amsterdam

Nishimatsu S, Iwao M, Nagai T, Oda S, Suzuki A, Asashima M, Murakami K, Ueno N (1992) A carboxyl-terminal truncated version of the activin receptor mediates activin signals in early *Xenopus* embryos. FEBS Lett 312: 169-173

Okada TS (1954a) Experimental studies on the differentiation of the endodermal organs in Amphibia. I. Significance of the mesenchymatous tissue to the differentiation of the presumptive endoderm. Mem Coll Sci Univ Kyoto Ser B 21: 1-6

Okada TS (1954b) Experimental studies on the differentiation of the endodermal organs in Amphibia. II. Differentiating potencies of the presumptive endoderm in the presence of the mesodermal tissues. Mem Coll Sci Univ Kyoto Ser B 21: 7-14

Okada TS (1960) Epithelio-mesenchymal relationships in the regional differentiation of the digestive tract in the amphibian embryo. Wilhelm Roux' Arch Entwicklungsmech Org 152:1-21

Okada YK, Hama T (1943) Examination of regional differences in the inductive activity of the organizer by means of transplantation into ectodermal vesicles. Proc Imp Acad (Tokyo) 19: 48-53

Okada YK, Ichikawa M (1947) Normal table of *Triturus pyrrphogaster*. Jpn J Exp Morphol 3: 1-6

Okada YK, Takaya H (1942a) Experimental investigation of regional differences in the inductive capacity of the organizer. Proc Imp Acad (Tokyo) 18: 505-513

Okada YK, Takaya H (1942b) Further studies upon the regional differentiation of the inductive capacity of the organizer. Proc Imp Acad (Tokyo) 18: 514-519

Orts-Llorca F, Gil DR (1965) Influence of the endoderm on heart differentiation. Wilhelm Roux' Arch Entwicklungsmech Org 156: 368-370

Rosa FM (1989) Mix. 1, a homeobox mRNA inducible by mesoderm inducers, is expressed mostly in the presumptive endodermal cells of *Xenopus* embryos. Cell 57: 965-974

Rosa F, Roberts AB, Danielpour D, Dart LL, Sporn MB, Dawid IB (1988) Mesoderm induction in amphibians: the role of TGF-ß2-like factors. Science 239: 783-785

Ruiz i Altaba A, Melton DA (1989) Interaction between peptide growth factor and homoeobox genes in the establishment of antero-posterior polarity in frog embryos. Nature 341: 33-38

Saxén L, Toivonen S (1962) Primary embryonic induction. Logos Press, London

Shiurba RA, Jing N, Sakakura T, Godsave SF (1991) Nuclear translocation of fibroblast growth factor during *Xenopus* mesoderm induction. Development 113: 487-493

Slack JMW, Isaacs HV (1989) Presence of basic fibroblast growth factor in the early *Xenopus* embryo. Development 105: 147-153

Slack JMW, Darligton BG, Heath JK, Godsave SF (1987) Mesoderm induction in early *Xenopus* embryos by heparin-binding growth factors. Nature 326: 197-200

Smith JC (1987) A mesoderm-inducing factor is produced by a *Xenopus* cell line. Development 99: 3-14

Smith JC, Price BMJ, Nimmen K van, Huylebroeck D (1990) Identification of a potent *Xenopus* mesoderm-inducing factor as a homologue of activin A. Nature 345: 729-731

Smith JC, Price BMJ, Green JBA, Weigel D, Herrmann BG (1991) Expression of a *Xenopus* homolog of *Brachyury (T)* is an immediate-early response to mesoderm induction. Cell 67: 79-87

Sokol S, Melton DA (1991) Pre-existent pattern in *Xenopus* animal pole cells revealed by induction with activin. Nature 351: 409-411

Sokol S, Melton DA (1992) Interaction of Wnt and activin in dorsal mesoderm induction in *Xenopus*. Dev Biol 154: 348-355

Sokol S, Wong GG, Melton DA (1990) A mouse macrophage factor induces head structures and organizes a body axis in *Xenopus*. Science 249: 561-564

Spemann H (1931) Über den Anteil von Implantat und Wirtskeim an der Orientierung und Beschaffenheit der induzierten Embryonalanlage. Wilhelm Roux' Arch Entwicklungsmech Org 123: 389-517

Spemann H, Mangold H (1924) Über Induktion von Embryonalanlagen durch Implantation artfremder Organisatoren. Arch Mikrosk Anat Entwicklungsmech 100: 599-638

Stanier DYR, Fishman MC (1994) The zebra fish as a model system to study cardiovascular development. TCM 4: 207-212

Sugi Y, Lough J (1995) Activin-A and FGF-2 mimic the inductive effects of anterior endoderm on terminal cardiac myogenesis *in vitro*. Dev Biol 168: 567-574

Suzuki AS, Yoshimura Y, Yano Y (1986) Neural-inducing activity of newly mesodermalized ectoderm. Roux's Arch Dev Biol 195: 168-172

Taguchi M, Uehara M, Asashima M, Pfeiffer CJ (1989) Development of the heartbeat during normal ontogeny and during long-term organ culture of hearts of the newt, *Cynops pyrrhogaster*. Cell Differ Develop 27: 95-102

Takaya H (1956a) Two types of neural differentiation produced in connection with mesenchymal tissue. Proc Jpn Acad 32: 282-286

Takaya H (1956b) On the types of neural tissue developed in connection with mesodermal tissues. Proc Jpn Acad 32: 287-292

Takaya H (1977) Types of neural tissues induced through the presumptive notochord of newt embryo. Differentiation 7: 187-192

