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In vitro induction systems for analyses of amphibian organogenesis and body patterning

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ABSTRACT The discovery that some well-known growth factors have inducing activity in embryogenesis has accelerated our understanding of embryonic induction. Relevant receptors, signal transduction pathways and patterns of gene expression have been characterized over the past decade. Amphibian embryos have provided an excellent model for analysis of embryonic induction because they are easily surgically manipulated and cultured *in vitro*, and with the addition of treatment with various inducing factors we have been able to control organogenesis and body patterning during early development *in vitro*. Activin A, a TGF-β family protein, has a potent mesoderm-inducing activity on the isolated ectoderm called the animal cap. Activin induces animal caps to differentiate into various mesodermal and endodermal tissues, including beating hearts, in a dose-dependent fashion. Activin, in combination with retinoic acid, also induces the formation of the pronephros, a primitive embryonic kidney. The *in vitro* induced kidney was confirmed to function *in vivo* in a transplantation experiment. Furthermore, the activin-induced animal caps organize heads or trunk-and-tails in exactly the same manner as the organizer. The potential use of *in vitro* induction systems to further our understanding of vertebrate organogenesis and body patterning will be discussed.

KEY WORDS: *activin, animal cap, body patterning, embryonic induction, growth factor, organizer, organogenesis.*

Introduction

Embryonic induction events are key regulatory events in organogenesis and body patterning during early vertebrate development. Amphibian embryos are particularly suitable for surgical manipulation, and isolated tissues can be easily cultured compared with many other vertebrates. In the classical induction experiment by Spemann and Mangold (1924), transplantation of the organizer (dorsal lip region of gastrula) into the ventral side of a host gastrula-stage embryo resulted in the formation of a secondary embryo. The neural tissues of the induced secondary embryo were almost entirely derived from the host ventral ectoderm, normally fated to become epidermis. This inductive event between the organizer and ectoderm is referred to as "neural induction". Another classical induction experiment performed by Nieuwkoop (1969) revealed that endodermal (vegetal) cells induce the formation of mesodermal tissues when they are combined with ectodermal (animal) cells. This inductive phenomenon generated by the recombination manipulation is called "mesoderm induction". The basic body plan is generally considered to be established as a result of these two major embryonic inductions.

As shown in classical induction experiments, the ectodermal region of blastula or gastrula embryos (animal cap) has pluripotency and can be induced to differentiate into neural tissue and mesoderm by exposure to an appropriate inducing agent. During the past decade, several growth factors belonging to the fibroblast growth factor (FGF) and transforming growth factor- β (TGF-β) families have been identified as strong candidate mesoderm inducing factors that can induce various mesodermal tissues in isolated animal caps (reviewed by Asashima *et al.*, 1999). Activin induces a variety of mesodermal and endodermal tissues in animal caps in a concentration-dependent manner (Ariizumi *et al.*, 1991a, b, 1999), and can also induce multiple organs when combined with other molecules. Treatment of animal caps with activin and retinoic acid results in the formation of a functionally active pronephros, the simplest embryonic kidney (Moriya *et al.*,

Abbreviations used in this paper: bpm, beats per minute.

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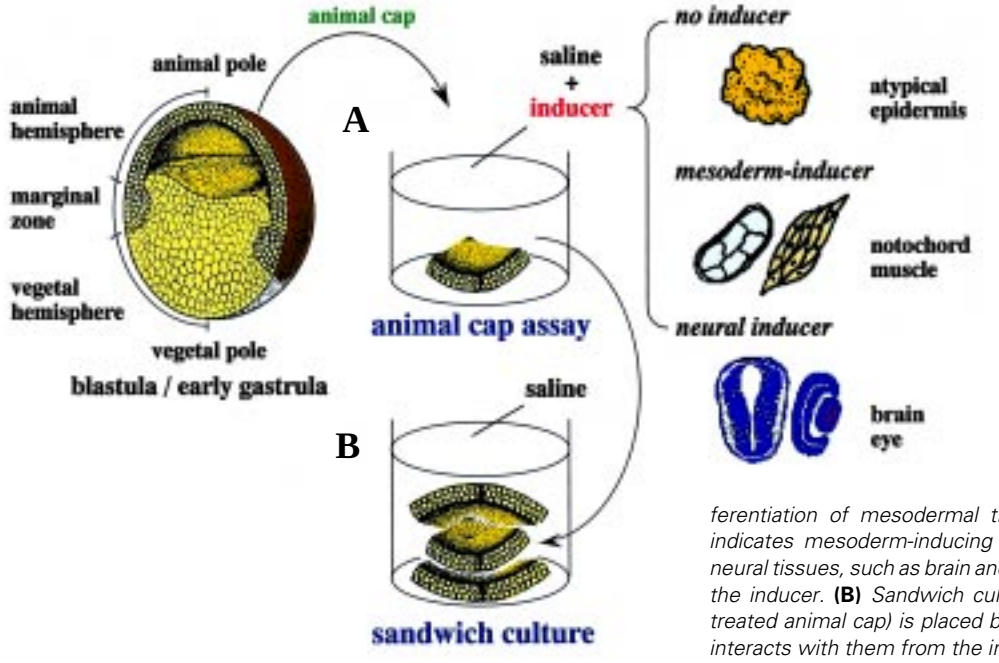

