Effects of Maturation Age of Porcine Oocytes on the Induction of Activation and Development *In Vitro* Following Somatic Cell Nuclear Transfer

Koji IKEDA¹⁾ and Yoshiyuki TAKAHASHI^{1)*}

¹⁾Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

(Received 21 March 2001/Accepted 1 June 2001)

ABSTRACT. To determine the effect of maturational age of porcine oocytes on the induction of activation and development following nuclear transfer, we investigated maturation rate and efficacy of oocyte activation treatment with an electrical stimulus (ES) and cycloheximide (CHX) at various timing of maturation culture. Most oocytes developed to the metaphase II (MII) stage after 32 hr of maturation culture. Both in newly matured (32 and 36 hr of age) and MII-arrested (42 and 48 hr of age) oocytes, ES followed by exposure to CHX for 6 hr caused higher rates of pronuclear formation than ES alone. Effect of maturational age of oocytes on the development of parthenotes activated with ES and CHX was then examined. The highest percentage of parthenotes developed to blastocysts was obtained when ES was added at 42 hr of culture. Finally, newly matured (33 hr of age) and MII-arrested oocytes (44 hr of age) were enucleated, fused with serum-starved pig fetal fibroblasts and activated with ES and CHX around suitable timing (39 to 40 hr of age) or later (50 to 51 hr of age), respectively. The frequencies of cleavage (58.8%) and blastocyst formation (13.6%) of newly matured oocytes were significantly ($P<0.05$) higher than those of MII-arrested oocytes (38.9 and 1.8%, respectively). These results demonstrated that the development of porcine nuclear transfer embryos can be improved by using complete matured oocytes as cytoplasts and activation treatment with ES followed by CHX treatment.

KEY WORDS: activation, cycloheximide, nuclear transfer, oocyte age, swine.

J. Vet. Med. Sci. 63(9): 1003–1008, 2001

Recently, successful nuclear transfers using fetal and adult somatic cells as donor karyoplasts were reported in sheep [31], mice [29], cattle [6, 11, 30], goats [1] and pigs [2, 18, 21]. In the pig, however, the rate of offspring per embryos transferred was very low (0.9 to 6.9%), indicating that the viability of *in vitro* produced embryo is still low.

Pig follicular oocytes reach the metaphase II (MII) stage after *in vitro* maturation culture for 36 hr [32]. Arrest at the MII stage for a longer period (oocyte aging) may cause deterioration of various cytoskeletal components [14], which might be responsible for the low efficiency of the nuclear transfer procedure and the poor developmental potential of the embryos obtained thereby in cattle [26] and pig [4]. The development of porcine nuclear transfer embryos could be improved by using newly matured oocytes (33 hr of maturation culture) compared to 44 hr-cultured ones as recipient cytoplasts [4]. However, newly matured porcine oocytes (36 hr of maturation culture) showed lower developmental potentials after *in vitro* fertilization compared to the oocytes cultured for 44 hr [8]. In addition, recently ovulated mouse oocytes [24] and newly matured bovine oocytes [7, 9] showed lower developmental potential after *in vitro* fertilization compared with the oocytes cultured for an additional culture period. It is unclear that the relationship of maturation culture period and acquisition of developmental potential in the pig.

Cycloheximide (CHX), a protein synthesis inhibitor, was useful for parthenogenetic activation of bovine [22] and por-

cine [10, 17, 19] oocytes. CHX can be used for activation of porcine nuclear transfer embryos [4]. However, previous reports [10, 17, 19] focused on the activation of oocytes cultured for 44 to 46 hr, which is easily activated by a single parthenogenetic stimulus [23], and did not test the activation and development of porcine oocytes at various time of maturation culture.

In this study, *in vitro* oocyte maturation rate and efficacy of oocyte activation by the treatment with an electrical stimulus and CHX were examined at various time of maturation culture. We also tested the effect of maturational age of oocytes on the *in vitro* development of parthenotes to determine suitable timing of activation. Finally, the development of nuclear transfer embryos reconstructed with newly matured oocytes was compared with that of oocytes cultured for 44 hr, which have been most frequently used in *in vitro* fertilization and nuclear transfer studies [2, 3, 8].

