CLONING AND STEM CELLS
Volume 8, Number 3, 2006

Brief Communication

In Vitro Development of Cloned Embryos Derived from Miniature Pig Somatic Cells after Activation by Ultrasound Stimulation

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ABSTRACT

The present study was carried out to examine the activation and development of cloned embryos produced by transferring miniature pig somatic cells into enucleated farm pig oocytes after exposing to ultrasound. The rates of the pronucleus-like structure formation and polar body-like structure extrusion in embryos exposed to ultrasound did not differ from those applied electric pulses. Although there was no significant difference in the blastocyst formation rates between different activation methods, the mean number of cells in the blastocysts developed from embryos activated by exposing to ultrasound was significantly ($p < 0.05$) higher than that obtained by applying electric pulses. The results of the present study showed that ultrasound stimulation can induce the activation and *in vitro* development of cloned embryos derived from miniature pig somatic cells.

INTRODUCTION

THE SUCCESSFUL DEVELOPMENT of cloned embryos produced using nuclear transfer depends on artificial stimulation-induced activation (Robl et al., 1992). Electric stimulation is the most common for activation of embryos in somatic cell nuclear transfer studies that have succeeded in producing cloned piglets (Onishi et al., 2000; Polejaeva et al., 2000; Bondioli et al., 2001; Park et al., 2001, 2002; Boquest et al., 2002; Dai et al., 2002; De Sousa et al., 2002; Lai et al., 2002a,b). However, the efficiency of cloning, when measured as development to offspring as a proportion of embryos transferred into recipient females, was less than 1% in most of the previous reports and widespread use of this technology in a number of animal agriculture and biomedical applications has been limited. The establishment of an effective activation protocol instead of electric stimulation would bring about improvement of pig cloning efficiency.

Recently, we have shown that ultrasound stimulation can induce the nuclear activation and parthenogenetic development of pig oocytes matured *in vitro* (Sato et al., 2005). Because blastocysts developed from oocytes exposed to ultrasound contained more cells compared with those applied electric pulses, ultrasound is considered to be effective artificial stimulation for activation of pig oocytes. Therefore, we hypothesized that this protocol would be effective to activate pig cloned embryos produced by somatic cell nuclear transfer.

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By combining the technology for homologous recombination in somatic cells with that of somatic cell nuclear transfer, it is possible to create specific modifications to the mammalian genome (McCreath et al., 2000). Pigs have been used in biomedical applications as a model for human disease processes, as a genetically defined model for surgery and transplantation, and as a source of human therapeutics (Prather et al., 2003). All of these uses could be enhanced by the ability to specifically modify the pig genome. One of the major applications is the production of pigs in which the α-1,3-galactosyl transferase (α-1,3-GT) locus is knocked out (Dai et al., 2002; Lai et al., 2002a). This enzyme is responsible for adding the xenogenetic sugar, galactose α-1,3-galactose, to the surface of pig cells. Therefore, α-1,3-GT knockout pigs are considered to be a donor of cells, tissues, and organs for transplantation into humans. Clawn miniature pigs were established at Kagoshima University and weigh 50–80 kg at 2-years-old, indicating that they have organs with the same size as those of humans. This means that α-1,3-GT knockout Clawn miniature pigs are useful alternative animals as a source of organs for xenotransplantation. To produce these animals, the establishment of an effective production system of cloned Clawn miniature pigs by somatic cell nuclear transfer is essential.

In the present study, therefore, somatic cells derived from a Clawn miniature pig were used as donor cells for production of cloned embryos, and it was examined whether the cloned embryos can be activated and develop into blastocysts following treatment with ultrasound stimulation.

METHODS

Donor cells

Donor cells were harvested from an ear skin biopsy that was obtained from a 2-year-old female Clawn miniature pig (Japan Farm Co., Kagoshima, Japan). The cells were cultured in DMEM/F-12 medium (Gibco BRL, Grand Island, NY) supplemented with 10% (v/v) fetal calf serum (FCS) under 5% CO_2 in air at 37°C. After reaching confluence, cells were passaged. Passage 2 cells were trypsinized, suspended in the culture medium with 10% (v/v) dimethyl sulfoxide (Sigma Chemical Co., St. Louis, MO), and stored as frozen aliquots. The cells were used as donors for nuclear transfer at passages 3–5 of culture. The cells were allowed to grow to confluency and continued to culture for an additional 5–6 days without a change of medium. A single cell suspension was prepared by standard trypsinization immediately prior to nuclear transfer.

