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Activin-treated Urodele Animal Caps: II. Inductive Interactions in Newt Animal Caps After Treatment with Activin A

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ABSTRACT—The inductive interactions between activin-induced and non-induced cells were investigated in newt animal cap explants. A wide range of concentrations of activin A (0.1-100 ng/ml) induced mesodermal tissues in the animal caps, but at generally low frequencies. Animal caps treated with 100 ng/ml of activin A, on the other hand, differentiated solely into nonspecific endoderm. At this concentration, various mesodermal tissues were induced in addition to endoderm as the animal caps increased in size. They were more frequently induced in explants in which the activin-treated animal caps were combined with untreated animal caps. Central nervous systems were frequently induced in sandwich explants with larger amounts of untreated animal caps. Differentiation of endodermal organs such as the liver, the pancreas and the intestine in the long-term cultured sandwich explants was confirmed by electron microscopy. Lineage tracing of the combination and sandwich explants revealed that the activin-treated animal caps mainly formed the endoderm and induced mesodermal and neural tissues in the untreated animal caps. These results suggest that activin A is capable of inducing the endoderm that can act as an initiator of further inductive interactions in early newt development.

INTRODUCTION

The animal cap, the presumptive ectodermal region of the blastula and gastrula, has provided an excellent model for the analysis of inductive interactions during early amphibian development. Isolated animal caps can be induced to differentiate into ectodermal (neural), mesodermal, and even endodermal tissues by the addition of various inductive agent(s) to the culture medium. This simple animal cap assay system (Yamada and Takata, 1961) contributed greatly to the discovery that peptide growth factors belonging to the fibroblast growth factor (FGF) and transforming growth factor-β (TGF-β) families have mesoderm-inducing activity (reviewed in Ariizumi and Asashima, 1995a).

Most studies based on the animal cap assay have been carried out with the anuran *Xenopus laevis*, because it has already been well characterized at the biochemical and molecular genetic level (reviewed in Klein and Melton, 1994). A variety of genes are expressed in *Xenopus* animal caps after treatment with peptide growth factors (reviewed in Ariizumi and Asashima, 1995a). However, embryonic induction was originally discovered in urodele newt embryos (Spemann and Mangold, 1924), and significant theories on embryonic induction have largely depended on experiments in urodele (Nakamura and Toivonen, 1978; Hamburger, 1988). While little is known about the molecular background of urodele embryos, they are particularly well-suited for microsurgery and histological analysis. The animal caps of the Japanese newt, *Cynops pyrrhogaster*, are composed of a single homogeneous cell layer, whereas *Xenopus* animal caps consist of heterogeneous cell layers. Thus, newt animal caps might be expected to reflect the activity of inductive agents more directly than *Xenopus* animal caps. Because of their simple structure, it is also possible to investigate inductive interactions between induced and non-induced cells by combining newt animal caps.

Activin A, a member of the TGF-β family, is known to induce various mesodermal tissues in *Xenopus* animal caps in a dose-dependent manner (Ariizumi *et al.*, 1991a, b). It can

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also induce mesodermal tissues such as the notochord and muscle in *Cynops* animal caps, but at low frequencies (Moriya and Asashima, 1992). However, when *Cynops* animal caps are treated with a high concentration of activin A they preferentially form a mass of yolk-laden cells. Based on their inductive properties, these cells are thought to be fated to form the anterior endoderm (Ariizumi and Asashima, 1995b; Ninomiya *et al.*, 1998). Takata and Yamada (1960) reported an observation that well-organized endodermal organs develop from *Cynops* animal caps under the influence of a mesoderm-inducing agent, guinea pig bone marrow. A long period of culture (more than one month) enabled histological identification of a variety of endodermal organs such as the pharynx, the stomach and the intestine.

In this study, we investigated the inductive interactions triggered by activin A in *Cynops* animal caps with the following series of experiments. First, we examined the differentiation pattern of animal caps after treatment with various concentrations of activin A (series I). Second, the inductive interactions of the activin-treated animal caps were investigated by employing three culture modes; *isolation culture*, using animal caps of different sizes, and *combination culture* and *sandwich culture*, both using untreated animal caps of different sizes (series II). The cell lineage of the activin-treated animal caps in the combination and sandwich explants was traced with a fluorescent dye. Differentiation of endodermal organs in the long-term cultured sandwich explants was also examined under transmission electron microscope.

MATERIALS AND METHODS

Embryos and activin A solution

Eggs of the Japanese newt, *Cynops pyrrhogaster*, were obtained by hormonal stimulation of females as described elsewhere (Ariizumi and Asashima, 1995b). Embryo staging was according to Okada and Ichikawa (1947). Early gastrulae (stage 11) were sterilized in 70% ethanol for 1 min. Jellycoats were dissolved in modified Holtfreter's solution (MHS; 60 mM NaCl, 0.7 mM KCl, 0.9 mM $CaCl_2$, 4.6 mM HEPES, 0.1 g/l kanamycin sulfate, pH 7.6) containing 1% sodium thioglycolate (pH 9.0). Vitelline membranes were removed with watchmaker's forceps. Human recombinant activin A, a gift from Dr. Y. Eto (Central Research Laboratories, Ajinomoto Co. Inc., Japan), was used as an inducer in all experimental series. Activin A was dissolved in MHS containing 0.1% BSA (A-7888, Sigma, USA) at concentrations of 0, 0.1, 0.5, 1, 5, 10, 50, and 100 ng/ml. Activin A solutions were placed in polystyrene dishes (Tissue Culture Dish 25000; Iwaki Glass, Japan).

