Diploid porcine parthenotes produced by inhibition of first polar body extrusion during *in vitro* maturation of follicular oocytes

Tamás Somfai^{1,2}, Manabu Ozawa¹, Junko Noguchi¹, Hiroyuki Kaneko¹, Katsuhiko Ohnuma¹, Ni Wayan Kurniani Karja⁴, Mokhamad Fahrudin¹, Naoki Maedomari³, András Dinnyés², Takashi Nagai⁴ and Kazuhiro Kikuchi¹

¹Reproductive Biology Unit, Department of Animal Science, National Institute of Agrobiological Sciences, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan, ²Micromanipulation and Genetic Reprogramming Group, Institute of Animal Biology, Agricultural Biotechnology Center, 2100 Gödöllo, Hungary, ³Graduate School, Azabu University, Sagami-hara, Kanagawa 229-8501, Japan and ⁴Research Support Center, National Institute of Livestock and Grassland Science, Tsukuba, Ibaraki 305-0901, Japan

Correspondence should be addressed to K Kikuchi; Email: kiku@nias.affrc.go.jp

Abstract

We investigated nuclear progression and *in vitro* embryonic development after parthenogenetic activation of porcine oocytes exposed to cytochalasin B (CB) during *in vitro* maturation (IVM). Nuclear progression was similar in control oocytes and oocytes matured in the presence of 1 µg/ml CB (IVM-CB group) by 37 h IVM; at this time the proportion of oocytes that had reached or passed through the anaphase-I stage did not differ significantly between the IVM-CB and the control groups (61.3 and 69.9% respectively; $P < 0.05$). After IVM for 37 h, no polar body extrusion was observed in the IVM-CB group. In these oocytes, the two lumps of homologous chromosomes remained in the ooplasm after their segregation and turned into two irregular sets of condensed chromosomes. By 41 h IVM, the double sets of chromosomes had reunited in 89.5% IVM-CB oocytes and formed a single large metaphase plate, whereas 68.8% of the control oocytes had reached the metaphase-II stage by this time. When IVM-CB oocytes cultured for 46 h were stimulated with an electrical pulse and subsequently cultured for 8 h without CB, 39.0% of them extruded a polar body and 82.9% of them had a female pronucleus. Chromosome analysis revealed that the majority of oocytes that extruded a polar body were diploid in both the control and the IVM-CB groups. However, the incidence of polyploidy in the IVM-CB group was higher than that in the control group ($P < 0.05$). *In vitro* development of diploid parthenotes in the control and the IVM-CB groups was similar in terms of blastocyst formation rates (45.8 and 42.8% respectively), number of blastomeres (39.9 and 44.4 respectively), the percentage of dead cells (4.3 and 2.9% respectively), and the frequency of apoptotic cells (7.3 and 6.3% respectively). Tetraploid embryos had a lower blastocyst formation rate (25.5%) and number of cells (26.2); however, the proportion of apoptotic nuclei (7.0%) was similar to that in diploid parthenotes. These results suggest that the proportion of homozygous and heterozygous genes does not affect *in vitro* embryo development to the blastocyst stage.

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Introduction

The production of parthenogenetic embryos is of great importance in studies of early embryonic development. Studies on uniparental embryos, such as gynogenotes, androgenotes, and parthenotes, are essential to our understanding of genome imprinting. Porcine parthenotes can be obtained by electrical stimulation of metaphase-II (M-II) oocytes (Hagen *et al.* 1991, Prather *et al.* 1991, Lee *et al.* 2004), by the treatment with chemicals, such as ionophores (Hagen *et al.* 1991, Wang *et al.* 1998), or by a combination of electrical activation and treatment with chemicals, such as protein synthesis

inhibitors (Nussbaum & Prather 1995), or protein kinase inhibitors (Dinnyes *et al.* 1999). The most effective and widely used method of activating oocytes is electrical stimulation. Whenever oocytes are activated by such treatments, the second polar body (2PB) extrudes, resulting in haploid parthenotes. Haploid mammalian parthenotes have compromised developmental competence compared with diploid parthenotes (Henery & Kaufman 1992, Kawarsky *et al.* 1996, Kim *et al.* 1997a, Liu *et al.* 2002). Therefore, for precise comparison of the development of parthenotes and fertilized embryos or diploid androgenotes, diploid parthenotes are needed.

Diploid parthenotes are usually obtained by inhibiting 2PB extrusion with a chemical such as cytochalasin B (CB) after the activation of M-II oocytes. CB, an inhibitor of actin polymerization, disrupts microfilaments, thus effectively inhibiting the extrusion of the 2PB without any effect on the formation and movement of pronuclei (Kim *et al.* 1997b). A short (2–5 h) treatment with this drug is widely used to diploidize parthenogenetic mammalian oocytes after an activation procedure (Balakier & Tarkowsky 1976, Fukui *et al.* 1992, Cha *et al.* 1997). On the other hand, diploid embryos can also be generated by inhibiting the extrusion of the first polar body (1PB) with cytochalasin D during *in vitro* maturation (IVM) of oocytes before the activation procedure (Kubiak *et al.* 1991). When parthenotes are produced by inhibition of 1PB extrusion before parthenogenetic activation of the oocytes obtained from heterozygous mice, the proportion of heterozygous embryos is higher than with the conventional method, in which 2PB extrusion is inhibited after the activation procedure. This clearly shows that the genotypes of parthenotes obtained by the two methods are not the same and suggests that ensuring the diploid status of parthenotes by the inhibition of 1PB (containing homologous chromosomes) extrusion, results in a higher frequency of heterozygous genes than that by the inhibition of 2PB (containing sister chromatids). The higher frequency of homozygous status after the inhibition of 2PB extrusion might entail the expression of recessive lethal, sublethal, and subvital genes in such embryos, which might affect early embryonic development. However, little is known about the effect of the frequency of homozygous genes on the development of porcine embryos to the blastocyst stage.

