In Vitro Organogenesis using Amphibian Pluripotential Cells

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ABSTRACT

In the embryonic development of vertebrate, sequential cleavage is followed by the gastrulation as the first dynamic event of morphogenesis. Mesodermal induction is the most important event for normal body patterning such as the gastrulation, neural induction and formation of various organs. In the study to search for the mesodermal inducing factor, we reported "activin" as a strong mesoderm-inducible factor by the "animal cap assay", the *in vitro* assay system using amphibian pluripotential cell mass. We found that activin has mesodemal and endodermal inducing activity in dose-dependent manner, and then established the *in vitro* induction system for various types of tissues and organs including craniofacial cartilage from animal cap cells by the treatments with activin and other inducing factors. Embryonic transplantation method showed that the treatments of animal cap cells for the induction of pronephros, beating heart were able to induce normally functional organs *in vivo*. These *in vitro* induction methods are useful for investigation of the molecular mechanisms of organ formation and body patterning in vertebrate development.

Key words: activin, organogenesis, animal cap, mesoderm induction, organizer

INTRODUCTION

Embryonic development is the dynamic event, which consist of sequential cell division from the single cell to the various many cells and the interaction among them. The fundamental question in this phenomenon is what kind of factors and mechanisms cause these continuous processes from the simplicity to the complexity. Fertilization is the first step on the morphogenesis in early embryonic development. In amphibian, fertilized egg as a single cell develops into the blastula by the progress of cleavage, three germ layers (endoderm, mesoderm, and ectoderm) are formed, and the gastrulation and neural induction proceed simultaneously. Various tissues are differentiated on the process of axial body patterning, and many organs are formed by the interaction of various types of cells and tissues.

As a monumental study in 1924, Spemann and Mangold (Spemann et al., 1924) found the ectopic neural axis formation from the host ectoderm by the transplantation of blastoporal lip (organizer) into the blastocoel, and showed experimentally the neural induction in ectoderm by mesoderm. Nieuwkoop showed the mesoderm induction directly by the experiment using microsurgical combination of the endodermal cells with ectodermal cells (Nieuwkoop, 1969). As showed in these results, the ectodermal cells of amphibian blastula had pluripotency, and these cell mass was called "animal cap" later. Mesoderm induction was considered as a key event in early development, and the characterization of mesoderm-inducing factor became an important issue in this field. Animal cap cells were used to evaluate the inducing activity of many candidates as mesodermal inducers.

In the end of 1980s, several growth factors were identified as candidates for mesoderm inducing factors. We reported first that activin (Asashima et al., 1989; Asashima et al., 1990) could induce all types of typical mesodermal tissues including notochord (the most dorsal part of mesodermal tissues) in a concentration-dependent manner (Nakano et al., 1990; Ariizumi et al., 1991) in animal cap cell mass. Then we have established *in vitro* induction methods and the appropriate culture conditions for animal cap cells to differentiate into various tissues and organs including heart, pronephros, pancreas, cartilage, eye, neural tissues, and other mesodermal and endodermal tissues using activin and other inducing factors (Fig. 1). These methods are stable and reliable in that it is possible to induce specific tissues reproducibly by simple manipulation of animal cap cells. In this report, we describe our recent study on the mechanism of body patterning and various organogenesis based on these experimental systems using amphibian embryos in combination with molecular biological methods.

RESULTS

Induction of mesodermal and endodermal tissues by animal cap assay

Amphibian have been used as experimental animal generally in the field of developmental biology because of its rapid embryonic development and easiness to observe, to cultivate *in vitro* with simple saline solutions, and to use for microsurgical manipulation. *Xenopus laevis* belongs to anura, and it is investigated well in molecular biological studies. A fertilized egg of *Xenopus laevis* is spherical with a diameter of approximately 1.2 mm, and develops into blastula by the sequential cleavage while about 7 hr after fertilization in 20℃. The blastula of *Xenopus laevis* consists of about 10,000 cells, and has large cavity named "blastocoel" in the animal hemisphere, which is the upper hemisphere of the spherical blastula. The blasto-

