

## THE USE OF DIFFERENT METHODS OF OOCYTE ACTIVATION FOR GENERATION OF PORCINE FIBROBLAST CELL NUCLEAR-TRANSFERRED EMBRYOS\*

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### Abstract

The present study was undertaken in order to examine the preimplantation developmental capacity of fibroblast cell cloned pig embryos produced applying different methods for activation of reconstituted oocytes. In Groups IA and IB, nuclear-transferred (NT) oocytes that had descended from either adult cutaneous or foetal fibroblast cells were subjected to simultaneous fusion and activation mediated by DC pulses. In Groups IIA and IIB, they underwent sequential physical and chemical activation involving treatment with DC pulses and subsequent exposure to 10  $\mu\text{M}$  calcium ionomycin, followed by incubation with 1 mM 6-dimethylaminopurine and 5  $\mu\text{g mL}^{-1}$  cycloheximide. The proportions of cloned embryos that reached the morula and blastocyst stages were 203/327 (62.1%) and 115/327 (35.2%) or 270/361 (74.8%) and 167/361 (46.3%) in Groups IA or IB, respectively. In turn, in Groups IIA and IIB, the morula and blastocyst formation rates yielded 147/298 (49.3%) and 79/298 (26.5%) or 228/359 (63.5%) and 118/359 (32.9%), respectively. In summary, the simultaneous fusion and electrical activation of NT oocytes reconstructed with both types of nuclear donor fibroblast cells resulted in considerably higher *in vitro* developmental outcomes of porcine cloned embryos than the sequential electrical and chemical activation. Moreover, to our knowledge, the use of electric pulses followed by an additional activation with calcium ionomycin at a double dose and subsequent exposure to the combination of 6-dimethylaminopurine and cycloheximide at the twofold decreased concentrations is the first to stimulate gilt or sow oocytes that had been reconstituted by somatic cell nuclear transfer.

**Key words:** pig, somatic cell nuclear transfer, clonal cybrid, simultaneous fusion and electrical activation, sequential electrical and chemical activation, DC pulse, calcium ionomycin, 6-dimethylaminopurine, cycloheximide

The commonly used source of nuclear recipient cells in the somatic cell cloning of pigs are *in vivo*-matured (post-ovulatory) oocytes (Boquest et al., 2002; De

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Sousa et al., 2002; Lee et al., 2003 a, b; Ramsoondar et al., 2003) or *in vitro*-matured oocytes (Samiec et al., 2003; Lee et al., 2003 a; Brunetti et al., 2008; Skrzyszowska et al., 2008), whose meiotic cell cycle is reversibly blocked at the second metaphase (MII) stage. One of the most important factors that significantly affect the developmental competences of porcine cloned embryos is the artificial activation of oocytes reconstructed with somatic cell nuclei. The ability of an artificial stimulus to activate MII stage-arrested oocytes and to initiate embryo development following resumption and completion of meiosis is essential for successful cloning by somatic cell nuclear transfer. This ability is especially important for species such as the pig where relatively little is known about early embryonic development and where *in vitro* handling procedures have not been optimized.

In the swine somatic cell cloning technology, commonly used activating stimuli are physical agents such as electric (DC) pulses (Park et al., 2002; Martinez Diaz et al., 2002, 2003; Im et al., 2004; Hölker et al., 2005), or chemical agents such as specific ionophore antibiotics (e.g., calcium ionomycin,  $\text{Ca}^{2+}$  ionophore A23187/calcimycin) (Betthausen et al., 2000; Boquest et al., 2002; Yin et al., 2002; Hyun et al., 2003) or thimerosal in combination with dithiothreitol (Tao et al., 2000; Hao et al., 2006; Im et al., 2006; Whitworth et al., 2009). The current intensive studies on improving activation methods of porcine nuclear-cytoplasmic hybrids (i.e., clonal cybrids) are chiefly aimed at optimizing technical parameters of electrical field involving strength, duration of DC pulses, number of pulses and time interval between them (Park et al., 2002; Martinez Diaz et al., 2002, 2003; Im et al., 2004). Alternatively, and more often, these investigations are focused on combining an activating stimulus/stimuli, most frequently calcium ionomycin or DC pulses with exogenous agents that non-specifically or specifically block the activity of cyclin-dependent protein kinases (CDKs), which include, e.g., maturation/meiosis-promoting factor (MPF) and cytostatic factor (CSF) activity-mediated mitogen-activated protein kinases, i.e., extracellular-regulated protein kinases (MAPKs/ERKs). This group of CDK repressory agents involves such members as 6-dimethylaminopurine (6-DMAP) (Betthausen et al., 2000; Boquest et al., 2002; Roh and Hwang, 2002; Hyun et al., 2003; Hölker et al., 2005) and butyrolactone I (BTRL-I) (Yin et al., 2002), which are, respectively, non-selective inhibitor of, among others, MPF- and MAPK/ERK-related  $\text{p34}^{\text{cdc}2}$ /CDK1 kinases and selective inhibitor of MPF-related  $\text{p34}^{\text{cdc}2}$ /CDK1 kinase. Another strategy is the treatment of reconstructed pig oocytes with activating factors (physical or chemical), followed by their exposure to the agents that reversibly inhibit protein synthesis. Here an example is cycloheximide (CHXM), which suppresses the re-translation of cyclin B (i.e., regulatory subunit of the heterodimeric MPF enzyme complex) following resumption of oocyte meiosis from metaphase II stage-arrest (Cheong et al., 2000; Yin et al., 2002; Martinez Diaz et al., 2002, 2003; Lee et al., 2003 a).