Fig. 1. *In vitro* induction systems using animal caps. (A) *An animal cap assay. Blastula or gastrula animal caps are immersed in saline containing various concentrations of inducer (e.g. activin). Animal caps form atypical epidermis in the absence of inducer. Differentiation of mesodermal tissues, such as notochord and muscle, indicates mesoderm-inducing activity of the inducer, whereas that of neural tissues, such as brain and eyes, indicates neural-inducing activity of the inducer.* **(B)** *Sandwich culture. The inducer (e.g. organizer, activin-treated animal cap) is placed between two non-treated animal caps and interacts with them from the inside of a vesicle.*

1993, Chan *et al.*, 1999). Moreover, activin is likely to facilitate formation of the organizer. The activin-induced animal cap displays organizer activities (Ariizumi and Asashima, 1995). In this article, we focus on activin as the first molecular signal and review the extent to which we can control organogenesis and fundamental body form *in vitro*.

In vitro induction systems using pluripotent embryonic tissues

As the cleavage of fertilized eggs proceeds, a large cavity called the blastocoel develops inside the animal hemisphere. The blastocoelic roof is known as the animal cap, and is composed of a few layers of ectodermal cells fated to develop into epidermis and neural tissues during normal development. The isolated animal caps from blastulae or early gastrulae, however, have pluripotency and can be induced to differentiate into a variety of neural, mesodermal and/or endodermal tissues by addition of inducers into the culture medium. Several induction systems have been devised based on the pluripotency of animal caps.

Fig. 1A illustrates the "animal cap assay" (Yamada and Takata, 1961), a basic system using animal cap as the responding tissue. This assay is easy to perform and has many advantages for estimating the inductive activity of soluble factors both qualitatively and quantitatively at the histological and molecular levels. The animal cap assay contributed greatly to the discovery of several growth factors that have mesoderm-inducing activity (reviewed by Asashima *et al.*, 1999). In the animal cap assay, the animal cap cultured in saline for a definite period (e.g., 2-3 days in *Xenopus*) forms atypical epidermis that shows no evidence of cytodifferentiation. It serves as a control for actual assays of prospective inductive substances. If the saline contains a prospective neural inducer, neural differentiation features can be recognized in the cultured animal caps. Differentiation of mesodermal tissues, such as notochord and muscle, in an animal cap indicates the mesoderm-inducing activity of an inductive substance. As described later, one mesoderm inducer, activin, can induce endodermal tissues as well as mesoderm at a high concentration. This phenomenon is called "vegetalization" because these tissues normally arise in the vegetal half of the embryo.

To reproduce the inductive interactions between induced and uninduced tissues, the "sandwich culture" is generally employed (Fig. 1B; Holtfreter, 1933). Inducer (e.g., dorsal lip, activin-induced animal cap) is placed between two pieces of uninduced animal cap and interacts with them from the inside of a vesicle. In the sandwich culture, well-organized heads and/or trunk-and-tails are induced when the dorsal lip (the organizer) is sandwiched as an inducer. By combining these two systems, we have succeeded in reproducing the fundamental embryonic body *in vitro* (Ariizumi and Asashima, 1995; also see the last section).

Activin and its inductive activity on animal caps

Various attempts have been made to isolate inductive factors that are active in the animal cap assay or sandwich culture. In the early studies, some adult vertebrate tissues such as guinea pig bone marrow (Toivonen, 1953) and carp swim bladder (Kawakami, 1976; Asashima *et al.*, 1987) were found to induce animal caps to form mesodermal tissues. In contrast, guinea pig liver was shown to have neural inducing activity and exclusively induce archencephalic structures such as forebrain, eye and nose (Toivonen, 1940). From the experiments using these "heterogeneous" inducers, hypotheses based on the gradients of inducers were proposed for embryonic body patterning (e.g., Toivonen and Saxén, 1955).

