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Changes in the adhesive properties of dissociated and reaggregated *Xenopus laevis* embryo cells

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Activin A is a member of the transforming growth factor β superfamily, and the strongest candidate mesoderm-inducer. The initial adhesive property changes in amphibians are likely to be mediated by mesoderm-inducers like activin A. The manner in which these changes actually occur, however, remains poorly understood. In the present study, the adhesive property changes mediated by activin A were directly demonstrated. Activin A functioned as a morphogen at low concentrations (less than 0.5 ng/mL), with no effect on the type A adhesive property. But at high concentrations (1 ng/mL), it induced another type of adhesive property, type N, and at very high concentrations (more than 10 ng/mL), it induced yet another type of adhesive property, type Y. Cells that have types A, N, and Y adhesive properties ultimately differentiated into atypical epidermis, notochord, and yolk-rich cells, respectively. It was also shown that these changes occurred between 5 and 10 h after induction by activin A. The implications of these results for the relationship between the adhesive property acquired during early and later stages of differentiation are also discussed.

Key words: activin A, adhesive property, animal cap, mesoderm-inducer, *Xenopus laevis*.

Introduction

Regulated changes in cell adhesion play an important role in establishing the embryonic body plan during vertebrate development. The initial changes in embryonic cell adhesion during amphibian development may be regulated by mesoderm-inducers, but these putative signals have not been isolated to date. One candidate molecule for these primary events is activin A, a member of the transforming growth factor (TGF)-β superfamily. Activin A has very potent mesoderm-inducing activity on *Xenopus* presumptive ectoderm and induces various mesodermal tissues in a dose-dependent manner. Presumptive ectoderm treated with activin A differentiates into ventral mesodermal tissue such as coelomic epithelium at low activin A concentrations, muscle at high concentrations, and the dorsalmost tissue, notochord, at very high concentrations (Asashima *et al.* 1990; Ariizumi *et al.* 1991a,b). In addition, cells dissociated from animal caps have several prospective cell fates dependent on distinct activin A concentration thresholds (Green *et al.* 1994).

In explantation experiments, ectoderm dissected from late morula–early gastrula stage embryos treated with a high concentration of activin A (100 ng/mL) formed only yolk-rich endodermal cells. When the explants were transplanted into the blastocele of other early gastrula, they formed part of the endoderm of the host embryo and induced a secondary axis with only posterior characteristics, including axial mesoderm and neural tissues. In contrast, whole secondary axes were induced when activin-treated ectoderm was transplanted into the ventral marginal zone (VMZ) of early blastula; the transplanted pieces invaginated and differentiated into foregut structures, including the pharynx, stomach, and liver. These phenomena were also observed in experiments in which presumptive foregut was used instead of activin-treated ectoderm. These findings show that activin-treated ectoderm undergoes changes in adhesive properties and can act as the single organizing center in *Cynops* (Ariizumi & Asashima 1995; Ninomiya *et al.* 1998). In addition, when presumptive epidermal explants from pigmented neurula embryos and neural plate explants from unpigmented neurula embryos were dissociated and mixed, the cells reaggregated such that epidermal cells covered other cell types (Townes & Holtfreter 1955).

In the present study, we used a new *in vitro* assay to determine how changes in cell–cell adhesion occur in the early stages of development and directly

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demonstrated that cells induced by activin A undergo changes in adhesive properties and subsequently differentiate.

Materials and Methods

Embryos and activin A solution

Xenopus laevis eggs were obtained from females by injecting them with 400 units of human chorionic gonadotropin (Gestron; Denka Seiyaku Co., Kawasaki, Japan), fertilized *in vitro* with minced testis, and cultured in 1 × Steinberg's solution (58 mM sodium chloride, 0.67 mM potassium chloride, 0.34 mM calcium nitrate, 0.83 mM magnesium sulfate, 100 mg/L kanamycin sulfate and 4.6 mM timza base, pH 7.4). Staging was according to Nieuwkoop and Faber (1967). The jelly coat was removed with 3% cysteine hydrochloride in Steinberg's solution (pH 7.8), and the vitelline membrane was manually removed with fine forceps. All operations were carried out under sterile conditions.

Human recombinant activin A (provided by Dr Yuzuru Eto, Ajinomoto Co. Inc., Kawasaki, Japan) was dissolved at concentrations of 0.05–100 ng/mL in 1 × Steinberg's solution containing 0.1% bovine serum albumin (BSA; A-7999; Sigma Chemical Co., St Louis, MO, USA).