MATERIALS AND METHODS

Oocyte collection and maturation culture *in vitro*: Prepubertal pig ovaries were collected from a local abattoir, and transported to our laboratory in physiological saline at 37°C within 2 hr of slaughter. *In vitro* maturation culture was performed as previously described [4]. Briefly, cumulus-oocyte complexes (COCs) were aspirated from antral follicles of 4 to 6 mm in diameter through an 18G needle attached to a 10-ml disposable syringe and put into Hepes-buffered TCM-199 (Sigma Co., St. Louis, MO, U.S.A.) supplemented with 0.1% polyvinyl alcohol (Sigma). The maturation medium was bovine serum albumin (BSA)-free NCSU-23 [20] supplemented with 10% porcine follicular

* CORRESPONDENCE TO: DR. TAKAHASHI, Y., Department of Veterinary Clinical Sciences, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan.

fluid (pFF), 0.57 mM cysteine (Sigma), 10 IU/ml equine chorionic gonadotropin (Serotropin, Teikoku Hormone Mfg. Co., Ltd., Tokyo, Japan) and 10 IU/ml human chorionic gonadotropin (Gonotropin, Teikoku Hormone). The pFF collected from prepubertal pig ovarian follicles of 4 to 6 mm in diameter was filtrated 2 times through 0.45- μ m filters, and stored at -40°C until use. Around 10 COCs with evenly granulated cytoplasm and intact cumulus cell layers were cultured in a 100- μ l drop of maturation medium covered with paraffin oil for 20 hr at 39°C under humidified atmosphere of 5% CO_2 in air. The COCs were then washed 3 times and transferred to a 100- μ l drop of maturation medium without hormones, and cultured for an additional 4 to 28 hr. After *in vitro* maturation culture, cumulus cells were removed by vortexing the COCs for 4 min in Dulbecco's PBS (DPBS) supplemented with 0.1% hyaluronidase (Sigma).

Oocyte activation: Oocytes with the first polar body were placed in a fusion medium consisting of 0.3 M mannitol, 0.1 mM CaCl_2 , 0.1 mM MgCl_2 and 0.1 mg/ml fatty acid-free BSA (Sigma) for 5 min at 37°C . They were placed between 2 platinum electrodes (1 mm apart), overlaid with 1 ml of fusion medium and subjected to a single DC pulse of 125 V/mm for 100 μ sec duration generated by a BTX Electro Cell Manipulator 2001 M (BTX, San Diego, CA, U.S.A.). After being allowed to settle for 2 min in the fusion medium, oocytes were washed 4 times and cultured for 6 hr in 30- μ l drops of NCSU-23 containing 4 mg/ml fatty acid-free BSA (NCSU-IVC) with or without 10 μ g/ml CHX (Sigma) in a humidified atmosphere of 5% CO_2 in air at 39°C .

Preparation and characterization of nuclear donor cells: Fetal fibroblasts obtained from an about 30-day-old porcine fetus [27], were used as nuclear donor source. The fetus was washed 3 times in physiological saline. After removal of the head, tail and intestinal organs, the remnant tissues were cut into small pieces and dispersed by exposure to DPBS containing 0.1% trypsin (Sigma) and 0.1% EDTA (Kanto Chemical Co., Inc., Tokyo, Japan). The dispersed tissues were cultured until confluence in a tissue culture flask (Falcon #3109, Becton Dickinson Labware, Franklin Lakes, NJ, U.S.A.) using Dulbecco's modified Eagle's medium/Ham's F12 (DMEM/F12, Gibco Laboratories, Grand Island, NY, U.S.A.) supplemented with 10% fetal calf serum (FCS, Gibco Laboratories) and 50 μ g/ml gentamycin (Sigma) under humidified atmosphere of 5% CO_2 in air at 37°C . After 6 passages, cells were frozen in DMEM/F12 containing 10% dimethyl sulfoxide (Kanto Chemical) and stored in liquid nitrogen until use. Frozen-thawed cells were cultured for 3 or 4 days in DMEM/F12 supplemented with 10% FCS at a concentration of 1×10^5 cells/ml in a 4-well culture plate (Nunc, Roskilde, Denmark). They were then further cultured for 3 or 4 days in DMEM/F12 supplemented with 0.5% FCS (serum-starvation). A cell suspension was prepared by trypsinization for a few minutes before use as nuclear donors.