The first molecule reported to possess potent inducing activity was isolated from chicken embryos (Born *et al.*, 1972; Geithe *et al.*, 1981), and was named vegetalizing factor because of its meso- and endoderm-inducing effect on the animal cap assay. A factor capable of inducing dorsal mesoderm was found in the culture medium of a *Xenopus* tadpole cell line (XTC cell) and designated

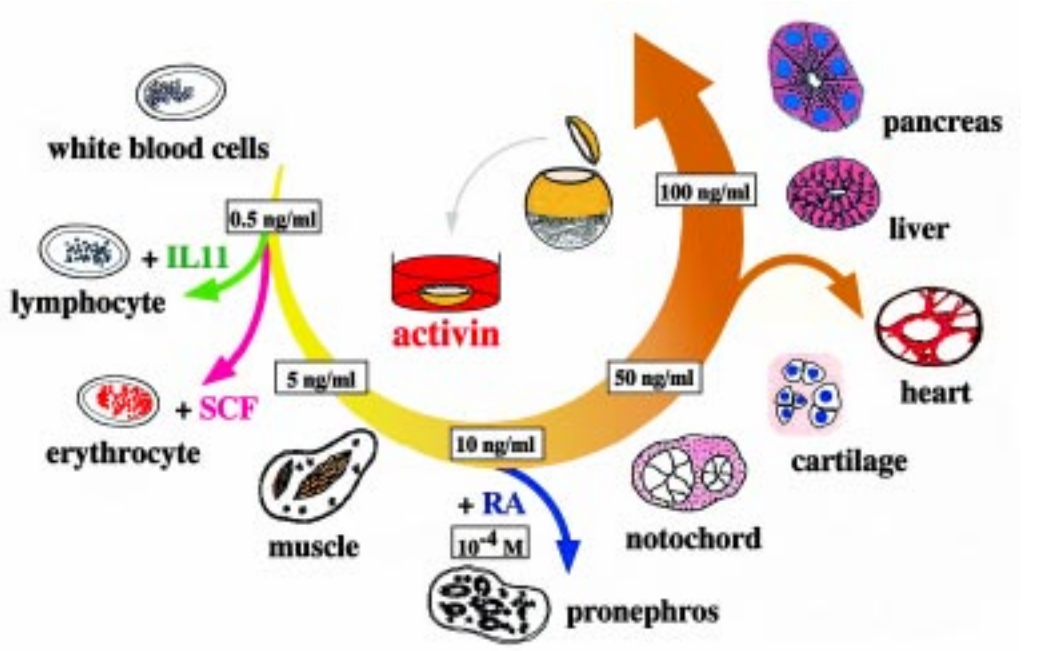

Fig. 2. Spectrum of tissue types induced in *Xenopus* and newt animal caps by activin. *Activin induces several types of mesoderm, depending upon its concentration. The induced mesoderm progressively changes from ventral type to dorsal type, as the concentration increases. Beating hearts and endodermal tissues are induced in newt animal caps by high concentrations of activin. Activin can also induce various types of blood cells or pronephros when it is combined with other bioactive molecules (IL11, interleukin 11; SCF, stem cell factor; RA, retinoic acid).*

XTC-MIF (Smith, 1987). However, in the past decade there has been marked progress in the characterization of inductive factors. Factors originally identified as cell growth factors have been shown to possess mesoderm-inducing activity on *Xenopus* animal caps. These include the FGF and TGF-β families of proteins. Slack *et al.* (1987) first reported that mammalian basic fibroblast growth factor (bFGF) has mesoderm-inducing activity. Animal caps treated with bFGF differentiate into ventral mesoderm such as blood cells and coelomic epithelium, and no dorsal mesoderm with notochord is detected. These results suggested that other factors are required for complete mesoderm formation. Knöchel *et al.* (1989) and Rosa *et al.* (1988) found that some proteins belonging to the transforming growth factor-β (TGF-β) family also have mesoderm-inducing activities.

