Ann. Anim. Sci., Vol. 10, No. 4 (2010) 385–398

PREIMPLANTATION DEVELOPMENTAL CAPABILITY OF CLONED PIG EMBRYOS DERIVED FROM DIFFERENT TYPES OF NUCLEAR DONOR SOMATIC CELLS*

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Abstract

The aim of our study was to assess the effect of different tissue phenotype and origin of nuclear donor cells on both the *in vitro* developmental outcome of porcine somatic cell cloned embryos and their morphological quality at the blastocyst stage, which was based on an average total cell number. Nuclear-transferred (NT) embryos were assigned to one of three subpopulations according to reconstruction of enucleated gilt or sow oocytes with either non-cultured cumulus cell nuclei via intracytoplasmic microinjection of karyoplasts (Group I) or cultured adult dermal fibroblast cells (Group II) or cultured foetal fibroblast cells (Group III) via their electrofusion with ooplasts. The morula and blastocyst yields were 152/341 (44.6%) and 66/341 (19.4%) in Group I, respectively. The frequencies of cloned embryos that developed to the morula and blastocyst stages were 208/335 (62.1%) and 112/335 (33.4%) in Group II, respectively. In Group III, NT embryos reached the morula and blastocyst stages at the percentages of 254/346 (73.4%) and 153/346 (44.2%), respectively. The overall mean cell counts in the cloned blastocysts generated that originated from Groups I, II and III were 24.6, 33.5 and 42.7, respectively. In conclusion, the abilities of cultured foetal or adult cutaneous fibroblast cell nuclei to support the *in vitro* development of NT-derived pig embryos to the morula/blastocyst stages have achieved much higher rates than the abilities of non-cultured cumulus cell nuclei. To our knowledge, this is one of only a few reports that so far have compared the preimplantation developmental competences of porcine cloned embryos, depending on more than two types of nuclear donor somatic cells.

Key words: pig, somatic cell cloning, nuclear-transferred embryo, cumulus cell, adult dermal fibroblast, foetal fibroblast

Pig genomic embryo engineering (i.e., transgenesis linked to somatic cell cloning) is a particularly important research field of assisted reproductive technologies

*This study was conducted as a part of research project no. N N311 315936, financed by the Polish Ministry of Science and Higher Education from 2009 to 2012.

due to not only generation of animal bioreactors providing biopharmaceuticals (Lee et al., 2003 a, 2005), but also an increasing role of the porcine organs in xenotransplantology (Fujimura et al., 2004; Harrison et al., 2004; Kolber-Simonds et al., 2004). Despite tremendous improvement of somatic cell nuclear transfer (SCNT) technique in pigs, high early-, mid- and late-gestation mortality rates of nuclear-transferred embryos/foetuses as well as numerous malformations of resultant cloned offspring still often appear in this species (Jiang et al., 2004; Kolber-Simonds et al., 2004; Koo et al., 2004; Park et al., 2004 a, b).

Generally, the main cause of low somatic cell cloning efficiency in pigs and other mammalian species, with many severe developmental anomalies (anatomo-histological disorders in foetal and extrafoetal/placental tissues, immune dysfunction) leading to high pregnancy loss and neonatal death, may be an incomplete rearrangement of gene expression patterns within the donor cell nuclear DNA during pre- and postimplantation phases of embryogenesis. Transcriptional activity of somatogenic nuclear genome during embryo pre- and/or postimplantation development as well as foetogenesis is correlated with the frequencies for spatial remodelling of chromatin architecture and reprogramming of cellular epigenetic memory. This former and this latter process include such covalent modifications as demethylation/*de novo* methylation of DNA cytosine residues and acetylation/deacetylation as well as demethylation/re-methylation of lysine and arginine residues of nucleosomal core-derived H3 and H4 histones (Kang et al., 2001; Santos and Dean, 2004; Zhu et al., 2004). In addition, intergenomic communication between heteroplasmically-transmitted nuclear DNA, maternally (ooplasmically) inherited copies of mitochondrial DNA (mtDNA) and nuclear donor cell-descended copies of mtDNA affects the profile of gene expression. It also affects the nuclear-ooplasmic interactions in cloned embryos and foetuses (Hiendleder et al., 2004; Shi et al., 2004). Understanding of the molecular mechanisms involving both the epigenetic transcriptional reprogramming of donor nuclear genome and the interactions between donor nuclei and recipient ooplasts (Kang et al., 2001; Shi et al., 2004) will be helpful to solve the problems resulting from swine SCNT and opens new possibilities for common application of this technology in human biomedicine (Hyun et al., 2003 a; Kolber-Simonds et al., 2004; Watanabe et al., 2005).