Recipient oocytes

Ovaries were collected from prepubertal crossbred gilts (Landrace × Large White × Duroc) at a local slaughterhouse and transported to the laboratory in saline at 36–38°C. Oocytes were aspirated from antral follicles (2–5 mm in diameter) with an 18-gauge needle fixed to a 5-mL syringe. The follicular contents were pooled in test tubes and maintained at 38.5°C. Oocytes that had an evenly granulated cytoplasm and were surrounded by at least three uniform layers of compact cumulus cells were recovered from the collected fluid. About 50 oocytes were transferred to 200 μL of TCM-199 (Gibco) supplemented with 3.05 mM D-glucose, 0.91 mM Na pyruvate, 0.57 mM L-cysteine, 10 ng/mL epidermal growth factor (Sigma), 10 IU/mL eCG (Teikoku-Zoki Co., Tokyo, Japan), 10 IU/mL hCG (Teikoku-Zoki), 0.1 mg/mL amikacin sulfate (Meiji Seika Co., Tokyo, Japan), and 0.1% (w/v) polyvinylalcohol (Abeydeera et al., 1998) that had been previously covered with paraffin oil (Nacalai Tesque Inc., Kyoto, Japan) and equilibrated in an atmosphere of 5% CO_2 in air at 38.5°C for 3–5 h. After culture of oocytes for 38–42 h under 5% CO_2 in air at 38.5°C, cumulus cells were removed by vortexing with 0.1% (w/v) hyaluronidase (Sigma). Oocytes with a polar body, indicating successful nuclear maturation, were selected for the experiments.

Nuclear transfer

In vitro–matured and denuded oocytes were transferred into HEPES-buffered TCM-199 with the osmolarity adjusted to 300 mOsm by adding sucrose supplemented with 7.5 μg/mL cytochalasin B and 10% FCS. The oocytes were enucleated by removing the first polar body and the metaphase II plate in a small amount (approximately 20% of total volume) of surrounding cytoplasm with a 15-μm-inner-diameter glass pipette. In the preliminary experiment, 62 of 67 (92.5%) oocytes were successfully enucleated with this system. A single donor cell was inserted into the perivitelline space of each enucleated

oocyte using the same glass pipette. Cell-oocyte complexes were transferred to porcine zygote medium-3 (Yoshioka et al., 2002) with a modification in which 0.05 mg/mL gentamicin was replaced with 0.1 mg/mL amikacin sulfate (mPZM-3) and kept in a CO_2 incubator adjusted to 5% CO_2 in air at 38.5°C until fusion.

The chamber for fusion was a 60-mm dish filled with 7 mL of fusion medium composed of 250.3 mM sorbitol, 0.5 mM $Mg(CH_3COO)_2$, 0.3 mM HEPES, and 0.2% (w/v) BSA. Two stainless-steel wires (100-μm-diameter) were used as electrodes, and they were attached to micromanipulators. The single cell-oocyte complex was sandwiched between the electrodes, and oriented with the contact surface between the cytoplast and the donor cell perpendicular to the electrodes. Membrane fusion was induced by applying a single direct-current pulse of 25 V for a duration of 20 μsec with a prepulse of alternating-current field of 5 V, 1 MHz for 2 sec using an LF 101 Fusion Machine (Nepa Gene Co., Chiba, Japan). Following the fusion pulse, the complexes were cultured for a period of 2 h in 100 μL of mPZM-3. Fusion was determined by microscopic examination at 1 h after applying the pulse.