Our objectives were to establish a method of producing diploid porcine parthenotes by the inhibition of 1PB extrusion during IVM, and to compare the *in vitro* development of diploid parthenotes obtained by the inhibition of homologous chromosome extrusion and by the inhibition of chromatid extrusion.

Materials and Methods

Collection and maturation of oocytes

Collection and IVM of porcine follicular oocytes were performed according to Kikuchi *et al.* (2002). Briefly, ovaries from prepubertal cross-bred gilts (Landrace × Large White) were collected at a local slaughterhouse and carried to the laboratory in Dulbecco's PBS (Nissui Pharmaceutical Co. Ltd, Tokyo, Japan) at 35–37 °C within 1 h. Cumulus–oocyte complexes (COCs) were collected by scraping of 3–6 mm follicles in a collection medium consisting of Medium 199 (with Hanks' salts; Sigma Chemical Co.) supplemented with 10% fetal bovine serum (Gibco and Invitrogen Corp.), 20 mM HEPES (Dojindo Laboratories, Kumamoto, Japan), and

antibiotics (100 units/ml penicillin G potassium (Sigma) and 0.1 mg/ml streptomycin sulfate (Sigma)). Maturation culture was performed in a modified North Carolina State University (NCSU)-37 solution (Petters & Wells 1993) containing 10% (v/v) porcine follicular fluid, 0.6 mM cysteine, 1 mM dibutyryl cAMP (dbcAMP, Sigma), 10 IU/ml eCG (PMS 1000 Tani NZ, Nihon Zenyaku Kogyo, Koriyama, Japan), and 10 IU/ml hCG (Puberogen, 500 U, Sankyo, Tokyo, Japan) in a four-well dishes (Nunc Multidishes, Nalge Nunc International, Roskilde Denmark) for 22 h in an atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 39 °C. Because of the presence of the dbcAMP, the oocytes remain at the germinal vesicle (GV) stage during this period (Funahashi *et al.* 1997). COCs were subsequently cultured in maturation medium without dbcAMP and hormones for an additional 24 h under the same atmosphere.

Parthenogenetic activation

After brief treatment of the COCs in collection medium supplemented with 0.1% (w/v) hyaluronidase, the oocytes were freed from the cumulus cells mechanically with a fine glass pipette in a hyaluronidase-free collection medium. The denuded oocytes were washed twice and transferred to an activation solution, which consisted of 0.28 M D-mannitol, 0.05 mM CaCl₂, 0.1 mM MgSO₄, and 0.01% (w/v) BSA, and washed three times. Then they were stimulated with a direct current pulse of 1.5 kV/cm for 100 µs by using a somatic hybridizer (SSH-10, Shimadzu, Kyoto, Japan).

In vitro culture

In vitro culture (IVC) of stimulated oocytes was performed according to the method of Kikuchi *et al.* (2002). In brief, two types of IVC media were prepared: (1) IVC-PyrLac consisted of NCSU-37 without glucose, but supplemented with 4 mg/ml BSA, 50 µM β-mercaptoethanol, 0.17 mM sodium pyruvate, and 2.73 mM sodium lactate. (2) IVC-Glu was NCSU-37 containing 5.55 mM glucose and supplemented with 4 mg/ml BSA and 50 µM β-mercaptoethanol. IVC was performed in 500 µl drops of IVC-PyrLac for days 0–2 (day 0 was defined as the day of electrical stimulation) and in IVC-Glu for days 2–6 in four-well dishes (Nunc Multidishes, Nalge Nunc International) under an atmosphere of 5% CO₂, 5% O₂, and 90% N₂ at 39 °C.

Oocyte and embryo evaluation with orcein staining

For evaluation of the meiotic stage of oocytes or their activation status after parthenogenetic activation, oocytes or embryos were mounted on glass slides and fixed with acetic alcohol (acetic acid:ethanol, 1:3) for at least 3 days, then stained with 1% (w/v) orcein in acetic

acid, destained in glycerol:acetic acid:water (1:1:3) and examined under a phase-contrast microscope with $\times 40$ and $\times 100$ objectives.

Blastocyst evaluation with live–dead nuclear staining

Blastocysts were transferred to PBS supplemented with 5 mg/ml BSA containing 1 μ g/ml fluorescein diacetate (FDA, Sigma), 50 μ g/ml propidium iodide (PI, Sigma), and 20 μ g/ml Hoechst 33342 (Calbiochem, EMD Biosciences, Inc., San Diego, CA, USA) and incubated for 10 min. Then the embryos were mounted on glass slides with coverslips. They were examined under UV light with an epifluorescence microscope (BX-51, Olympus, Tokyo, Japan). Live blastomeres (FDA positive and PI negative) appeared green with blue nuclei (labeled with Hoechst only), whereas dead blastomeres were FDA negative and their nuclei were labeled with both Hoechst and PI, thus appearing red (Somfai *et al.* in press).

