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CREATION OF CLONED PIG EMBRYOS USING CONTACT-INHIBITED OR SERUM-STARVED FIBROBLAST CELLS ANALYSED *INTRA VITAM* FOR APOPTOSIS OCCURRENCE*

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Abstract

Somatic cell cloning efficiency is determined by many factors. One of the most important factors is the structure-functional quality of nuclear donor cells. Morphologic criteria that have been used to date for qualitative evaluation of somatic cells may be insufficient for practical application in the cloning. Biochemical and biophysical changes that are one of the earliest symptoms in the transduction of apoptotic signal may be not reflected in the morphologic changes of somatic cells. For this reason, adult cutaneous or foetal fibroblast cells that, in our experiments, provided the source of genomic DNA for the cloning procedure had been previously analysed for biochemical and biophysical proapoptotic alterations with the use of live-DNA (YO-PRO-1) and plasma membrane (Annexin V-eGFP) fluorescent markers. In Groups IA and IB, the generation of nuclear-transferred (NT) embryos using non-apoptotic/non-necrotic contact-inhibited or serum-starved adult cutaneous fibroblast cells yielded the morula and blastocyst formation rates of 125/231 (54.1%) and 68/231 (29.4%) or 99/237 (41.8%) and 43/237 (18.1%), respectively. In Groups IIA and IIB, the frequencies of embryos reconstituted with non-apoptotic/non-necrotic contact-inhibited or serum-starved foetal fibroblast cell nuclei that reached the morula and blastocyst stages were 171/245 (69.8%) and 97/245 (39.6%) or 132/227 (58.1%) and 63/227 (27.8%), respectively. In conclusion, contact inhibition of migration and proliferative activity among the subpopulations of adult dermal fibroblast cells and foetal fibroblast cells resulted in considerably higher morula and blastocyst formation rates of *in vitro* cultured cloned pig embryos compared to serum starvation of either type of fibroblast cell line. Moreover, irrespective of the methods applied to artificially synchronize the mitotic cycle of nuclear donor cells at the G0/G1 phases, developmental abilities to reach the morula/blastocyst stages were significantly higher for porcine NT embryos that had been reconstructed with non-apoptotic/non-necrotic foetal fibroblast cells than those for NT embryos that had been reconstructed with non-apoptotic/non-necrotic adult dermal fibroblast cells. To our knowledge, the generation of cloned pig embryos using abattoir-derived oocytes receiving cell nuclei descended from contact-inhibited or serum-deprived somatic cells undergoing comprehensive vital diagnostics for the absence of biochemical and biophysical proapoptotic alterations within their plasmalemmas has not been reported so far.

Key words: pig, somatic cell cloning, adult cutaneous fibroblast cell, foetal fibroblast cell, contact inhibition, serum starvation, apoptosis, nuclear-transferred embryo

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The selection of nuclear donor cells that possess excellent structural and functional quality plays a pivotal role in the somatic cell nuclear transfer (SCNT) technique, determining to a high degree its efficiency. The environmental stress factors imposed by physico-chemical conditions of *in vitro* culture that affect to a high degree the cellular processes can be related to different methods of artificially synchronizing the mitotic cycle of somatic cells at desirable phase/phases prior to their use for the SCNT procedure in pigs (Samiec, 2004; Samiec and Skrzyszowska, 2005 a, b). Strategies such as drastic serum deprivation (Kues et al., 2000, 2002; Anger et al., 2003; Hyun et al., 2003; Lee et al., 2003 a; Brunetti et al., 2008) or contact inhibition of cell migration and proliferative growth under the conditions of a total confluence (Boquest et al., 2002; Im et al., 2004; Lee et al., 2003 b; Watanabe et al., 2005; Samiec and Skrzyszowska, 2010 a; Samiec et al., 2012) that are commonly applied to synchronize the cell cycle of *in vitro* cultured nuclear donor cells at the G0/G1 stages can lead to unscheduled decrease in the cellular viability. As a result, the molecular scenario of apoptosis can be initiated in the artificially synchronized somatic cell lines.

