

—Technology Report—

## Enhancement of Cytoplasmic Maturation of *In Vitro*-Matured Pig Oocytes by Mechanical Vibration

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**Abstract.** The effects of mechanical vibration during *in vitro* maturation and/or *in vitro* culture after artificial activation of pig oocytes on maturation and development were examined. In addition, the optimal conditions were applied to *in vitro* production of blastocysts derived from miniature pig somatic cell nuclear transfer (SCNT) embryos. Mechanical vibration during *in vitro* maturation did not affect the rates ( $60.5 \pm 1.9$ – $69.5 \pm 2.2\%$ ) of oocytes reaching the metaphase-II stage. However, the blastocyst formation rates after activation of oocytes matured with mechanical vibration for 5 sec at intervals of 30–60 min or for 10 sec at intervals of 60 min were significantly ( $P < 0.05$ ) higher than those of oocytes matured without mechanical vibration ( $25.7 \pm 2.0$ – $28.1 \pm 2.7\%$  vs.  $12.3 \pm 1.4\%$  and  $25.8 \pm 1.8\%$  vs.  $15.7 \pm 1.9\%$ , respectively). In contrast, mechanical vibration during *in vitro* culture after activation did not affect the blastocyst formation ( $11.6 \pm 5.2$ – $16.5 \pm 3.0\%$ ) of oocytes. Mechanical vibration for 5 sec at intervals of 60 min during *in vitro* maturation of oocytes did not affect fusion ( $66.8 \pm 3.5$ – $72.1 \pm 3.1\%$ ) with miniature pig somatic cells after enucleation. However, the blastocyst formation rate of SCNT embryos was improved ( $P < 0.05$ ) by mechanically vibrating recipient oocytes for 5 sec at intervals of 60 min during *in vitro* maturation, regardless of the presence or absence of the same treatment during *in vitro* culture ( $17.6 \pm 2.5\%$  vs.  $9.4 \pm 0.9\%$  and  $13.0 \pm 0.3\%$  vs.  $7.4 \pm 0.9\%$ , respectively). The results indicated that mechanical vibration enhances the cytoplasmic maturation of *in vitro*-matured pig oocytes, resulting in improvement of their parthenogenetic development. In addition, it was shown that *in vitro* maturation of oocytes with mechanical vibration can be applied to efficient production of blastocysts derived from miniature pig SCNT embryos.

**Key words:** *In vitro* development, *In vitro* maturation, Mechanical vibration, Nuclear transfer, Parthenogenetic activation

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The low cloning efficiency associated with the development of somatic cell nuclear transfer (SCNT) embryos to offspring remains the major obstacle to use of this technology in various fields of animal science and biomedical applications. Establishment of systems to support efficient production of blastocysts from SCNT embryos *in vitro* is quite important for basic research to clarify the mechanism controlling the development of SCNT embryos, which will bring about improvement of cloning efficiency. In cattle and pigs, *in vitro* oocyte maturation systems produce an abundant and stable supply of recipient oocytes for SCNT because immature oocytes can be obtained from slaughtered animals. *In vitro*-matured oocytes have been commonly used for production of cloned calves [1–3] and pigs [4–8]. The oocyte maturation process is a crucial step for the generation of oocytes capable of being fertilized and undergoing normal embryonic development into blastocysts after *in vitro* fertilization [9]. Therefore, efficient production of blastocysts from SCNT embryos requires optimization of both recipient oocyte maturation systems and SCNT embryo culture systems.

Oocyte maturation and early embryonic development after fertilization take place in the ovaries, oviducts and uteri of live females, which continually move around. In addition, oocytes and embryos

are transported in the oviduct by ciliary beating of oviductal epithelial cells and contraction of oviductal smooth muscle [10–12]. For *in vitro* maturation and *in vitro* culture, however, oocytes and embryos are transferred into microdroplets of a medium under paraffin oil in dishes, and then the dishes are kept static in an incubator with a controlled temperature and atmosphere for adequate durations [13]. Therefore, we hypothesized that mechanical vibration of dishes containing oocytes and embryos during *in vitro* maturation and *in vitro* culture might improve the production efficiency of blastocysts from SCNT embryos. To test this hypothesis, in the present study, the effects of mechanical vibration during *in vitro* maturation and/or *in vitro* culture after artificial activation of pig oocytes on their maturation and development were examined. In addition, the optimal conditions were applied to *in vitro* production of blastocysts derived from miniature pig SCNT embryos.