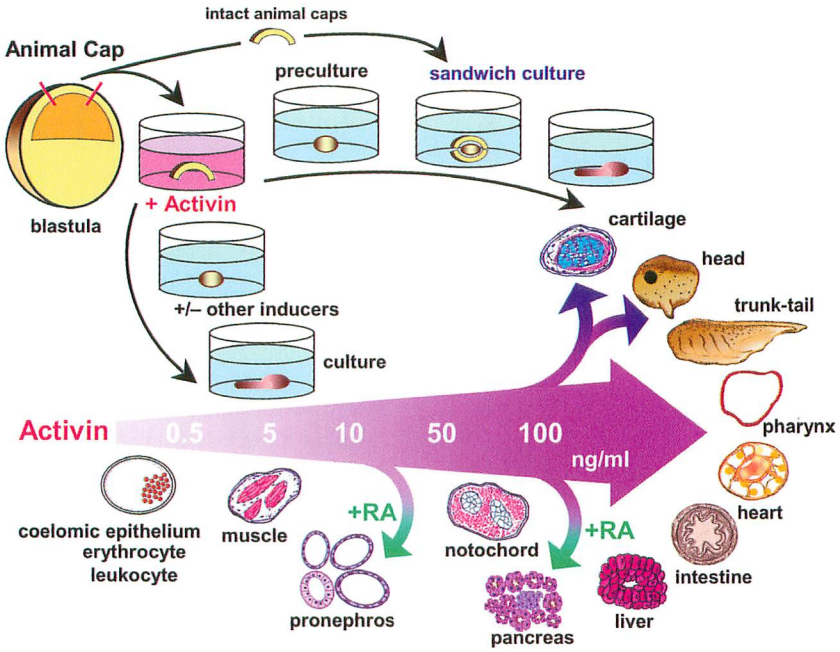

Fig. 1. *In vitro* tissue/organ induction using animal caps.
The schematic diagram of animal cap assay. The "animal cap", blastocoelic roof of amphibian blastula, is excised and cultured in saline containing inducers such as activin and retinoic acid (RA). Differentiated explants are examined by histological and immunohistological analysis, and the expression of molecular markers.

coelic roof is called "animal cap" region, consist of a few layers of presumptive ectodermal cells, which can be easily excised by the watchmakers' forceps and the sharp needles with stereoscopic microscope. Animal cap cells differentiate into ectodermal tissues such as neural tissues or epidermis in normal development of amphibian. When the animal cap cell mass is excised from the blastula and cultivated in a saline solution containing appropriate induction factors, it can be directed to differentiate into various types of tissues including mesoderm- and endoderm-derived tissues as well as ectoderm-derived tissue (Fig. 1, Animal cap assay.). An animal cap cell mass cultivated *in vitro* is also called an "explant". The explants can be cultivated stably for a week or longer, the time that is sufficient for the normal organogenesis in a tadpole of *Xenopus laevis*. Highly differentiated tissues and organs with three-dimensional structures can be formed in explants, and this fact indicate the effectiveness of the animal cap assay as a tool for the investigation of the mechanism of embryonic development and organ generation *in vitro*.

Using animal cap assay, we isolated mesoderm-inducing factor from conditioned medium of the human K-562 cell line (Nakano et al., 1990), and found it was activin (EDF) (Asashima et al., 1989). Activin treatment caused the differentiation of the animal cap cells into various mesodermal tissues in a concentration-dependent manner (Fig. 1). Untreated animal caps form atypical epidermis when it cultured for a few days. In the culture medium with 0.3-1 ng/mL activin, animal caps were differentiated into ventral mesoderm-derived cells/tissues such as blood cells, coelomic epithelium and mesenchymal cells. The animal caps cultured in 5-10 ng/mL of activin solutions showed elongation movement and muscle differentiation. Treatment with 50 ng/mL of activin induced animal cap to differentiate into notochord, the most dorsal mesoderm, and endodermal tissue that contains yolk abundantly. These results suggest that the gradient concentration of activin can regulate the differentiation of the animal cap cells into the mesodermal tissues along with dorsoventral axis (Asashima et al., 1990; Ariizumi et al., 1991). Activin could induce the tissues of endodermal organs including liver, pancreas and intestine in newt animal caps by the combined cultivation of intact animal caps and an animal cap treated with 100 ng/mL of activin (Ariizumi et al., 1999).