The studies on somatic cell cloning in pigs and other mammalian species confirmed that pre- and postimplantation development of nuclear transfer (NT)-derived embryos/foetuses depends to a high degree on the preservation of appropriate conditions in all steps of SCNT procedure (Yin et al., 2002; Kawakami et al., 2003; Kurome et al., 2003; Lee et al., 2003 b). Some of the most important factors that determine the somatic cell cloning efficiency are: 1) the phenotype; 2) tissue-, organ-, body part-related topographical lineage; 3) the status of both *in vivo* or *in vitro* structuro-functional cytodifferentiation and cytodifferentiation at the molecular level; 4) ability for dedifferentiation and redifferentiation or transdifferentiation in *in vivo* or *ex vivo* conditions, as well as 5) specient-specific origin, anatomical provenance and histological derivation of nuclear donor cells. Moreover, crucial requirements are: 6) mitotic cycle stage; 7) structuro-functional quality; 8) *in vivo* and *in vitro* replicative senescence; 9) cellular, tissue-, organ- and specient-specific aging, as well as

10) proliferative and migration activity. Other prerequisites involve: 11) susceptibility and resistance to pronecrotic or proapoptotic stimuli; 12) limitation of life-span and number of population doublings during *in vitro* culture; 13) cellular viability and mortality, as well as 14) epigenetic (telomeric, i.e. telomere attrition- and telomerase activity-associated) age of nuclear donor cells. All the latter are highly dependent on the circumstances of their *in vitro* culture (both primary culture and establishment of clonal cell lines), cell cycle synchronization, selection and preparation prior to SCNT procedure (Xu and Jang, 2001; Kues et al., 2002; Lee et al., 2003 a, b; Jang et al., 2004; Skrzyszowska et al., 2008). It is beyond any doubt that the degree of both nuclear-cytoplasmic and epigenomic maturity of recipient oocytes, whose cell cycle has been blocked at metaphase II stage of meiosis, plays also a significant role (Hyun et al., 2003 b; Lee et al., 2003 b). Furthermore, the proper coordination between nuclear donor cell phenotype and cell cycle stage, methods of maternal chromosome elimination, oocyte reconstruction techniques, artificial activation procedures and *in vitro* culture systems of cloned embryos seem to be also significant (Hyun et al., 2003 a; Im et al., 2004; Kurome et al., 2003; Lee et al., 2003 a).

In the pig, the results of the studies aimed at estimation of *ex vivo* development of nuclear transfer-derived embryos are still unsatisfactory. So far, both the relatively low rates of morula and/or blastocyst formation and poor morphological quality of generated morulae as well as blastocysts, which was evaluated on the basis of quantitative cytological analysis, have been frequently demonstrated. For that reason, many investigations have been focused on improving the *in vitro* developmental outcome of porcine cloned embryos. In these examinations, the cell nuclei of foetal fibroblasts (Boquest et al., 2002; De Sousa et al., 2002; Hyun et al., 2003 a, b; Kawakami et al., 2003; Martinez Diaz et al., 2003) or adult ear skin-derived fibroblasts (Miyoshi et al., 2002; Park et al., 2002; Roh and Hwang, 2002; Lee et al., 2003 a), whose mitotic cell cycle had previously been synchronized by the methods of serum starvation or contact inhibition after their reaching a total confluency under the *in vitro* culture conditions, were most frequently used for reconstruction of enucleated oocytes by SCNT. In contradistinction to the cultured foetal or adult cutaneous fibroblast cells, adult ovarian follicle-derived cells such as cultured mural granulosa cells (Lee et al., 2003 a) as well as non-cultured cumulus cells (De Sousa et al., 2002; Lee et al., 2003 a; Samiec et al., 2003; Skrzyszowska et al., 2005) or cultured cumulus cells (Cheong et al., 2000; Martinez Diaz et al., 2002; Lee et al., 2003 b) were only sporadically the source of donor nuclei in the somatic cell cloning of pigs. Nonetheless, the research groups that have already compared the cloning competence among nuclear donor cells of different origin and/or phenotype, often reported the contradictory or inconsistent results (De Sousa et al., 2002; Lee et al., 2003 a, b; Martinez Diaz et al., 2003; Park et al., 2002; Skrzyszowska et al., 2008). Because of these discrepancies, our study was conducted to examine the effect of nuclear donor cell type on the *in vitro* developmental capacity of cloned pig embryos.