The cytophysiological proapoptotic alterations can be detected by different fluorescent methods including either vital or non-vital diagnostics of the cells. One of the *intra vitam* diagnostic methods is based on the use of highly labile, yellowy-green DNA fluorochrome YO-PRO-1 that displays the ability to easily diffuse through the microchannels arising in the plasma membrane and the megachannels in the semipermeable membranes of intracellular compartments (Estaquier et al., 1995; Idziorek et al., 1995; Skrzyszowska and Samiec, 2005; Skrzyszowska et al., 2006). The second method is aimed at confirming the exposure of the phosphatidylserine (PS) residues on the surface of plasmalemma, to which the moieties of the anti-coagulative, amphitropic, calcium-dependent and low molecular weight (35.8 kDa) glycoprotein called Annexin V that is conjugated with the bioluminescent *Aequorea victoria* hydrozoan-derived fluorochrome protein, known as enhanced green fluorescent protein (eGFP), has high affinity (Fadok et al., 1992 a; Martin et al., 1995; Engeland et al., 2001; Skrzyszowska and Samiec, 2008, Skrzyszowska et al., 2007). Because the YO-PRO-1 or Annexin V-eGFP labelling of nuclear donor somatic cells was performed without the use of propidium iodide to maintain live-DNA or plasma membrane dyeing properties, the cells that fluoresced bright green represented the sum of the apoptotic and necrotic cells. The YO-PRO-1- or Annexin V-eGFP-mediated vital and non-differential method of proapoptotic alteration detection in the cultured somatic cells enables a qualitative evaluation of the random (representative) samples that were collected from the overall population of analysed cell lines undergoing serum starvation or contact inhibition after reaching the total confluence state. In other words, it allows determining the percentage of low-quality apoptotic and/or necrotic cells in relation to the total number of analysed cells originating from representative samples, without quantification of their early-apoptotic, late-apoptotic and/or necrotic cells (Samiec and Skrzyszowska, 2012 a; Skrzyszowska et al., 2006).

This study was conducted to explore whether adult cutaneous and foetal fibroblast cells that have been evaluated *intra vitam* as morphologically normal before their selection for SCNT procedure can exhibit biochemical and biophysical changes in

the plasma membrane related to the early and middle phases of apoptosis. Moreover, the effect of two methods (contact inhibition and serum starvation) used for fibroblast cell cycle synchronization at the G0/G1 phases on both the apoptosis occurrence among somatic cell lines and the *in vitro* developmental capability of porcine cloned embryos that were derived from YO-PRO-1- and Annexin V-eGFP-negative nuclear donor cells was determined.

Material and methods

Collection and *in vitro* meiotic maturation of porcine oocytes

Unless otherwise mentioned, all chemicals used in this study were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Slaughterhouse ovaries were collected from both prepubertal female pigs (gilts) and postpubertal female pigs (gilts and sows). Cumulus-oocyte complexes (COCs) were recovered by aspiration of follicular fluid from 2- to 6-mm antral ovarian follicles using an 18-gauge needle attached to a 10-mL disposable syringe. The COCs were collected into Tissue Culture Medium 199 (TCM 199-HEPES; Gibco BRL, Life Technologies Inc., Grand Island, NY, USA) that was buffered with 25 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (N-(2-hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid); HEPES, $C_8H_{18}N_2O_4S$) and supplemented with 5 IU mL^{-1} heparin. The COCs, with evenly granulated ooplasm and several uniform layers of compact cumulus oophorus cells, were washed three times in TC 199-HEPES medium with the addition of 4 mg mL^{-1} bovine serum albumin (fraction V; BSA-V) and selected for *in vitro* maturation under atmospheric conditions. Immature COCs were transferred into the single wells of Nunclon polystyrene four-well multidishes (Nunclon Δ Surface; Nunc A/S, Nalge Nunc International, Thermo Fisher Scientific, Roskilde, Denmark) filled with 500 μL of TC 199 maturation medium that had been previously overlaid with light mineral oil and equilibrated at 39ºC in atmosphere of 5% CO_2 in air for 1 to 3 h. The maturation medium was comprised of 25 mM HEPES- and 26.2 mM sodium bicarbonate-buffered Tissue Culture Medium 199 (Gibco BRL) and supplemented with 10% porcine follicular fluid (pFF), 0.6 mM *L*-cysteine, 10 ng mL^{-1} recombinant human epidermal growth factor (rhEGF), 1 mM dibutyryl cyclic adenosine monophosphate (db-cAMP; bucladesine) and 0.1 IU mL^{-1} human menopausal gonadotropin (hMG). Approximately 50 to 60 COCs were cultured in the db-cAMP- and hMG-supplemented medium for 20 h at 39ºC 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 and hMG (Samiec and Skrzyszowska, 2010 b; Skrzyszowska et al., 2008).

After maturation, expanded cumulus and corona cells were completely removed by vigorous pipetting of COCs in the presence of 0.5 mg mL^{-1} (50 IU mL^{-1}) bovine testis-derived hyaluronidase in 500 μL of HEPES-buffered TCM 199 for 1 to 2 min. *In vitro*-matured, metaphase II (MII)-stage oocytes, selected on the basis of accepted morphological criteria, and first of all with evenly granulated, dark cytoplasm and an

intact plasma membrane, and with distinctly extruded first polar bodies (polocytes) provided a source of recipient cells for exogenous cell nuclei for the somatic cloning procedure (Samiec and Skrzyszowska, 2012 a, b).