Nuclear-transferred (NT) pig oocytes are artificially stimulated using one of the three experimental protocols: 1) electrical, chemical or physicochemical delayed activation (i.e., post-activation); 2) simultaneous fusion and electrical activation (SF-EA) or simultaneous electrofusion and physicochemical activation, as well as 3) sequential (combined) electrical and chemical activation. In the first activation

protocol, the cell nuclei of whole donor somatic cells or somatic cell-descended karyoplasts at G0/G1 or G2/M stages of cell cycle are introduced via electrofusion or intracytoplasmic microinjection into enucleated Metaphase II oocytes (MII ooplasts), which are activated by physical, chemical or combined (physicochemical) stimuli 30 minutes to several hours after nuclear transfer (Boquest et al., 2002; Lee et al., 2003 a; Ramsoondar et al., 2003; Hyun et al., 2003; Samiec et al., 2003; Skrzyszowska et al., 2005; Brunetti et al., 2008). In the second activation protocol, the cell nuclei of whole somatic cells at G1 or G0 phase of mitotic cycle are introduced into non-activated MII-staged host ooplasm via DC pulse-induced fusion. The clonal nuclear-cytoplasmic hybrids generated in that way are simultaneously electroactivated (Dai et al., 2002; Lai et al., 2002; Park et al., 2002; Hyun et al., 2003; Lee et al., 2003 b; Skrzyszowska et al., 2008) or activated through the use of electric pulses, which had previously triggered fusion of ooplast-nuclear donor cell couplets, followed by their treatment with chemical agents such as, e.g., CHXM or 6-DMAP (Roh and Hwang, 2002; Martinez Diaz et al., 2002, 2003). In turn, the third activation protocol includes the SF-EA followed by an additional treatment of the reconstituted oocytes with chemical factors, which is initiated after a 1–2-h delay (Bethausser et al., 2000; Hyun et al., 2003; Skrzyszowska et al., 2008). The concentration of calcium cations in the fusion/activation medium affects not only the transition from meiotic to mitotic control of cell cycle of clonal cybrids, but also the degree of ploidy of reconstructed zygotes as a result of both emission of second polar body and formation of pseudopronucleus/pseudopronuclei. It has been recently reported that increasing the  $\text{Ca}^{2+}$  concentration in fusion/activation medium from 0.05 mM or 0.1 mM to 1.0 mM enhances blastocyst formation rates of NT embryos originating from foetal fibroblast cells (Cheong et al., 2002; Im et al., 2004; Lee et al., 2005).

This is the first report in which the effect of different protocols of oocyte artificial activation on the preimplantation development of cloned pig embryos was examined depending on the type of nuclear donor fibroblast cells. The origin and type of nuclear donor cells as well as *in vitro* techniques utilized to prepare viable donor nuclei with a relatively high cloning competence are significant aspects that determine the efficiency of generating the embryos and/or offspring by somatic cell nuclear transfer (SCNT) technology. To date, there are many studies aimed at using the fibroblast cells in the swine SCNT technology. Among them, foetal fibroblast cells have already been applied to the production of nuclear-transferred embryos/offspring because of their rapid growth rate and potential for multiple mitotic cell divisions before replicative senescence under *in vitro* culture circumstances (Boquest et al., 2002; Lee et al., 2003 b, 2005; Im et al., 2004). In turn, among adult somatic cells, only ear-derived cutaneous fibroblast cells have been utilized in the generation of porcine cloned embryos and/or offspring (Park et al., 2002; Roh and Hwang, 2002; Lee et al., 2003 a, b). Nevertheless, none of the studies on somatic cell cloning in pigs have compared the *ex vivo* developmental capacity between SCNT embryos descended from oocytes receiving cell nuclei of fibroblast cells of different lineages and then stimulated by using different activation strategies. These strategies involved simultaneous fusion and electrical activation (SF-EA) and sequential (combined) electrical and chemical activation. To our knowledge, the pro-