Material and methods

Preparation of nuclear recipient pig oocytes originating from *in vitro* maturation

Slaughterhouse ovaries were collected from pre- and postpubertal gilts as well as 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 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 ($NaHCO_3$)-buffered TC 199 medium (Gibco BRL), supplemented with 10% porcine follicular fluid (pFF), 0.6 mM *L*-cysteine (Sigma-Aldrich), 10 ng mL^{-1} recombinant human epidermal growth factor (rhEGF, Sigma-Aldrich), 1 mM dibutyryl cyclic adenosine monophosphate (db-cAMP, Sigma-Aldrich), 10 IU mL^{-1} equine chorionic gonadotropin/pregnant mare serum gonadotropin (eCG/PMSG, Sigma-Aldrich) and 10 IU mL^{-1} human chorionic gonadotropin (hCG, Sigma-Aldrich). Approximately 50 to 60 COCs were cultured in the db-cAMP- and eCG + hCG-supplemented medium for 20 h at 39°C in a 100% water-saturated atmosphere of 5% 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, eCG and hCG. After maturation, expanded cumulus cells and corona cells were completely removed by vigorous pipetting of the COCs in the presence of 0.1% bovine testis-derived hyaluronidase (Sigma-Aldrich) in 500 µL 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.

Preparation of porcine nuclear donor somatic cells

Isolation of cumulus cells

The freshly collected cumulus cells were dissociated from expanded cumulus masses of *in vitro*-matured COCs by vortexing them for 1 to 2 min in TCM 199-HEPES supplemented with 0.1% hyaluronidase and washing three times in the medium with addition of 4 mg mL^{-1} BSA-V.

Isolation and culture of fibroblast cells

Foetal fibroblast cells were isolated from conceptuses (7.0 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 unknown. Adult fibroblast cells were collected from an ear-skin biopsy obtained from a 12-month-old sow. 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^{-1} 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 twice. 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). After reaching the 100% confluency, the cells underwent the contact inhibition of their migration and proliferative growth for 24–48 h in order to synchronize their mitotic cycle at the G1/G0 stages.

Before use for somatic cell nuclear transfer, the clonal fibroblast cell lines (following 2–4 passages), which had been subjected to the contact inhibition of mitotic activity under the conditions of the total confluency, were trypsinized and subsequently centrifuged at 300 × g for 5 min. The supernatant was removed, and 50 µL of manipulation medium was added.

Production of nuclear-transferred embryos

All the microsurgical operations, including enucleation of nuclear recipient oocytes and somatic cell injection under their zonae pellucidae (creation of ooplast-somatic cell couplets before their electrofusion) or injection of somatic cell-descended karyoplasts directly into host ooplasm, were performed by using a micromanipulator (Leitz, Ernst Leitz Wetzlar GmbH, Germany) equipped with an inverted microscope (Olympus, Tokyo, Japan).

Enucleation of oocytes

Cumulus-denuded oocytes were incubated in the maturation medium supplemented with 0.4 µg mL^{-1} demecolcine (DMCC, Sigma-Aldrich) and 0.05 M L^{-1} sucrose (Sigma-Aldrich) for 40–50 min at 39ºC. Afterwards, the treated oocytes were transferred into a glass micromanipulator chamber filled with TCM 199 containing 4 mg mL^{-1} BSA-V, 5 µg mL^{-1} cytochalasin B (CB, Sigma-Aldrich) and 0.4 µg mL^{-1}

DMCC. Maternal chromosomes (metaphase plates), which had been allocated into chemically induced protrusion of plasma membrane, were removed microsurgically (Yin et al., 2002; Kawakami et al., 2003). Enucleation was accomplished by gently aspirating the ooplasmic cone, which contained condensed chromosome cluster, with the aid of a bevelled micropipette of 20- to 25-μm external diameter. Following enucleation, the resulting cytoplasts (ooplasts) were washed extensively in HEPES-buffered TCM 199/BSA and held in this CB- and DMCC-free medium until microinjection of donor nuclei.