The cell-cycle phase of serum-starved fetal fibroblasts was determined by measuring the DNA content of individ-

ual cells (more than 5×10^3 viable cells) using flow cytometry as described previously [16]. Briefly, trypsinized cells were washed with DPBS and fixed in 70% (v/v) ethanol at -20°C for at least 24 hr. Fixed cells were washed twice with DPBS and incubated with 200 μ g/ml RNase A (Boehringer Mannheim GmbH, Mannheim, Germany) at 37°C for 30 min. The cells were then washed with DPBS and stained with 50 μ g/ml propidium iodide (Sigma). They were filtered through nylon mesh with 50- μ m pores (Kysin Riko, Tokyo, Japan) and analyzed using a flow cytometer (EPICS XL-MCL, Beckman Coulter Inc., Fullerton, CA, U.S.A.). The distribution of the cells in various phases of the cell cycle was determined using forward scattered light separately gated on small, medium and large cells (<15 μ m, 16 to 20 μ m and >21 μ m, respectively). Gating for different cell sizes was based on microscopically measured cell-size plotted histograms. Subsequent calculation of the percentages of cells in G_0/G_1 -, S- and G_2/M -phase was performed using Epics XL system II software.

Nuclear transfer procedure: Cumulus-free oocytes were enucleated by removing the first polar body and approximately 20% of the adjacent cytoplasm by micromanipulation in a 40- μ l drop of Hepes-buffered TCM-199 supplemented with 10% FCS and 5 μ g/ml cytochalasin B (CCB, Sigma). After enucleation, cytoplasts were incubated in TCM-199 containing 5 μ g/ml Hoechst 33342 (Sigma) for 15 min at 37°C . Enucleation was assessed by exposing the oocytes under UV light for a few seconds using an inverted microscope (Diaphot-TMD, Nikon, Tokyo, Japan) equipped with epifluorescence and a UV-2A filter block (330–380 nm excitation and 420 nm emission). Medium-sized (16 to 20 μ m in diameter) fetal fibroblasts were inserted in the perivitelline space of the enucleated oocytes.

Manipulated couplets were placed between 2 platinum electrodes overlaid with 1 ml of fusion medium and then manually aligned with Pasteur pipette so that the contact surface between the enucleated oocyte and the fibroblast was parallel to the electrodes. Fusion was electrically induced by a single DC pulse of 150 V/mm for 25 μ sec. After fusion treatment, reconstituted embryos were cultured for 1.5 hr in NCSU-IVC under humidified atmosphere of 5% CO_2 , 5% O_2 and 90% N_2 at 39°C . They were then treated to induce activation by an electric pulse followed by CHX treatment as described above. All of nuclear transfer procedures from the removal of cumulus cells to oocyte activation by electric pulse required for 6 to 7 hr.

Evaluation of nuclear maturation, activation and premature chromosome condensation: Oocytes or fused couplets were mounted on a glass slide, fixed with an acetic acid and ethanol mixture (1:3) for at least 48 hr, and stained with 1% aceto-orcin. Oocytes with first polar body and metaphase chromosomes were considered to be in the MII stage. Oocytes beyond the MII stage were considered to be activated. Fused couplets with condensed chromatin were considered to have premature chromosome condensation (PCC).

Evaluation of embryo development in vitro: *In vitro* development of parthenotes and nuclear transfer embryos was examined by culturing for 7 days in NCSU-IVC under 5% CO₂, 5% O₂ and 90% N₂ at 39°C. The percentages of cleaved embryos and blastocysts were recorded on Day 2 and 7, respectively (Day 0=day of activation). The blastocyst cell number was assessed on Day 7 of culture using an air-dry method [25].

Experimental design: In experiment 1, the oocyte maturation rate was examined every 4 hr between 20 and 48 hr of *in vitro* maturation culture.