duction of porcine nuclear-transferred embryos with the use of three-grade physical and chemical activation of reconstituted oocytes through treatment with DC pulses, followed by their delayed exposure to increased dosage of 10  $\mu\text{M}$  calcium ionomycin for an extended incubation time of 7 to 9 min and subsequently to combination of 6-dimethylaminopurine/cycloheximide at diminished concentrations (1 mM and 5  $\mu\text{g mL}^{-1}$ , respectively) for 2 h has not yet been reported. In this paper we investigated the effect of oocyte activation methods on the *in vitro* developmental competences of cloned pig embryos that had originated from cell nuclei of adult ear skin-retrieved or foetal fibroblasts.

## Material and methods

### Collection of pig oocytes and their *in vitro* maturation

Abattoir-derived ovaries were collected from prepubertal and postpubertal gilts and sows. Cumulus-oocyte complexes (COCs) were recovered by aspiration of follicular fluid from 2- to 6-mm antral ovarian follicles. The COCs, with evenly granulated ooplasm and several uniform layers of compact cumulus cells, were washed three times in HEPES-buffered Tissue Culture Medium 199 (TCM 199-HEPES; Gibco BRL, Life Technologies Inc., Grand Island, NY, USA) with the addition of 4  $\text{mg mL}^{-1}$  bovine serum albumin (fraction V; BSA-V, Sigma-Aldrich Chemical Co., St. Louis, MO, USA). The COCs were selected for *in vitro* maturation under atmospheric conditions. The maturation medium comprised 25 mM HEPES and 26.18 mM sodium bicarbonate ( $\text{NaHCO}_3$ )-buffered TC 199 medium (Gibco BRL), supplemented with 10% porcine follicular fluid (pFF), 0.6 mM *L*-cysteine (Sigma-Aldrich), 10  $\text{ng mL}^{-1}$  recombinant human epidermal growth factor (rhEGF, Sigma-Aldrich), 1 mM dibutyryl cyclic adenosine monophosphate (db-cAMP, Sigma-Aldrich) and 0.1 IU  $\text{mL}^{-1}$  human menopausal gonadotropin (hMG, Sigma-Aldrich). Approximately 50 to 60 COCs were cultured in the db-cAMP- and hMG-supplemented medium for 20 h at 39°C in a 100% water-saturated atmosphere of 5%  $\text{CO}_2$  and 95% air. The oocytes were then cultured for 22 to 24 h in fresh maturation medium that did not contain db-cAMP and hMG. After maturation, expanded cumulus cells and corona cells were completely removed by vigorous pipetting of the COCs in the presence of 0.5  $\text{mg mL}^{-1}$  hyaluronidase (Sigma-Aldrich) in 500  $\mu\text{L}$  of HEPES-buffered TCM 199 for 1 to 2 min. The metaphase II-staged oocytes, which had been selected on the basis of accepted morphological criteria involving evenly granulated, dark ooplasm and the presence of distinctly expelled first polar bodies, provided a source of recipient cells for exogenous cell nuclei in the somatic cloning procedure.

### Establishment of porcine fibroblast cell lines

Foetal fibroblast cells were isolated from conceptuses (11.0 cm in length), following the removal of their heads and internal organs. The foetuses were obtained from a slaughterhouse; therefore, the age and breed of the foetuses were un-