Animal cap can be dissociated to single cells easily by the incubation in Ca^{2+}/Mg^{2+}-free culture medium. Activin treatments with 0-0.5 ng/mL, 1-5 ng/mL and 2.5-100 ng/mL caused dissociated cells to differentiate into atypical epidermis, notochord and yolk-rich tissue respectively, when they reaggregated after the activin treatments (Kuroda et al., 1999). When the two groups of animal cap cells that treated with different concentrations of activin were cultured as a mixed aggregate, cells migrate in the aggregate and form separated clusters of the cells treated with the same concentrations of activin, within 5-10 hr after the treatments. This autonomous cell sorting in the mixed aggregate indicates that activin treatment caused the change in the adhesive properties of animal cap cells. This result suggests that there is important relation between the function of cell adhesion molecules and various tissue inductions by activin treatments using animal cap cells (Kuroda et al., 2002).

Animal caps acquire organizer-like activity by the treatment with activin

Transplantation of the newt animal cap treated with activin into the ventral side of newt gastrula caused the formation of well-organized secondary axis, as well as classical experiment of organizer transplantation (Ninomiya et al., 1998). When a newt animal cap was treated with activin 100 ng/mL for 1 hr and cultured for additional 6-12 hr or 18-24 hr ("preculture"), then sandwiched between intact animal caps for culturing, the combined animal cap cell mass (explant) differentiated into the head structure containing hindbrain with otic vesicle or forebrain with eyes, respectively. If the preculture step was omitted in this procedure, the explant differentiated into the trunk-and-tails structure including axial structures such as spinal cord, notochord and somites (Ariizumi et al., 1995) (Fig. 1). These results show that an animal cap treated with activin acquires organizer-like activity. We found actually that one of the organizer-related genes, newt homolog of chordin (*Cychd*), was expressed in both the organizer region of embryos and head-inducible animal caps treated with activin (Yokota et al., 1998).

Maxillofacial cartilage formation *in vitro*

Based on the induction method for the head-like structure from animal caps using activin, we tried to establish the procedure for inducing maxillofacial cartilage at a high rate. When *Xenopus* animal caps were treated with