Reaggregation experiment

Embryos at the 2-cell stage were injected with a total volume of 12 nL of 1% Texas red-dextran-amine (TRDA; D-1863; Molecular Probes, Eugene, OR, USA) or 1% fluorescein-dextran-amine (FDA, D-1820; Molecular Probes).

The 0.4 × 0.4 mm *X. laevis* presumptive ectoderm was cut from stage 8.5 embryos in 3% agar-coated culture dishes (35 mm Tissue Culture Dish, 3000–035; Iwaki Glass, Chiba, Japan) containing calcium-magnesium-free Dulbecco's phosphate-buffered saline (CMF-PBS, D-5652; Sigma) using tungsten needles.

The presumptive ectodermal regions were transferred to another 3%-agar-coated culture dish filled with CMF-PBS. The outer layer of cells, which is difficult to dissociate, was discarded, and the inner layer was disaggregated into a single-cell suspension by gentle pipetting.

Cells from 10 caps were incubated in 5 mL diluted activin A solution with Steinberg's solution containing 0.1% BSA. After 1 h, the cells were swirled into the center of the dish, the activin A solution was replaced with fresh Steinberg's solution, and the cells were gently resuspended. This operation was performed three times. Reaggregates were prepared by gentle swirling until initiation of reaggregation or by gentle centrifugation at 100 *g* and cultured for 5, 10, 20 h or 3 days. (Fig. 1).

The reaggregates were fixed in phosphate-buffered 10% commercial formalin for 5 h. They were then dehydrated through an ethanol series, embedded in paraffin, and sectioned at 6 µm. After deparaffinization, the sections were mounted in PermaFluor Aqueous Mounting Medium (434990; Immunon, Pittsburgh, USA) and examined under an epifluorescence microscope.

Histological examination

Reaggregates were fixed with Bouin's fluid for 3 h, dehydrated with ethanol, embedded in paraffin wax, sectioned at 6 µm and stained with hematoxylin and eosin (HE).

Results

Activin A has more potent and distinct effects on dissociated cells than on intact animal caps

Activin A dose-dependently induces animal caps to form several different types of cell, with higher doses eliciting more dorsal tissues (Asashima *et al.* 1990; Ariizumi *et al.* 1991a,b). Preliminary experiments were

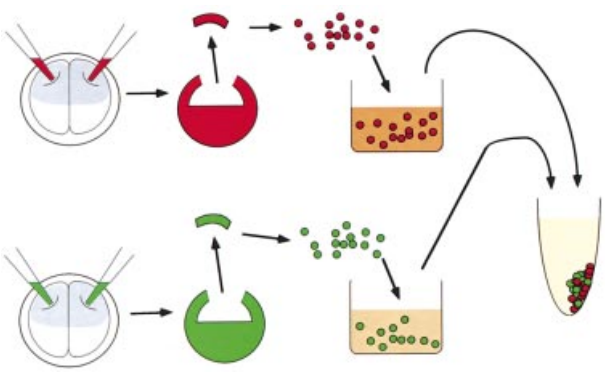

Fig. 1. Schematic diagram of the experimental procedures. *Xenopus laevis* embryos at the 2-cell stage were injected with a total volume of 12 nL of 1% Texas red-dextran-amine (TRDA, shown in red), or with a total volume of 12 nL of 1% fluorescein-dextran-amine (FDA, shown in green). The embryos were grown to stage 8.5, and a 0.4 × 0.4 mm segment of the presumptive ectoderm (called animal caps) was then cut from the embryos. The animal caps were then transferred to dishes filled with calcium-magnesium-free solution. The outer layer of the animal cap, which is difficult to dissociate, was discarded and the inner layer was disaggregated into a single-cell suspension by gentle pipetting. Dissociated cells derived from five caps and labeled with TRDA or FDA were incubated in 5 mL of medium with various concentrations of activin A: 0, 0.5, 1, 10, or 100 ng/mL. After 1-h incubation each dish of cells was washed, and the cells were reaggregated by centrifugation at 100 *g*, washed again, and incubated for 5, 10, 20 h or 3 days.

performed to establish the overall dose-response of dissociated animal cap cells to form differentiated tissue. The effect of different activin A concentrations on the adhesive properties of dissociated *Xenopus* animal cap cells was examined (Fig. 2). The results suggested a simple model for the effects of activin A on dissociated cells. Low concentrations of activin A, less than 0.5 ng/mL, induced only atypical epidermis (Fig. 2A–E). High concentrations, 0.75–5 ng/mL, induced mostly notochord (Fig. 2F–I), and in particular, 1 ng/mL induced only notochord. Very high concentrations, exceeding 7.5 ng/mL, induced only yolk-rich