### Materials and Methods

#### *In vitro* maturation of oocytes

Ovaries were collected from prepubertal gilts at a local slaughterhouse and transported to the laboratory in saline at 32–35 C. The follicular contents were recovered by aspiration from follicles (2–5 mm in diameter) using an 18-gauge needle and a 5-ml disposable syringe. The cumulus-oocyte complexes (COCs) were gathered from the follicular contents and washed twice with HEPES (Nacalai Tesque, Kyoto, Japan)-buffered Tyrode-lactate-pyruvate-polyvinyl alcohol (PVA; Sigma-Aldrich Chemical, St. Louis, MO,

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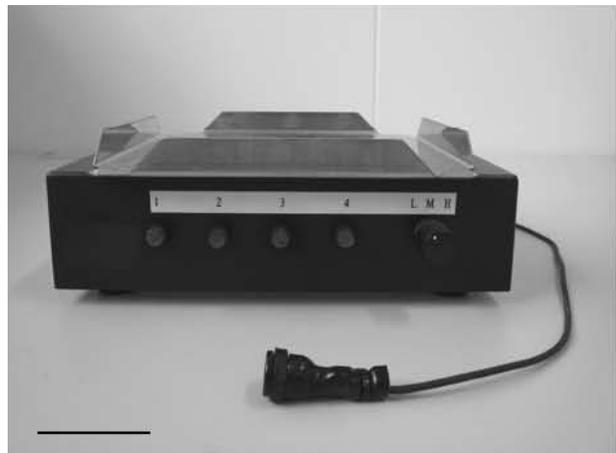
USA) and maturation medium, respectively. The maturation medium consisted of 90% (v/v) TCM-199 with Earle's salts (Gibco BRL, Grand Island, NY, USA) supplemented with 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 0.57 mM cysteine hydrochloride hydrate, 10 ng/ml epidermal growth factor, 10 IU/ml eCG (Aska Pharmaceutical, Tokyo, Japan), 10 IU/ml hCG (Aska), 100  $\mu$ g/ml amikacin sulfate (Meiji Seika, Tokyo, Japan), 0.1% (w/v) PVA and 10% (v/v) pig follicular fluid. Only COCs possessing a compact cumulus mass and evenly granulated ooplasm were selected. COCs in groups of 40–50 were transferred to a droplet of the maturation medium (200  $\mu$ l) under paraffin oil (Nacalai Tesque) in a 35-mm polystyrene dish (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and cultured at 38.5 C in an atmosphere of 5% CO<sub>2</sub> in air. During culture, mechanical vibrations at a frequency of 20 Hz and accelerations of  $\pm 0.33$  G and  $\pm 0.11$  G in the x-axis and y-axis directions, respectively, were applied to the COCs for different durations at different intervals using an MS-1000 (Fig. 1; Nepa Gene, Chiba, Japan). After 40–42 h of culture, cumulus cells were removed by pipetting in HEPES-buffered Tyrode-lactate-pyruvate-PVA supplemented with 0.1% (w/v) hyaluronidase. Some oocytes 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 in ethanol and examined for meiotic progression under a Nomarski differential interference microscope. Oocytes with a polar body were selected from the remainder for the experiments.

#### Donor cells

Fetal fibroblasts were obtained from a female Clawn miniature pig fetus on Day 33 of pregnancy. Body tissues were cut into small pieces and cultured in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Gibco) supplemented with 100  $\mu$ g/ml amikacin sulfate and 10% (v/v) fetal calf serum (FCS; Equitech-Bio, Kerrville, TX, USA) under 5% CO<sub>2</sub> in air at 37 C. After reaching confluence, cells were passaged. Cells at passage 8 were trypsinized, suspended in Cell Banker 1 (Wako Pure Chemical Industries, Osaka, Japan) and stored as frozen aliquots. After thawing, the cells were cultured in HEPES-buffered TCM-199 with Earle's salts (Gibco) supplemented with 100  $\mu$ g/ml amikacin sulfate and 10% FCS and used as donors for nuclear transfer at passages 9 and 10 of culture. The cells were allowed to grow to confluency, and culture was continued 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.