At that time, we succeeded in isolating potent mesoderm-inducing factors from conditioned medium of the human K-562 cell line (Nakano *et al.*, 1990), which were found to be closely related to activin A (Asashima *et al.*, 1990). Activin A is a TGF-β family protein and was originally identified as a gonadal hormone that promotes the release of FSH from the anterior pituitary gland (Ling *et al.*, 1986; Vale *et al.*, 1986). Later, mesoderm-inducing factors derived from different sources were shown to be identical to activin A or to be activin homologues (reviewed by Asashima, 1994). These included vegetalizing factor, XTC-MIF, WEHI-MIF from murine leukemia cells and PIF from a mouse macrophage cell line. We have focused on activin as a model mesoderm-inducer, although there are clearly likely to be a host of such factors in different animals or tissues.

Activin can induce almost all types of mesoderm and endoderm from animal caps. The effect of activin on *Xenopus* animal caps is distinctly dose-dependent, with induction of more dorsal mesoderm as the concentration increases (Ariizumi *et al.*, 1991a, b). Concentrations of 0.3-1 ng/ml result in ventral mesoderm such as blood cells, coelomic epithelium and mesenchyme. After treatment with 5-10 ng/ml of activin, animal caps begin to elongate and differentiate into muscle. This elongation is considered to mimic the convergent extension of dorsal mesoderm during gastrulation. Cytochalasin B, an inhibitor of actin polymerization, disrupts the organization of actin filaments and inhibits the elongation of the activin-treated explants. Expression of the muscle specific marker genes is also inhibited and explants never exhibit muscle differentiation (Tamai *et al.*, 1999). At 50 ng/ml of activin, notochord, the most dorsal mesoderm, is often induced along with yolk-laden endoderm. These phenomena suggest that differences in the concentration gradient of activin in the embryo may regulate the pattern of mesoderm along the dorsoventral body axis. Activin induces newt animal caps to solely form endodermal tissues. We have confirmed the differentiation of anterior endoderm such as liver, pancreas and intestine in long-term cultured explants by electron microscopy (Ariizumi *et al.*, 1999). Treatment of newt animal caps with a high concentration of activin also induces beating hearts along with anterior endoderm (Ariizumi *et al.*, 1996). Fig. 2 illustrates the spectrum of tissue types that are induced in animal caps cultured in increasing concentrations of activin.

In vitro kidney induction system and *in vivo* transplantation

In vertebrates, retinoic acid (RA), a derivative of vitamin A, is widely involved in pattern formation including the antero-posterior specification of the limb bud and the central nervous system. Endogenous RA has been detected in early *Xenopus* embryos in a concentration gradient along the antero-posterior axis (Chen *et al.*, 1994). Phased truncation of the head structures is observed following the addition of RA to *Xenopus* blastula-stage embryos (Durston *et al.*, 1989). RA does not display any inducing activity in the *Xenopus* animal cap assay, but it can modify the action of mesoderm inducers on mesoderm pattern formation.

In 1993, we devised a simple kidney induction system in which we treated *Xenopus* animal caps with 10 ng/ml of activin and 10^{-4} M of RA (Moriya *et al.*, 1993). As described above, animal caps