Chromosome analysis

Chromosome samples of porcine oocytes and embryos were prepared by the method described previously (Yoshizawa *et al.* 1998, Somfai *et al.* 2005). Briefly, after IVC for 5 days, expanding blastocysts (≤ 140 μ m in diameter) were cultured for 14–17 h in IVC-Glu containing vinblastine sulfate 60 ng/ml (Wako Pure Chemical Industries, Ltd, Osaka, Japan). In the case of oocytes, chromosome preparation was performed without vinblastine treatment. The blastocysts/oocytes were then washed and incubated in 1% (w/v) sodium citrate solution for 15 min, and fixed mildly by pouring 0.02 ml acetic alcohol (acetic acid:methanol, 1:1) into 0.4 ml hypotonic solution of sodium citrate. A blastocyst was placed on a glass slide, immediately covered with a very small droplet of acetic acid to separate each cell, and then refixed with several drops of acetic alcohol (acetic acid:methanol, 1:3). After being dried completely, chromosome samples were stained with 2% (w/w) Giemsa solution (Merck KgaA) for 10 min. They were evaluated under a microscope with a $\times 100$ objective.

Evaluation of DNA fragmentation by TUNEL assay

Apoptosis in embryos was assessed according to the method by Karja *et al.* (2004). Briefly, on day 6, IVC blastocysts were washed four times in PBS containing 3 mg/ml polyvinylalcohol (PBS-PVA), and then fixed at 4 °C overnight in 3.7% (w/v) paraformaldehyde diluted in PBS. After fixation, the embryos were washed three times in PBS-PVA, permeabilized in 0.1% Triton-X-100 (diluted in PBS) for 60 min, and incubated at 4 °C overnight in a blocking solution, which was PBS containing 10 mg/ml BSA. The embryos were then

washed four times in PBS-PVA and incubated in fluorescein-conjugated dUTP and TdT (TUNEL reagent, Roche Diagnostics) for 1 h at 38.5 °C and 5% CO₂ in air. As positive controls, before each TUNEL analysis, two embryos were incubated in 1000 IU/ml DNase I (DNase I, Sigma) for 20 min at 38.5 °C and 5% CO₂ in air, then washed three times in PBS-PVA. After TUNEL staining, embryos were exposed to 50 μ g/ml RNase for 60 min at room temperature, then stained with 50 μ g/ml PI for 20 min. Finally, embryos were washed three times in PBS-PVA, then mounted on glass slides in anti-fade solution. Labeled nuclei were examined under a confocal laser-scanning microscope (IX-71, Olympus) fitted with 25/40 $\times$ PL Fluotar/0.75 oil objectives and an argon/krypton laser, which was used for excitation at wavelengths of 488 and 568 nm for detection of TUNEL reaction and PI respectively. A complete Z series of 20–27 optical sections at 3–4 μ m intervals was acquired from each embryo, and the images were stacked. The images were reconstructed using FluoView software (Olympus). Cells labeled by TUNEL were judged to be apoptotic. The apoptotic index of the embryos was calculated as the percentage of apoptotic cells relative to the total number of cells.

Experimental design

Experiment 1

To determine whether CB had any side effects on germinal vesicle breakdown (GVBD), and to confirm its effectiveness in inhibiting the extrusion of the 1PB, porcine oocytes were subjected to IVM in the presence of 0, 1, 3, or 5 μ g/ml CB in the second half (from 22 h) of IVM. The nuclear status of oocytes was evaluated at 33 and 44 h IVM.

Experiment 2

The nuclear progression of oocytes exposed to CB during IVM was studied. COCs were matured *in vitro* in the absence (control) or presence (IVM-CB) of 1 μ g/ml CB from 22 h IVM. To evaluate nuclear status, oocyte samples were fixed at 33, 35, 37, 39, 41, or 43 h IVM. To compare chromosome morphologies, some control oocytes at 33 h (at the presumed metaphase-I (M-I) stage) and 44 h IVM (at the M-II stage) and IVM-CB oocytes at 44 h IVM were subjected to chromosome analysis.

Experiment 3

To study their ability to be activated, oocytes matured in the absence (control) or presence (IVM-CB) of 1 μ g/ml CB from 22 h IVM, were parthenogenetically activated at 46 h IVM. From the control group, only M-II oocytes

(with a visible 1PB) were subjected to parthenogenetic activation. After they had received the electrical pulse, the oocytes from this group were cultured *in vitro* in the presence of 5 µg/ml CB to inhibit extrusion of the 2PB (and thus to avoid haploidization of the activated egg). After 44 h IVM, all the IVM-CB oocytes without polar bodies (PBs) were cultured in CB-free IVC medium for 2 h and then subjected to parthenogenetic activation. After electrical stimulation, the oocytes in this group were cultured without CB to allow extrusion of 1PB for diploidization of the oocytes. After 8 h IVC, the stimulated oocytes were fixed. Activation status (presence of pronuclei) in polar body-bearing IVM-CB (IVM-CB PB+) and control oocytes was compared.

Experiment 4

In vitro embryonic development of IVM-CB and control oocytes was compared in this experiment. IVM-CB and control oocytes were activated as described in Experiment 3. In the IVM-CB group oocytes with (IVM-CB PB+ group) and without (IVM-CB PB− group) extruded PBs were selected under a stereomicroscope after 5 h IVC and cultured separately. On day 2, only cleaved embryos (two to six cells) were considered to be activated oocytes. On day 6 of IVC, the proportions and quality of blastocysts resulting from IVM-CB PB+, IVM-CB PB− and control oocytes were compared. Blastocyst quality was described by the number of blastomeres and the ratio of live and dead cells in the blastocyst.

Experiment 5

To verify the ploidy of parthenotes (especially the diploid status of PB extruded oocytes), day 6 blastocysts generated from IVM-CB PB+, IVM-CB PB−, and control oocytes were subjected to chromosome analysis.

Experiment 6

The frequencies of apoptotic cell nuclei in day 6 blastocysts were compared among the control, IVM-CB PB+ and IVM-CB PB− groups by TUNEL DNA fragmentation assay.