Reconstruction and activation of oocytes

Reconstruction of enucleated oocytes was achieved by intracytoplasmic microinjection of cumulus cell-derived karyoplasts (Group I) or by electrofusion of whole (foetal or adult dermal) fibroblast cells with ooplasts (Groups II and III). Nuclear donor cells were selected under differential interference contrast (DIC) optics according to their size and shape. For cumulus cells, the cells with a smooth, intact plasma membrane surface were chosen as donor nuclei to be injected into enucleated oocytes. By using an injection pipette, whose sharp, bevelled tip had an external diameter about half smaller than the diameter of the selected cumulus cells, the plasma membrane was broken by gentle, repeated aspiration of the entire cells into and out of the pipette. Then the injection pipette was introduced mechanically through the zona pellucida (using the same slit as made during enucleation) and up to halfway into the ooplasm of each cytoplast and the tiny karyoplast was quickly deposited with the cytoplasm. The reconstructed oocytes were incubated in NCSU-23 (North Carolina State University-23) medium with 4 mg mL^{-1} BSA-V at 39°C in a 100% humidified atmosphere of 5% CO_2 in air for 1.5 h before activation. Reconstructed oocytes were equilibrated in 500 μL of electroporation medium for 5 min at 39°C and then transferred to electroactivation chamber with two wire electrodes 0.5 mm apart overlaid with 10 mL of the same medium. The electroporation medium was isotonic dielectric solution consisting of 0.3 M *D*-mannitol (Sigma-Aldrich), 1.0 mM $CaCl_2$ (Sigma-Aldrich), 0.1 mM $MgSO_4$ (Sigma-Aldrich), and 0.2 mg mL^{-1} fatty acid free BSA (FAF-BSA, Sigma-Aldrich). Activation of NT-derived oocytes was induced with two successive DC pulses of 1.2 kV cm^{-1} for 60 μs each, delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA, USA). Immediately after electroactivation, nuclear-transferred embryos were left once more in the electroporation medium for 5 min at 39°C, before being transferred to the NCSU-23/BSA medium supplemented with 5 μg mL^{-1} CB for 2 h.

For fibroblast cells, single nuclear donor cells were inserted into perivitelline space of previously enucleated oocytes. The resulting somatic cell-ooplast couplets were transferred to a fusion/activation chamber filled with electroporation medium consisting of 0.3 M *D*-mannitol supplemented with 1.0 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.2 mg mL^{-1} FAF-BSA. Fibroblast cell-ooplast complexes were simultaneously fused and activated with two consecutive DC pulses of 1.2 kV cm^{-1} for 60 μs each.

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

Cloned embryos were cultured in 50-μL droplets of NCSU-23 medium supplemented with 4 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, dividing embryos were transferred into a 50-µL drop of NCSU-23/BSA medium supplemented with 10% FBS for an additional 72 h. The reconstructed embryos were incubated at 39°C, in a 100% water-saturated atmosphere of 5% 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.

Quantitative cytological analysis of embryos for morphological quality

Nuclear transfer-derived blastocysts were stained *intra vitam* with 5 µg mL^{-1} bisbenzimide (Hoechst 33342 fluorochrome), to analyse the total number of interphase cell nuclei, metaphase plates or anaphase/telophase chromosome clusters using the epi-fluorescent microscopy (Olympus, Tokyo, Japan). The mean overall/summed number of inner cell mass (ICM) and trophectoderm (TE) cells in each blastocyst was estimated on the basis of double counting.

Statistical analysis

In order to compare the number of successfully reconstructed oocytes, the number of embryos undergoing cleavage divisions and the number of embryos at morula and blastocyst stages between different groups including the types of nuclear donor somatic cells, the χ^2 test was used.

In order to compare the morphological quality of the cloned blastocysts, which had been evaluated via mean total cell number, between three nuclear donor cell type-related groups, the Student's *t*-test-based one-way analysis of variance (ANOVA) and Duncan's multiple range test were performed using the General Linear Model (GLM) procedure in the Statistical Analysis System (SAS v.8.2) software (SAS Institute, Inc., Cary, NC). A probability (*P*) value that was less than 0.05 was considered to be statistically significant.