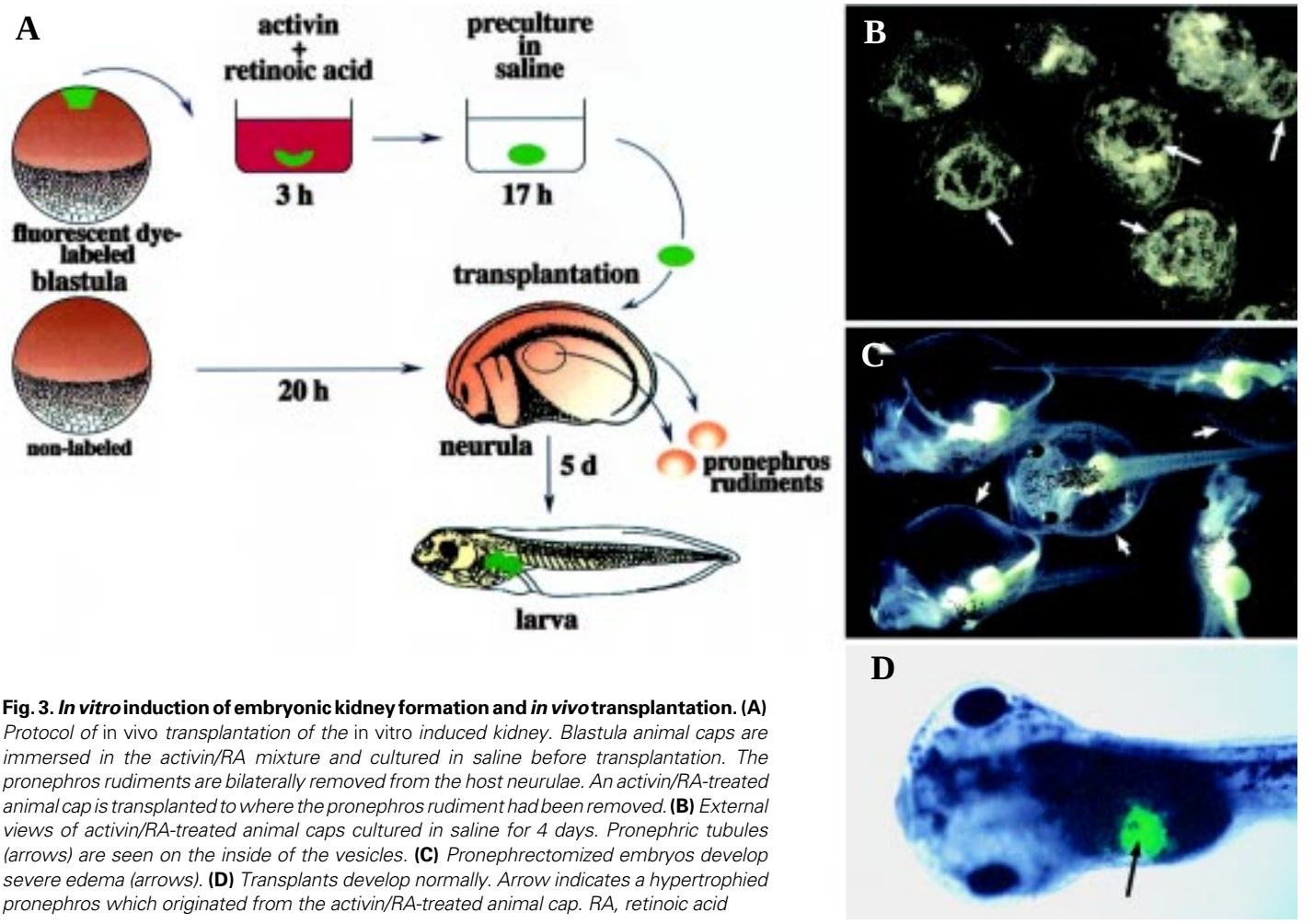

Fig. 3. *In vitro* induction of embryonic kidney formation and *in vivo* transplantation. (A) *Protocol of* in vivo *transplantation of the* in vitro *induced kidney. Blastula animal caps are immersed in the activin/RA mixture and cultured in saline before transplantation. The pronephros rudiments are bilaterally removed from the host neurulae. An activin/RA-treated animal cap is transplanted to where the pronephros rudiment had been removed.* **(B)** *External views of activin/RA-treated animal caps cultured in saline for 4 days. Pronephric tubules (arrows) are seen on the inside of the vesicles.* **(C)** *Pronephrectomized embryos develop severe edema (arrows).* **(D)** *Transplants develop normally. Arrow indicates a hypertrophied pronephros which originated from the activin/RA-treated animal cap. RA, retinoic acid*

treated with 10 ng/ml of activin differentiate predominantly into muscle, a dorsal mesoderm. However, pronephros, a lateral plate mesoderm, develops in the animal cap at a high frequency (100%) when 10^{-4} M RA is added to the activin solution. The pronephros is the simplest and earliest form of vertebrate kidney, and provides an attractive system for studying many of the features of kidney organogenesis (reviewed by Chan and Asashima, 2000). The *in vitro* induced kidney displays a cytodifferentiation pattern typical of a normal functioning pronephros under electron microscopy. It also expresses several genes found in pronephros rudiments of the normal embryos at exactly the same developmental stages (Uochi and Asashima, 1996). Furthermore, we have cloned several genes expressed during kidney organogenesis using this *in vitro* kidney induction system (Uochi and Asashima, 1998; Uochi *et al.*, 1997).