The effects of maturational age of oocytes and CHX treatment on oocyte activation were examined in the next experiment. After 32, 36, 42 or 48 hr of maturation culture, oocytes were activated by electrical stimulation and cultured with or without CHX. Treated oocytes were washed 4 times and cultured for an additional 6 hr in CHX-free NCSU-IVC. As a control, some oocytes were exposed to fusion medium for 7 min without an electric pulse and treated with or without CHX to determine if the exposure to fusion medium or CHX treatment itself could activate the MII stage oocytes. To examine the effect of maturational age on the development of parthenotes, oocytes were activated by electrical stimulation at 32, 36, 42 or 48 hr of culture. They were then exposed to CHX and 5 µg/ml CCB for 6 hr to generate diploid parthenotes before being cultured in NCSU-IVC.

In experiment 3, the effect of the maturational age of the recipient oocytes on the developmental potential of nuclear transfer embryos was examined. Cumulus-free oocytes enucleated at 33 or 44 hr of maturation culture were used as recipient cytoplasts. Fused couplets were subjected to an electric pulse at 39 to 40 hr (33 hr-cultured oocytes) or 50 to 51 hr (44 hr-cultured oocytes). Some fused couplets were fixed after 4.5 hr of fusion without activation treatment, and examined for the presence of PCC to determine whether they had entered interphase.

Statistical analysis: Data were subjected to one-way ANOVA followed by Fisher's protected least significant difference test (Experiment 1 and the development of parthenotes in Experiment 2), chi-square test with Yates' correction (Experiment 2) or Student's *t*-test (Experiment 3) by using StatView software for Macintosh (Abacus Conceptus Inc., Berkeley, CA, U.S.A.).

RESULTS

Experiment 1: As shown in Fig. 1, MII stage oocytes were first observed at 28 hr of maturation culture (5.1 ± 5.3%). Most oocytes (74.3 ± 2.6%) reached the MII stage at 32 hr of culture, although oocytes reached the MII stage were further increased at 36 hr of culture (94.3 ± 2.4%, *P*<0.05). There were no significant differences in the percentages of MII stage oocytes cultured for longer than 36 hr (*P*>0.1).

Experiment 2: In the absence of an electrical stimulus, the frequencies of spontaneous activation were low (0 to 12.7%,

Fig. 1. Nuclear maturation rate of pig follicular oocytes cultured *in vitro*. a, b, c) The values (means ± SD of 3 or 4 replicates) with different letters differ significantly (*P*<0.05).

3 to 6 replicates) regardless of the oocyte maturational age and CHX treatment. In all experimental groups, the combination of electrical stimulation and CHX improved the rate of pronuclear formation (Table 1). In the 32 hr-cultured oocytes, the formation of pronucleus(ei) was significantly lower (*P*<0.05) compared with other groups when they were activated with electrical stimulation and CHX. The proportion of the oocytes having pronucleus(ei) and a second polar body after treatment with the electric pulse and CHX was significantly higher (*P*<0.05) in 42 hr- (51.0%) and 48 hr- (61.6%) cultured oocytes than in 32 hr- (12.7%) and 36 hr- (16.0%) cultured oocytes. As shown in Table 2, the development of parthenotes to the blastocyst stage was higher when oocytes were activated at 42 hr compared to those activated at 32 and 48 hr.

Experiment 3: The proportions of fetal fibroblasts in the small-, medium- and large-sized range were 14.7 ± 5.5, 48.7 ± 5.0 and 36.7 ± 3.3%, respectively (4 replicates, 200 cells per replicate). The percentages of the medium-sized cells in G₀/G₁-, S- and G₂/M-phase of the cell cycle were 79.7 ± 8.1, 2.3 ± 0.1 and 18.1 ± 7.9%, respectively. The percentage of oocytes with a first polar body was lower after 33 hr of maturation culture (74.3 ± 8.5%, *P*<0.05) than that of oocytes cultured for 44 hr (94.6 ± 3.3%). Enucleation rates of oocytes cultured for 33 (72.8 ± 10.6%) and 44 hr (64.3 ± 11.5%) were not different (*P*>0.1).

There was no significant difference in the fusion rate between the two recipient oocyte groups (63.3 ± 18.6 and 69.9 ± 14.1%, respectively). As shown in Table 3, the frequencies of cleavage and blastocyst formation in nuclear transfer embryos constructed with 33 hr-cultured oocytes were higher (*P*<0.05) than those in embryos constructed with 44 hr-cultured oocytes. Almost all fused couplets constructed with 33 hr-cultured (96.7 ± 5.8%, *n*=30) and 44 hr-cultured oocytes (89.3 ± 12.9%, *n*=33) showed PCC.