Results

The preimplantation developmental outcomes of cloned embryos such as cleavage activity, morula and blastocyst formation rates and morphological quality of the SCNT-derived blastocysts on the basis of vital quantitative fluorocytochemical analysis were compared between three experimental groups, which differed in nuclear donor somatic cell type (Table 1). Group I included the NT oocytes derived from freshly-collected *in vitro* matured COC-derived cumulus cells. In Group II, the enucleated oocytes were reconstructed with cell nuclei of *in vitro* cultured adult ear skin-descended fibroblast cells, and in Group III – with cell nuclei of *in vitro* cultured foetal fibroblast cells. The effect of the somatic cell type on the frequencies of cleavage divisions of NT embryos reconstructed with adult dermal and foetal fibroblast cells was not significant ($P \geq 0.05$). However, the cleavage activity of cloned embryos ori-

ginating from the oocytes receiving cumulus cell nuclei was significantly lower than that for both NT embryos derived from adult cutaneous fibroblast cells ($P<0.001$) and those reconstructed with foetal fibroblast cells ($P<0.001$). The highest and the lowest morula and blastocyst formation rates have been indicated in Group III and Group I, respectively. There were significant differences in the developmental capabilities of SCNT-derived embryos to reach the morula and blastocyst stages between Groups I, II and III. The competences of foetal fibroblast cell nuclei to direct the development of cloned embryos to the morula stage were significantly higher than those for both adult dermal fibroblast cell nuclei ($P<0.01$) and cumulus cell nuclei ($P<0.001$). The percentage of the morulae obtained was also significantly higher in the population of NT embryos reconstructed with adult ear skin-derived fibroblast cells compared to the group of those originating from cumulus cells ($P<0.001$). Furthermore, the cloned embryos derived from either foetal fibroblast cell nuclei or adult cutaneous fibroblast cell nuclei were characterized by significantly higher blastocyst yields than those for NT embryos originating from cumulus cells ($P<0.001$, and $P<0.001$, respectively). In turn, the abilities of adult dermal fibroblast cell nuclei to support the development of cloned embryos to blastocyst stage were significantly lower than the abilities of foetal fibroblast cell nuclei ($P<0.01$).

The overall mean cell counts that were assessed in all the blastocysts developing from *in vitro* cultured SCNT embryos were 24.6, 33.5 and 42.7 in Groups I, II and III, respectively. The statistical analysis revealed that average total cell numbers in SCNT-descended blastocysts varied significantly between Groups I, II and III ($P<0.001$).

Table 1. Effect of somatic cell type/origin on the *in vitro* developmental competences of porcine cloned embryos

Type/Lineage of nuclear donor cells	No. of oocytes/embryos			Development to	
	reconstructed (microinjected* or electrofused**)	cultured (%)	cleaved (%)	morulae (%)	blastocysts (%)
Freshly collected (non-cultured) cumulus cells	394*	341/394 (86.5) a	204/341 (59.8) A	152/341 (44.6) A	66/341 (19.4) A
Cultured adult dermal fibroblast cells	373**	335/373 (89.8) a	259/335 (77.3) aB	208/335 (62.1) C	112/335 (33.4) C
Cultured foetal fibroblast cells	367**	346/367 (94.3) a	281/346 (81.2) aB	254/346 (73.4) B	153/346 (44.2) B

A, B – Values with different letters within the same column differ significantly ($P<0.001$, χ^2 test).
A, C – Values with different letters within the same column differ significantly ($P<0.001$, χ^2 test).
B, C – Values with different letters within the same column differ significantly ($P<0.01$, χ^2 test).
No. of replicates ≥ 8.

Discussion

The purpose of the present study was to compare the cloning competence of different types of nuclear donor somatic cells, which turned out to be necessary for improvement of preimplantation developmental potential of porcine SCNT-derived embryos. The results of our study clearly demonstrate that the phenotype and origin of nuclear donor cells are some of the most important factors affecting the efficiency of somatic cell cloning in pigs as has been measured with the percentage of the embryos achieving the morula/blastocyst stages in relation to the number of successfully reconstructed oocytes. The cell nuclei of *in vitro* cultured foetal fibroblasts exhibited the highest abilities for supporting the development of porcine cloned embryos to blastocyst stage. These abilities were over twofold higher than the cloning competences of freshly collected and non-cultured cumulus cells and almost one-and-a-half times higher than those for cultured adult cutaneous fibroblast cells. This may be elucidated partially by the fact that *in vitro* cultured fibroblast cell lines established from foetal body tissues are considerably less differentiated cell lineages than their counterparts derived from the explants of adult skin tissue or non-cultured ovarian follicle-derived cumulus cells. That is why, the capability of nuclear chromatin/genome from foetal fibroblast cells to be architecturally remodelled and epigenetically reprogrammed first in the cytoplasmic microenvironment of reconstructed oocytes and then in the descendant blastomeres of dividing cloned embryos is higher than that for more differentiated fibroblast cells retrieved from adult dermal tissue and freshly collected in vitro-matured COC-retrieved cumulus cells (Lee et al., 2003 a, b; Jang et al., 2004; Jiang et al., 2004; Santos and Dean, 2004; Zhu et al., 2004).