If the *in vitro* induced kidney is functionally active, it should act as an excretory organ in the embryos. We have confirmed this possibility by employing a unique functional test (Chan *et al.*, 1999). Fig. 3A illustrates the protocol of transplantation of an *in vitro* induced kidney into pronephrectomized embryos. Animal cap grafts are isolated from late blastulae, immersed in the activin and RA mixture, and then precultured in saline for transplantation. These grafts develop pronephric tubules if they are cultured in saline for an additional 4 days (Fig. 3B). Severe edema is recognized after the bilateral removal of the pronephros primordia from the host neurula-stage embryos (Fig. 3C). All the pronephrectomized embryos die within 9 days. However, the same embryos develop normally if the activin/RA-treated graft is transplanted to the site at which the pronephric primordia had been removed. Although the percentage is very low, some of the transplants survive for more than one month, and hypertrophy of the pronephros is observed in some of the recipients (Fig. 3D). These results demonstrate that the *in vitro* induced kidney can function *in vivo* to maintain water balance, suppress the onset of edema, and has sufficient function to promote normal development. Although there are many barriers to applying these results to mammals including humans, our *in vitro* kidney induction system and its transplantation protocol will serve as the first step for future organ engineering.

In vitro heart induction system

The heart is an attractive organ to study, and its dynamic and rhythmical beating is conspicuous in developing larvae. Many fundamental studies on amphibian heart development were performed in the 1960s using newt and salamander embryos (Jacobson, 1960, 1961; Jacobson and Duncan, 1968). The inductive interactions in heart development have been well characterized:

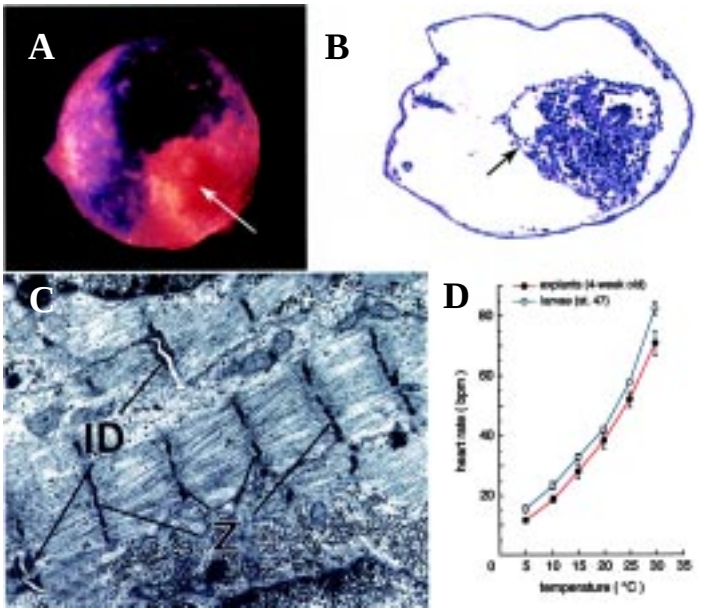

Fig. 4. *In vitro* induced beating hearts. (A) *External view of an activin-induced beating heart (arrow).* **(B)** *Heart tube (arrow) is seen inside the vesicle.* **(C)** *Transmission electron microscopy of the induced cardiac muscle (Z, Z band; ID, intercalated discs).* **(D)** *Heartbeats of the induced heart are parallel to those of the normal embryonic heart. bpm, beats per minute.*

anterior endoderm contacts heart rudiment (presumptive heart mesoderm; PHM) and stimulates its differentiation and morphogenesis. This process is considered to be initiated by the early neurula stages. Heart differentiation can be easily assayed since the cardiac muscle proteins are well characterized and cytodifferentiation is easy to recognize under electron microscopy. However, the molecular basis of inductive events and the genes expressed during the earliest phases of differentiation remain largely unknown. A suitable *in vitro* heart induction system would greatly facilitate studies of the mechanisms of cardiogenesis.

We have succeeded in raising beating hearts *in vitro* by treating newt animal caps with high concentrations of activin (50-100 ng/ml). Although most explants differentiated into endodermal cell masses, up to 30% of them developed beating hearts (Fig. 4, Ariizumi *et al.*, 1996). Heartbeats of the induced heart were almost identical to those of the normal embryonic heart, showing a clear temperature-dependency. A cytodifferentiation pattern typical of a functioning heart was also observed on transmission electron microscopy. It is likely that the other explants also displayed heart differentiation to some extent, but never developed to a fully functional state. That is, if less rigorous criteria (such as the presence of cardiac muscle proteins) were employed, the frequency would likely exceed more than 30% of specimens.