Table 1. Effects of maturational age and cycloheximide (CHX) treatment on parthenogenetic activation of *in vitro* matured porcine oocytes

Maturational age of oocytes (hr)	Without CHX treatment			With CHX treatment		
	No. of examined	% of Act	% of PN	No. of examined	% of Act	% of PN
32	60	10.0 ^{a)}	5.0 ^{a)}	55	67.3 ^{a)*}	61.8 ^{a)*}
36	65	33.8 ^{b)}	13.8 ^{a)}	75	92.0 ^{b)*}	88.0 ^{b)*}
42	53	88.7 ^{c)}	62.3 ^{b)}	51	96.1 ^{b)}	96.1 ^{b)*}
48	106	86.8 ^{c)}	68.9 ^{b)}	86	93.0 ^{b)}	90.7 ^{b)*}

Act: Activated oocytes. PN: Oocytes with pronucleus(ei). Data were pooled from 3 to 6 replicates.

a,b,c) Values with different superscripts in the same column differ significantly ($P < 0.05$).

* Values differ significantly from control (without CHX treatment, $P < 0.05$).

Table 2. Effect of maturational age of oocytes on the *in vitro* development of parthenotes

Maturational age of oocytes (hr)	No. of examined (replicates)	% of embryos developed to		Cell no. in blastocysts (n)
		≥ 2 -cell on Day 2	blastocysts on Day 7	
32	61 (4)	$41.8 \pm 10.4^a)$	$11.8 \pm 9.4^a)$	25.1 ± 12.4 (7)
36	75 (4)	$67.7 \pm 16.3^b)$	$28.3 \pm 18.3^{a,b)}$	33.9 ± 9.8 (21)
42	84 (5)	$68.9 \pm 5.7^b)$	$35.8 \pm 10.0^b)$	29.8 ± 9.3 (30)
48	83 (4)	$64.9 \pm 5.1^b)$	$15.3 \pm 9.3^a)$	24.6 ± 7.3 (13)

a,b) Values (means $\pm$ SD) with different superscripts differ significantly ($P < 0.05$).

Table 3. Effect of maturational age of recipient oocytes on the *in vitro* development of nuclear transfer embryos

Maturational age of oocytes (hr) ^{a)}	Timing of activation (hr) ^{a)}	No. of couplets cultured (replicates)	% of embryos developed to		Cell no. in blastocysts
			≥ 2 -cell on Day 2	blastocysts on Day 7	
33	39–40	73 (6)	$58.8 \pm 12.1^b)$	$13.6 \pm 11.5^b)$	$32.8 \pm 7.9^d)$
44	50–51	97 (6)	$38.9 \pm 9.5^c)$	$1.8 \pm 2.8^c)$	35, 17

a) Hours after the initiation of oocyte maturation culture.

b,c) Values (means $\pm$ SD) with different superscripts differ significantly ($P < 0.05$).

d) Mean $\pm$ SD of 10 blastocysts.

DISCUSSION

Under the present maturation culture conditions, most of the oocytes reached the MII stage after 32 hr of maturation culture, which is slightly earlier than TCM-199 based maturation medium [32]. The oocytes cultured for 32 to 36 hr were therefore considered to be newly matured oocytes and the oocytes cultured for 44 to 48 hr, which have been most frequently used in *in vitro* fertilization and nuclear transfer studies [2, 3, 8], were served as controls in the following experiments.

Various stimuli have been used for the activation of pig oocytes. Electrical stimulation seems to be most effective for pig oocyte activation [3]. Newly matured pig oocytes, however, are hardly activated by a single electric pulse alone [23], probably due to the re-activation of maturation-promoting factor (MPF) after the parthenogenetic activation [15]. CHX, a protein synthesis inhibitor, was reported to

efficiently prevent the re-activation of MPF in newly matured cattle oocytes after activation treatment [22]. However, the effects of CHX on pig oocyte activation were inconsistent. An electric pulse followed by CHX treatment improved the pronuclear formation [17], while no beneficial effect was observed [3] in 44 hr-cultured oocytes. This might be due to differences in the experimental conditions, such as the media used for the maturation culture or activation protocols. In this study, most (92 to 96%) oocytes cultured for longer than 36 hr were activated by a combination of electrical stimulation and CHX. It is confirmed that CHX treatment after electrical stimulation improves the activation of both newly matured and MII-arrested porcine oocytes.