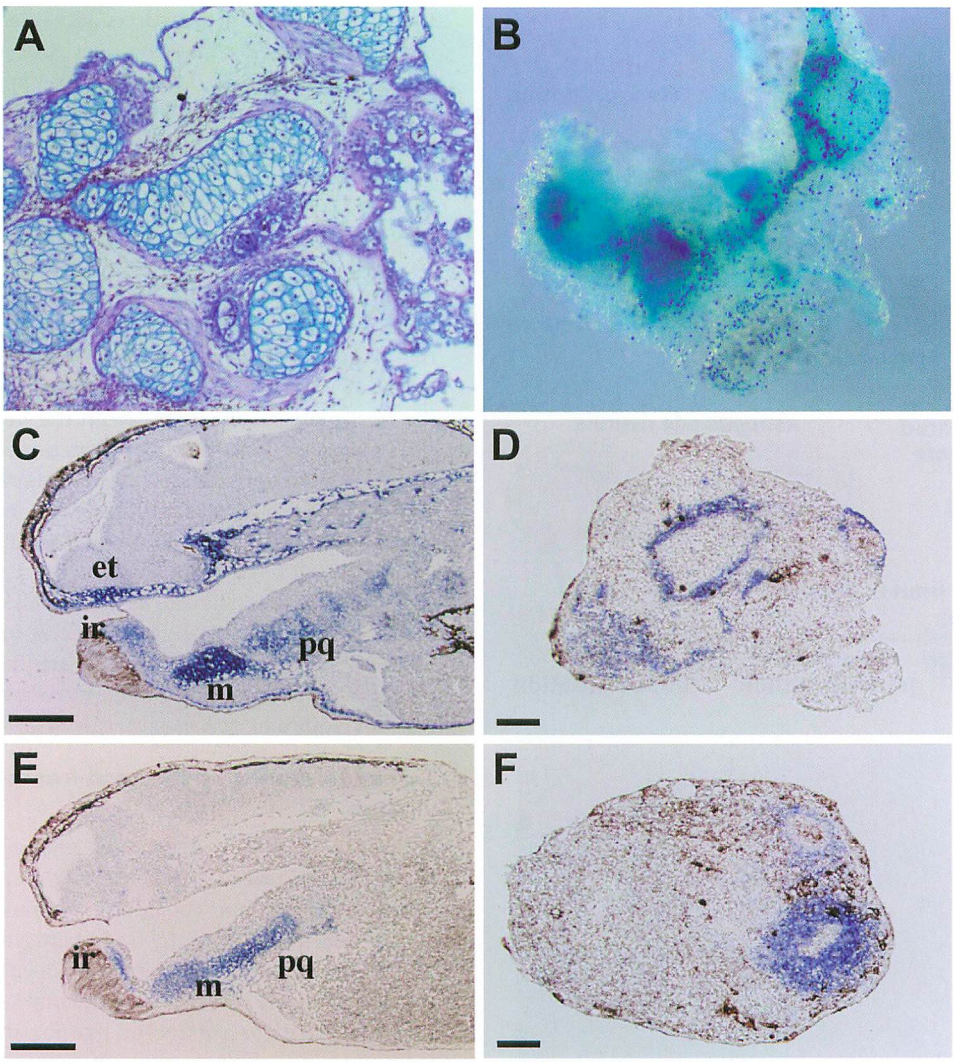

Fig. 2. *In vitro* induction of maxillofacial cartilage in animal caps.
(A) Histological section of the animal cap-derived explant differentiated into cartilage at 7-days after the treatment with activin (dissociation-reaggregation method). PAS/Alcian blue staining. (B) Whole-mount alcian blue staining of the explant treated the same condition as (A). (C,D) The expression pattern of *Collagen type II* mRNA. (C), Sagittal section in the cephalic region of the normal *Xenopus* tadpole (Stage 40). (D), Section of the explant at 4-days after the treatment. (E,F) The expression pattern of *goosecoid* mRNA. (E), Sagittal section in the cephalic region of the normal *Xenopus* tadpole (Stage 40). (F), Section of the explant at 4-days after the treatment. Abbreviations, et: ethmoid-trabecular cartilage, ir: infrarostal cartilage, m: Meckel's cartilage, pq: palatoquadrate cartilage. Scale bar: 100 μm.

activin 100 ng/mL for 1 hr, precultured for 1hr and sandwiched between untreated animal caps, formation of the chondrocytes were the most frequently observed in the explant after the 7-days culture (Fig. 2A) (Furue *et al.*, 2002). For maxillofacial cartilage induction, the method using dissociated animal cap cells were also developed as follows; dissociated animal cap cells were treated with 25 ng/mL of activin for 1hr, and mixed with untreated cells in a ratio of 1:5 to form an aggregate, then cultured for 7days as an explant (Myoishi *et al.*, 2004) (Fig. 2B). *Col*2 and *Cart*-1, the marker genes expressed in the process of cartilage differentiation, were expressed in these explants in a similar way to the normal development of maxillofacial cartilage. *Goosecoid* and *X-dll*4, the marker of cephalic ventral mesenchyme and anterior ectoderm, were also expressed in these explants (Fig. 2C-F). Ectopic tooth germ-like structures were observed in the host embryo by the transplantation of the explant made by dissociation-reaggregation method into the abdominal region of the embryo (Myoishi *et al.*, 2004). These results strongly suggest that the maxillofacial cartilage was induced in *Xenopus* animal cap explant by activin treatment.