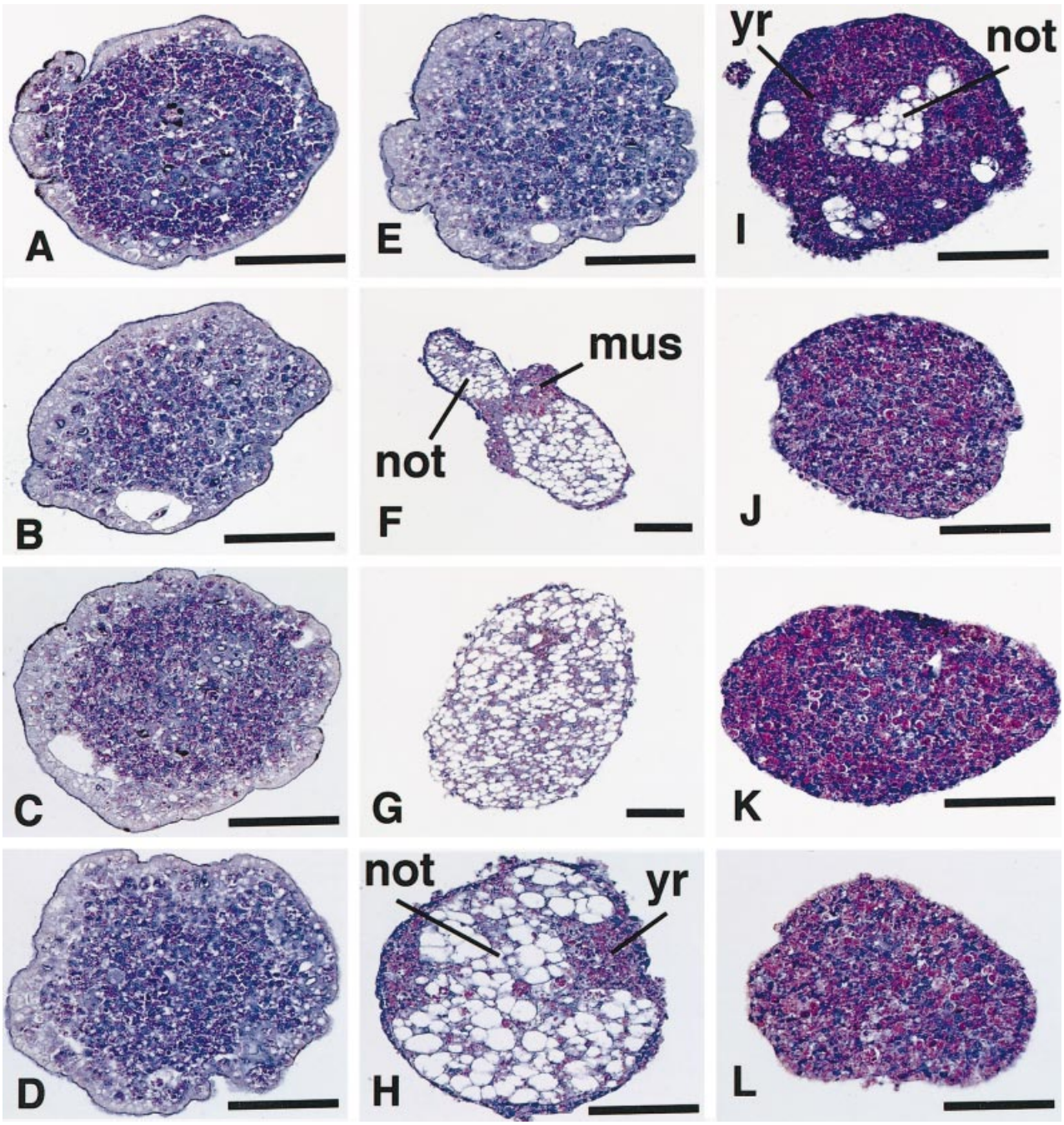

Fig. 2. Effects of activin A on dissociated animal cap cells. Reaggregates treated with 0 (A), 0.05 (B), 0.1 (C), 0.25 (D), or 0.5 ng/mL activin A (E) differentiated into atypical epidermis. Reaggregates treated with 0.75 ng/mL (F) activin A differentiated into notochord, muscle, and atypical epidermis. Reaggregates treated with 1 ng/mL (G) activin A differentiated only into notochord. Reaggregates treated with 2.5 (H) or 5 ng/mL (I) differentiated into notochord and yolk-rich tissue. Reaggregates treated with 7.5 (J), 10 (K) or 100 ng/mL (L) differentiated only into yolk-rich tissue. mus, muscle; not, notochord; yr, yolk-rich tissue. Bars, 100 µm.

tissue (Fig. 2J–L). These results indicate that activin A has only two functions, induction of notochord and induction of yolk-rich tissue. Thus, the effect of activin A on the adhesive properties of *Xenopus* animal cap cells was examined at concentrations of 0.5, 1, 10, and 100 ng/mL.