Statistical analysis

Each experiment was replicated at least three times. Statistical analyses in Experiment 1 were carried out by χ^2 -test. Data on nuclear progression, parthenogenetic activation and IVC, and TUNEL assay were analyzed by ANOVA followed by Duncan's multiple range test by using the GLM procedures of the Statistical Analysis System (SAS Institute, Inc., Cary, NC, USA). Percent data were transformed into arcsine before statistical analysis.

Results

Experiment 1

After 33 h IVM, there was no significant difference in the nuclear status of oocytes cultured in the absence or presence of 1, 3, or 5 µg/ml CB (Table 1; $P < 0.05$). In general, most of the oocytes were at the M-I stage (63.7–76.1%) or prometaphase-I stage (9.5–15.9%). The proportion of oocytes that passed through the anaphase-I (A-I) stage was not remarkable. After 44 h IVM, 76.7% control oocytes were at the M-II stage. However, most of the oocytes matured with CB had a single metaphase plate with no extruded PB (Table 2). The proportion of oocytes arrested at this M-I-like stage did not differ significantly among the groups treated with 1, 3, or 5 µg/ml CB (77.7, 91.0, and 82.6% respectively; $P < 0.05$). Almost no oocytes reached the M-II stage in the CB-treated groups. The frequency of degeneration and the proportion of oocytes with two metaphase plates or abnormal chromosome distribution did not differ significantly between the control and the treatment groups ($P < 0.05$).

Experiment 2

At 33, 35, and 37 h IVM, the proportion of oocytes that had reached or passed through the A-I stage (with two distinguishable sets of segregated chromosomes) did not differ significantly between the control and the CB-treated groups (Fig. 1; $P < 0.05$). Confirming the results of Experiment 1, the majority of oocytes were at the M-I stage at 33 h IVM (Figs 1, 2A and B). At 35 h IVM, an increased frequency of chromosome segregation was detected in both groups, but this rate did not differ between the control and the CB-treated groups (22.7 and 16.3% respectively; $P < 0.05$; Fig. 1). At this time, oocytes with segregating chromosomes were generally at the A-I stage in both groups (Fig. 2C). After 37 h IVM, the proportion of oocytes with segregated homologues peaked in both the IVM-CB and the control groups, with no significant difference (61.3 and 69.9% respectively; $P < 0.05$; Fig. 1). In both the control and the IVM-CB groups, most of the oocytes were at the telophase-I (T-I) stage. Nevertheless, protrusion of the 1PB was not observed in IVM-CB oocytes (Fig. 2D and E). In a large number of oocytes in the IVM-CB group, the two lumps of homologous chromosomes remained after their segregation and turned into two irregular sets of condensed chromosomes (Fig. 2G and H). By 39 h IVM, the proportion of oocytes with segregated chromosomes did not change in the control group (in this group they were mostly at the M-II stage; Fig. 2F, 72.6%). However, in the IVM-CB group, the percentage was significantly lower (35.4%; Fig. 1; $P < 0.05$). By this time, a remarkable number of IVM-CB oocytes had a

Table 1 Nuclear status of oocytes cultured for 33 h with CB at different concentrations.

CB in IVM ($\mu\text{g/ml}$)	Total no. of oocytes	No. (%) of oocytes at							No. (%) of oocytes with abnormal chromosome distribution	No. (%) of degenerated oocytes
		GV	GVBD	pM-I	M-I	A-I	T-I	M-II		
0	181	8 (4.7 $\pm$ 1.8)	1 (0.5 $\pm$ 0.5)	19 (9.8 $\pm$ 3.6)	138 (76.1 $\pm$ 3.6)	7 (3.9 $\pm$ 2.5)	3 (1.6 $\pm$ 0.7)	0	1 (0.7 $\pm$ 0.7)	4 (2.2 $\pm$ 1.2)
1	89	5 (5.7 $\pm$ 0.9)	1 (1.0 $\pm$ 0.7)	8 (9.5 $\pm$ 3.4)	66 (73.5 $\pm$ 3.2)	2 (2.3 $\pm$ 0.8)	1 (1.0 $\pm$ 0.7)	0	5 (5.4 $\pm$ 2.6)	1 (1.0 $\pm$ 0.7)
3	83	4 (4.9 $\pm$ 1.5)	0	13 (15.9 $\pm$ 2.1)	59 (70.4 $\pm$ 5.3)	2 (2.5 $\pm$ 1.2)	2 (2.3 $\pm$ 1.1)	0	2 (2.4 $\pm$ 1.2)	1 (1.3 $\pm$ 1.3)
5	119	15 (11.2 $\pm$ 4.1)	6 (5.4 $\pm$ 1.0)	17 (15.4 $\pm$ 2.6)	75 (63.7 $\pm$ 2.1)	5 (3.3 $\pm$ 1.9)	1 (0.5 $\pm$ 0.5)	0	0	0

GV, germinal vesicle; GVBD, germinal vesicle breakdown; pM-I, prometaphase-I; M-I, metaphase-I; A-I, anaphase-I; T-I, telophase-I; M-II, metaphase-II.

Table 2 Nuclear status of oocytes cultured for 44 h with CB at different concentrations.