Experimental series

Schematic diagrams of the experimental series are shown in Fig. 1. All operations were performed under sterile conditions at 20°C.

Series I

A 1.0 mm × 1.0 mm explant containing 989 ± 41 cells of the animal cap of stage 11 embryos (about 2.3 mm in diameter) was dissected out. They were incubated in the activin A solutions (0-100 ng/ml) for 1 hr with their surface cell side face down. After washing in two changes of MHS with gentle pipetting, explants were cultured for 2 weeks at 20°C in 96-well plates (SUMILON®; MS-309UR, Sumitomo Bakelite, Japan) filled with MHS containing 0.1% BSA.

Series II

In the *isolation culture* mode, animal caps were dissected from the stage 11 embryos to sizes of 0.3 × 0.3 mm, 0.6 × 0.6 mm, 0.9 × 0.9 mm, 1.2 × 1.2 mm and 1.5 × 1.5 mm and incubated in 100 ng/ml of activin A solution for 1 hr. In the *combination culture* mode, the activin-treated animal caps (0.6 × 0.6 mm) were combined with untreated animal caps measuring 0.6 × 0.6 mm, 0.9 × 0.9 mm, 1.2 × 1.2 mm, and 1.5 × 1.5 mm. In the *sandwich culture* mode, the activin-treated animal caps (0.6 × 0.6 mm) were sandwiched between two pieces of untreated animal caps measuring 0.9 × 0.9 mm, 1.2 × 1.2 mm, and 1.5 × 1.5 mm. Explants were cultured for 2 weeks (for light microscopy) or 5 weeks (for electron microscopy) as described in series I.

Histology

For light microscopy, explants were fixed in Bouin's fluid for 12 hr, dehydrated in a graded series of ethanol, cleared in xylene, embedded in paraffin, and cut into 6-μm thick sections. Sections were stained with Delafield's hematoxylin/eosin. For transmission electron microscopy, 5-week-old larvae (stage 50) and sandwich explants were fixed in 3% paraformaldehyde/2.5% glutaraldehyde/0.1 M cacodylate buffer (pH 7.4) for 24 hr, post-fixed in 1.0% OsO_4 and buffer (pH 7.4) for 2 hr, dehydrated in a graded series of ethanol and acetone, and embedded in epoxy resin. Sections were stained with uranyl acetate/lead citrate and examined under transmission electron microscope (JEM-100C; JEOL, Japan).

Cell lineage tracing

Embryos at the 2-cell stage were labeled with fluorescein-dextran-amine (FDA, D-1820, Molecular Probes, USA) as described elsewhere (Ariizumi and Asashima, 1995b). They were grown to stage 11 in 1/10 MHS. After animal caps dissected from FDA-labeled embryos (0.6 × 0.6 mm) were treated with 100 ng/ml of activin A solution for 1 hr, they were combined or sandwiched with untreated animal caps as described in series II. Explants were cultured in MHS for 2 weeks at 20°C and examined under an epifluorescence microscope as described elsewhere (Ariizumi and Asashima, 1995b).

RESULTS

Series I: Differentiation pattern of animal caps treated with various concentrations of activin A

Typical sections of explants are shown in Fig. 2, and the results of histological analysis are summarized in Table 1. All of the control explants, that were not exposed to activin A, formed irregular-shaped atypical epidermis (36 explants, Fig. 2A). Similar explants that did not show any other differentiation were obtained by treatment with 0.1 ng/ml of activin A (44 out of 48 explants). As the concentration of activin A increased, the frequency of atypical epidermis gradually decreased. At concentrations higher than 0.5 ng/ml, several different tissues belonging to the ectoderm and the mesoderm were induced in the explants. Epidermis and mesenchyme were consistently induced, regardless of the concentration of activin A. Nonspecific neural tissues and mesodermal tissues (notochord, muscle, coelomic epithelium and blood cells) were also consistently induced in the explants, though at very low rates. In contrast, most of the explants treated with activin A at concentrations higher than 10 ng/ml formed a mass of yolk-laden cells (79-95%, Fig. 2D), classified as "nonspecific endoderm" based on its histological features. These cells were also seen in animal caps treated with lower concentrations of activin A,