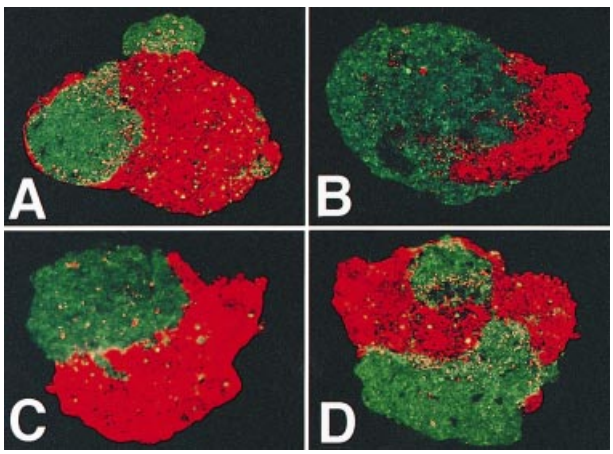

Fig. 3. 'Sorting by separation'. Reaggregates formed by treating cells with activin A concentrations that induce yolk-rich tissue and concentrations that induce atypical epidermis underwent 'sorting by separation'. (A) Reaggregates of cells treated with 0 (green cells) or 100 ng/mL (red cells) activin A. (B) Reaggregates of cells treated with 0.5 (green cells) or 100 ng/mL (red cells) activin A. (C) Reaggregates of cells treated with 0 (green cells) or 10 ng/mL (red cells) activin A. (D) Reaggregates of cells treated with 0.5 (green cells) or 10 ng/mL (red cells) activin A.

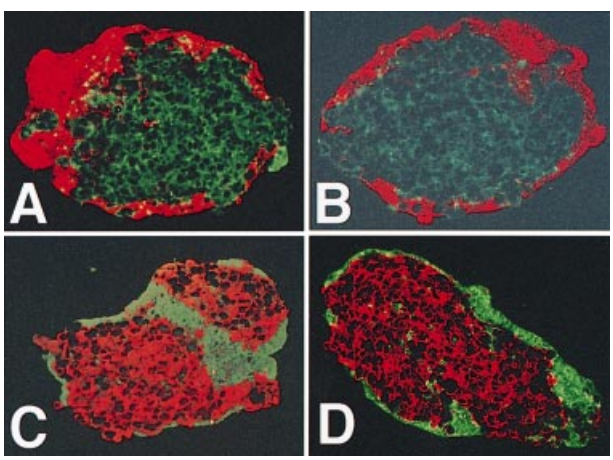

Fig. 4. 'Inner–outer sorting'. Reaggregates formed from cells treated with activin A concentrations that induce notochord, or yolk-rich tissue or atypical epidermis, underwent 'inner–outer sorting'. (A) Reaggregates of cells treated with 1 (green cells) or 100 ng/mL (red cells) activin A. (B) Reaggregates of cells treated with 1 (green cells) or 10 ng/mL (red cells) activin A. (C) Reaggregates of cells treated with 0.5 (green cells) or 1 ng/mL (red cells) activin A. (D) Reaggregates of cells treated with 0 (green cells) or 1 ng/mL (red cells) activin A.

'Sorting by separation' occurs in reaggregated mixed animal cap cells induced to form atypical epidermis or yolk-rich tissues

Cells treated with 10 or 100 ng/mL activin A to induce yolk-rich tissue were labeled with TRDA, and cells treated with 0.5 ng/mL activin A and untreated cells, which form atypical epidermis, were labeled with FDA. Labeled cells were mixed, and reaggregates were incubated until control embryos had reached stage 38. Reaggregates at this stage showed clear separation of TRDA-labeled and FDA-labeled cells (Fig. 3; Table 1). For convenience, we refer to this type of sorting as 'sorting by separation'. Stained sections of fixed reaggregates showed that TRDA-labeled cells had differentiated into yolk-rich tissue and FDA-labeled cells into atypical epidermis (data not shown).