known. Adult fibroblast cells were collected from an ear-skin biopsy obtained from a 4-month-old (i.e., prepubertal) gilt. Foetal body-retrieved or adult dermal tissue samples were cut into small pieces using a tissue chopper (0.5 mm), and tissue explants were placed in a culture flask with a small volume of Dulbecco's Modified Eagle Medium (DMEM, Gibco Invitrogen Co., UK). This volume was enough to wet the bottom of the flask, but not too wet so as to cause the tissue pieces to float. For the first 2 to 3 days of incubation, a few more drops of medium were added every 2 to 3 hours, and then gradually more medium was added when pieces had firmly attached. Cultures were replenished 2 to 3 times per week. For the primary cultures of dermal fibroblasts, modified Dulbecco's Minimum Essential Medium, which had been supplemented with 10% foetal bovine serum (FBS, Sigma-Aldrich), 5 ng mL<sup>-1</sup> recombinant human basal fibroblast growth factor (rh-bFGF, Sigma-Aldrich), 2 mM non-essential amino acids (NEAA, Sigma-Aldrich), 2 mM *L*-glutamine (Sigma-Aldrich), 0.36 mM sodium pyruvate (Sigma-Aldrich) and 1% antibiotic-antimycotic solution (Sigma-Aldrich), was used. After removal of the explants (Days 5 to 6), monolayers of fibroblast cells were harvested using DMEM supplemented with 0.25% trypsin-EDTA (Sigma-Aldrich). Cells were subsequently cultured up to a total confluency, and then passaged at least three times. Cells harvested from flasks by trypsinization were washed in 10 mL HEPES-buffered Tissue Culture Medium 199 (TCM 199-HEPES, Sigma-Aldrich) with 10% FBS and centrifuged at 200 × *g* for 10 min. The cell pellet was then suspended in FBS containing 9% dimethyl sulfoxide (DMSO, Sigma-Aldrich) before freezing in a Minicool freezer. Cryopreserved donor cells were thawed at 37°C and 200 µL of FBS was added. The suspension was kept at room temperature for 10 min, and then 800 µL of cell culture medium was added before the cells were cultured up to a total confluency state (in the medium supplemented with 10% FBS) in order to synchronize their mitotic cycle at the G1/G0 stages through 24–48-h contact inhibition of their migration and proliferative growth.

Prior to use for somatic cell nuclear transfer, the cultured clonal fibroblast cell lines (between passages 3 and 5), which had undergone the contact inhibition of mitotic divisions after they reached the 100% confluency, were trypsinized, followed by their centrifugation at 300 × *g* for 5 min. The supernatant was removed, and 50 µL of manipulation medium was added.

### **Creation of nuclear transfer-derived pig embryos**

Cumulus-denuded oocytes were incubated in the maturation medium supplemented with 0.4 µg mL<sup>-1</sup> demecolcine (DMCC, Sigma-Aldrich) for 50–60 min at 39°C. Afterwards, the treated oocytes were transferred into a glass micromanipulator chamber filled with TCM 199 containing 4 mg mL<sup>-1</sup> BSA-V and 7.5 µg mL<sup>-1</sup> cytochalasin B (CB, Sigma-Aldrich). Maternal chromosomes (metaphase plates), which had been allocated into the chemically induced protrusion of the plasma membrane, were removed microsurgically. Enucleation was accomplished by gently aspirating the ooplasmic cone, which contained the condensed chromosome mass, with the aid of a bevelled micropipette of 20- to 25-µm external diameter. Following enucleation, the resulting cytoplasts were washed extensively in HEPES-buffered TCM 199/BSA

and held in this CB- and DMCC-free medium until microinjection of donor nuclei. Reconstruction of enucleated oocytes was achieved by electrofusion of whole (foetal or adult dermal) fibroblast cells with ooplasts. Single nuclear donor cells were inserted into the perivitelline space of previously enucleated oocytes. The resulting somatic cell-ooplast couplets were transferred to a fusion/activation chamber filled with electroporation medium. Nuclear transfer-derived oocytes were artificially stimulated using one of the two experimental protocols: simultaneous fusion and electrical activation (SF-EA) or sequential (combined) electrical and chemical activation. In the first activation protocol, the complexes of enucleated oocytes and fibroblast cells were simultaneously fused and activated by application of two successive DC pulses of  $1.2 \text{ kV cm}^{-1}$  for  $60 \text{ }\mu\text{sec}$ , which were delivered with the use of a BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA). The fusion/activation medium consisted of  $0.3 \text{ M D-mannitol}$  (Sigma-Aldrich) supplemented with  $1.0 \text{ mM CaCl}_2$  (Sigma-Aldrich),  $0.1 \text{ mM MgSO}_4$  (Sigma-Aldrich) and  $0.2 \text{ mg mL}^{-1}$  fatty acid free BSA (FAF-BSA, Sigma-Aldrich). In turn, the second activation protocol included the SF-EA followed by an additional treatment of the reconstituted oocytes with chemical factors (calcium ionomycin and 6-dimethylaminopurine/cycloheximide combination). The SF-EA were induced using the DC pulses of the same technical parameters as for the first activation protocol. Nevertheless, the process of SF-EA occurred in the dielectric solution with a standard level of extracellular calcium cations, i.e., with the minimal threshold dose of  $0.05 \text{ mM CaCl}_2$ . Two-grade chemical activation of clonal cybrids was initiated after a 1–2-h delay. The nuclear-cytoplasmic hybrids were exposed to  $10 \text{ }\mu\text{M}$  calcium ionomycin in NCSU-23 (North Carolina State University-23) medium for 7 to 9 min, and were then washed three times in  $500 \text{ }\mu\text{L}$  of NCSU-23 medium containing 0.4% BSA. The clonal cybrids were then incubated in  $1 \text{ mM}$  6-dimethylaminopurine (6-DMAP, Sigma-Aldrich) and  $5 \text{ }\mu\text{g mL}^{-1}$  cycloheximide (CHXM, Sigma-Aldrich) for 2 h at  $39^\circ\text{C}$  in humidified air with 5%  $\text{CO}_2$  in NCSU-23 medium supplemented with  $4 \text{ mg mL}^{-1}$  BSA.