Newly matured oocytes showed lower developmental potentials after *in vitro* fertilization compared with the oocytes cultured for an additional 8 hr in pigs [8] and cattle [7, 9]. This might be due to the immaturity of the metaphase spindle and/or inadequate cytoplasmic maturation of MII

stage oocytes immediately after MII-arrest [7]. In the present study, oocytes cultured for 32 and 36 hr failed to extrude the second polar body more frequently than those cultured for 42 and 48 hr after the activation treatment, suggesting that spindles had not yet fully developed in newly matured oocytes. These results suggest that the complete nuclear and cytoplasmic maturation of oocytes need additional culture after reaching the MII stage.

We adopted parthenote models to determine the developmental potential of oocytes. The developmental potential of parthenotes increased gradually by the time, and reached to the maximum rate at 42 hr-cultured oocytes (Table 2). The developmental potential of nuclear transfer embryos constructed with 33 hr-cultured oocytes and received electrical stimulation around 39 to 40 hr from the initiation of maturation culture was therefore higher than that of embryos constructed with 44 hr-cultured oocytes and received electrical stimulation around 50 to 51 hr (Table 3). Forty-four hours of maturation culture and the subsequent 6 to 7 hr required for the nuclear transfer procedure might induce oocyte aging and result in a low development to blastocysts. Indeed, parthenotes derived from oocytes cultured for 48 hr, as a model of 44 hr-cultured recipients for nuclear transfer, decreased the developmental potential to blastocysts. The mechanisms of acquisition and reduction of activation and developmental potential were not fully understood, although MPF may play a pivotal role on oocyte aging [13]. Furthermore, the developmental potential of newly matured oocytes (32 to 36 hr of maturational age) could not be examined by our current nuclear transfer procedure because it took 6 to 7 hr to complete all of the nuclear transfer procedures and the timing of activation was 39 to 40 hr of maturation culture at the soonest. Further experiments are needed to determine the activation and developmental potential of nuclear transfer embryos constructed with newly matured oocytes by using different nuclear transfer procedure.

In somatic cell nuclear transfer, fusion of karyoplasts with cytoplasts before activation treatment was effective for the development of nuclear transfer embryos [29, 30]. Nuclear donor cells in the G₁-phase of the cell cycle showed nuclear envelope breakdown and PCC when they were fused with recipient cytoplasts with high MPF activity. PCC was essential for the full-term development of mouse nuclear transfer embryos reconstituted with karyoplasts in the G₀/G₁-phase of the cell-cycle [5, 29]. In the pig, cloned piglets were obtained when fusion was applied before activation treatment [2, 18], while no piglet was born when fusion and activation were applied simultaneously [28]. We expected that maturational age could affect the formation of PCC because MPF activity gradually decreased [12] and sensitivity to the electric pulse also gradually increased [23] during MII-arrest. However, PCC of donor nuclei occurred in almost all fused couplets regardless of the maturational age of the recipient cytoplast. As observed in a previous report [4] and present study, the decrease in the development to blastocysts in 44 hr-cultured recipients might be due

to oocyte aging during MII-arrest or other factors that responsible for the complete reprogramming of transferred nuclei.

In conclusion, the present study demonstrated that newly matured pig oocytes could be activated with an electric pulse followed by CHX treatment. However, the complete maturation of oocytes needed additional culture after reaching the MII stage. The *in vitro* development of nuclear transfer embryos to blastocysts can be improved by using complete matured oocytes as recipient cytoplasts.

ACKNOWLEDGMENTS. We thank the staff of the Ebetsu Meat Inspection Office for supplying the ovaries. This study was supported by Grant-in-Aid for scientific research (Nos. 10556058 and 12480248) from the Japan Society for the Promotion of Science.