#### Nuclear transfer

The osmolarity of HEPES-buffered TCM-199 supplemented with 10% FCS was adjusted to 300 mOsm by adding 20 mM sucrose. This medium was used as the basic medium (BM) for nuclear transfer. *In vitro*-matured and denuded oocytes were cultured in 100  $\mu$ l of BM supplemented with 0.2  $\mu$ g/ml demecolcine (Sigma) and 20 mM sucrose for 0.5–1 h [14]. Oocytes with a protruding cytoplasm were transferred into BM supplemented with 0.2  $\mu$ g/ml demecolcine and 5  $\mu$ g/ml cytochalasin B. The protruding part of the ooplasm and the first polar body were removed by aspi-



**Fig. 1.** An MS-1000 used for applying mechanical vibrations. Dishes containing cumulus-oocyte complexes, oocytes or somatic cell nuclear transfer embryos were set on the machine and vibrated in an incubator. Scale bar=5 cm.

ration with a 15- $\mu$ m inner diameter glass pipette. 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 BM and kept in 5% CO<sub>2</sub> 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<sub>3</sub>COO)<sub>2</sub>, 0.3 mM HEPES and 0.2% (w/v) BSA. Two stainless steel wires (100  $\mu$ m in diameter) were used as electrodes and were attached to micromanipulators. The single cell-oocyte complex was sandwiched between the electrodes and oriented with the contact surface between the cytoplasm and 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  $\mu$ sec with a prepulse of a 5-V, 1-MHz alternating current field for 2 sec using an LF 101 Fusion Machine (Nepa Gene). Following the fusion pulse, the complexes were cultured for 2 h in 100  $\mu$ l of modified PZM-3 (mPZM-3) [15] until activation. Success of fusion was determined by microscopic examination at 1 h after applying the pulse.

#### Activation and culture of oocytes and embryos

*In vitro*-matured and denuded oocytes or fused embryos in groups of 30–60 were washed twice in activation medium composed of 250.3 mM sorbitol, 0.5 mM Ca(CH<sub>3</sub>COO)<sub>2</sub>, 0.5 mM Mg(CH<sub>3</sub>COO)<sub>2</sub> and 0.1% BSA [16] and then transferred to a well of a 4-well plate (Nunc, Roskilde, Denmark) containing 800  $\mu$ l of the same medium. The ultrasound probe (8 mm in diameter) of a KTAC-3000 Sonopore (Nepa Gene) was inserted directly into the activation medium, and the oocytes were exposed to 2872-kHz ultrasound at an intensity of 45 V for 30 sec with a 10-Hz burst rate and 30% duty cycle. The embryos were exposed to the same ultrasound except that the intensity and duty cycle were changed to 65 V and 10%, respectively. A miniature stirrer was placed within the well and spun at 300 rpm during ultrasound exposure. After exposure, the oocytes or embryos were transferred into 50  $\mu$ l of mPZM-

**Table 1.** Parthenogenetic development of pig oocytes matured *in vitro* with mechanical vibration for 5 sec at different intervals

Intervals of vibration (min)	No. of oocytes cultured	No. (mean % $\pm$ SEM) of oocytes developed to		Mean no. $\pm$ SEM of cells in blastocysts
		$\geq$ 2-Cell	Blastocyst	
Control	90	39 (43.8 $\pm$ 5.3)	11 (12.3 $\pm$ 1.4) <sup>a</sup>	53.0 $\pm$ 6.1
10	90	48 (54.3 $\pm$ 8.0)	16 (17.8 $\pm$ 0.6) <sup>a,b</sup>	50.6 $\pm$ 3.6
30	90	54 (60.4 $\pm$ 4.6)	23 (25.7 $\pm$ 2.0) <sup>b,c</sup>	50.7 $\pm$ 3.4
60	90	53 (59.1 $\pm$ 4.9)	25 (28.1 $\pm$ 2.7) <sup>c</sup>	50.2 $\pm$ 3.2
90	89	37 (41.8 $\pm$ 4.3)	14 (16.0 $\pm$ 4.6) <sup>a</sup>	43.4 $\pm$ 2.5

<sup>a-c</sup> Values with different superscripts are significantly different (at least  $P < 0.05$ ).