'Inner–outer sorting' occurs in reaggregated mixed animal cap cells induced to form notochord or other tissues

Cells induced by 10 ng/mL activin A to form yolk-rich tissue were labeled with TRDA, and cells treated with 1 ng/mL activin A to form only notochord were labeled with FDA. In reaggregates composed of the mixture of these cells, the FDA-labeled cells were located in a central mass, surrounded by TRDA-labeled cells. We referred to this type of sorting as 'inner–outer sorting' (Fig. 4A,B; Table 1). Fixing and staining of reaggregates confirmed that the TRDA-labeled cells had differentiated into yolk-rich tissue and the FDA-labeled cells into notochord (data not shown).

Table 1. Cell-lineage analysis of the composition of reaggregates

Activin concentration (ng/mL)		Number			
TRDA labeled cells	FDA labeled cells	Total	IOS	SBS	NS
100	100	5	0	0	5
100	10	5	0	0	5
100	1	7	7	0	0
100	0.5	5	0	5	0
100	0	11	0	11	0
10	10	5	0	0	5
10	1	5	5	0	0
10	0.5	5	0	5	0
10	0	8	0	8	0
1	1	5	0	0	5
1	0.5	5	5	0	0
1	0	8	8	0	0
0.5	0.5	5	0	0	5
0.5	0	5	0	0	5
0	0	9	0	0	9

IOS, Inner–outer sorting; SBS, sorting by separation; NS, not sorting.

In a second experiment, cells induced by 1 ng/mL activin A to form only notochord were labeled with TRDA, and another part of cells treated with 0.5 ng/mL activin A or untreated, which differentiated into only atypical epidermis, were labeled with FDA. In this case, 'inner–outer sorting' occurred in reaggregates resulting from mixing of these cells, and the TRDA-labeled cells were located in a central mass, surrounded by FDA-labeled cells (Fig. 4C,D; Table 1). Histological examination confirmed that the TRDA-labeled cells had formed notochord and the FDA-labeled cells atypical epidermis (data not shown). In addition, although it is usually difficult to obtain histological information from fluorescent sections, we had no difficulty detecting the vacuolation characteristics of notochord differentiation in these preparations.

Sorting does not occur in reaggregates prepared by mixing histologically identical cells

The TRDA-labeled and FDA-labeled cells in reaggregates did not sort when they had been treated with yolk-inducing tissue concentrations of activin A (10 or 100 ng/mL), (Fig. 5A–C; Table 1), the notochord-inducing concentration of activin A (1 ng/mL), (Fig. 5D; Table 1), or the atypical epidermis-inducing concentration of activin A (0.5 ng/mL), or when they were untreated (Fig. 5E–G; Table 1). Regions in which red and green cells were well mixed are shown as yellow in Fig. 5. These results refute the hypothesis that different concentrations (here 0 and 0.5 ng/mL, and 10 and 100 ng/mL) result in formation of the same tissue, but induce different adhesive properties. Rather, these results indicate that yolk-rich cells and notochord cells induced by activin A, as well as atypical epidermal cells unexposed to activin A, have a single type of adhesive property.

Adhesive properties of animal cap cells may change 5–10 h after induction mediated by mesoderm-inducers

When do changes in the adhesive properties of cells induced by a mesoderm-inducer occur *in vivo*? To answer this question, we examined the time course of reaggregate formation (from initiation of regeneration, 5, 10, 20 h, and 3 days after induction with activin A). There are two general methods to gather dissociated cells. One is the centrifugation method, which we predominantly employed, and the other is the swirling method, which we used to gather cells immediately upon initiation of regeneration (Fig. 6D,I,N). Sorting did not occur in reaggregates between the initiation of regeneration (Fig. 6D,I,N) and 5 h (Fig. 6E,J,O). However, by 10 h, sorting was evident (Fig. 6F,K,P), and was sustained through 20 h (Fig. 6G,L,Q) up to 3 days (Fig. 6H,M,R). In control experiments, no sorting occurred up to 3 days (Fig. 6A–C). These results indicate that adhesive property changes occur 5–10 h after induction by mesoderm-inducers, at the gastrula stage. This is the first study to directly demonstrate that adhesive property changes occur at this stage.