Tandler B (1990) Improved sectionability of paraffin-embedded specimens that initially are fixed in EM fixatives. J Electron Microsc Tech 14: 287-288

Thomsen G, Woolf T, Whitman M, Sokol S, Vaughan J, Vale W, Melton DA (1990) Activins are expressed early in *Xenopus* embryogenesis and can induce axial mesoderm and anterior structures. Cell 67: 485-493

Tiedemann H (1990) Cellular and molecular aspects of embryonic induction. Zool Sci 7: 171-186

Tiedemann H, Lottspeich F, Davids M, Knöchel S, Hoppe P, Tiedemann H (1992) The vegetalizing factor: a member of the evolutionarily highly conserved activin family. FEBS Lett 300: 123-126

Toivonen S (1938) Spezifische Induktionsleistungen von abnormen Induktoren in Implantatversuch. Ann Soc Zool Bot Fenn Vanamo 6: 1-12

Toivonen S (1940) Über die Leistungsspezifität der abnormen Induktoren in Implantatversuch bei Triton. Ann Acad Sci Fenn Ser A 55: 1-150

Toivonen S (1953) Bone-marrow of the guinea-pig as a mesodermal inductor in implantation experiments with embryos of *Triturus*. J Embryol exp Morph 1: 97-104

Toivonen S, Saxén L (1955a) The simultaneous inducing action of liver and bone-marrow of the guinea pig in implantation and explantation experiments with embryos of *Triturus*. Exp Cell Res Suppl 3: 346-357

Toivonen S, Saxén L (1955b) Über die Induktion des Neuralrohrs bei *Triturus* Keimen als simultane Leistung des Leber- und Knochenmarkgewebes vom Meerschweinchen. Ann Acad Sci Fenn A IV 30: 1-29

Uchiyama H, Nakamura T, Komazaki S, Takio K, Asashima M, Sugino H (1994) Localization of activin and follistatin proteins in the *Xenopus* oocyte. Biochem Biophy Res Commun 202: 484-489

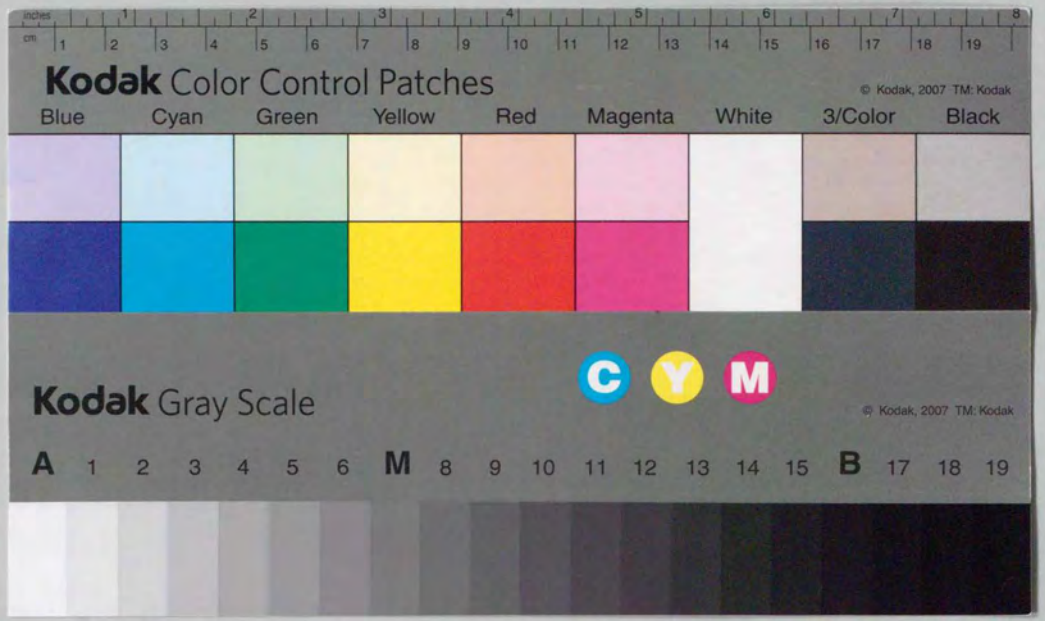
Uehara M, Taguchi M, Asashima M, Pfeiffer CJ (1989) Developmental physiology of cardiac contraction in the Japanese newt *in vivo* and *in vitro*. J Mol Cell Cardiol 21: 709-718

Ueno N, Ling L, Ying SY, Esch F, Shimasaki S, Guillemin R (1987) Isolation and partial characterization of follistatin: a single-chain Mr 35,000 monomeric protein that inhibits the release of follicle-stimulating hormone. Proc Natl Acad Sci USA 84: 8282-8286

Vale W, Rivier J, Vaughan J, McClitock R, Corrigan A, Woo W, Karr D, Spiess Y (1986) Purification and characterization of a FSH-releasing protein from porcine ovarian follicular fluid. Nature 321: 776-779

Wright CVE, Schnegelsberg P, De Robertis EM (1988) XlHbox 8: a novel *Xenopus* homeo protein restricted to a narrow band of endoderm. Development 104: 787-794

Yamada T, Takata K (1961) A technique for testing macromolecular samples in solution for morphogenetic effects on the isolated ectoderm of the amphibian gastrula. Dev Biol 3: 411-423


inches
cm
Kodak Color Control Patches
© Kodak, 2007 TM: Kodak
Blue
Cyan
Green
Yellow
Red
Magenta
White
3/Color
Black
Kodak Gray Scale
C
Y
M
© Kodak, 2007 TM: Kodak
A 1 2 3 4 5 6 M 8 9 10 11 12 13 14 15 B 17 18 19