### ***In vitro* culture of reconstructed embryos**

Cloned embryos were cultured in  $50\text{-}\mu\text{L}$  droplets of NCSU-23 medium supplemented with  $4 \text{ mg mL}^{-1}$  BSA-V that had been overlaid with light mineral oil. The number of embryos per droplet of culture medium ranged from 15 to 20. After 72 to 96 h of *in vitro* culture, cleaved embryos were transferred into a  $50\text{-}\mu\text{L}$  drop of NCSU-23/BSA medium supplemented with 10% FBS for an additional 72 h. The reconstructed embryos were incubated at  $39^\circ\text{C}$  in a 100% water-saturated atmosphere of 5%  $\text{CO}_2$  and 95% air. At the end of the *in vitro* culture period (Days 6 to 7), embryos were evaluated morphologically for morula/blastocyst formation rates.

### **Statistical analysis**

In order to compare the number of successfully reconstructed oocytes, the number of dividing embryos and the number of embryos at morula and blastocyst stages between different groups, including both the strategy of NT oocyte activation and the type/origin of nuclear donor fibroblast cells, the  $\chi^2$  test was used.

## Results

The preimplantation developmental capacity of porcine SCNT-derived embryos reconstituted with different types of donor fibroblast cells was compared according to different oocyte activation treatments. The overall developmental rates of cloned embryos from two activation groups are presented in Tables 1 and 2. The effect of the activation protocol on the cleavage activity as well as morula and blastocyst yields turned out to be statistically significant among subpopulations of nuclear-transferred embryos descended from adult dermal and foetal fibroblast cells (Tables 1 and 2). Moreover, regardless of the strategies used to artificially activate the reconstructed oocytes, the competences of foetal fibroblast cell nuclei to support the development of cloned embryos to the morula/blastocyst stages were significantly higher than the competences of adult dermal fibroblast cell nuclei (Tables 3 and 4).

Table 1. Effect of oocyte activation protocols on the *in vitro* developmental capability of NT embryos derived from adult cutaneous fibroblast cells

Activation strategy	No. of oocytes/embryos			Development to	
	enucleated	fused (%)	cleaved (%)	morulae (%)	blastocysts (%)
SF-EA	365	327/365 (89.6) a	249/327 (76.1) A	203/327 (62.1) A	115/327 (35.2) a
Sequential electrical and chemical	341	298/341 (87.4) a	188/298 (63.1) B	147/298 (49.3) B	79/298 (26.5) b

a, b – Values with different letters within the same column differ significantly ( $P < 0.05$ ,  $\chi^2$  test).

A, B – Values with different letters the same column differ significantly ( $P < 0.001$ ,  $\chi^2$  test).

No. of replicates = 8.

Table 2. Effect of oocyte activation protocols on the *in vitro* developmental capability of NT embryos derived from foetal fibroblast cells

Activation strategy	No. of oocytes/embryos			Development to	
	enucleated	fused (%)	cleaved (%)	morulae (%)	blastocysts (%)
SF-EA	386	361/386 (93.5) a	322/361 (89.2) a	270/361 (74.8) A	167/361 (46.3) C
Sequential electrical and chemical	397	359/397 (90.4) a	281/359 (78.3) b	228/359 (63.5) B	118/359 (32.9) D

a, b – Values with different letters within the same column differ significantly ( $P < 0.05$ ,  $\chi^2$  test).

A, B – Values with different letters within the same column differ significantly ( $P < 0.01$ ,  $\chi^2$  test).

C, D – Values with different letters within the same column differ significantly ( $P < 0.001$ ,  $\chi^2$  test).

No. of replicates  $\geq$  8.