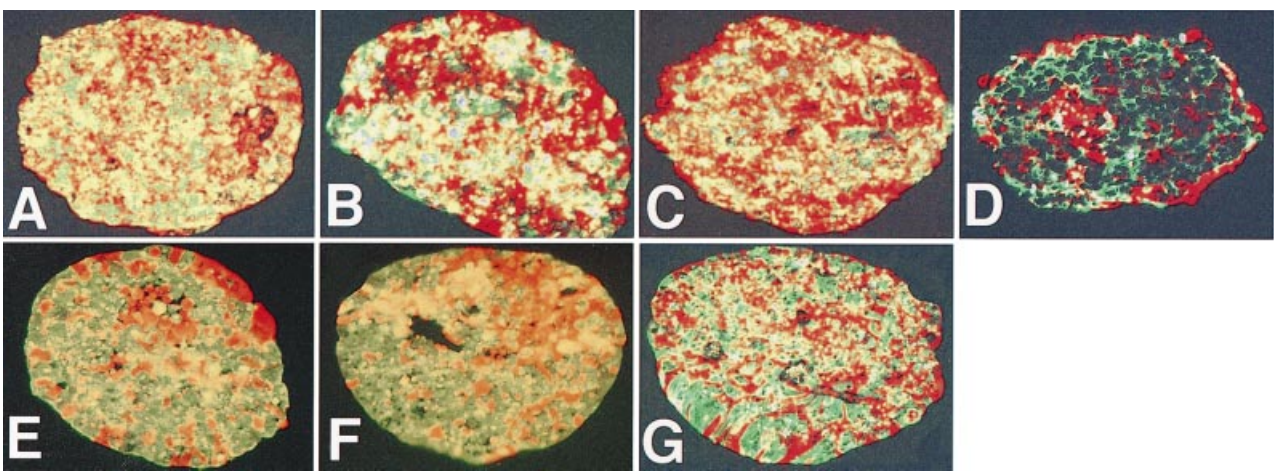

Fig. 5. Sorting did not occur when reaggregates were composed of two cell types having the same fate. (A) Reaggregates in which both red and green cells were treated with 100 ng/mL activin A. (B) Reaggregates of cells treated with 10 (green cells) or 100 ng/mL (red cells) activin A. (C) Reaggregates in which both red and green cells were treated with 10 ng/mL. (D) Reaggregates in which both red and green cells were treated with 1 ng/mL. (E) Reaggregates in which both red and green cells were treated with 0.5 ng/mL. (F) Reaggregates of cells untreated (green cells) or treated with 0.5 ng/mL (red cells) activin A. (G) Reaggregates in which both red and green cells were untreated with 0 ng/mL activin A (red cells). Yellow regions in Fig. 5 indicate the region of mixed red and green cells.