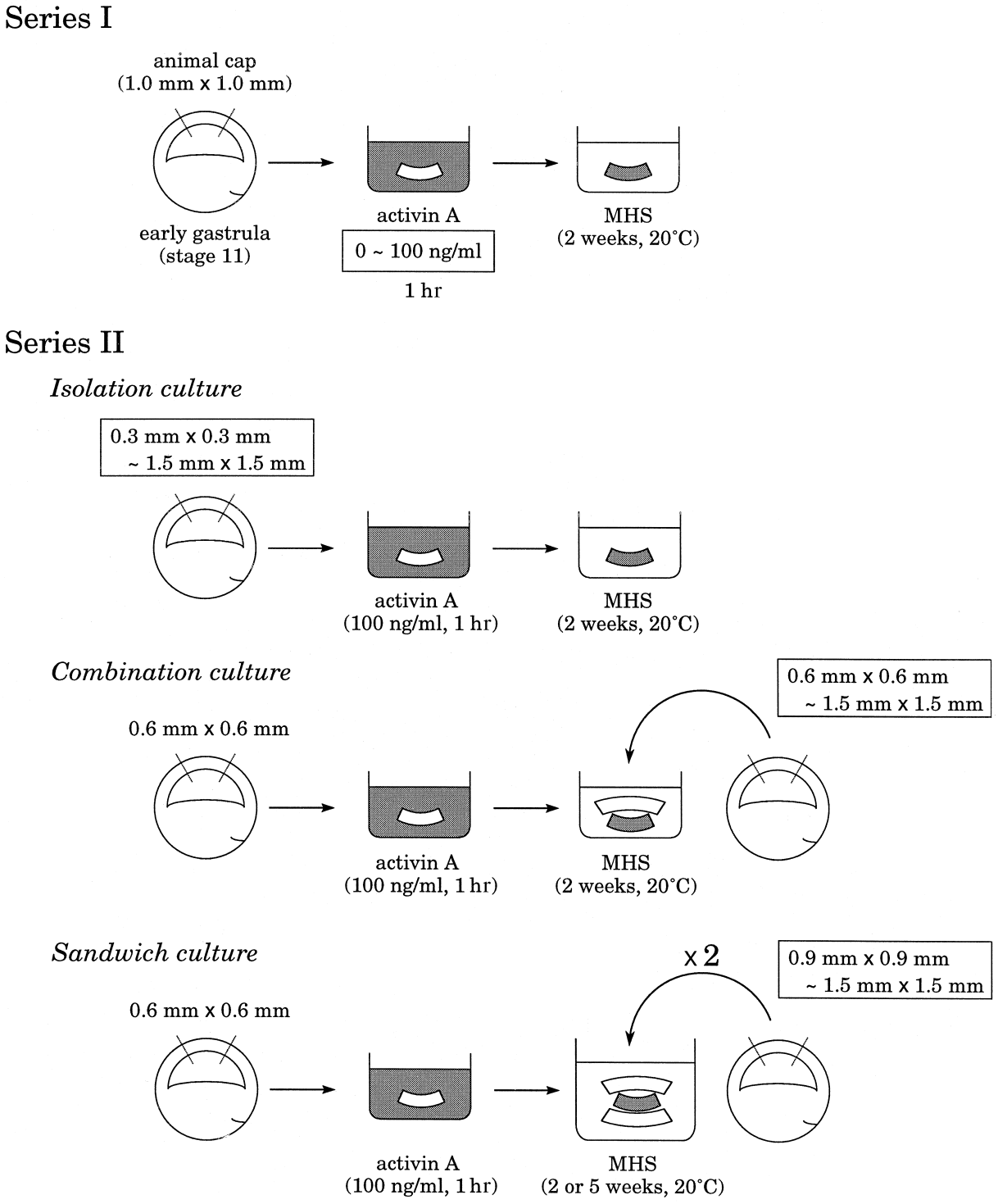

Fig. 1. Schematic diagrams of the experimental series.

usually accompanied by a variety of mesodermal tissues (Fig. 2B, C).

Series II: Differentiation patterns of activin-treated animal caps of different sizes and different combinations

Typical sections of explants are shown in Fig. 3, and the results of histological analysis are summarized in Tables 2-4.

In the *isolation culture* mode (Table 2), nonspecific endoderm was always induced at high frequencies (95-100%) irrespective of the size of the explants that were treated with 100 ng/ml of activin A for 1 hr. It was predominant in the small explants (less than 0.9 × 0.9 mm, Fig. 3A). As the size of animal caps increased, the frequencies of differentiation of both ectodermal and mesodermal tissues increased slightly.