Activation and culture of embryos

At 2 h after fusion, fused embryos were activated by electric or ultrasound stimulation. For electric activation, embryos were washed twice in activation medium composed of 250.3 mM sorbitol, 0.1 mM $Ca(CH_3COO)_2$, 0.5 mM $Mg(CH_3COO)_2$, and 0.1% BSA and then placed between two wire electrodes (1 mm apart) of the chamber slide with 15 mL of the medium. Direct-current pulses of 100 V/mm were applied twice to the embryos for a duration of 50 μsec at intervals of 30 min using an LF 101 Fusion Machine. For ultrasound activation, embryos were washed twice in the activation medium and then transferred to a well of a four-well plate (Nunc, Roskilde, Denmark) containing 700 μL of the medium. The ultrasound probe (0.5 mm in diameter) of a Sonitron 1000 (Rich Mar Inc., Inola, OK) was inserted directly into the activation medium and the embryos were exposed to 1.0–MHz ultrasound at an intensity of 2.0 W/cm^2 for 30 sec with 10% duty cycle. A miniature stirrer was placed within the well and spun at 300 rpm during ultrasound exposure. These protocols had been optimized and routinely used for activation of oocytes and/or cloned embryos in our laboratory (Miyoshi et al., 2005; Sato et al., 2005). After each treatment, the embryos were transferred into 100 μL of mPZM-3 supplemented with 2.2 μg/mL cytochalasin B to prevent extrusion of a polar body–like structure. After incubation for 2 h under 5% CO_2, 5% O_2, and 90% N_2 at 38.5°C, the embryos were transferred into 100 μL of mPZM-3 without cytochalasin B and continued to culture. At 12–14 h after activation, some embryos were mounted, fixed for 48–72 h in 25% (v/v) acetic acid in ethanol at room temperature, stained with 1% (w/v) orcein in 45% (v/v) acetic acid, and examined for polar body–like structure extrusion and pronucleus-like structure formation under a Nomarski differential interference microscope (Olympus Co., Tokyo, Japan). The embryos with a pronucleus-like structure were regarded as activated embryos. The remainder were assessed for cleavage and blastocyst formation at 2 and 7 days of culture, respectively. At the end of the culture period, nuclei were counted in blastocyst stage embryos after staining with Hoechst. The blastocysts were placed on slides with a drop of mounting medium consisting of glycerol and PBS (9:1) containing 0.1 mg/mL Hoechst 33342 (Sigma). A cover slip was placed on top of the blastocysts, and the edge was sealed with nail polish. The number of nuclei was counted under ultraviolet light.

Statistical analysis

All percentage data were subjected to an arcsin transformation in each replicate. The transformed values and the numbers of cells in blastocysts were analyzed by using one-way analysis of variance (ANOVA), followed by Fisher's protected least significant difference test. A probability of $p < 0.05$ was considered statistically significant.

RESULTS

The rates of activated embryos (90.0–92.3%) were not affected by different activation methods (Table 1). There was no significant difference in the rates of activated embryos without a polar body–like structure (91.7–95.6%) between different methods.

There was no significant difference in the rates of cleaved embryos (26.3–36.3%) between differ-

TABLE 1. ACTIVATION STATUS IN MINIATURE PIG CLONED EMBRYOS APPLIED ELECTRIC PULSES OR EXPOSED TO ULTRASOUND[a]

Method	No. of embryos examined	No. (%)[b] of embryos activated	No. (%)[c] of activated embryos					
			Without PB				With 1PB	
			1PN	2PN	3PN≤	Total	1PN	2PN
Electric pulses	50	45 (90.0)	22 (48.9)	17 (37.8)	4 (8.9)	43 (95.6)	1 (2.2)	1 (2.2)
Ultrasound	52	48 (92.3)	24 (50.0)	17 (35.4)	3 (6.3)	44 (91.7)	4 (8.3)	0 (0)

[a]Experiments were repeated three times.
[b]Percentage per embryos examined.
[c]Percentage per embryos activated.
PB, polar body-like structure; PN, pronucleus-like structure.

ent activation methods (Table 2). Similarly, the rates of embryos (16.2–18.7%) developed to the blastocyst stage (Fig. 1) were not affected by different activation methods. When embryos were activated by exposing to ultrasound, however, the mean number of cells in the blastocysts (59.1 cells) was significantly ($p < 0.05$) higher than that (43.3 cells) obtained by applying electric pulses (Fig. 2).

DISCUSSION

The results of the present study show that ultrasound stimulation can induce the activation and *in vitro* development of cloned embryos derived from miniature pig somatic cells. Because blastocysts developed from embryos exposed to ultrasound contained more cells compared with those with applied electric pulses, ultrasound is considered to be effective artificial stimulation for activation of miniature pig cloned embryos.