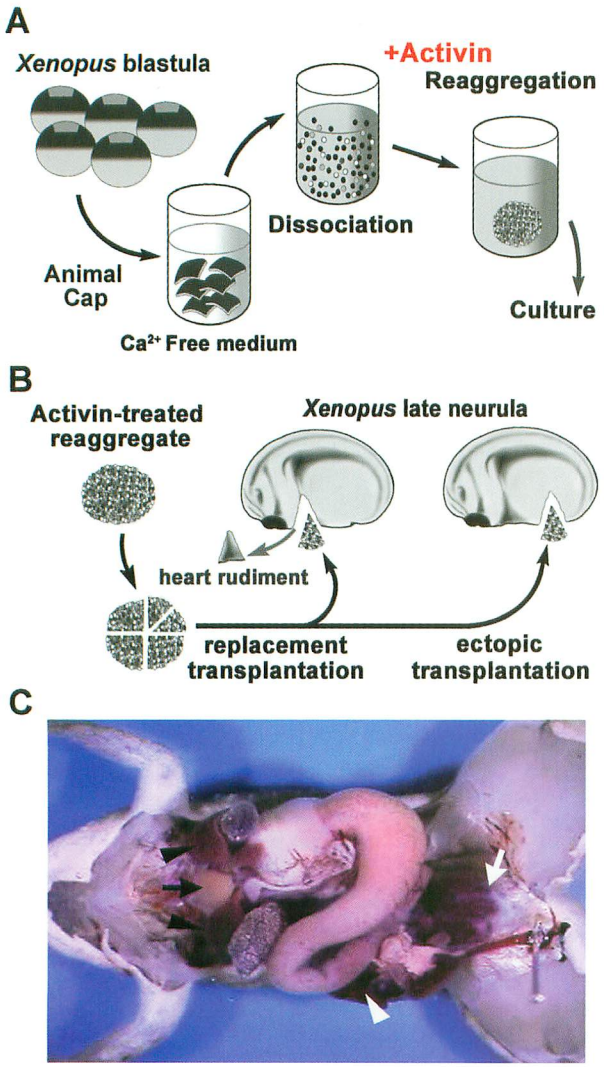

Fig. 3. *In vitro* induction of heart rudiment in animal caps. (A) The schematic diagram of the method to induce cardiac tissues in animal cap cells of *Xenopus*. (B) The schematic diagram of the transplantation experiment. (C) An 1-year-old frog with a well-developed ectopic heart that was caused by ectopically transplantation of the activin-treated reaggregate into the abdominal region at the late neurula stage. (black arrow; original heart, white arrow; ectopic heart, black arrowhead; original liver, white arrowhead; ectopic liver)

Heart formation *in vitro* and transplantation in embryo

We found the *in vitro* induction method to induce a heart-like structure in a newt animal cap. When the newt animal caps was treated with 100 ng/mL of activin, the explants differentiate into anterior endoderm, but about 20%-30% of explants differentiate into the beating heart-like structures (Ariizumi et al., 1996). These explants were anti-α-sarcomeric actin positive, and the characteristic microstructures of myocardium, Z band and intercalated discs, were also observed on electron microscopy. In our recent study, we succeed to establish the optimal induction method of beating heart using *Xenopus* animal cap cells as follows; 5 animal caps (approx. 1,000 cells total) were dissociated and treated with 100ng/mL of activin for 5 hr, then reaggregated and cultured for 3days as an explant (Fig. 3A). The expression of *XGATA-4*, the early marker for heart and anterior endoderm, was prolonged in this dissociation-reaggregation explant as compared to the intact animal cap treated with the same concentration of activin. The expression of *XNkx2.5*, the early heart-field marker, also increased in this explant. When the original heart primordium of the host embryo was replaced with this explant ("replacement transplantation"), the host embryo developed with the heart derived from donor explant, and the heart functioned normally (Fig. 3B).. When the explant was transplanted into the abdomen region of the host embryo ("ectopic transplantation"), the host embryo developed into the frog with two hearts derived from original heart primordium and donor explant (Ariizumi et al., 2003) (Fig. 3C). These results suggest that these dissociation-reaggregation methods to induce the beating heart-like structures may reproduce the induction of heart primordium in the normal development of *Xenopus*.

Kidney formation *in vitro* and transplantation in embryo

Retinoic acid (RA) is distributed endogenously in a concentration gradient along the antero-posterior axis in *Xenopus* embryo, and considered as one of the candidates for the determinants of the body patterning in embryonic development. In *Xenopus* animal cap assay, RA has the activity to affect mesoderm induction and to modify its fate to lateral or posterior property. We found the method to induce pronephros formation *in vitro* effectively in animal caps using the combination of RA and activin as inducers. By simultaneous treatment with 10 ng/mL of activin and 10^{-4} M of RA, pronephric tubules were formed in animal cap explants at a high frequency (Moriya et al., 1993) (Fig. 4A). The pronephros-specific marker genes were also expressed in these explants as well as the normal embryo (Uochi et al., 1996). Immunohistological staining and histological examination using electron microscopy revealed that these