3 supplemented with 2.2  $\mu$ g/ml cytochalasin B to prevent extrusion of a second polar body or polar body-like structure. After incubation for 2 h under 5% CO<sub>2</sub> in air at 38.5 C, the oocytes or embryos were transferred into 50  $\mu$ l of mPZM-3, and culture was continued. During culture, the same mechanical vibrations described above were applied to the oocytes or embryos for 5 sec at different intervals. The oocytes or embryos were assessed for cleavage and blastocyst formation at 2 and 7 days of culture, respectively. At the end of culture, blastocysts were placed on slides with a drop of mounting medium consisting of glycerol and PBS (9:1) containing 100  $\mu$ g/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.

#### Experimental designs

In experiment 1, the effects of intervals of mechanical vibration during *in vitro* maturation on the meiotic progression and parthenogenetic development of oocytes were examined. COCs were cultured with mechanical vibration for 5 sec at intervals of 10, 30, 60 or 90 min. Control COCs were cultured without mechanical vibration. After culture, some oocytes were examined for *in vitro* maturation. The remainder were activated and cultured without mechanical vibration to examine their development.

In experiment 2, the effects of durations of mechanical vibration during *in vitro* maturation on the nuclear maturation and parthenogenetic development of oocytes were examined. COCs were cultured with mechanical vibration for 5, 10 or 30 sec at intervals of 60 min. Control COCs were cultured without mechanical vibration. After culture, some oocytes were examined for meiotic progression. The remainder were activated and cultured without mechanical vibration to examine their development.

In experiment 3, the effects of intervals of mechanical vibration during *in vitro* culture after activation on the parthenogenetic development of oocytes were examined. Oocytes matured without mechanical vibration were activated and cultured with mechanical vibration for 5 sec at intervals of 10, 30, 60, 180, 360 or 720 min. Control oocytes were cultured without mechanical vibration.

In experiment 4, the effects of mechanical vibration during *in vitro* maturation of recipient oocytes and/or *in vitro* culture after reconstruction on the development of SCNT embryos were examined. Oocytes matured with or without mechanical vibration for 5 sec at intervals of 60 min were enucleated and fused with donor cells. Fused embryos were cultured with or without mechanical

vibration for 5 sec at intervals of 60 min.

#### Statistical analysis

Experiments were replicated three times. All percentage data were subjected to an arcsine transformation in each replicate. The transformed values and numbers of cells in blastocysts were analyzed by one-way or two-way ANOVA followed by Fisher's protected least significant difference test. A probability of  $P < 0.05$  was considered statistically significant.

## Results

#### Experiment 1

Mechanical vibration at different intervals did not affect the rates (control, 64.3  $\pm$  1.3%; 10 min, 66.8  $\pm$  0.8%; 30 min, 69.5  $\pm$  2.2%; 60 min, 66.9  $\pm$  3.4%; 90 min, 60.5  $\pm$  1.9%) of oocytes reaching the metaphase-II stage. As shown in Table 1, the cleavage rates (41.8  $\pm$  4.3–60.4  $\pm$  4.6%) of the oocytes were not affected by mechanical vibration at the different intervals. However, the blastocyst formation rates (25.7  $\pm$  2.0–28.1  $\pm$  2.7%) of the oocytes vibrated at intervals of 30–60 min were significantly ( $P < 0.05$ ) higher than those (12.3  $\pm$  1.4–16.0  $\pm$  4.6%) of the oocytes vibrated at intervals of 90 min or matured without mechanical vibration. In addition, the blastocyst formation rate (28.1  $\pm$  2.7%) of the oocytes vibrated at intervals of 60 min was significantly ( $P < 0.05$ ) higher than that (17.8  $\pm$  0.6%) of the oocytes vibrated at intervals of 10 min. The mean numbers of cells (43.4  $\pm$  2.5–53.0  $\pm$  6.1 cells) in the blastocysts were not affected by mechanical vibration at the different intervals.