CB in IVM ($\mu\text{g/ml}$)	Total no. of oocytes	No. (%) of oocytes at					No. (%) of oocytes with 2 chromo- some plates	No. (%) of oocytes with abnormal chromosome distribution	No. (%) of degen- erated oocytes
		1 metaphase plate (M-I like stage)	A-I+T-I	M-II					
0	220	28 (13.1 $\pm$ 2.8) ^b	2 (0.9 $\pm$ 0.6)	170 (76.7 $\pm$ 3.2) ^a	2 (1.0 $\pm$ 0.6)	2 (1.1 $\pm$ 0.7)	7 (3.2 $\pm$ 1.2)		
1	79	62 (77.7 $\pm$ 7.7) ^a	1 (1.3 $\pm$ 1.3)	1 (1.1 $\pm$ 1.1) ^b	6 (8.0 $\pm$ 4.6)	0	4 (5.3 $\pm$ 2.6)		
3	91	83 (91.0 $\pm$ 2.2) ^a	1 (1.1 $\pm$ 1.1)	1 (1.1 $\pm$ 1.1) ^b	1 (1.2 $\pm$ 1.2)	1 (1.2 $\pm$ 1.2)	2 (1.9 $\pm$ 1.9)		
5	121	100 (82.6 $\pm$ 2.5) ^a	2 (1.0 $\pm$ 1.0)	0	3 (2.2 $\pm$ 1.1)	2 (1.5 $\pm$ 0.8)	2 (1.1 $\pm$ 1.1)		

^{a,b}Different superscript letters denote significant differences ($P<0.01$). **Figure 1** Percentage of oocytes that had reached/or passed through the anaphase-I (A-I) stage with two distinguishable sets of segregated chromosomes. Numbers of oocytes examined at different culture periods in the two treatment groups are given in parentheses. Asterisks denote significant difference ($P<0.05$).

single set of chromosomes and were very often at the stage of reunion of previously segregated bunches of chromosomes (Fig. 2I). After 41–44 h IVM, the nuclear status of the control oocytes had not changed significantly, 74.8–76.9% oocytes in this group were at the M-II stage. However, in the IVM-CB group, the proportion of oocytes with two sets of segregated chromosomes dropped to its minimum level (9.1% at 41 h and 4.8% at 44 h IVM; Fig. 1). By 41 h IVM, most of the IVM-CB oocytes had a single set of metaphase chromosomes, usually arranged irregularly, but the appearance of microtubules (the initiation of spindle formation) was observed even with orcein staining under a phase-contrast microscope (Fig. 2J). By 44 h IVM, the formation of a meiotic spindle was completed in most of these oocytes, resulting in a single large metaphase plate with 38 chromosomes (Fig. 2K and L).

Experiment 3

In the control group, 73.5% of the oocytes became diploid (described by the presence of a PB) during IVM for 46 h; after electrical stimulation, the proportion of diploid embryos in the IVM-CB group was significantly lower (39%; Table 3). Eight hours after electrical stimulation, a higher proportion of PB+ oocytes in the IVM-CB group than in the control had female pronuclei (Table 3; $P<0.05$). Interestingly, 63.7% (62 out of 97) of the PB– IVM-CB oocytes had also formed female pronuclei. These embryos were considered to be tetraploid owing to the failure of diploidization.

Figure 2 Nuclear morphology of oocytes during *in vitro* maturation (IVM). Scale bar represents 10 µm. (A) Metaphase-I (M-I) stage nucleus of IVM-CB oocyte at 33 h IVM; (B) M-I stage nucleus of a control oocyte at 33 h; (C) A-I stage IVM-CB oocyte at 35 h; (D) a control oocyte at the telophase-I (T-I) stage at 37 h. Note the protrusion of the future polar body; (E) IVM-CB oocyte at the T-I stage at 37 h. Despite chromosome segregation, the polar body is not extruded; (F) M-II-stage control oocyte at 39 h; (G) and (H) IVM-CB oocytes with two bunches of segregated chromosomes inside at 37 h. *Note:* the meiotic spindle is not visible by orcein staining; (I) an IVM-CB oocyte at 39 h – reunion of the two sets of chromosomes; (J) an IVM-CB oocyte at 41 h. The metaphase chromosomes are in one group. Microtubules are visible, but the positions of the chromosomes are irregular; (K) and (L) IVM-CB oocytes at 44 h. The chromosomes are arranged in a single large metaphase plate. *Note:* unlike the real M-I stage (see above) 38 chromosomes can be distinguished in this metaphase plate.

Experiment 4

On day 2, the proportions of cleaved embryos did not differ significantly between the control and IVM-CB PB+ oocytes (78.6 and 80% respectively) after activation (Table 4). However, IVM-CB PB– oocytes had a significantly lower (51.0%) cleavage rate than control and IVM-CB PB+ oocytes ($P<0.05$; Table 4). Similar to the cleavage rates, the blastocyst formation rates of the cleaved embryos on day 6 were similar between the control and IVM-CB PB+ oocytes (45.8 and 42.8% respectively), and both were significantly higher than in IVM-CB PB– oocytes (25.5%; $P<0.05$). No significant difference was detected in blastocyst characteristics between embryos obtained from control and IVM-CB PB+ oocytes in terms of total cell numbers (39.9 and 44.4 respectively) and the proportion of dead blastomeres (4.3 and 2.9% respectively) in blastocysts ($P<0.05$; Table 4). In

contrast, the total cell number was significantly lower in embryos obtained from IVM-CB PB– oocytes than in those obtained from control or IVM-CB PB+ oocytes ($P<0.05$).

Table 3 Activation status of oocytes 8 h after stimulus.

Treatment	All oocytes matured	No. (%) of diploid oocytes (PB+)	Female pronucleus (% diploid oocytes)
Control	188	139 (73.5 ± 2.6) ^a	93 (66.9 ± 7.8) ^b
IVM-CB PB+	166	69 (39.0 ± 4.5) ^b	57 (82.9 ± 5.7) ^a

^{a,b}Different superscript letters denote significant difference ($P<0.05$). Control, diploid parthenote obtained from IVM oocytes at M-II (with extruded polar body), activated and *in vitro* cultured in the presence of CB. IVM-CB PB+, oocyte matured in the presence of CB and with an extruded polar body after activation. Thus such embryos are presumably diploid.