Isolation, *in vitro* culture and mitotic cycle synchronization of porcine fibroblast cells

Foetal cells were isolated from foetuses (9.0 cm in length) following removal of their heads and internal organs. The foetuses were obtained from a slaugtherhouse, that is why the age and breed of the foetuses were unknown. Adult fibroblast cells were collected from an ear-skin biopsy obtained from a 5-month-old gilt. Foetal body-derived or adult tissue samples were cut into small pieces using a tissue chopper (0.5 mm). The resultant tissue explants were placed in a 12.5-cm^2 polystyrene BD Falcon cell culture flask (Becton Dickinson Co., Franklin Lakes, NJ, USA) filled with a small volume of Dulbecco's Modified Eagle's Medium (DMEM, Gibco Invitrogen Co., Paisley, Scotland, UK) to wet the bottom of the flask, but not enough volume to cause the tissue pieces to float. During the first 2 to 3 days of incubation, a few more drops of the medium were added every 2 to 3 h, and more medium was added gradually when the pieces had firmly attached to the bottom of the culture flask. The cultures were replenished 2 to 3 times per week. Primary cultures of dermal fibroblasts were grown in modified Dulbecco's Minimum Essential Medium that was formulated to contain high concentration of *D*-glucose (4 mg mL^{-1}). This medium was supplemented with 10% heat-inactivated foetal bovine serum (FBS), 5 ng mL^{-1} recombinant human basic fibroblast growth factor (rh-bFGF), 2 mM *L*-glutamine (*L*-Gln), 0.36 mM sodium pyruvate, 1% antibiotic-antimycotic solution (AAS) and 2 mM iso-nitrogenous non-essential amino acid (NEAA) solution based on Eagle's Minimum Essential Medium (MEM). After removal of the explants (Days 5 to 6), monolayers of fibroblast cells were detached from the bottom of the culture flask and disaggregated/digested into a single cell suspension using DMEM that was deprived of such cell chemoattractants as 10% FBS and supplemented with the cell detachment/digestion buffer consisting of 0.25% trypsin and 0.01% ethylenediaminetetraacetic acid (EDTA). The cells were subsequently cultured to total confluence and then passaged at least once. The cells harvested from the flasks by trypsinization were washed in 10-mL manipulation medium (HEPES-buffered Tissue Culture Medium 199) enriched with 10% FBS and centrifuged at 200 × g for 10 min. The cell pellet was then suspended in FBS containing 9% dimethyl sulfoxide (DMSO) before freezing in a Minicool freezer (Minicool 40 PC; Air Liquide, Bussy-Saint-Georges, France). The cryopreserved donor cells were thawed at 37°C, and 200 μL of FBS was added. The suspension was incubated at room temperature for 10 min, and then 800 μL of cell culture medium was added (Samiec and Skrzyszowska, 2010 a, 2012 a; Skrzyszowska et al., 2008).

Before their use for somatic cell nuclear transfer, the frozen/thawed clonal cell lines (cell strains) of foetal or adult dermal fibroblasts were cultured *in vitro* to total confluence (in the medium supplemented with 10% FBS) to synchronize their mitotic cycle at the G1/G0 stages by initiating 24- to 48-h contact inhibition of their migration and proliferative growth (Samiec and Skrzyszowska, 2010 b; Samiec et

al., 2012). The second method used for synchronization of the cell cycle at the G0/G1 stages was the culture of the subconfluent fibroblast cell lines under the conditions of 3-4-Day serum deprivation/starvation (i.e., reduction of FBS concentration in the medium from 10% up to 0.5%) (Skrzyszowska et al., 2006).

After the cell cycle synchronization had been completed, the cultured clonal fibroblast cell lines (between passages 1 and 5) were trypsinized, followed by centrifugation at 300 × g for 5 min. The supernatant was subsequently removed, and 50 µL of the manipulation medium enriched with 4 mg mL^{-1} BSA-V was added.