#### Experiment 2

Mechanical vibration for different durations did not affect the rates (control, 62.9  $\pm$  3.2%; 5 sec, 66.9  $\pm$  3.4%; 10 sec, 65.9  $\pm$  4.4%; 30 sec, 63.4  $\pm$  1.9%) of oocytes reaching the metaphase-II stage. As shown in Table 2, the cleavage rates (47.8  $\pm$  10.7–61.3  $\pm$  6.4%) of the oocytes were not affected by mechanical vibration for the different durations. However, the blastocyst formation rates (25.8  $\pm$  1.8–27.1  $\pm$  3.5%) of the oocytes vibrated for 5–10 sec were significantly ( $P < 0.05$ ) higher than those (9.0  $\pm$  2.0–15.7  $\pm$  1.9%) of the oocytes vibrated for 30 sec or matured without mechanical vibration. The mean numbers of cells (45.3  $\pm$  4.2–53.9  $\pm$  3.5 cells) in the blastocysts were not affected by mechanical vibration for the different durations.

**Table 2.** Parthenogenetic development of pig oocytes matured *in vitro* with mechanical vibration for different durations at intervals of 60 min

Duration of vibration (sec)	No. of oocytes cultured	No. (mean % $\pm$ SEM) of oocytes developed to		Mean no. $\pm$ SEM of cells in blastocysts
		$\geq 2$ -Cell	Blastocyst	
Control	96	48 (50.4 $\pm$ 6.7)	15 (15.7 $\pm$ 1.9) <sup>a</sup>	53.9 $\pm$ 3.5
5	90	51 (57.1 $\pm$ 5.0)	24 (27.1 $\pm$ 3.5) <sup>b</sup>	51.8 $\pm$ 3.4
10	93	57 (61.3 $\pm$ 6.4)	24 (25.8 $\pm$ 1.8) <sup>b</sup>	47.3 $\pm$ 2.4
30	88	41 (47.8 $\pm$ 10.7)	8 (9.0 $\pm$ 2.0) <sup>a</sup>	45.3 $\pm$ 4.2

<sup>a-b</sup> Values with different superscripts are significantly different (at least  $P < 0.05$ ).

**Table 3.** Effects of mechanical vibration for 5 sec at different intervals during *in vitro* culture on parthenogenetic development of pig oocytes

Intervals of vibration (min)	No. of oocytes cultured	No. (mean % $\pm$ SEM) of oocytes developed to		Mean no. $\pm$ SEM of cells in blastocysts
		$\geq 2$ -Cell	Blastocyst	
Control	91	42 (46.0 $\pm$ 6.5)	11 (12.1 $\pm$ 1.0)	46.8 $\pm$ 3.6
10	90	48 (53.3 $\pm$ 7.7)	14 (15.6 $\pm$ 7.3)	43.4 $\pm$ 2.9
30	90	52 (57.8 $\pm$ 7.3)	11 (12.2 $\pm$ 4.0)	43.4 $\pm$ 3.6
60	89	36 (40.5 $\pm$ 1.2)	11 (12.4 $\pm$ 1.0)	59.0 $\pm$ 6.1
180	94	49 (52.1 $\pm$ 4.1)	13 (13.8 $\pm$ 3.7)	47.0 $\pm$ 1.9
360	90	41 (46.0 $\pm$ 6.6)	15 (16.5 $\pm$ 3.0)	47.2 $\pm$ 3.5
720	95	48 (50.5 $\pm$ 2.6)	11 (11.6 $\pm$ 5.2)	39.7 $\pm$ 2.8

**Table 4.** Effects of mechanical vibration for 5 sec at intervals of 60 min during *in vitro* maturation (IVM) of recipient oocytes and/or *in vitro* culture (IVC) after reconstruction on development of miniature pig somatic cell nuclear transfer embryos