Table 4 *In vitro* development and quality of embryos.

Treatment	No. of oocytes subjected to activation	Day 2	Day 6		
		Cleaved (%)	No. of blastocysts (% cleaved)	No. of cells in blastocyst	Percentage of dead cells
Control	282	222 (78.6 ± 1.7) ^a	100 (45.8 ± 4.6) ^a	39.9 ± 2.1 ^a	4.3 ± 0.2
IVM-CB PB+	238	189 (80.0 ± 1.8) ^a	83 (42.8 ± 6.6) ^a	44.4 ± 2.5 ^a	2.9 ± 0.2
IVM-CB PB−	406	212 (51.0 ± 1.9) ^b	54 (25.5 ± 3.3) ^b	26.2 ± 1.2 ^b	4.9 ± 0.1

^{a,b}Different superscript letters denote significant difference ($P < 0.05$).

Control, embryos are diploid; IVM-CB PB+, embryos are presumably diploid; IVM-CB PB−, oocyte matured in the presence of CB and with no extruded polar body after activation. These embryos were considered to be tetraploids.

Experiment 5

The majority (80.0%) of control embryos were found to be diploid (Table 5). However, some embryos had haploid status (8.5%) and occasionally polyploid embryos were found in the control group. Similarly, diploid status was the most frequent (51.7%) in IVM-CB PB+ embryos, but the frequency of polyploidy (especially triploidy) was higher than in the control group (Table 5). Although most of those oocytes that failed to extrude a PB (IVM-CB PB−) after activation were polyploid (73.5%; mainly tetraploid) or mixoploid (15.7%), a few diploid (10.5%) embryos were also found in this group, probably as a result of failure of PB detection (Fig. 3).

Experiment 6

After TUNEL staining of day 6 blastocysts, the proportion of apoptotic cells did not differ significantly between the control, IVM-CB PB+, and IVM-CB PB− embryos (7.3, 6.3, and 7.0% respectively; Table 6 and Fig. 6).

Discussion

The method used most widely to obtain diploid mammalian parthenotes is the activation of mature (at the M-II stage) oocytes followed by inhibition of 2PB extrusion. However, the experiments of Kubiak *et al.* (1991) showed that diploid status of parthenotes could also be achieved in mice by the inhibition of 1PB extrusion using cytochalasin D before the activation procedure. Our results show that, in porcine oocytes, a CB concentration of 1 µg/ml is enough to inhibit extrusion of the 1PB. The inhibitory effect of CB on meiosis has been shown in several papers. Wassarman *et al.* (1976) reported that CB treatment causes meiotic

arrest of mouse oocytes at the M-I stage without any effect on the GVBD, chromatin condensation, or spindle formation. However, Kubiak *et al.* (1991) showed that when mouse oocytes were exposed to cytochalasin D during IVM, segregation of homologous chromosomes occurred without extrusion of the PB, resulting in two meiotic spindles in the oocytes. Later these spindles merged into one single spindle. We used CB instead of cytochalasin D during IVM, and pigs instead of mice, and we confirmed that the effect was almost the same (Fig. 4). Our results showed that in pigs under the influence of CB, as in mice under cytochalasin D, segregation of homologous chromosomes occurs in the same manner as in the control groups. However, following the T-I stage, the spindle disappears (becomes invisible upon phase-contrast microscopic observation) and the chromosomes are then located in two irregular bunches within the oocyte. These chromosome sets, then unite into a single and irregular bunch of chromosomes. As shown in Fig. 1, reunion of the homologous chromosome sets occurs in a short time (2–3 h). The mechanism of this phenomenon is not yet clear. During this time, no signs of spindle formation were observed in the oocytes by phase-contrast microscopy. After reunion of homologous chromosomes, at about 3–5 h, a single meiotic spindle was formed, with an evenly arranged metaphase plate. Despite their similar appearances and ploidy, the structure of this spindle was different from an M-I spindle. A real M-I spindle bears 19 pairs of attached homologous chromosomes and 19 (n) (or fewer) bivalents can be distinguished in the metaphase plate (Fig. 2A and B); however, in the metaphase plates of oocytes matured for 44 h in the presence of CB, 38 chromosomes could be distinguished (Fig. 2L). This clearly suggests that this metaphase plate forms a plane of the two chromatid metaphase chromosomes arranged next to each other. This metaphase plate has a structure

Table 5 Ploidy of parthenogenetic blastocysts produced by different methods.

Treatment	No. of blastocysts	n	$2n$	$3n$	$4n$	$> 4n$	Mixoploid
Control	35	3 (8.5%)	28 (80.0%)	1 (2.8%)	1 (2.8%)	2 (5.7%)	0
IVM-CB PB+	29	3 (10.3%)	15 (51.7%)	5 (17.2%)	3 (10.3%)	2 (6.8%)	1 (3.4%)
IVM-CB PB−	19	0	2 (10.5%)	1 (5.2%)	10 (52.6%)	3 (15.7%)	3 (15.7%)

Figure 3 Metaphase chromosome sets prepared from control and IVM-CB-derived blastomeres. (A) Haploid ($n=19$); (B) diploid ($2n=38$); (C) triploid ($3n=57$); (D) tetraploid ($4n=76$); and (E) decaploid ($10n=190$) chromosome sets.

similar to that of a metaphase plate at the M-II stage, but it contains twice as many chromosomes (Figs 2A, B, and L and 5B and C). Comparison of the chromosome morphologies of oocytes matured in the presence of CB and control oocytes matured for 33 (at the M-I stage) and 44 h (at the M-II stage), confirmed this finding. Real M-I oocytes possessed 19 ring-shaped, symmetrical complexes of homologous chromosomes (Fig. 5A), but both M-II- and CB-treated oocytes displayed similar separate chromosomes with two chromatids. M-II oocytes had 19 two-chromatid chromosomes (here, the chromosome sets of the oocyte and the PB could be clearly distinguished), whereas, the number of chromosomes in the CB-treated oocytes was double in the M-II oocytes (Fig. 5B and C).