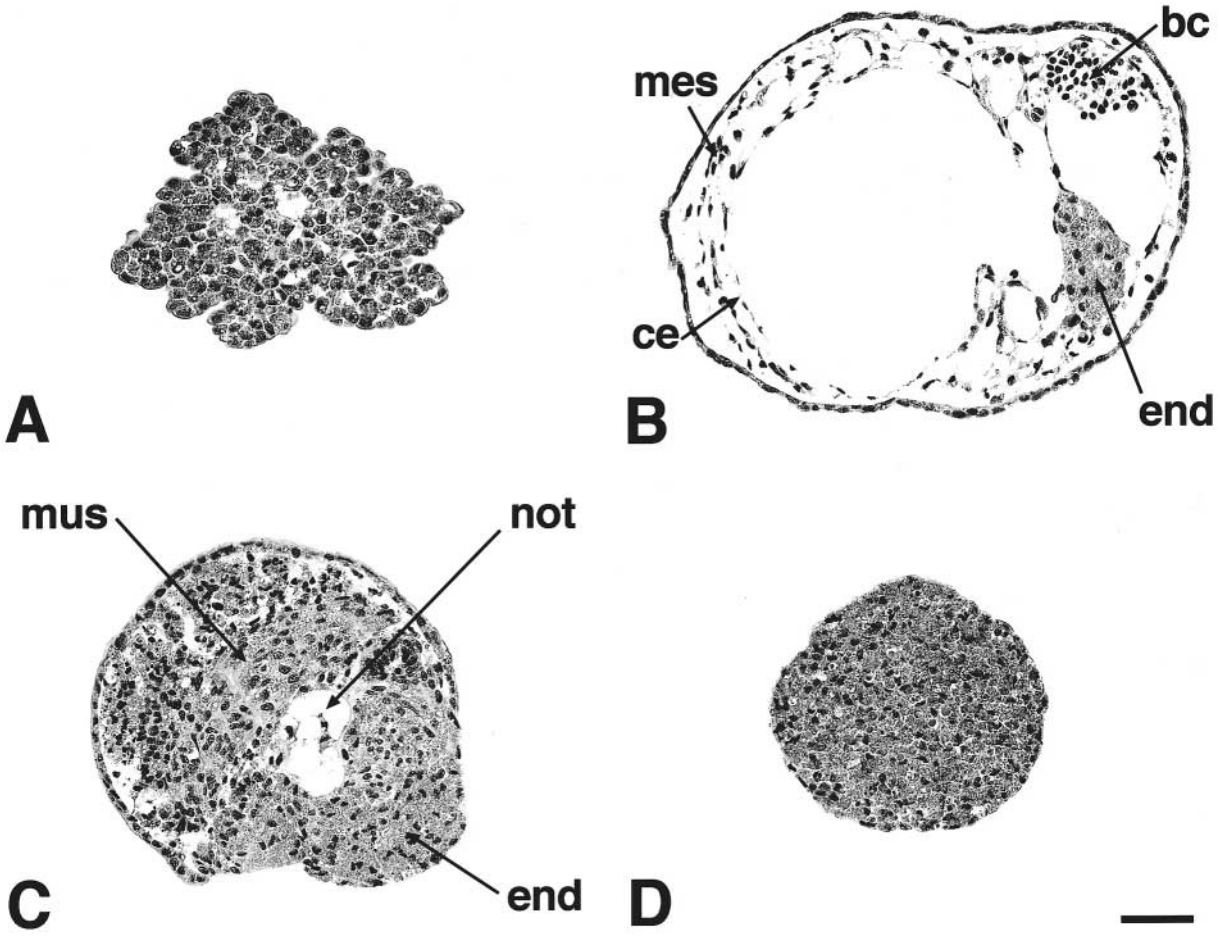

Fig. 2. Histological sections of animal caps treated with various concentrations of activin A. Animal caps (1.0 × 1.0 mm, containing about 1,000 cells) were treated with 0-100 ng/ml of activin A for 1 hr and cultured for 2 weeks. (A) Control explants cultured without activin A became highly wrinkled, and developed into atypical epidermis. (B) Explant with ventral mesoderm obtained by treatment with 0.5 ng/ml of activin A. Ventral mesoderm such as the blood cells (bc), the coelomic epithelium (ce) and the mesenchyme (mes) was induced along with nonspecific endoderm (end). (C) Explant with dorsal mesoderm induced by 5 ng/ml of activin A. Dorsal mesoderm, i.e. notochord (not) and somitic muscle (mus), was induced together with nonspecific endoderm. (D) Animal caps treated with 10-100 ng/ml of activin A differentiated exclusively into a mass of nonspecific endoderm. Bar, 100 μm.

Table 1. Differentiation of animal caps treated with various concentrations of activin A.

Activin A (ng/ml)	0	0.1	0.5	1	5	10	50	100
Total no. of explants	36	48	47	48	47	58	42	55
Negative explants[†]	36	44	30	24	20	5	1	0
Positive explants[‡]	0	4	17	24	27	53	41	55
Epidermis	0*	6	34	29	38	36	40	31
Nonspecific neural tissue	0	2	6	27	9	9	17	5
Notochord	0	0	4	8	9	7	7	4
Muscle	0	0	4	4	2	2	2	2
Mesenchyme	0	4	26	33	32	22	31	13
Coelomic epithelium	0	2	9	8	6	16	26	4
Blood cells	0	2	6	2	9	7	7	5
Nonspecific endodermal tissue	0	4	4	2	17	79	88	95

[†]Explants with atypical epidermis alone. [‡]Explants with any of the tissues listed below.
*Figures are percentages of the total number of explants.