Vibration during IVM	No. of complexes examined	No. (mean % $\pm$ SEM) of complexes fused	No. of embryos cultured	No. (mean % $\pm$ SEM) of embryos developed to		Mean no. $\pm$ SEM of cells in blastocysts	
				$\geq 2$ -Cell	Blastocyst		
-	-	206	138 (66.8 $\pm$ 3.5)	66	31 (47.7 $\pm$ 5.4)	5 (7.4 $\pm$ 0.9) <sup>a</sup>	34.8 $\pm$ 1.0
-	+			65	39 (60.6 $\pm$ 3.6)	6 (9.4 $\pm$ 0.9) <sup>a,b</sup>	35.7 $\pm$ 1.5
+	-	192	138 (72.1 $\pm$ 3.1)	69	44 (63.6 $\pm$ 4.1)	9 (13.0 $\pm$ 0.3) <sup>b,c</sup>	36.8 $\pm$ 1.0
+	+			68	37 (54.5 $\pm$ 3.4)	12 (17.6 $\pm$ 2.5) <sup>c</sup>	38.0 $\pm$ 1.4

<sup>a-c</sup> Values with different superscripts are significantly different (at least  $P < 0.05$ ).

### Experiment 3

Mechanical vibration at different intervals during *in vitro* culture did not affect the cleavage rates (40.5  $\pm$  1.2–57.8  $\pm$  7.3%) of the oocytes (Table 3). Similarly, the blastocyst formation rates (11.6  $\pm$  5.2–16.5  $\pm$  3.0%) of the oocytes and mean numbers of cells (39.7  $\pm$  2.8–59.0  $\pm$  6.1 cells) in the blastocysts were not affected by mechanical vibration at the different intervals.

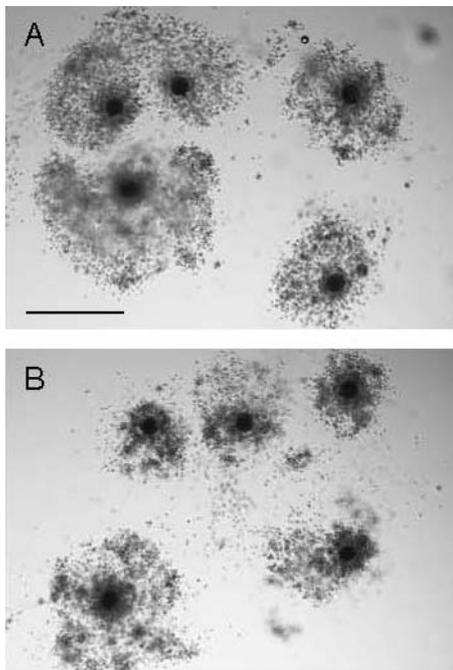
### Experiment 4

Mechanical vibration during *in vitro* maturation of recipient oocytes did not affect the fusion rates (66.8  $\pm$  3.5–72.1  $\pm$  3.1%) of the cell-oocyte complexes (Table 4). The cleavage rates (47.7  $\pm$  5.4–63.6  $\pm$  4.1%) of the embryos were not affected by mechanical vibrations during *in vitro* maturation of the recipient oocytes and/or *in vitro* culture after reconstruction. However, the blastocyst formation rates (13.0  $\pm$  0.3–17.6  $\pm$  2.5%) of the embryos vibrated during *in vitro* maturation were significantly ( $P < 0.05$ ) higher than that (7.4  $\pm$  0.9%) of the embryos matured and cultured without mechanical vibration. In addition, the blastocyst formation rate

(17.6  $\pm$  2.5%) of the embryos vibrated during both *in vitro* maturation and *in vitro* culture was significantly ( $P < 0.01$ ) higher than that (9.4  $\pm$  0.9%) of the embryos vibrated during only *in vitro* culture. The mean numbers of cells (34.8  $\pm$  1.0–38.0  $\pm$  1.4 cells) in the blastocysts were not affected by mechanical vibrations during *in vitro* maturation and/or *in vitro* culture.

## Discussion

The results of the present study showed that mechanical vibration during *in vitro* maturation enhances the parthenogenetic development *in vitro* of pig oocytes, although it does not affect their nuclear maturation. In addition, it was demonstrated that the *in vitro* development of miniature pig SCNT embryos is also improved by vibrating recipient oocytes during maturation *in vitro*. In contrast, mechanical vibration during *in vitro* culture after activation did not affect the development of pig oocytes and miniature pig SCNT embryos. These results indicate that mechanical vibration enhances the cytoplasmic maturation of *in vitro*-matured pig



**Fig. 2.** Cumulus-oocyte complexes cultured *in vitro* for 40 h with (A) or without (B) mechanical vibration for 5 sec at intervals of 60 min. Scale bar=500  $\mu$ m.

oocytes.