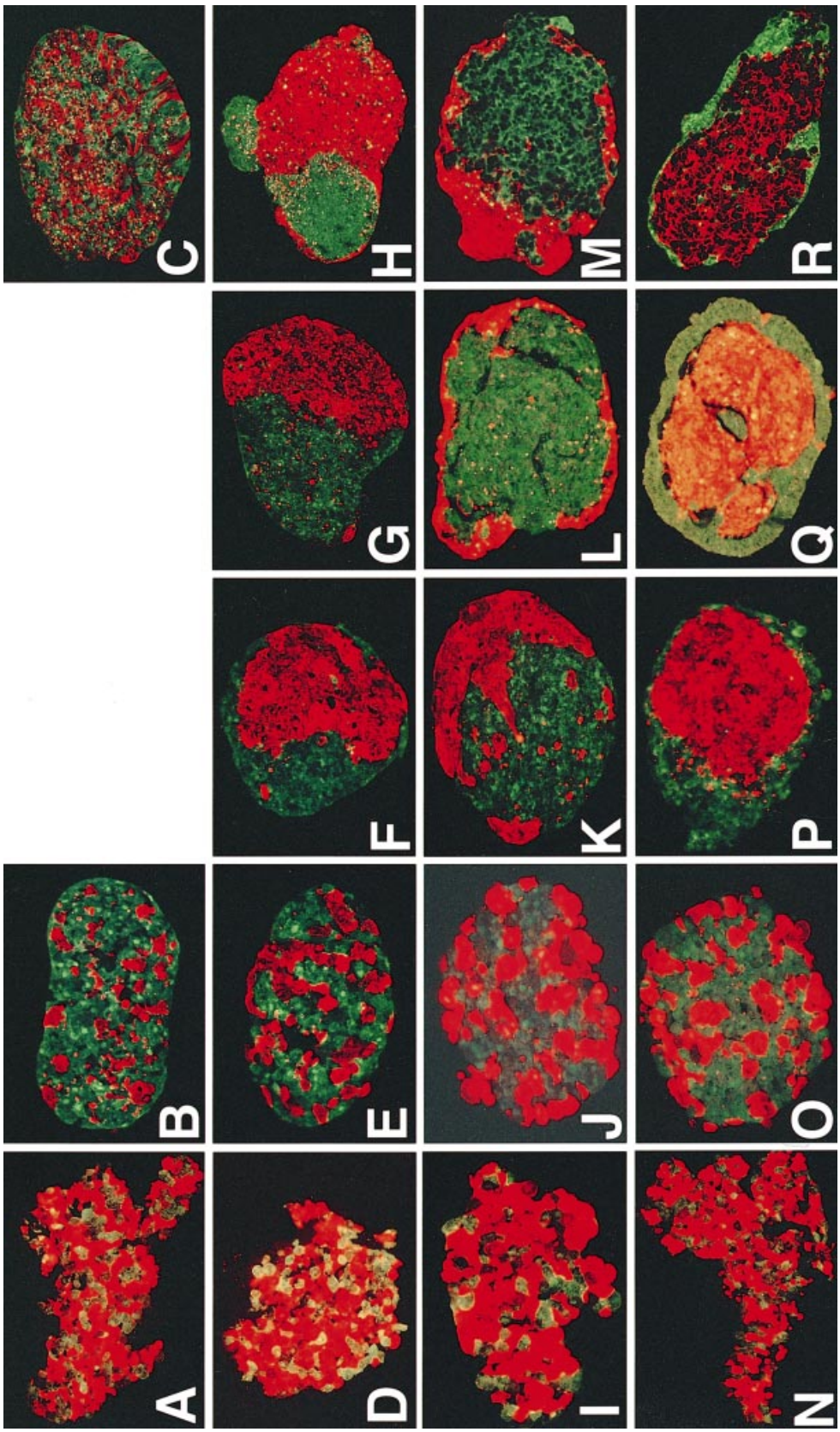

Fig. 6. Time course of reaggregate formation. All panels are sections of reaggregates. (A–C) Reaggregates of untreated cells. (D–H) Reaggregates of cells treated with 100 ng/mL (red cells) and 0 ng/mL activin A (green cells). (I–M) Reaggregates of cells treated with 100 ng/mL (red cells) and 1 ng/mL activin A (green cells). (N–R) Reaggregates of cells treated with 1 ng/mL (red cells) and 0 ng/mL activin A (green cells). (A,D,I,N) Immediately upon initiation of reaggregation (≈ 2–3 h); (B,E,J,O) 5 h after reaggregation; (F,K,P) 10 h after reaggregation; (G,L,Q) 20 h after reaggregation; (C,H,M,R) 3 days after reaggregation.

Discussion

The finding that mesoderm-inducers change adhesive properties in amphibians was predicted, but the mechanism underlying this phenomenon remains to be elucidated. As there is no apparent difference between the cells induced or not induced by mesoderm-inducers, it is difficult to demonstrate these changes. In the present study, we used activin A, which is widely regarded as the most promising candidate mesoderm-inducer, plus fluorescent molecules and dissociated cells, and directly demonstrated that activin A changes adhesive properties of blastomeres. We also showed that activin A exerts this effect dose dependently and that the change occurs 5–10 h after activin A treatment. These results imply that mesoderm-inducers change cell adhesive properties at the gastrula stage.

Two types of adhesive properties are induced by activin A

Reaggregates composed of untreated animal cap cells or of cells treated with less than 0.5 ng/mL activin A differentiate into atypical epidermis, while reaggregates composed of cells treated with 0.75–5 ng/mL activin A differentiate into notochord. Cells treated with more than 7.5 ng/mL activin A differentiate into yolk-rich cells. In addition, we found that these three cell types have specific and distinct adhesive properties. The adhesive properties of the cells treated with less than 0.5 ng/mL, with 0.75–5 ng/mL, and with more than 7.5 ng/mL of activin A are, for convenience, referred to as type A (atypical epidermis), type N (notochord), and type Y (yolk-rich tissue), respectively. In other words, activin A induces animal cap cells to dose-dependently manifest distinct adhesive properties, with low, high, and very high doses eliciting types A, N, and Y, respectively.

According to the thermodynamic model of cell–cell interaction (Steinberg 1964), the binding activity of cells with type N adhesion appears to be very strong, because the cells move into the central region of a reaggregate whenever it is composed of cells with type A or Y adhesion. This is an important concept for understanding the formation of the notochord *in vivo*, as discussed below. The binding activity of cells with type Y adhesion, however, may be weak because reaggregates composed of cells treated with a high concentration of activin A differentiate into yolk-rich tissue and become smaller and the reaggregate ultimately disintegrates (data not shown). In light of these results and the thermodynamic model (Steinberg 1964), we advocate the former hypothesis (expressing the binding activity between cells having type A and type N adhesivity as 'S (a-n)', where:

$$S(n\text{-}n) > S(a\text{–}n) \doteqdot S(n\text{–}y) \geq S(a\text{–}a) > S(y\text{–}y) \geq S(a\text{–}y)$$