When donor cells are transferred into metaphase II recipient oocytes that have high maturation/meiosis/mitosis-promoting factor activity, the membrane of the donor nucleus is broken and the chromosomes are prematurely condensed. After successful activation, the membrane reforms and the pronucleus-like structure appears (Campbell et al., 1993). In the present study, the pronucleus-like structures were observed in most of the miniature pig cloned embryos exposed to ultrasound, and their formation rate did not differ from that of embryos with applied electric pulses. These results indicate that ultrasound stimulation can induce the activation of cloned embryos as well as standard electric stimulation. In addition, few cloned embryos extruded a polar body–like structure after exposure to ultrasound, suggesting that most of them have a diploid nucleus, like the electric-activated embryos (Wakayama et al., 1998).

It has been known since the 1980s that ultrasound can enhance passage of genes across the membrane of mammalian cells (Fechheimer et al., 1987; Kim et al., 1996; Wasan et al., 1996; Newman et al., 2001). Although the exact mechanisms that cause this phenomenon are still unclear, ultrasound is thought to permeabilize cell membranes, allowing passive diffusion of plasmid into

TABLE 2. EFFECT OF ACTIVATION METHODS ON THE DEVELOPMENT OF MINIATURE PIG CLONED EMBRYOS[a]

Method	No. of embryos cultured	No. (%)[b] of embryos developed to		Mean no. ± SEM of cells in blastocysts
		≥2-Cell (2)[c]	Blastocyst (7)[c]	
Electric pulses	99	26 (26.3)	16 (16.2)	43.3 ± 2.4[d]
Ultrasound	91	33 (36.3)	17 (18.7)	59.1 ± 3.9[e]

[a]Experiments were repeated three times.
[b]Percentage per embryos cultured.
[c]Numbers in parentheses indicate the time of examination (days of culture).
[d,e]Values with different superscripts are significantly different ($p < 0.05$).

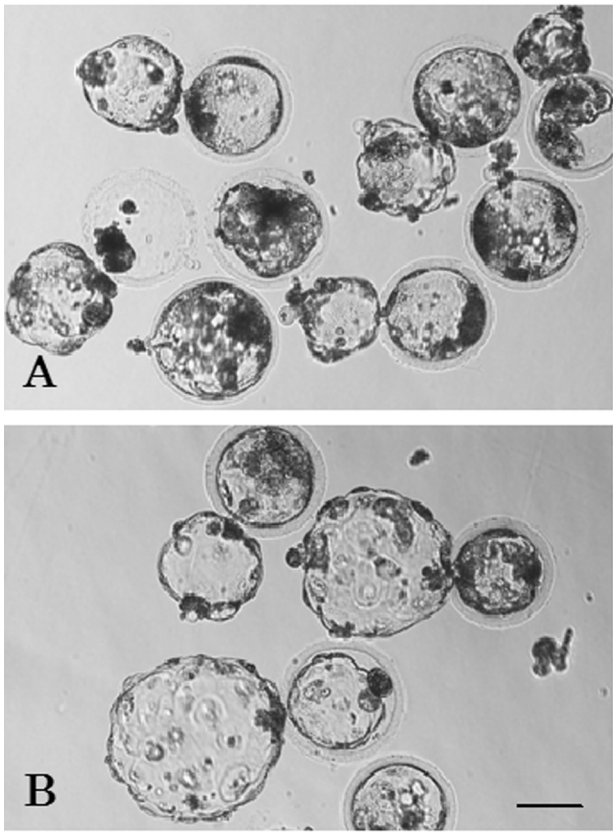

FIG. 1. Blastocysts developed from cloned embryos reconstituted with miniature pig somatic cells and activated by applying electric pulses (**A**) or exposing to ultrasound (**B**). Scale bar = 100 μm.

the cells (Kim et al., 1996). Ultrasound energy can initiate inertial cavitation when the acoustic pressures are high enough to fracture the liquid or grow bubble nuclei. The resulting microbubbles oscillate violently a few times in response to the ultrasound before being destroyed. The associated pressures are very high (Brayman and Miller, 1992) and can certainly breach cell membranes. Electric stimulation of oocytes results in the formation of pores in the plasma membrane, which facilitate the uptake of extracellular calcium by the oocytes (Onodera and Tsunoda, 1989; Ozil, 1990; Collas et al., 1993). This influx of calcium leads to an increase in intracellular calcium concentration and oocyte activation (Sun et al., 1992). On the basis of these reports, it is suggested that miniature pig cloned embryos are activated by an influx of extracellular calcium via ultrasound-produced breaches in the plasma membrane.