In the pig, although spontaneous nuclear maturation of competent oocytes *in vitro* upon liberation from follicles results in morphologically normal secondary oocytes, the developmental ability of those oocytes after *in vitro* fertilization is still low compared with those matured and fertilized *in vivo* [17]. Polyspermy is a major problem encountered in pig oocytes matured and fertilized *in vitro* [18]. Furthermore, only a low proportion of *in vitro*-matured and *in vitro*-fertilized pig oocytes are capable of transforming sperm into the male pronucleus [19]. These deficiencies are attributed to abnormalities in cytoplasmic maturation of *in vitro*-matured pig oocytes even though apparently normal nuclear maturation is observed. The cytoplasmic maturation of recipient oocytes seems to be important for successful development of pig SCNT embryos [20]. Studies on the maturation of pig oocytes *in vitro* by co-culture with either follicular cells or follicular fluid [17, 21] indicate that follicular cells secrete factors that play a crucial role in supporting oocyte cytoplasmic maturation.

Mechanical vibrations seem to affect the proliferation and secretory competence of somatic cells *in vitro*. Recently, Kaupp and Waldman [22] reported that mechanical vibrations increase the proliferation of bovine articular chondrocytes. In addition, Puig *et al.* [23] indicated that the release of interleukin-8 in human bronchial epithelial cells is enhanced by subjecting the cells to mechanical vibrations during culture. The optimal mechanical vibrations during *in vitro* maturation seem to stimulate cumulus expansion in COCs (Fig. 2). With this as the background, we hypothesize that mechanical vibrations would enhance the proliferation and/or

secretory competence of cumulus cells during *in vitro* maturation, resulting in increased factors that play a crucial role in supporting the cytoplasmic maturation of oocytes.

The results of the present study indicated that mechanical vibration for 5–10 sec at intervals of 60 min is suitable for cytoplasmic maturation of *in vitro*-matured pig oocytes. In contrast, mechanical vibration for 5 sec at intervals of 10 min or for 30 sec at intervals of 60 min did not enhance cytoplasmic maturation, suggesting that an overabundance of vibrations is not effective. One possible explanation for this phenomenon is that overabundant vibrations increase factors derived from cumulus cells but, on the other hand, dilute the factors around oocytes by scattering them in the droplet of medium.

In conclusion, we have established an effective production system for blastocysts from miniature pig SCNT embryos *in vitro*. This system would be useful not only for basic research to clarify the mechanism controlling the development of SCNT embryos, but also for nonsurgical transfer of such embryos at the morula and blastocyst stages to recipient females [24]. It would be of interest to examine whether mechanical vibration during *in vitro* maturation of pig oocytes can enhance development after *in vitro* fertilization or intracytoplasmic sperm injection.

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### References

1. Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de León FA, Robl JM. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* 1998; 280: 1256–1258.
2. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of a single adult. *Science* 1998; 282: 2095–2098.
3. Gibbons J, Arat S, Rzucidlo J, Miyoshi K, Waltenburg R, Respass D, Venable A, Stice S. Enhanced survivability of cloned calves derived from roscovitine-treated adult somatic cells. *Biol Reprod* 2002; 66: 895–900.
4. Betthausen J, Forsberg E, Augenstein M, Childs L, Eilertsen K, Enos J, Forsythe T, Golueke P, Jurgella G, Koppang R, Lesmeister T, Mallon K, Mell G, Misica P, Pace M, Pfister-Genskow M, Stelchenko N, Voelker G, Watt S, Thompson S, Bishop M. Production of cloned pigs from *in vitro* systems. *Nat Biotech* 2000; 18: 1055–1059.
5. Walker SC, Shin T, Zaunbrecher GM, Romano JE, Johnson GA, Bazer FW, Piedrahita JA. A high efficient method for porcine cloning by nuclear transfer using *in vitro*-matured oocytes. *Cloning Stem Cells* 2002; 4: 105–112.
6. Lai L, Kolber-Simonds D, Park KW, Cheong HT, Greenstein JL, Im GS, Samuel M, Bonk A, Rieke A, Day BN, Murphy CN, Carter DB, Hawley RJ, Prather RS. Production of  $\alpha$ -1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* 2002; 295: 1089–1092.
7. Dai Y, Vaught TD, Boone J, Chen SH, Phelps CJ, Ball S, Monahan JA, Jobst PM, McCreath KJ, Lamborn AE, Cowell-Lucero JL, Wells KD, Colman A, Polejaeva IA, Ayares DL. Targeted disruption of the  $\alpha$ 1,3-galactosyltransferase gene in cloned pigs. *Nat Biotech* 2002; 20: 251–255.
8. Miyoshi K, Inoue S, Himaki T, Mikawa S, Yoshida M. Birth of cloned miniature pigs derived from somatic cell nuclear transferred embryos activated by ultrasound treatment. *Mol Reprod Dev* 2007; 74: 1568–1574.
9. Wang WH, Abeydeera LR, Cantley TC, Day BN. Effects of oocyte maturation media on development of pig embryos produced by *in vitro* fertilization. *J Reprod Fertil* 1997;