Characterization of IVM-CB oocytes as M-I stage arrested oocytes only by the presence of a single spindle and the absence of a PB can, therefore, be misleading. Nevertheless, a number of papers have reported the M-I arrest of mouse (Wassarman *et al.* 1976, Soewarto *et al.* 1995) and porcine (Wang *et al.* 2000) oocytes after IVM in the presence of cytochalasin D. In our *in vitro* porcine embryo production system, meiotic arrest after GVBD, but before the M-II stage occurs in about 20% of the oocytes during IVM (Kikuchi *et al.* 1999, Somfai *et al.* 2005). Previously, when the nuclear status of oocytes was evaluated after 44 h IVM by whole mounting and orcein staining, oocytes bearing a metaphase plate, but without PB, were considered and referred to as M-I arrested eggs. However, as described earlier, the presence of a single meiotic spindle and the lack of a PB do not necessarily indicate that the oocyte was arrested at the definite M-I stage. It is possible that, during the meiotic arrest of IVM oocytes, a similar actin-depolymerization-related phenomenon of chromosome reunion and rearrangement occurs by an obscure mechanism after successful segregation of the homologous chromosomes, resulting in an 'M-I like' nuclear stage, which is also characterized by a single metaphase plate with no PB. This hypothesis is supported by the finding that under certain culture conditions, the polymerization of actin filaments is insufficient in porcine embryos (Wang *et al.* 1999).

Table 6 Rate of DNA fragmentation in parthenogenetic blastocysts produced by different methods on day 6 of in IVC.

Treatment (ploidy)	No. of embryos examined	Total no. of cells (mean $\pm$ S.E.M.)	Percentage of cells with apoptotic nuclei (mean $\pm$ S.E.M.)
Control ($2n$)	38	39.3 $\pm$ 2.5 ^a	7.3 $\pm$ 2.3
IVM-CB PB+ ($2n$)	38	43.5 $\pm$ 0.9 ^a	6.3 $\pm$ 0.4
IVM-CB PB- ($4n$)	31	29.7 $\pm$ 1.9 ^b	7.0 $\pm$ 1.0

^{a,b}Different superscript letters denote significant difference ($P<0.05$).

Figure 4 Scheme of the two methods used in this study to produce diploid parthenotes. (A) Traditional method. GV oocytes (1) are cultured *in vitro* without CB for 46 h. After about 33 h IVM, the homologous paternal and maternal chromosomes form bivalents (M-I stage, 2). At this stage, the oocyte is tetraploid. Later segregation of the homologous chromosomes occurs (3) with extrusion of the first polar body (M-II stage, 4), so the oocyte becomes diploid. After electrical stimulation, the M-II oocytes are cultured in the presence of CB for 3 h. During this period, the sister chromatids of the chromosomes segregate, but without extrusion of the second polar body (5); thus the embryo retains its diploid status (6). (B) Parthenogenesis with the inhibition of homologous chromosome segregation. After maturation for 22 h in CB-free medium (7), GV oocytes are matured in the presence of CB for an additional 22 h. The M-I stage (8) is followed by the segregation of homologues (9), but extrusion of the polar body is inhibited, so that all chromosomes remain in the oocyte (10). During the last phase of IVM, the segregated chromosomes rearrange into an M-I-like metaphase plate (11). After 2 h culture in CB-free medium, the oocytes are electrically stimulated and subsequently cultured in CB-free medium. After the stimulation, segregation of sister chromatids (12) and extrusion of the polar body (13) occur, thus the tetraploid oocyte becomes diploid (14). Paternal and maternal chromosomes are represented by red and black respectively. Note that the proportions of paternal and maternal chromosomes are not similar after application of the two methods (see 5 and 13).

This suggestion might have importance not only in porcine *in vitro* embryo production systems, but also in human reproduction, as maturation arrest of oocytes before the M-II stage is considered to be an important cause of infertility (reviewed by Mrazek & Fulka 2003). Further studies are needed to clarify the exact process of post-GVBD meiotic arrest in mammalian oocytes during maturation.

After parthenogenetic activation, pronuclear formation in control and IVM-CB oocytes occurred at similar frequencies, suggesting that the cytoplasmic maturation required for oocytes to be activated was not compromised by CB treatment during IVM. However, only a relatively low (39.0%) proportion of the IVM-CB oocytes extruded PBs (Table 3), and the rest retained their complete chromatin sets and remained tetraploid. This was confirmed by ploidy analysis of the blastocysts obtained from these oocytes (Table 5). Failure of PB extrusion after activation of IVM-CB oocytes might occur as a result of unknown side effects of CB. In our experiments, after the last 22 h IVM in the presence of CB, oocytes were washed in CB-free IVC medium and additionally cultured for 2 h without CB. Thus, it is likely that this interval is not long enough for the oocytes to fully recover from the effects of the CB. After long-term exposure (in our case 22 h) of oocytes to CB,

Figure 5 Morphology of porcine chromosomes. (A) Chromosomes prepared from an M-I stage oocyte at 33 h IVM. Note: the homologous chromosomes form bivalents; (B) chromosomes prepared from an M-II stage control oocyte at 44 h IVM. Chromosomes incorporated by the polar body are shown in the small micrograph; (C) chromosomes prepared from a porcine oocyte matured *in vitro* for 44 h in the presence of CB.