The presence of endodermal cells induced by activin in animal caps seems to be requisite for the formation of beating hearts. These cells form anterior endoderm such as pharynx and liver when they are cultured for more than one month (Ariizumi *et al.*, 1999). In urodele heart development, PHM differentiates into a heart under the influence of the anterior endoderm. The activin-treated animal cap (which mainly differentiates into endodermal cells) acts as a positive agent for cardiogenesis, in the same manner as the anterior endoderm. Indeed, we recently confirmed that activin-treated animal caps could facilitate formation of beating hearts in the predetermined PHM of the late gastrulae (Ariizumi *et al.*, unpublished data). The frequency of beating heart formation reached more than 60% in these specimens. On the other hand, activin does not seem to directly induce PHM in the animal caps. Recent results showed that activin might act in combination with other growth factors in eliciting and regulating heart differentiation (Muslin and Williams, 1991; Sugi and Lough, 1995). It will be necessary to induce animal caps into PHM to increase the frequency of heart formation. By combining such animal caps with the activin-induced animal caps, a more reliable heart induction system may finally be established.

In vitro control of embryonic body plan by artificial organizer

The organizer, the dorsal lip region of gastrulae, plays a major role in the establishment of fundamental body plan. As demonstrated by Spemann and Mangold (1924), a transplanted organizer graft can induce a complete secondary embryo on the ventral side of a host embryo. The dorsal lip from an early gastrula-stage donor induces mostly secondary heads, while that from a late gastrula-stage donor induces trunk-and-tails (Mangold and Spemann, 1927). Thus, the early dorsal lip has been termed the "head organizer" and the later lip the "trunk/tail organizer" (Spemann, 1931). Mangold (1933) further refined this distinction in neurula-stage embryos. The anterior region of the archenteron roof, corresponding to the invaginated head organizer, behaved as a head inducer, and the posterior region, the trunk/tail organizer, induced trunk-and-tails.

Later, Japanese groups found that the inducing activity of the early dorsal lip (head organizer) is not established at the early

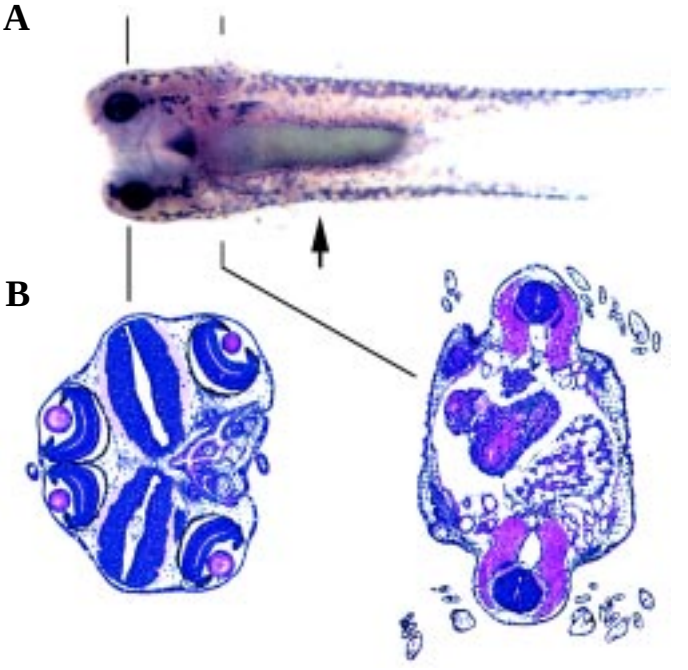

Fig. 5. Secondary embryos induced by the activin-treated animal cap. *The activin-treated animal cap acts as the organizer and induces a well-organized secondary embryo (arrow) when it is transplanted into the ventral side of a host gastrula embryo.* **(A)** *External view.* **(B)** *Histological section.*