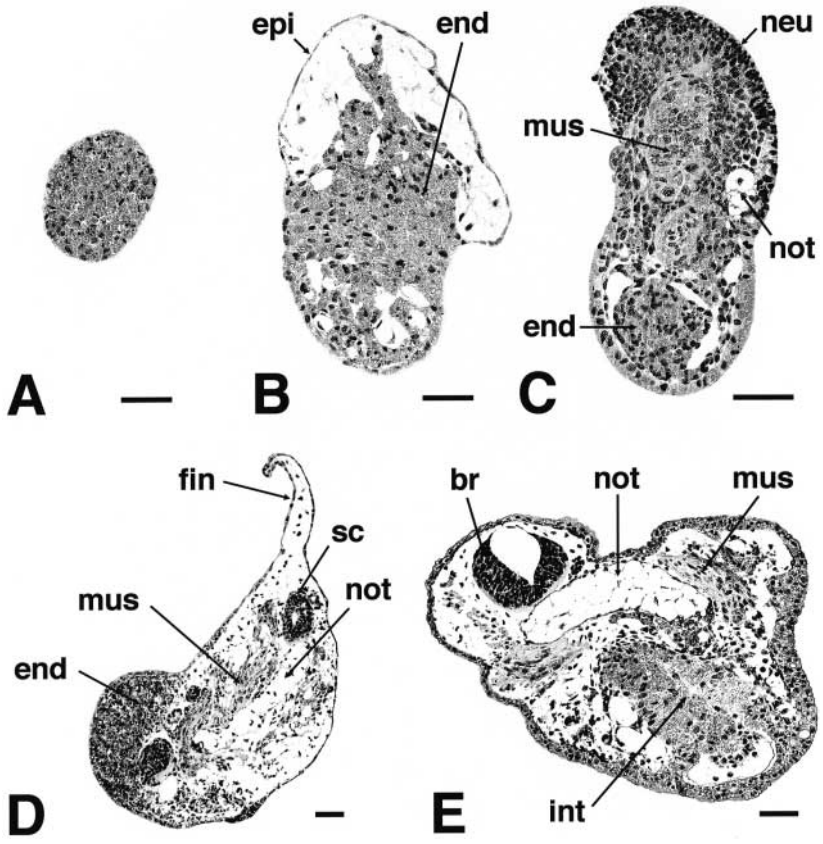

Fig. 3. Histological sections of activin-treated animal cap explants of different sizes and combinations. Animal caps of different sizes were treated with 100 ng/ml of activin A for 1 hr and cultured with or without various sizes of untreated animal caps for 2 weeks. (A) The small isolation explants (0.6 × 0.6 mm) differentiated preferentially into an endodermal cell mass. (B) Epidermis (epi) was seen in the large isolation explants (1.2 × 1.2 mm), in addition to nonspecific endoderm (end). (C) The combination explant in which the activin-treated animal cap (0.6 × 0.6 mm) was combined with untreated animal cap of the same size differentiated into dorsal mesoderm (notochord, not; somitic muscle, mus) accompanied by nonspecific neural tissue (neu) and nonspecific endoderm. (D) Central nervous system tissues (spinal cord, sc) were induced as the proportion of untreated animal cap increased (1.2 × 1.2 mm). (E) Sandwich explants formed a trunk-tail with dorsal mesoderm and central nervous system (hindbrain, br). Intestine (int) consisting of yolk-rich columnar cells was often induced in these explants. Bars, 100 μm.

Table 2. Differentiation of animal cap explants in the isolation culture mode.

Size of AA[†] (mm^2)	0.3 × 0.3	0.6 × 0.6	0.9 × 0.9	1.2 × 1.2	1.5 × 1.5
Total no. of explants	47	62	65	58	62
Ectodermal tissues					
Epidermis	17*	21	32	33	48
CNS (hindbrain, spinal cord)	0	0	0	0	2
Nonspecific neural tissue	9	11	31	19	31
Mesodermal tissues					
Notochord	9	2	3	12	21
Muscle	4	2	9	14	27
Pronephros	0	0	0	2	2
Mesenchyme	9	10	26	28	37
Coelomic epithelium	0	6	8	7	10
Blood cells	0	0	0	2	2
Endodermal tissues					
Digestive organ (pharynx, liver, pancreas, intestine)	0	0	0	0	2
Nonspecific endodermal tissue	100	95	98	100	100

[†]100 ng/ml activin-treated animal caps.
*Figures are percentages of the total number of explants.

Epidermis often developed in addition to the nonspecific endoderm (Fig. 3B). Dorsal axial mesoderm such as the notochord and the muscle were also induced though at relatively low rates (21 and 27%, respectively) even when the largest animal caps (1.5 × 1.5 mm) were treated. Well-developed central nervous system and digestive organs were rarely induced in this culture mode (2%).

In the *combination culture* mode (Table 3), nonspecific endoderm was also induced in most of the explants (66-85%). Although the percentages were low (less than 8%), endodermal organs such as the liver and the intestine were also induced in each combination. Ectodermal tissues (epidermis and nonspecific neural tissues) and mesodermal tissues (notochord, muscle, and mesenchyme) were more frequently induced than in the isolation culture mode (Fig. 3C). As the proportion of untreated animal caps increased, the frequencies of differentiation of the dorsal axial mesoderm (notochord and muscle) and the central nervous system (hindbrain and spinal cord) increased, and explants often formed a trunk-tail with fins (Fig. 3D).

Table 3. Differentiation of animal cap explants in the combination culture mode.

Size of AA† (mm^2)	0.6 × 0.6			
Size of UA‡ (mm^2)	0.6 × 0.6	0.9 × 0.9	1.2 × 1.2	1.5 × 1.5
Total no. of explants	55	52	50	43
Ectodermal tissues				
Epidermis	67*	87	88	100
CNS (hindbrain, spinal cord)	2	2	4	28
Nonspecific neural tissue	36	40	52	42
Mesodermal tissues				
Notochord	16	42	52	72
Muscle	20	37	58	70
Pronephros	0	0	0	0
Mesenchyme	62	62	80	98
Coelomic epithelium	25	21	32	23
Blood cells	11	6	2	0
Endodermal tissues				
Digestive organ (pharynx, liver, pancreas, intestine)	4	4	8	5
Nonspecific endodermal tissue	85	83	66	74

†100 ng/ml activin-treated animal caps. ‡Untreated animal caps.
*Figures are percentages of the total number of explants.

Table 4. Differentiation of animal cap explants in the sandwich culture mode.

Size of AA† (mm^2)	0.6 × 0.6		
Size of UA‡ (mm^2 × 2 pcs.)	0.9 × 0.9	1.2 × 1.2	1.5 × 1.5
Total no. of explants	31	28	30
Ectodermal tissues			
Epidermis	97*	86	100
CNS (hindbrain, spinal cord)	39	61	73
Nonspecific neural tissue	45	18	23
Mesodermal tissues			
Notochord	77	71	83
Muscle	77	75	90
Pronephros	6	7	0
Mesenchyme	90	75	87
Coelomic epithelium	26	39	43
Blood cells	10	7	7
Endodermal tissues			
Digestive organ (pharynx, liver, pancreas, intestine)	10	36	60
Nonspecific endodermal tissue	81	79	43

†100 ng/ml activin-treated animal caps. ‡Untreated animal caps.
*Figures are percentages of the total number of explants.

In the *sandwich culture* mode (Table 4), explants often formed a trunk-tail composed of the central nervous system, the notochord and the somitic muscle (Fig. 3E). The central nervous system induced in the explants was identified as the hindbrain and the spinal cord based on histological features. Archencephalic structures such as the forebrain and the eyes were never detected in these explants. Endodermal tissues were also induced in these explants, and well-developed digestive organs (liver and intestine) formed more frequently than in other culture modes. As the proportion of untreated animal caps increased, the frequencies of differentiation of both the central nervous system and digestive organs increased markedly. Differentiation of digestive organs was also confirmed by transmission electron microscopy (Fig. 4). Typical cytodifferentiation patterns expected for the functioning liver, the pancreas and the intestine were seen in the 5-week old sandwich explants, in which the activin-treated animal caps were sandwiched with 1.5×1.5 mm untreated animal caps.