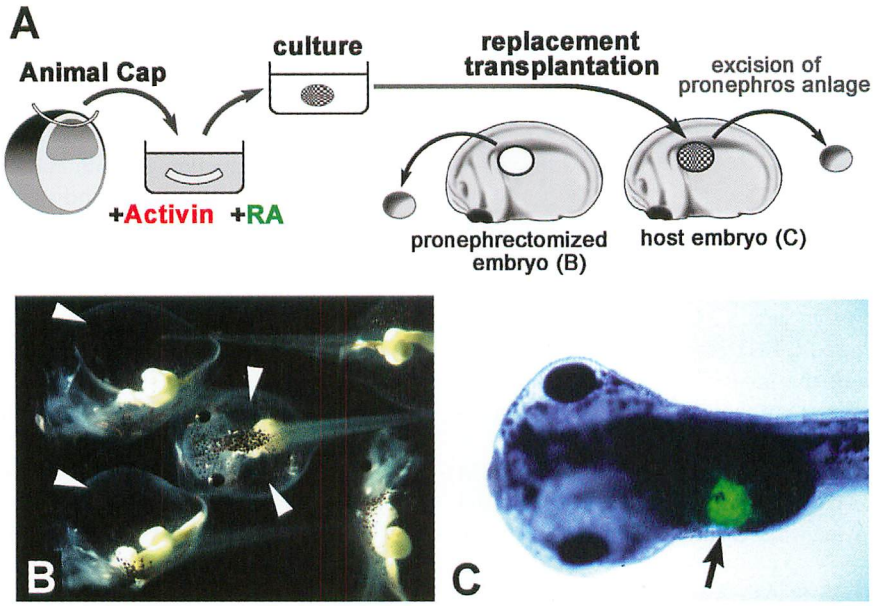

Fig. 4. In vitro induction of pronephric tubules in animal caps.
(A) The schematic diagram of the induction method of pronephros in animal caps and transplantation experiment into the late neurula of *Xenopus*. (B) Embryos from which the pronephric primordium had been excised were unable to eliminate water and showed clear edemas (arrowheads). (C) An embryo transplanted with a pronephric explant showed normal development. Green fluorescent dye previously introduced into the explant revealed that it had been incorporated into the pronephric area (arrow).

explants contained all the three components of a nephron, glomeruli, pronephric tubules, and the pronephric duct (Osafune et al., 2002). When *Xlim-1*, the essential gene for the differentiation of pronephros in normal development, was inhibited in animal cap cells, pronephros formation was suppressed in the animal caps treated with acticvin and RA. These results indicate the method for pronephric induction in animal caps may replicates well the normal kidney development in *Xenopus*. By the replacement transplantation of the presumptive pronephros region of *Xenopus* embryo with the explant treated with activin and RA, we showed that the donor explant was integrated into the host embryo, and the normally functional pronephros was formed (Chan et al., 1999) (Fig. 4A-C). We investigated the detailed expression of gene markers for pronephric development in the pronephric structure formed in the explant, and found that the timing of their expression was the same as that in normal development.

Pancreas formation *in vitro*

By the modification of the timing and the length of the treatment with activin and RA, we established the method to differentiate animal cap cells into pancreatic tissue as follows; animal cap was treated with 100 ng/mL of activin and cultured for 5 hours and then treated with 10^{-4} M RA (Moriya et al., 2000). The induction mechanism of the pancreatic tissue in this method can be explain that anterior endodermal tissue induced by high concentration of activin was posteriolized by RA, and differentiated into pancreas. This explant contained normal pancreatic structure including both exocrine regions and endocrine regions. Glucagon and insulin were also detected by immunohistological staining.

DISCUSSION

As mentioned above, we established the artificial induction methods for various tissues and organs using amphibian animal cap cells. These are simple, stable and reproducible methods for *in vitro* organogenesis, and useful tools to investigate the mechanisms of various organ formations in normal embryonic development of vertebrate. We showed these *in vitro*-induced tissues and organs are nearly the same as normal ones of the embryo from histological and molecular biological standpoints. Using the differential screening with the cDNA library constructed from these explants induced by activin and/or RA, we actually isolated many novel specific genes for organ formation and body patterning (Uochi et al., 1997; Uochi et al., 1998; Eisaki et al., 2000; Satow et al., 2002; Sogame et al., 2003; Nitta et al., 2004; Li et al., 2005), and found one of these were essential for the normal kidney development in mice (Onuma et al., 1999; Nishinakamura et al., 2001). By the improvement of these methods and detailed analysis of induction mechanisms in the explants, we are trying the application of these methods into pluripotent cell lines of mammal to contribute to regenerative medicine.

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