Incubation *in vitro* has a negative effect on the development of pig embryos, and blastocysts developed *in vitro* contain fewer number of cells compared with those *in vivo* (Macháty et al., 1998). Therefore, one approach to evaluate the quality of blastocysts obtained *in vitro* is to count the number of cells in them. In the present study, the blastocyst formation rates of activated embryos were not different between conditions of ultrasound exposure and applied electric pulses. However, blastocysts developed from embryos exposed to ultrasound contained more cells than those exposed to electric pulses. Relatively low levels of ultrasound (lower than a level that should cause biologic damage) appear to be sufficient to facilitate passage of molecules across the membrane of cultured cells because such levels of ultrasound enhanced gene expression from liposomal transfection (Unger et al., 1997). Therefore, it is suggested that ultrasound might reduce the damage of embryos during activation.

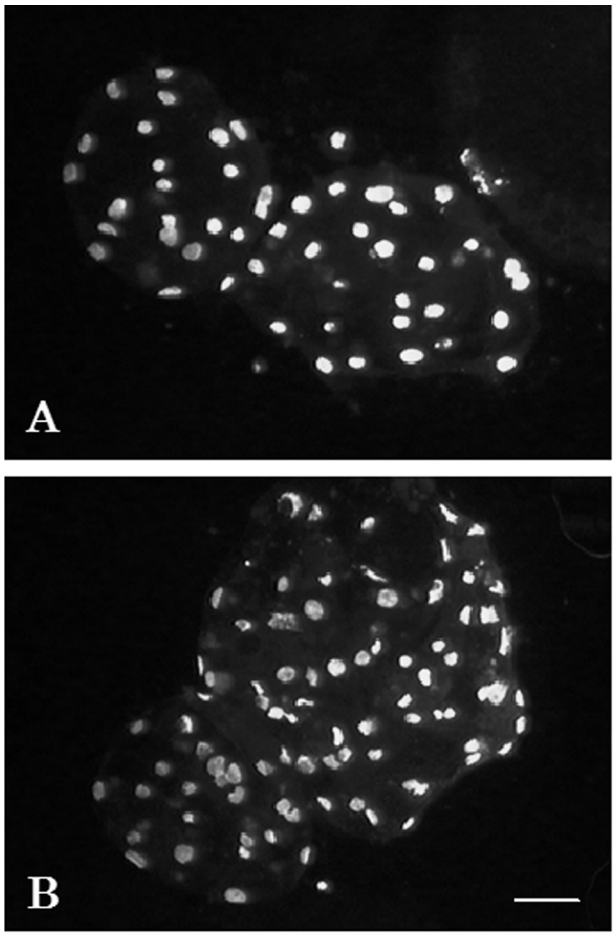

FIG. 2. Blastocysts developed from cloned embryos reconstituted with miniature pig somatic cells that were activated by applying electric pulses (**A**) or exposing to ultrasound (**B**) and stained with Hoechst 33342. Scale bar = 50 μm.

An additional advantage of the activation protocol by ultrasound may be to treat more embryos at once compared to that by electric pulses. When cloned embryos are activated by applying electric pulses, the number of embryos treated at once is limited to 20–30 because they must be placed in a line between two wire electrodes. Therefore, embryos were transferred to a well of a four-well plate in groups of 16–33 and exposed to ultrasound in the present study. However, we verified that the blastocyst formation rate and mean number of cells in the blastocysts of oocytes exposed to ultrasound in groups of 90 do not differ from those exposed in groups of 30 (Miyoshi et al., unpublished data), suggesting that more embryos can be activated at once. This advantage is valuable in somatic cell nuclear transfer studies in which we have to activate many cloned embryos.

In conclusion, we have established a novel activation protocol using ultrasound for cloned embryos produced by somatic cell nuclear transfer.

ACKNOWLEDGMENTS

We express gratitude to the staff of the Kagoshima Meat Inspection Office and Meat Center Kagoshima, Inc., Kagoshima, for supplying pig ovaries. The present study was supported by a Grant-in-Aid for Scientific Research to K.M. (no. 16688008) from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and a Research Grant for Cardiovascular Diseases to M.Y. (no. 15A-1) from the Ministry of Health, Labour, and Welfare of Japan.

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