The cell lineage of the activin-treated animal caps in the combination and sandwich explants was traced using a fluorescent dye (FDA). The results of histological analysis are summarized in Table 5. Nonspecific endoderm was always found in the explants regardless of the size of untreated animal caps. Ectodermal tissues (epidermis and neural tissues including CNS) and various mesodermal tissues were induced at high frequencies when the proportion of untreated animal caps increased. Cell lineage analysis of these explants revealed that most ectodermal and mesodermal tissues were derived from untreated animal caps. In contrast, the activin-treated animal caps differentiated mainly into endodermal tis-

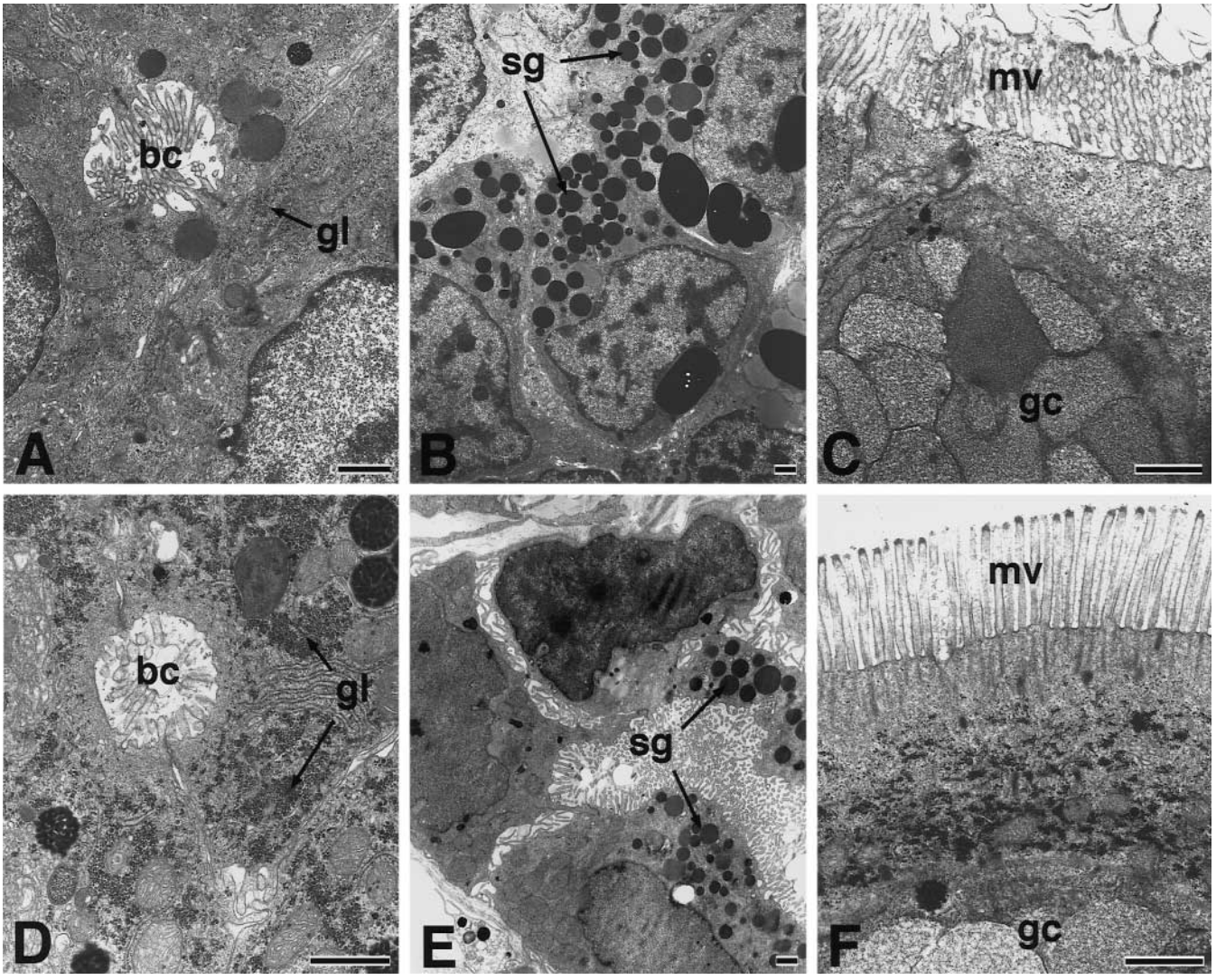

Fig. 4. Transmission electron microscopy of activin-induced endodermal cells in the 5-week old sandwich explants. (A)-(C) Portions of hepatocytes, exocrine cells of the pancreas, and epithelial and goblet cells in the intestine from the 5-week old larvae (stage 50), respectively. (D) Portions of activin-induced liver from the 5-week old sandwich explants. A large pool of glycogen (gl) has accumulated in the cytoplasm. The bile capillary (bc), a characteristic finding in the liver, is present in the intercellular space. (E) Portions of activin-induced pancreas. Secretory granules (sg) can be seen facing the glandular cavity. (F) Portions of activin-induced intestine. The goblet cell (gc) is present under the microvilli (mv)-lined epithelial cell. Bars, 1 μm.

Table 5. Cell lineage analysis of the combination and sandwich explants.