So far, only the studies by Lee et al. (2003 b) and Yin et al. (2002) have compared, similar to the present study, the abilities of more than two types of nuclear donor cells for directing the *in vitro* development of cloned embryos to blastocyst stage. Similarly to the results of our study, Lee et al. (2003 b) reported that the capability of SCNT embryos reconstructed with cultured foetal fibroblast cells to reach the blastocyst stage was significantly higher than the capability of cloned embryos derived from cell nuclei of either cultured adult dermal fibroblasts or cultured cumulus cells. Nonetheless, although the adult dermal fibroblast cells had a slightly higher cloning competence compared to the competence of cumulus cells, the differences between these two nuclear donor cell groups were not statistically significant (Lee et al., 2003 b). However, we obtained a considerably higher percentage of blastocysts originating from NT embryos reconstituted with either foetal or adult ear skin-retrieved fibroblasts or cumulus cells. The possible explanation of lower cavitation rates in the study by Lee et al. (2003 b) may be that in contradistinction to our study, in which non-cultured cumulus cells or cultured (100%-confluent) fibroblast cells undergoing 1–2-day contact inhibition of their migration and proliferative growth provided a source of nuclear donor cells, Lee and co-workers applied 3–5-day serum starvation to synchronize the mitotic cycle of three types of *in vitro* cultured somatic cells at the G0/G1 phases. Such long-term trophic deprivation of the clonal cell lines could lead to increase of apoptosis occurrence, promotion of advanced epigenetic alterations (e.g., DNA methylation, histone deacetylation), onset and/or progression

of mutagenetic changes (i.e., chromosomal abnormalities), diminishment of telomerase activity and acceleration of telomere length shortening, subsequent potentiation of replicative senescence, strengthening of cytophysiological aging and thereby decrease of survival rates and proliferative activities (Xu and Jang, 2001; Kues et al., 2002; Lee et al., 2003 a; Jang et al., 2004; Jiang et al., 2004). In contrast to the results of the current investigation and to the study by Lee et al. (2003 b), Yin et al. (2002) reported that the *in vitro* developmental capacity of cloned embryos that had been derived from foetal or adult cutaneous fibroblast cell nuclei or cumulus cell nuclei, did not differ significantly between these three nuclear donor cell groups. In the study by De Sousa et al. (2002), porcine NT embryos that had been reconstructed with foetal fibroblast cells were also characterized by blastocyst yields comparable to those for cloned embryos originating from cumulus cell nuclei. Moreover, we have found discrepancies between the results of our study and the study by Lee et al. (2003 a), in which *ex vivo* developmental outcome to the blastocyst stage for NT embryos created using non-cultured cumulus cells was not significantly different from that for NT embryos produced using cultured fibroblast cells from dermal tissue biopsy of transgenic sow. Additionally, Park et al. (2002) have shown that genetically transformed nuclear-transferred embryos, which had developed from oocytes receiving replication-defective retroviral vector-transfected foetal fibroblast cells, exhibited a blastocyst formation rate similar to that for cloned embryos derived from transgenic pig-descended dermal fibroblast cell nuclei. These data are inconsistent with the results of both our present and previous studies (Skrzyszowska et al., 2008) in which the cloning competence of non-transgenic and transgenic (lipofected or nucleofected) foetal fibroblasts, as measured with *ex vivo* developmental capability to blastocyst stage, was considerably higher than the competence of non-transfected or transfected adult ear skin-retrieved fibroblasts. However, similarly as has been indicated in our present study, Uhm et al. (2000) have found that abilities of foetal fibroblast cell nuclei to direct the *in vitro* development of NT embryos to the blastocyst stage were severalfold higher than the competences of cumulus cell nuclei. For NT-descended embryos reconstituted with non-cultured cumulus cells, the *in vitro* developmental outcome to blastocyst stage ranged from approximately 5% to 7% (Uhm et al., 2000; De Sousa et al., 2002; Samiec et al., 2003; Skrzyszowska et al., 2005) and for NT-descended embryos reconstituted with cultured cumulus cells, it ranged from about 0.5% to 14% (Cheong et al., 2000; Martinez Diaz et al., 2002; Yin et al., 2002; Lee et al., 2003 b). In our present study and the study by Lee et al. (2003 a), the percentage of cloned blastocysts generated from oocytes receiving the freshly collected (non-cultured) cumulus cell nuclei was over two-and-a-half times or almost fourfold higher. Nevertheless, in the experiments by Uhm et al. (2000), De Sousa et al. (2002), Park et al. (2002) and Yin et al. (2002), NT embryos that had derived from each nuclear donor cell group (foetal fibroblasts, adult cutaneous fibroblasts and cumulus cells) developed to the blastocyst stage at much lower rate than those in our study.