Effects of activin A on dissociated cells

Activin A induces animal caps to form several different types of cells in a dose-dependent manner. These are ventral mesoderm in the form of coelomic epithelium, blood corpuscles, and mesenchyme at very low concentrations (0.1 ng/mL), neural tissue and muscle at a low concentration (1 ng/mL), dorsal mesoderm in the form of notochord at a high concentration (10 ng/mL), and endoderm in the form of yolk-rich tissue at a very high concentration (100 ng/mL). In dissociated cells, however, we have detected only two tissue types: notochord and yolk-rich tissue. While we also recognized some muscle, it may have formed secondary to notochord induction. Why are other tissues not induced, and why is the effect of activin A on dissociated cells much stronger than on animal caps? We propose two hypotheses. One is that dissociated cells have more receptors and bind activin A more readily than animal cap cells, and each dissociated cell has an almost equal number of activin A receptors, making the induced tissue nearly homogeneous. The second is that reaggregates may no longer possess the molecules to function competitively or cooperatively with activin A. The most probable candidate for the competitive molecule is bone morphogenetic protein-4 (BMP-4) (Dale *et al.* 1992; Jones *et al.* 1992; Fainsod *et al.* 1994). The BMP-4 is a secreted protein that binds to the dorsalization and neuralization inducer proteins chordin and noggin (Piccolo *et al.* 1996; Zimmerman *et al.* 1996), which are induced by activin A. Thus, it seems reasonable to assume that these proteins are removed by dissociation, thereby enhancing activin A activity. The most probable candidate for the cooperative molecule is fibroblast growth factor (FGF). The FGF is a secreted protein, and it is a highly effective inducer of mesoderm, equivalent to activin A. We propose that activin does not have multiple functions as a morphogen, as previously thought, but that its sole function is simply to induce notochord or endoderm, and muscle may be induced cooperatively by activin A and FGF. The many types of tissue induced by treating the animal cap with activin A may result from induction by other cofactors, or from secondary induction of another cell type induced by activin A or other cofactors.

Mechanism of notochord formation in vivo

The results of the present study suggest the following model for the mechanism of early development of *X. laevis*. Cells from the Nieuwkoop center region involute along with the dorsal mesoderm during gastrulation and serve as a continuing source of an activin A-like signal resulting in generation of notochord and other structures (Vodicka & Gerhart 1995). Initially, the cells

are positioned in the proper region by gastrulation. Then, 5–10 h after induction by an appropriate level of activin A-like signal, the cells acquire a type N adhesive property, are sorted from other cells, and stabilize in one position. Finally, the cells differentiate from the notochord structure and undergo secondary neural induction.

One intriguing finding in the present study was that cells treated with 1 ng/mL activin A only differentiated into notochord and showed strong adhesive properties. It is possible that presumptive ectoderm cells induced by a specific level of activin A-like signal *in vivo* assemble to form a prenotochord thread at the site of subsequent notochord formation. It is not surprising that cells forming the notochord, a key tissue in the developing vertebrate, should be directed by very strong intercellular bonds.

Molecular basis of activin-induced changes in cell adhesive properties

In the present study we have shown that more than 0.75 ng/mL activin A induces changes in the adhesive properties of *Xenopus* animal cap cells. There are two candidate molecules for mediation of these adhesive properties, a heterophilic molecule like the extracellular matrix glycoprotein (ECM), or a homophilic molecule like cadherin.

The most recent studies of activin-induced changes in cell adhesiveness have examined changes in adhesion to fibronectins (Smith *et al.* 1990; Howard & Smith 1993; Whittaker & DeSimone 1993; Symes *et al.* 1994; Ramos & DeSimone 1996). According to those studies, blastomeres derived from induced animal pole regions are able to spread and migrate on a fibronectin-coated surface in the same manner as marginal zone cells, but unlike control animal pole blastomeres. Dispersed animal pole cells are also able to respond to activin A in this way (Smith *et al.* 1990). However, we found no differences in the expression of fibronectin or other ECM molecules between activin-treated cells and untreated cells by immunohistochemical staining (data not shown).

The present results are generally consistent with a homophilic alteration in adhesive properties induced by activin A, rather than a heterophilic alteration. Cadherins, Ca^{2+}-dependent cell–cell adhesion receptors (Nose *et al.* 1988; Takeichi 1991), were found to be equally expressed in activin-treated cells and untreated cells (data not shown). Thus, there must be some other as yet unidentified molecules mediating these changes in adhesive properties.

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