Size of AA† (mm^2)	0.6×0.6					
Size of UA‡ (mm^2)	0.6×0.6		1.2×1.2		$1.2 \times 1.2 \times 2$ pcs.	
Total no. of explants	20		20		33	
Ectodermal tissues						
Epidermis	45*	(0)**	95	(0)	97	(0)
CNS (hindbrain, spinal cord)	0	(0)	25	(0)	85	(0)
Nonspecific neural tissue	15	(0)	25	(0)	67	(0)
Mesodermal tissues						
Notochord	10	(5)	25	(15)	85	(9)
Muscle	0	(0)	20	(10)	85	(3)
Pronephros	5	(5)	0	(0)	24	(0)
Mesenchyme	40	(0)	90	(0)	94	(0)
Coelomic epithelium	0	(0)	10	(0)	12	(0)
Blood cells	0	(0)	0	(0)	0	(0)
Endodermal tissues						
Digestive organ (pharynx, liver, pancreas, intestine)	0	(0)	0	(0)	9	(9)
Nonspecific endodermal tissue	100	(45)	100	(85)	100	(76)

†100 ng/ml activin-treated animal caps. ‡Untreated animal caps.
*Figures are percentages of the total number of explants.
**Figures in parentheses are percentages of tissues derived from activin-treated animal caps alone.

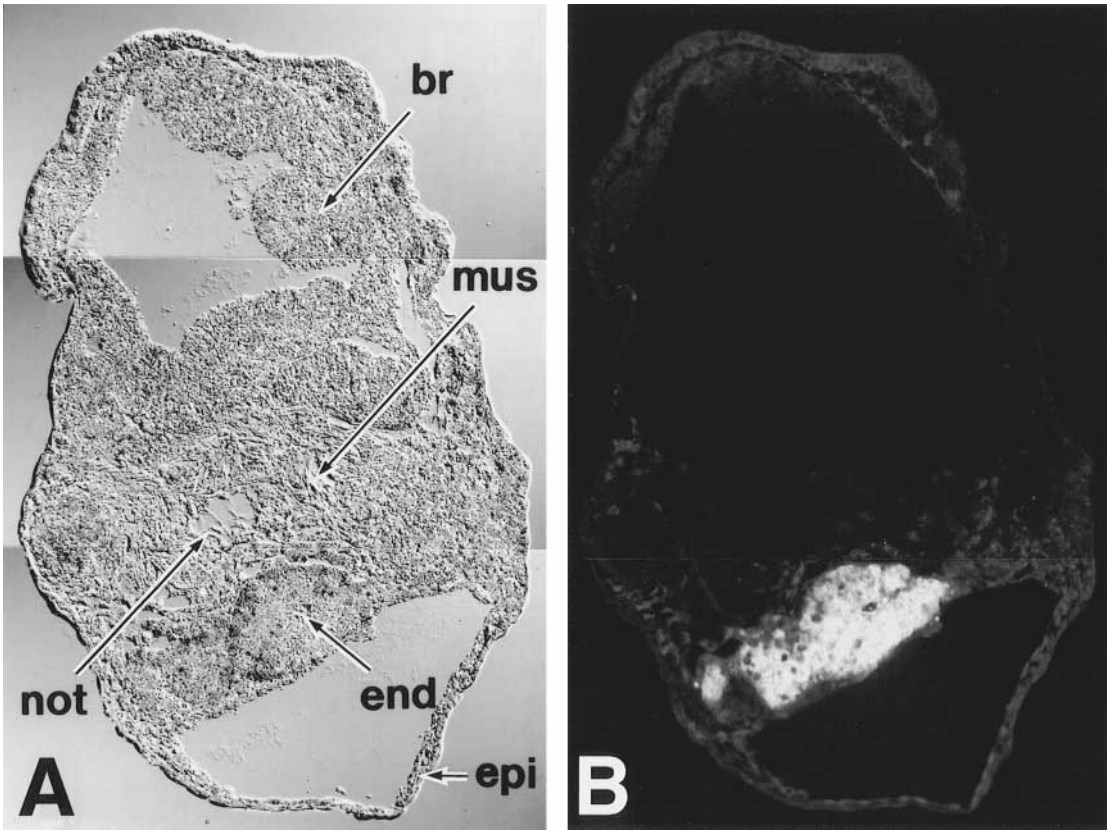

Fig. 5. Cell lineage of activin-treated animal cap in the sandwich explant. The activin-treated animal caps (0.6×0.6 mm) dissected from FDA-labeled embryos were sandwiched between non-labeled animal caps (1.2×1.2 mm). After 2 weeks of culture, they were sectioned and observed under an epifluorescence microscope. (A) Differentiation of brain (br), epidermis (epi), muscle (mus), notochord (not), and endodermal tissue (end) can be seen in the bright field. (B) It is obvious in the dark field that the activin-treated animal cap (FDA-labeled) differentiated into endodermal tissue alone.

sues, including digestive organs (Fig. 5).