- 111: 101–108.
10. Halbert SA, Tam PY, Blandau RJ. Egg transport in the rabbit oviduct: the roles of cilia and muscle. *Science* 1976; 191: 1052–1053.
  11. Halbert SA, Becker DR, Szal SE. Ovum transport in the rat oviductal ampulla in the absence of muscle contractility. *Biol Reprod* 1989; 40: 1131–1136.
  12. Croxatto HB. Physiology of gamete and embryo transport through the fallopian tube. *Reprod Biomed Online* 2002; 4: 160–169.
  13. Brinster RL. *In vitro* culture of mammalian embryos. *J Anim Sci* 1968; 27: 1–14.
  14. Miyoshi K, Mori H, Yamamoto H, Kishimoto M, Yoshida M. Effects of demecolcine and sucrose on the incidence of cytoplasmic protrusions containing chromosomes in pig oocytes matured *in vitro*. *J Reprod Dev* 2008; 54: 117–121.
  15. Sato K, Yoshida M, Miyoshi K. Utility of ultrasound stimulation for activation of pig oocytes matured *in vitro*. *Mol Reprod Dev* 2005; 72: 396–403.
  16. Miyoshi K, Fujimoto Y, Mori H, Yoshida M. Activation and parthenogenetic development of pig oocytes exposed to ultrasound in media containing different concentrations of Ca<sup>2+</sup>. *J Reprod Dev* 2008; 54: 42–45.
  17. Niwa K. Effectiveness of *in vitro* maturation and *in vitro* fertilization techniques in pigs. *J Reprod Fertil Suppl* 1993; 48: 49–59.
  18. Cran DG, Cheng WTK. The cortical reaction in pig oocytes during *in vivo* and *in vitro* fertilization. *Gamete Res* 1986; 13: 241–251.
  19. Nagai T, Niwa K, Iritani A. Effect of sperm concentration during preincubation in a defined medium on fertilization *in vitro* of pig follicular oocytes. *J Reprod Fertil* 1984; 70: 271–275.
  20. Solter D. Mammalian cloning: advances and limitations. *Nat Rev Genet* 2000; 1: 199–207.
  21. Sirard MA, Dubuc A, Bolamba D, Zheng Y, Coenen K. Follicle-oocyte-sperm interactions *in vivo* and *in vitro* in pigs. *J Reprod Fertil Suppl* 1993; 48: 3–16.
  22. Kaupp JA, Waldman SD. Mechanical vibrations increase the proliferation of articular chondrocytes in high-density culture. *Proc Inst Mech Eng H* 2008; 222: 695–703.
  23. Puig F, Rico F, Almendros I, Montserrat JM, Navajas D, Farre R. Vibration enhances interleukin-8 release in a cell model of snoring-induced airway inflammation. *Sleep* 2005; 28: 1312–1316.
  24. Martinez EA, Caamaño JN, Gil MA, Rieke A, McCauley TC, Cantley TC, Vazquez JM, Roca J, Vazquez JL, Didion BA, Murphy CN, Prather RS, Day BN. Successful nonsurgical deep uterine embryo transfer in pigs. *Theriogenology* 2004; 61: 137–146.