Table 3. Effect of the nuclear donor cell type on the *in vitro* developmental capability of cloned embryos originating from oocytes subjected to the SF-EA

Type of somatic cells	No. of oocytes/embryos			Development to	
	enucleated	fused (%)	cleaved (%)	morulae (%)	blastocysts (%)
Adult cutaneous fibroblasts	312	284/312 (91.0) a	221/284 (77.8) A	185/284 (65.1) C	99/284 (34.9) C
Foetal fibroblasts	295	278/295 (94.2) a	254/278 (91.4) B	215/278 (77.3) D	130/278 (46.8) D

A, B – Values with different letters within the same column differ significantly ( $P < 0.001$ ,  $\chi^2$  test).

C, D – Values with different letters within the same column differ significantly ( $P < 0.01$ ,  $\chi^2$  test).

No. of replicates  $\geq 6$ .

Table 4. Effect of the nuclear donor cell type on the *in vitro* developmental capability of cloned embryos originating from oocytes subjected to the sequential electrical and chemical activation

Type of somatic cells	No. of oocytes/embryos			Development to	
	enucleated	fused (%)	cleaved (%)	morulae (%)	blastocysts (%)
Adult cutaneous fibroblasts	323	287/323 (88.9) a	185/287 (64.5) A	146/287 (50.9) C	78/287 (27.2) a
Foetal fibroblasts	334	305/334 (91.3) a	234/305 (76.7) B	195/305 (63.9) D	109/305 (35.7) b

a, b – Values with different letters within the same column differ significantly ( $P < 0.05$ ,  $\chi^2$  test).

A, B – Values with different letters within the same column differ significantly ( $P < 0.01$ ,  $\chi^2$  test).

C, D – Values with different letters within the same column differ significantly ( $P < 0.001$ ,  $\chi^2$  test).

No. of replicates = 7.

## Discussion

It has been found that the methods applied to artificially activate the porcine SCNT-descended oocytes affect, to a high degree, the *in vitro* developmental capability of cloned pig embryos, depending on the type of nuclear donor fibroblast cells used to reconstruct the enucleated oocytes. The foetal or adult dermal fibroblast cells had a considerably higher cloning competence following the SF-EA of reconstituted oocytes compared to the competence of cells, whose nuclei were transplanted into oocytes undergoing sequential electrical and chemical activation. On the contrary, Yin et al. (2002) reported that the *ex vivo* developmental potential of nuclear-transferred embryos, which had been derived from foetal fibroblasts or adult heart tissue-retrieved cells, ranged from 6% to 11% and did not vary significantly between the five strategies utilized for oocyte stimulation. These different strategies involved delayed activation of clonal cybrids using different stimuli: physical (electric pulses), chemical (calcium ionomycin and 6-DMAP) or physico-chemical (DC pulses/6-DMAP, DC pulses/butyrolactone I and DC pulses/CHXM). However, in the experiments by Hyun et al. (2003), porcine transgenic NT embryos, which had



been reconstructed with foetal fibroblast cells and oocytes subjected to sequential electrical and chemical activation using DC pulses prior to their incubation with 15  $\mu\text{M}$  calcium ionomycin for 4 min and then with 2 mM 6-DMAP for 2 h, were characterized by considerably lower blastocyst formation rates than those for transgenic NT embryos originating from oocytes simultaneously fused and electrically activated. Nonetheless, applying either the combination of electrical and chemical activation or simultaneous fusion and electroactivation to stimulate the oocytes receiving transfected foetal fibroblast cell nuclei, Hyun et al. (2003) have shown about twofold or almost threefold lower blastocyst yields than those achieved by us (Table 2). Similarly, Betthausen et al. (2000) have demonstrated that following sequential electrical and chemical activation of clonal cybrids *in vitro* developmental competences of cloned embryos reconstructed with foetal body or genital ridge cell nuclei to blastocyst stage ranged from 4% to 8% and was severalfold lower than those for NT embryos created in our study using foetal fibroblast cells (Table 2). In the paper by Betthausen et al. (2000), the activation protocol included the application of DC pulse in the presence of 0.1 mM  $\text{Ca}^{2+}$  ions before 20-min treatment of reconstituted oocytes with 15  $\mu\text{M}$  ionomycin 4 h later and subsequently their incubation with 1.9 mM 6-DMAP for 3–4 h. Moreover, in our experiments, the cloned embryos produced using nuclear donor foetal fibroblast cells and oocytes stimulated by DC pulses, followed by their exposure to 15  $\mu\text{M}$  calcium ionomycin for 7 to 9 min and then incubation with 6-DMAP/CHXM mixture for 2 h, reached the blastocyst stage at a nearly one-and-a-half times higher rate (Table 2) than those in the study by Boquest et al. (2002). However, Boquest and co-workers applied chemical post-activation to SCNT-derived pig oocytes treated with 5  $\mu\text{M}$  ionomycin for 5 min and then with 2 mM 6-DMAP for 3 h.