DISCUSSION

Mesoderm differentiation in activin-treated animal caps

In the present study, we first investigated the patterns of response of animal caps to activin A treatment (series I). Although the animal caps were treated for as short as 1 hr, activin A was effective at 0.5 ng/ml, a dose similar to that required for mesoderm induction in *Xenopus* animal caps (Ariizumi *et al*., 1991a, b). However, the response patterns of *Cynops* animal caps were also markedly different from *Xenopus* in some regards. At the range of concentrations examined (0-100 ng/ml), the frequencies of mesoderm differentiation in the *Cynops* early gastrula animal caps were generally low, which is consistent with our previous report (Moriya and Asashima, 1992) on late blastula animal caps of this species. On the other hand, *Xenopus* animal caps exhibit dose-dependent differentiation of mesoderm, varying from ventral to dorsal types, with clear dose thresholds (Ariizumi *et al*., 1991a, b). These discrepancies in mesoderm differentiation suggest the following possibilities. First, the role of activin A in these two species may differ, with activin A acting as an endogenous mesoderm inducer in *Xenopus*, but endoderm inducer in *Cynops*. Second, the structural differences between animal caps of the two animals may be related to the frequency of mesoderm differentiation. As already mentioned, the *Cynops* animal cap consists of a monolayer of ectodermal cells, while the *Xenopus* animal cap is multi-layered with each layer composed of different types of cells. The remarkable differentiation of mesodermal tissues in *Xenopus* may be due to the heterogeneity of the animal caps. Activin A may affect only the inner layer cells of blastocoel lining the animal caps. Mesodermal tissues are subsequently induced in the outer layer cells as a result of the secondary inductive interaction with the activin-induced blastocoelic cells. The latter possibility is also supported by the results of our present study (series II) and of a study with salamander animal caps, which also consists of heterogeneous cell layers (Ariizumi *et al*., 1998).

Endoderm differentiation in activin-treated animal caps

The *Cynops* animal caps exposed to higher concentrations of activin A (10-100 ng/ml) differentiated mainly into yolk-laden tissues. We consider that these tissues are the developing endoderm because they sometimes showed a columnar appearance, like the endodermal epithelium, after 2 weeks of culture. This tendency was clearer when they were induced together with the ventral mesoderm such as the blood cells and the coelomic epithelium (Fig. 2B). As reported by Takata and Yamada (1960), histological diagnosis of the endodermal organs that develop from animal caps requires more than one month of culture, during which time further inductive interactions occur between the developing endoderm and the coexisting tissues. Okada (1954a, b, 1960) reported that the presence of mesodermal tissues is a prerequisite for further development of the endoderm. In the present study (series II), well-organized endodermal organs were frequently induced in the sandwich explants together with a variety of mesodermal tissues. Differentiation of a liver, a pancreas and an intestine was confirmed by electron microscopic examination of the 5-week cultured sandwich explants. Thus, it appears probable that activin A can switch the fate of animal caps from ectoderm to endoderm. However, the development of well-organized endodermal organs requires further inductive interactions with the mesodermal tissues coexisting in the explants.

The results of several studies using molecular markers support the possibility that activin A also has endoderm-inducing ability in *Xenopus* animal caps. Genes normally expressed in the presumptive endoderm region of the embryo are directly induced in the animal caps by activin A (reviewed in Ariizumi and Asashima, 1995a). Jones *et al*. (1993) confirmed the presence of endodermal tissues in the activin-treated animal caps using a monoclonal antibody. Judging from these reports and the present study using *Cynops* animal caps, it seems probable that the activin-induced cells (i.e., inner blastocoelic cells) are fated to form endoderm in *Xenopus* animal caps.

Activin-induced endoderm as an initiator of inductive interactions

In series II, yolk-laden endodermal tissues were induced in almost all explants because of the high dose of activin A (100 ng/ml). As shown in the cell lineage analysis of the combination and sandwich explants, cells determined to differentiate into endoderm induced mesodermal tissues from the adjacent non-induced (untreated) cells as a secondary inductive event. This is corroborated by the results of isolation culture in which mesodermal tissues were more frequently induced as the size of animal caps increased. Non-induced cells probably coexist with activin-induced cells in larger animal caps. Differentiation of neural tissues including the central nervous system could be explained as the result of further inductive interactions between the mesodermalized cells and the remaining non-induced cells (Kanéda and Suzuki, 1983; Suzuki *et al*. 1986). Activin A is thus capable of inducing endoderm that can act as an initiator of a chain of induction events in *Cynops*.

In *Cynops* early gastrulae, the presumptive trunk organizer exhibits ectodermal characteristics and forms atypical epidermis when cultured in isolation (Kanéda and Hama, 1979). During gastrula stages, the self-differentiation capacity for dorsal mesoderm and the neural-inducing activity are established in the trunk organizer under the influence of the adjacent presumptive prechordal plate (Kanéda, 1980, 1981; Kanéda and Suzuki, 1983). The isolated presumptive prechordal plate, on the other hand, differentiates mainly into the endodermal cell mass (Kanéda, 1981). In the present study (series II), the animal caps treated with 100 ng/ml of activin A also formed yolk-laden endoderm and induced the adjacent untreated animal caps to differentiate into dorsal mesoderm and neural tissues. These results suggest that activin A can

induce the endoderm including the prechordal plate involved in the formation and determination of the organizer during early *Cynops* development.

In the combination and sandwich culture modes, explants often formed a trunk-tail equipped with the dorsal mesoderm (notochord and somitic muscle) and the posterior central nervous system (hindbrain and spinal cord). However, explants with head structures such as the forebrain and the eyes were never seen in these culture modes. Takaya (1977) reported that the anterior-posterior patterning of the central nervous system depends on the type and quantity of surrounding mesodermal tissues. The posterior central nervous system differentiates together with the dorsal mesoderm, whereas the forebrain differentiates in the presence of a few mesenchyme cells. We have succeeded in inducing heads *in vitro* by sandwich culture of activin-treated and untreated *Cynops* animal caps (Ariizumi and Asashima, 1995b). The activin-treated animal caps immediately sandwiched with untreated animal caps induced well-organized trunk-tails in exactly the same manner as the present study. On the other hand, heads were frequently induced when the activin-treated animal caps were precultured in saline (MHS) for more than 12 hr before being sandwiched. These findings suggest that the timing of the inductive interactions between the induced and non-induced cells is also important in the regulation of inductive events during early embryogenesis.

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