REFERENCES

1. Baguisi, A., Behboodi, E., Melican, D. T., Pollock, J. S., Destrempe, M. M., Cammuso, C., Williams, J. L., Nims, S. D., Porter, C. A., Midura, P., Palacios, M. J., Ayres, S. L., Deniston, R. S., Hayes, M. L., Ziomek, C. A., Meade, H. M., Godke, R. A., Gavin, W. G., Overström, E. W. and Echelard, Y. 1999. Production of goats by somatic cell nuclear transfer. *Nature Biotechnol.* **17**: 456–461.
2. 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., Strelchenko, N., Voelker, G., Watt, S., Thompson, S. and Bishop, M. 2000. Production of cloned pigs from *in vitro* systems. *Nature Biotechnol.* **18**: 1055–1059.
3. Cha, S. K., Kim, N. H., Lee, S. M., Baik, C. S., Lee, H. T. and Chung, K. S. 1997. Effect of cytochalasin B and cycloheximide on the activation rate, chromosome constituent and *in vitro* development of porcine oocytes following parthenogenetic stimulation. *Reprod. Fertil. Dev.* **9**: 441–446.
4. Cheong, H. T., Ikeda, K., Martinez Diaz, M. A., Katagiri, S. and Takahashi, Y. 2000. Development of reconstituted pig embryos by nuclear transfer of cultured cumulus cells. *Reprod. Fertil. Dev.* **12**: 15–20.
5. Cheong, H. T., Takahashi, Y. and Kanagawa, H. 1994. Relationship between nuclear remodeling and subsequent development of mouse embryonic nuclei transferred to enucleated oocytes. *Mol. Reprod. Dev.* **37**: 138–145.
6. Cibelli, J. B., Stice, S. L., Golueke, P. J., Kane, J. J., Jerry, J., Blackwell, C., Ponce de León, F. A. and Robl, J. M. 1998. Cloned transgenic calves produced from nonquiescent fetal fibroblasts. *Science* **280**: 1256–1258.
7. Dominko, T. and First, N. L. 1997. Timing of meiotic progression in bovine oocytes and its effect on early embryo development. *Mol. Reprod. Dev.* **47**: 456–467.
8. Grupen, C. G., Nagashima, H. and Nottle, M. B. 1997. Asynchronous meiotic progression in porcine oocytes matured *in vitro*: a cause of polyspermic fertilization? *Reprod. Fertil. Dev.* **9**: 187–191.
9. Gutierrez-Adán, A., Pérez-Garnelo, J., Granados, J., Garde, J. J., Pérez-Guzmán, M., Pintado, B. and De La Fuente, J. 1999. Relationship between sex ratio and time of insemination according to both time of ovulation and maturational state of oocyte. *Zygote* **7**: 37–43.