Generally, the *in vitro* developmental capacity to blastocyst stage for porcine nuclear-transferred embryos that had been reconstructed with one of the two types of cultured fibroblast cells (foetal or adult dermal) was about twofold higher than

that for embryos reconstructed with non-cultured cumulus cells. Moreover, we have shown that the use of cultured foetal fibroblast cells as the source of donor nuclei for reconstruction of enucleated oocytes by SCNT resulted in the percentage of blastocysts obtained being increased nearly one and a half times compared to the use of cultured adult dermal fibroblast cells. Nonetheless, further research is necessary to establish whether the cell nuclei of three types of nuclear donor cells tested in our laboratory for the purposes of creating cloned piglets enable the development of the SCNT-derived embryos to be supported by them not only to the blastocyst stage but also up to term. Future studies are also required to assess the cell nuclei of which type of somatic cells can be characterized by the highest cloning competence as measured with producing viable offspring.

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Accepted for printing 5 X 2010

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Przedimplantacyjny potencjał rozwojowy klonalnych zarodków świni zrekonstruowanych z jąder komórek somatycznych różnego pochodzenia

STRESZCZENIE

Celem pracy było określenie wpływu różnych rodzajów i rodowodu komórek somatycznych na zdolności rozwojowe *in vitro* zarodków klonalnych świni oraz jakość morfologiczną uzyskanych blastocyst, ocenianą przyżyciowo na podstawie ilościowej analizy cytologicznej. W procedurze klonowania somatycznego źródłem dawców jąder do zabiegu rekonstrukcji enukleowanych oocytów loszek lub loch metodą docytoplazmatycznej mikroiniekcji karioplastów były komórki wzgórka jajonośnego, otaczające dojrzałe *ex vivo* oocyty (grupa I). Z kolei w grupach II i III enukleowane oocyty rekonstruowane były

z jąder komórkowych hodowanych *in vitro* fibroblastów tkanki skórno-powłokowej dorosłych osobników lub płodów, przy wykorzystaniu metody elektrofuzji kompleksów cytoplast-komórka-dawca jądra. W grupie I stadium moruli i blastocysty osiągnęło odpowiednio 152/341 (44,6%) i 66/341 (19,4%) hodowanych zarodków klonalnych. Natomiast w grupach II oraz III uzyskano odpowiednio 208/335 (62,1%) i 112/335 (33,4%) oraz 254/346 (73,4%) i 153/346 (44,2%) morul i blastocyst. Wartości średniej arytmetycznej z sumarycznej liczby komórek węzła zarodkowego oraz trofoblastu wynosiły odpowiednio 24,6, 33,5 oraz 42,7 w blastocystach klonalnych pochodzących z grup I, II oraz III. Podsumowując, kompetencje jąder komórkowych hodowanych fibroblastów układu skórno-powłokowego płodów lub dorosłych osobników do pokierowania rozwojem *in vitro* klonalnych zarodków świni do stadium moruli/blastocysty były znacznie wyższe niż kompetencje jąder komórek wzgórka jajonośnego wyizolowanych z dojrzałych *ex vivo* kompleksów oocyt-*cumulus oophorus*. Praca ta należy do jednych z nielicznych w ciągu ostatnich lat publikacji z zakresu klonowania somatycznego świń, w których badano przedimplantacyjny potencjał rozwojowy zrekonstytuowanych zarodków, w zależności od wykorzystania jako dawców-jąder więcej niż dwóch typów komórek somatycznych.