the oocytes/embryos probably retain the effects of the CB for a long time, even in CB-free medium. It is probable that the ability of IVM-CB oocytes to extrude PB would increase with a longer CB-free culture interval before the oocyte activation. However, extended IVM culture would raise the problem of oocyte aging, which might cause a high frequency of oocyte fragmentation and thus a decreased blastocyst formation rate (Kikuchi *et al.* 1995). The majority (80.0%) of the control embryos were found to be diploid, but in the IVM-CB PB+ group, only 51.7% of blastocysts were diploid and the rest had triploid or tetraploid chromosome sets. Cytokinesis with abnormal karyokinesis in porcine embryos has been reported under the influence of cytochalasin D, resulting in binucleated blastomeres (Wang *et al.* 2000). Thus, the alterations in chromosome numbers can also be due to side effects of long-term CB treatment, which may result in abnormal segregation of chromosomes during PB extrusion and/or embryonic division, followed by irregular distribution of chromosomes in the sister cells. Those IVM-CB oocytes that failed to extrude PBs had lower rates of female pronucleus formation than did PB-extruded oocytes. This is probably because this group included some oocytes arrested at the GV stage, which could not be activated. This might explain the decreased cleavage rate in this group as well.

During IVC of parthenotes, the developmental rates of cleaved embryos to the blastocyst stage were similar in the diploid control and IVM-CB (IVM-CB PB+) groups, and significantly higher than those in the IVM-CB PB- group; the number of cells in the blastocysts were significantly lower in this last group than in the other groups. These low numbers may be caused by the tetraploid status of the embryos in this group. Supporting this suggestion, a lower rate of development to the blastocyst stage of tetraploid porcine embryos generated by the fusion of two cultured cell embryos, compared with diploid embryos, was published recently (Prochazka *et al.* 2004).

Our results show the similarities in developmental competence of diploid parthenotes obtained from the inhibition of 1PB extrusion or 2PB extrusion. The differences between the two methods are shown schematically in Fig. 4. Although both the methods result in diploid embryos, the genotype of the parthenotes is not the same. Considering the fact that the inhibition of 2PB extrusion maintains the diploid status of the embryo by preventing polar body extrusion after the segregation of sister chromatids from M-II oocytes (Fig. 4A). The genomes of such embryos contain genes that are mainly in a homozygous state despite crossover, which occurs in the prophase of the first meiotic division and may alter the genotypes of the sister chromatids. This is

supported by the results of Kubiak *et al.* (1991), who found that the majority of mouse parthenotes produced by the inhibition of 2PB extrusion from activated oocytes of F1 (C57Bl/DBA2) mice were homozygous for the glucose-phosphate isomerase gene. On the other hand, inhibition of 1PB extrusion prevents the extrusion of homologous chromosomes (Fig. 4B). Diploidization of such oocytes can be achieved by ensuring sister chromatid segregation, and their extrusion with a polar body after activation. In such embryos, the proportion of paternal and maternal chromosomes is the same as in the oocyte (and also the oocyte donor). These parthenotes have been referred to as true genetic clones of the oocyte donor animals (Kubiak *et al.* 1991). Nevertheless, the effect of crossover has to be reckoned as a factor that can modify the genotype of the oocyte during prophase. Little is known about the frequency and significance of crossover in oocytes. In sheep, an average of 1.3 chiasmata has been reported per bivalent, whereas this value is 1.19 per bivalent in bovines (Jagiello *et al.* 1974). We were unable to find any literature on chiasma frequency in porcine oocytes.

Development to the blastocyst stage of diploid parthenotes produced by the inhibition of homologous chromosome extrusion or sister chromatid extrusion was similar and no difference was found in the proportions of dead and apoptotic cells. This suggests that early development and cell death in diploid porcine embryos up to the blastocyst stage are not affected by the frequency of occurrence of homozygous genes. Previous papers have reported the relationship between abnormal ploidy and the rate of apoptosis in embryos. The correlation between the two has been suggested in human embryos (Delimitreva *et al.* 2005), and haploid status has been proven to induce apoptosis in mice (Liu *et al.* 2002). Interestingly, we found that despite their low developmental capacity, tetraploid embryos (IVM-CB PB-) had a rate of apoptosis similar to that of diploid parthenotes (Table 6, Fig. 6).

In conclusion, we have reported the production of diploid porcine parthenotes by the inhibition of 1PB extrusion. Inhibition of actin polymerization by CB during IVM did not affect homologous chromosome segregation, but prevented the extrusion of their polar body and led to their rearrangement into an 'M-I like' spindle. The similar *in vitro* development of diploid parthenotes obtained by the inhibition of homologous chromosome or sister chromatid extrusion suggests that heterosis does not affect *in vitro* embryo development to the blastocyst stage. Further studies on the events of cytoskeletal malfunction related to meiotic arrest are needed in future to improve our understanding of the maturation arrest of porcine oocytes during IVM.

Figure 6 Parthenogenetic porcine blastocysts after TUNEL staining on day 6 IVC. (A) Diploid blastocyst obtained by the inhibition of sister chromatid extrusion (control); (B) diploid blastocyst obtained by inhibition of homologous chromosome segregation (IVM-CB PB+); a tetraploid blastocyst obtained by the inhibition of homologous chromosome segregation (IVM-CB PB-). Chromatin content is stained red with PI. Yellow represents DNA fragmentation as a combination of PI staining and a positive (green) TUNEL reaction. Original magnification, $\times 400$.

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