10. Jílek, F., Hüttelová, R., Petr, J., Holubová, M. and Rozinek, J. 2000. Activation of pig oocytes using calcium ionophore: effect of protein synthesis inhibitor cycloheximide. *Anim. Reprod. Sci.* **63**: 101–111.
11. Kato, Y., Tani, T., Sotomaru, Y., Kurokawa, K., Kato, J., Doguchi, H., Yasue, H. and Tsunoda, Y. 1998. Eight calves cloned from somatic cells of a single adult. *Science* **282**: 2095–2098.
12. Kikuchi, K., Izaike, Y., Noguchi, J., Furukawa, T., Daen, F. P., Naito, K. and Toyoda, Y. 1995. Decrease of histone H1 kinase activity in relation to parthenogenetic activation of pig follicular oocytes matured and aged *in vitro*. *J. Reprod. Fertil.* **105**: 325–330.
13. Kikuchi, K., Naito, K., Noguchi, J., Shimada, A., Kaneko, H., Yamashita, M., Aoki, F., Tojo, H. and Toyoda, Y. 2000. Maturation/M-phase promoting factor: A regulator of aging in porcine oocytes. *Biol. Reprod.* **63**: 715–722.
14. Kim, N. H., Moon, S. J., Prather, R. S. and Day, B. N. 1996. Cytoskeletal alteration in aged porcine oocytes and parthenogenesis. *Mol. Reprod. Dev.* **43**: 513–518.
15. Liu, L., Ju, J. C. and Yang, X. 1998. Differential inactivation of maturation-promoting factor and mitogen-activated protein kinase following parthenogenetic activation of bovine oocytes. *Biol. Reprod.* **59**: 537–545.
16. Mohamed Nour, M. S., Ikeda, K. and Takahashi, Y. 2000. Bovine nuclear transfer using cumulus cells derived from serum-starved and confluent cultures. *J. Reprod. Dev.* **46**: 85–92.
17. Nussbaum, D. J. and Prather, R. S. 1995. Differential effects of protein synthesis inhibitors on porcine oocyte activation. *Mol. Reprod. Dev.* **41**: 70–75.
18. Onishi, A., Iwamoto, M., Akita, T., Mikawa, S., Takeda, K., Awata, T., Hanada, H. and Perry, A. C. 2000. Pig cloning by microinjection of fetal fibroblast nuclei. *Science* **289**: 1188–1190.
19. Petr, J., Grocholová, R., Rozinek, J. and Jílek, F. 1996. Activation of *in vitro* matured pig oocytes by combined treatment of ethanol and cycloheximide. *Theriogenology* **45**: 1473–1478.
20. Petters, R. M. and Wells, K. D. 1993. Culture of pig embryos. *J. Reprod. Fertil. (Suppl.)* **48**: 61–73.
21. Polejaeva, I. A., Chen, S. H., Vaught, T. D., Page, R. L., Mullins, J., Ball, S., Dai, Y., Boone, J., Walker, S., Ayares, D. L., Colman, A. and Campbell, K. H. 2000. Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature (Lond.)* **407**: 86–90.
22. Presicce, G. A. and Yang, X. 1994. Nuclear dynamics of parthenogenesis of bovine oocytes matured *in vitro* for 20 and 40 hours and activated with combined ethanol and cycloheximide treatment. *Mol. Reprod. Dev.* **37**: 61–68.
23. Procházka, R., Kaňka, J., Šutovský, P., Fulka, J. and Motlík, J. 1992. Development of pronuclei in pig oocytes activated by a single electric pulse. *J. Reprod. Fertil.* **96**: 725–734.
24. Schroeder, A. C., Downs, S. M. and Eppig, J. J. 1988. Factors affecting the developmental capacity of mouse oocytes undergoing maturation *in vitro*. *Ann. New York Acad. Sci.* **54**: 197–204.
25. Takahashi, Y. and First, N. L. 1992. *In vitro* development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. *Theriogenology* **37**: 963–978.
26. Takano, H., Koyama, K., Kozaki, C., Kato, Y. and Tsunoda, Y. 1993. Effect of aging of recipient oocytes on the development of bovine nuclear transfer embryos *in vitro*. *Theriogenology* **39**: 909–917.
27. Ullrey, D. E., Sprague, J. I., Becker, D. E. and Miller, E. R. 1965. Growth of the swine fetus. *J. Anim. Sci.* **24**: 711–717.
28. Verma, P. J., Du, Z. T., Crocker, L., Faast, R., Grupen, C. G., McIlfatrick, S. M., Ashman, R. J., Lyons, I. G. and Nottle, M. B. 2000. *In vitro* development of porcine nuclear transfer embryos constructed using fetal fibroblasts. *Mol. Reprod. Dev.* **57**: 262–269.
29. Wakayama, T., Perry, A. C., Zuccotti, M., Johnson, K. R. and Yanagimachi, R. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature (Lond.)* **394**: 369–374.
30. Wells, D. N., Misica, P. M. and Tervit, H. R. 1999. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol. Reprod.* **60**: 996–1005.
31. Wilmut, I., Schnieke, A. E., McWhir, J., Kind, A. J. and Campbell, K. H. 1997. Viable offspring derived from fetal and adult mammalian cells. *Nature (Lond.)* **385**: 810–813.
32. Yoshida, M., Bamba, K. and Kojima, Y. 1989. Effects of gonadotropins and estradiol-17 β on the timing of nuclear maturation and cumulus mass expansion in pig oocytes cultured *in vitro*. *Jpn. J. Anim. Reprod.* **35**: 86–91.