The *ex vivo* developmental outcomes of non-transgenic NT embryos derived from non-transfected foetal or adult dermal fibroblast cells were also considerably higher in our current investigation, compared to our previous study (Skrzyszowska et al., 2008), in which the generation of transgenic cloned embryos originating from the same types of transfected nuclear donor cells has been reported. For sequential electrical and chemical activation of nuclear-cytoplasmic hybrids descended from foetal fibroblast cells or adult cutaneous fibroblast cells, blastocyst formation rates yielded approximately 33% or 27% (Tables 1 and 2) and 23% or 14%, respectively, in our present and previous study. In the case of SF-EA protocol, these proportions were about 46% or 35% (Tables 1 and 2) and 30% or 16%, respectively, in our present and previous study. But, in our previous paper, the strategy of sequential electrical and chemical activation was different from that in our current investigation, because it involved the SF-EA of reconstituted oocytes, followed by their 5–7-min exposure to 15  $\mu\text{M}$  calcium ionomycin 1.5–2 h later, and then treatment with 10  $\mu\text{g mL}^{-1}$  CHXM for 3 h. In turn, the cavitation rate of porcine embryos generated from NT oocytes that had been reconstructed with ear skin-retrieved fibroblast cells and then were sequentially activated with DC pulses before treatment with calcium ionomycin and 6-DMAP/CHXM combination was also incomparably higher in our study (Table 1) than in other studies (Miyoshi et al., 2001, 2002; Roh and Hwang, 2002). In these other studies, the oocytes were

activated following the application of electric pulses only or in combination with 6-DMAP.

Using the confluent fibroblast cell nuclei, the percentage of porcine cloned embryos that were able to reach the morula and blastocyst stages under *in vitro* culture circumstances was relatively high compared with the percentage of morulae and blastocysts obtained by others (Boquest et al., 2002; Park et al., 2002; Hyun et al., 2003; Lee et al., 2003 b, 2005). We have demonstrated that regardless of the methods used to artificially activate the clonal cybrids, the competences of cell nuclei of the foetal fibroblast cells to support the preimplantation development of SCNT-derived pig embryos to morula and blastocyst stages were considerably higher than those for adult cutaneous fibroblast cell nuclei. Similarly, Lee et al. (2003 b) reported that following the SF-EA of reconstituted oocytes the cloning capability of foetal fibroblast cells, as measured with blastocyst yield, was twofold higher than the capability of adult ear skin-retrieved fibroblast cells. Nonetheless, applying the same activation strategy, almost one-and-a-half times higher frequency of blastocysts developing from nuclear-transferred embryos derived from either foetal or adult dermal fibroblast cells has been achieved by us (Table 3). On the contrary, in the experiments by Park et al. (2002), genetically transformed cloned embryos originating from oocytes that had received transfected foetal fibroblast cell nuclei and were stimulated by the SF-EA, exhibited a blastocyst formation rate similar to that for NT embryos descended from dermal fibroblast cells of transgenic pig. However, in the study by Hyun et al. (2003), the SF-EA of gilt and sow oocytes, which had been reconstituted with transfected foetal fibroblast cell nuclei, contributed to the blastocyst stage being reached by approximately 18% to 26% transgenic cloned embryos, respectively. This *ex vivo* developmental outcome was almost twofold higher than that achieved by Park et al. (2002), but about one and one-half times or nearly twofold lower than that obtained in the current investigation.

Furthermore, the novel method of sequential electrical and chemical activation of porcine nuclear-cytoplasmic hybrids, which has recently been developed by us, resulted also in relatively high percentages of embryos at morula and blastocyst stages. In the group of NT oocytes originating from adult cutaneous fibroblast cells, the morula and blastocyst formation rates yielded approximately 51% and 27% (Table 4), respectively. In turn, for subpopulation of oocytes receiving foetal fibroblast cell nuclei these rates were significantly higher (Table 4). However, in contrast to the results of our study, Hyun et al. (2003) reported that following combined electrical and chemical stimulation of clonal cybrids derived from transfected foetal fibroblast cell nuclei the frequencies of transgenic cloned embryos that developed to morula and blastocyst stages decreased considerably and ranged from approximately 18% to 27% and from 12% to 16%, respectively. These discrepancies in the *in vitro* developmental capacity of cloned embryos can result from differences in oocyte activation strategies between our study and the study by Hyun et al. (2003). In our protocol of sequential electrical and chemical activation, the SCNT couplets descended from fibroblast cells and enucleated oocytes were subjected to the SF-EA in the dielectric solution with low level of 50  $\mu\text{M}$  calcium cations. One to two hours later, they underwent stimulation via twofold elevated dose of 10  $\mu\text{M}$  calcium ionomycin