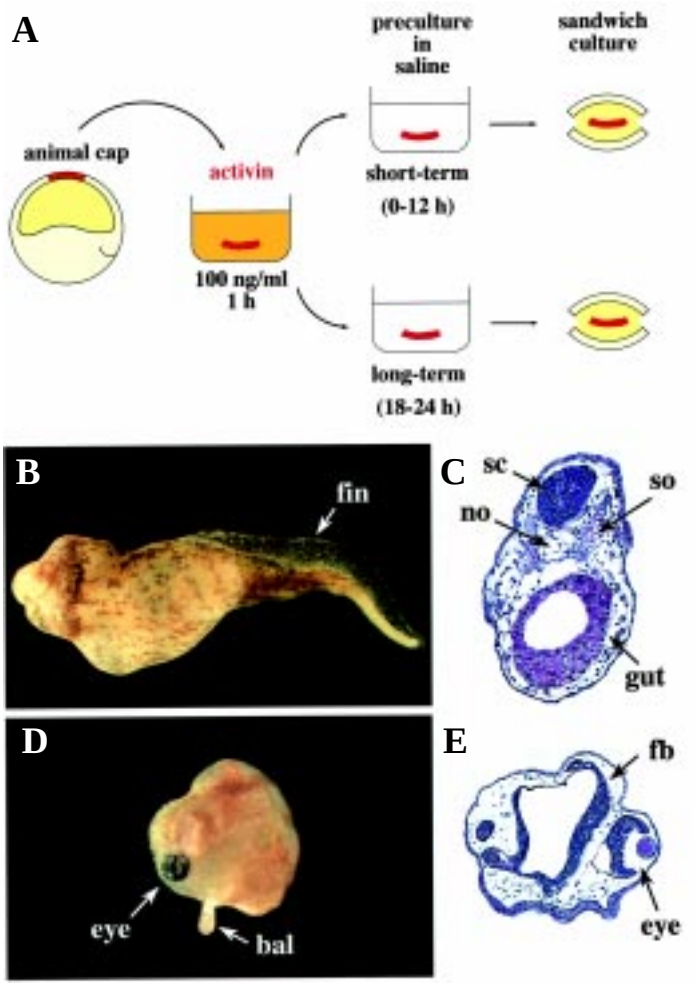

Fig. 6. *In vitro* formation of heads and trunk-and-tails by the artificial organizer. (A) *Protocol of sandwich culture of the activin-treated animal cap. Animal caps are treated with a high concentration of activin (100 ng/ml) and precultured in saline for various periods before sandwich culture.* **(B,C)** *Induction activity of the activin-treated animal cap, the artificial organizer, changes autonomously during preculture periods. The activin-treated animal cap induces trunk-and-tail structures when it is immediately combined with non-treated animal caps.* **(D,E)** *On the other hand, the long-term (more than 18 h) precultured artificial organizer induces head structures. bal, balancer; fb, forebrain; no, notochord; sc, spinal cord; so, somite.*

gastrula-stage from extensive transplantation and sandwich culture experiments (Okada and Takaya, 1942a, b; Okada and Hama, 1943; Hama *et al.*, 1985). The early lip mainly induces trunk-and-tails immediately upon contacting ectoderm, the reacting tissue. However, the same region facilitates the formation of heads after being aged in saline for the period required for gastrulation (about 20 h). This phenomenon means that the regional induction activities of the organizer change autonomously during gastrulation. Although these findings cast an important light on the establishment of the body plan, further investigation has long been awaited.

It is likely that activin not only induces endo-mesodermal tissues but also elicits organizer activity in animal caps. Exactly as in the organizer transplantation experiment, the activin-induced animal caps further induce a well-organized secondary embryo when transplanted into the ventral side of early gastrulae (Fig. 5; Ninomiya *et al.*, 1998). If activin invests animal caps with a complete organizer-activity, it may be that the activin-induced animal cap, the artificial organizer, also exhibits regional induction activity, as shown in the classical organizer experiments. We have confirmed this possibility by employing the sandwich culture technique on newt activin-induced animal caps (Fig. 6A; Ariizumi and Asashima, 1995).

As already mentioned above, newt animal caps themselves mainly differentiated into anterior endoderm by the addition of a high concentration of activin (100 ng/ml) to the culture medium. They induce trunk-and-tails equipped with well-organized axial structures such as spinal cord, notochord and somites, when they are immediately sandwiched with untreated animal caps (Fig. 6B). As the duration of “preculture” in saline is prolonged (6-12 h), hindbrain with ears is induced in the sandwich explants at high rates. Heads that consist of forebrain and eyes are eventually induced by a long-term (18-24 h) precultured artificial organizer (Fig. 6C). Cell-lineage tracing of the sandwich explants revealed that the activin-induced animal caps differentiate into anterior endoderm and induce axial mesoderm and central nervous system in the adjacent untreated animal caps. We further compared the temporal patterns of expression of the organizer-related genes in the activin-induced animal caps and the early gastrula dorsal lip (Yokota *et al.*, 1998). Among the various known organizer-related genes, *Cychd* (a partial *chordin* clone in newt) was up-regulated both in the organizer and activin-induced animal cap when they exhibit head-forming activities. As in *Xenopus* development, the *chordin* gene is likely to act as a key mediator of head formation. The mechanism of regional specification by the organizer is very complex and remains largely unknown at the molecular level. However, the experimental systems mentioned in this section should serve as a model system to analyze this problem.

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