for 7 to 9 min, followed by their 2-h exposure to 6-DMAP and CHXM at concentrations that were diminished by half to 1 mM and 5  $\mu\text{g mL}^{-1}$ , respectively. In turn, the activation protocol of Hyun et al. (2003) included SF-EA of reconstituted oocytes in the medium containing 0.1 mM  $\text{Ca}^{2+}$  ions, followed by their 5-min stimulation with threefold increased concentration of 15  $\mu\text{M}$  ionomycin after a 2-h delay and then treatment with 2 mM 6-DMAP for 2 h.

In conclusion, both the simultaneous fusion and electrical activation and sequential electrical and chemical activation of nuclear-transferred pig oocytes, which had originated from adult cutaneous or foetal fibroblast cells, led to the achievement of relatively high morula and blastocyst formation rates by cloned embryos under *in vitro* culture conditions. It has been also indicated that three-step physical and chemical activation via DC pulses followed by calcium ionomycin and 6-dimethylaminopurine/cycloheximide combination was applied to artificially stimulate the porcine reconstructed oocytes for the first time. Nevertheless, future studies are needed to estimate whether the two oocyte activation strategies utilized in our laboratory allow the ability of SCNT-descended pig embryos for reaching term to be preserved.

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**Zastosowanie różnych metod aktywacji oocytów świni zrekonstruowanych z jąder komórek fibroblastycznych do uzyskiwania zarodków klonalnych**

STRESZCZENIE

Badania podjęto w celu oceny przedimplantacyjnych zdolności rozwojowych klonalnych zarodków świni w zależności od wykorzystania różnych strategii sztucznej aktywacji oocytów, do cytoplazmy których wprowadzono na drodze elektrofuzji jądra komórkowe hodowanych *in vitro* fibroblastów. W grupach I A oraz I B enukleowane oocyty poddawano elektrofuzji z fibroblastami tkanki skórno-powłokowej dorosłych osobników lub płodów, a impulsy prądu stałego zastosowane do rekonstrukcji ooplastów z jąder komórek somatycznych były jednocześnie bodźcami aktywującymi formujące się hybrydy jądrowo-cytoplazmatyczne. Natomiast w grupach II A i II B, program rozwojowy cybryd klonalnych uzyskanych z fibroblastów układu skórno-powłokowego dorosłych osobników lub płodów inicjowano w następstwie sekwencyjnej aktywacji fizykochemicznej. Obejmowała ona równoczesną fuzję i aktywację zrekonstruowanych oocytów przy wykorzystaniu impulsów prądu stałego, a następnie opóźnioną aktywację chemiczną *de novo* hybryd jądrowo-ooplazmatycznych z użyciem 10  $\mu\text{M}$  jonomycyny wapnia oraz mieszaniny 1 mM 6-dimetyloaminopuryny (6-DMAP) i 5  $\mu\text{g mL}^{-1}$  cykloheksimidu (CHXM). Udział moruli i blastocyst w populacji hodowanych *in vitro* zarodków klonalnych utrzymywał się w grupach I A oraz I B na poziomie, odpowiednio 203/327 (62,1%) i 115/327 (35,2%) oraz 270/361 (74,8%) i 167/361 (46,3%). Z kolei w grupach II A i II B odsetek zarodków w stadiach moruli i blastocysty wynosił odpowiednio 147/298 (49,3%) i 79/298 (26,5%) oraz 228/359 (63,5%) i 118/359 (32,9%). Reasumując, jednoczesna fuzja i aktywacja elektryczna oocytów zrekonstruowanych z jąder dwóch typów komórek fibroblastycznych skutkowała wyższym potencjałem rozwojowym *in vitro* klonalnych zarodków świni niż sekwencyjna aktywacja elektryczna i chemiczna. Ponadto strategia trzystopniowej aktywacji fizykochemicznej, z użyciem impulsów prądu stałego, a następnie, kolejno, roztworu jonomycyny wapnia o podwojonym stężeniu molowym oraz kombinacji 6-DMAP i CHXM w dwukrotnie obniżonych dawkach została wykorzystana po raz pierwszy do stymulacji zrekonstruowanych oocytów w badaniach nad klonowaniem somatycznym świń.