Preparation and subsequent analysis of nuclear donor cells for apoptosis

Live-DNA or plasma membrane labelling of fibroblast cells

To detect the early-phase and middle-to-late-phase apoptotic changes in the cultured gilt ear skin-derived or foetal fibroblast cells (harvested by trypsin-mediated digestion), a single nuclear donor cell suspension (adjusted to a density of 1×10^5 to 1×10^6 cells per 1 mL of medium) was subjected to two *intra vitam* tagging methods with the use of different diagnostic markers. In the first method, the cells, which had been previously suspended in 1000 µL of TC 199-HEPES medium supplemented with 4 mg mL^{-1} BSA-V, were dyed with 1 µg mL^{-1} yellowy-green live-DNA fluorochrome YO-PRO-1 (Vybrant Apoptosis Assay Kit 4; Molecular Probes, Eugene, OR, USA) at a concentration of 100 µM in DMSO solution (Idziorek et al., 1995; Samiec and Skrzyszowska, 2012 a). In the second method, the cells were labelled with the conjugate of Annexin V and eGFP protein (ApoAlert Annexin V-EGFP Apoptosis Kit; Clontech; Becton, Dickinson and Co., BD Biosciences, Palo Alto, CA, USA), which has a high affinity for PS residues exposed on the surface of the plasmalemma (Fadok et al., 1992 b; Martin et al., 1995; Skrzyszowska et al., 2006). The reactive mixture was comprised of 5 µL of diagnostic conjugate at a concentration of 40 µg mL^{-1} in phosphate-buffered saline (PBS) solution and 200 µL of 10% FBS-enriched binding buffer, which chelates a surplus of Annexin V-eGFP reagent molecules preferentially linked to the negatively charged PS moieties. The reactive buffer solution (pH = 7.4) consisted of 10 mM HEPES sodium salt (HEPES/NaOH; $C_8H_{17}N_2NaO_4S$), 140 mM NaCl, 2 mM $CaCl_2$, 5 mM KCl and 1 mM $MgCl_2$. After a 15- to 20-min incubation of the somatic cells in one of the two diagnostic solutions, any proapoptotic alterations in the plasma membranes were assessed in the dark on an epi-fluorescent microscope (Olympus IMT-2, Tokyo, Japan). The nuclear donor cells were evaluated for apoptosis after a short (approximately 10- to 20-sec) excitation of the somatic cell-descended DNA and YO-PRO-1 complexes or PS and Annexin V-eGFP complexes with blue light (at an excitation/absorption maximum wavelength of λ_{max} = 488 nm). Approximately 50 YO-PRO-1- or Annexin V-eGFP-tagged representative (random) cell samples ranging from 0.5×10^3 to 1×10^3 cells per sample were collected from the populations of cultured/trypsinized adult dermal or foetal fibroblast cells whose mitotic cell cycle had been synchronized at the G0/G1 phases by the contact inhibition of their migration and proliferative growth or 3–4-Day serum deprivation. The cells that did not emit the yellowy-green YO-PRO-1-derived or green eGFP-derived fluorescence under the emission maximum wavelength λ_{max} = 530 nm were classified as non-apoptotic/non-necrotic and

used for the SCNT procedure (Samiec and Skrzyszowska, 2012 a; Skrzyszowska and Samiec, 2005, 2008; Skrzyszowska et al., 2006, 2007).

Production of porcine nuclear-transferred embryos

Enucleation of oocytes

Cumulus-denuded MII-stage oocytes were incubated in the maturation medium supplemented with 0.4 μg mL^{-1} demecolcine (DMCC; colcemid, N-deacetyl-N-methylcolchicine) and 0.05 M sucrose for 1 h at 39ºC. Afterwards, the treated oocytes were transferred into a glass micromanipulation chamber filled with TCM 199 with addition of 4 mg mL^{-1} BSA-V, 5 μg mL^{-1} cytochalasin B (CB) 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-V and held in this CB- and DMCC-free medium until the subzonal microinjection of intact nuclear donor cells was performed.

Reconstruction and activation of oocytes

Reconstruction of enucleated oocytes (ooplasts) was achieved by electrofusion of whole (adult dermal or foetal) fibroblast cells with ooplasts (Groups I and II). Nuclear donor cells were selected under Nomarski prism-based differential interference contrast (DIC) optics according to their size and shape. Only the fibroblast cells that exhibit a spherical shape and a smooth, intact surface of plasma membrane were microinjected subzonally into perivitelline space of the previously enucleated oocytes. The resulting whole somatic cell-ooplast couplets were transferred to a BTX fusion/activation chamber (BTX Model 450; Harvard Apparatus, Holliston, MA, USA), which was positioned between two parallel wire electrodes 0.5 mm apart. The chamber was filled with electroporation medium, which was composed of 0.3 M *D*-mannitol enriched with 1.0 mM $CaCl_2$, 0.1 mM $MgSO_4$ and 0.2 mg mL^{-1} fatty acid-free BSA (FAF-BSA). Fibroblast cell-ooplast complexes were simultaneously fused and electrically activated through the application of two consecutive DC pulses of 1.2 kV cm^{-1} for 60 μs each that were delivered by a BTX Electro Cell Manipulator 200 (BTX ECM Model 200; Biotechnologies & Experimental Research, Inc., San Diego, CA, USA) (Samiec and Skrzyszowska, 2012 b; Samiec et al., 2012).

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

Cloned embryos were cultured in 50-μL droplets of North Carolina State University-23 (NCSU-23) medium supplemented with 4 mg mL^{-1} BSA-V, 1% MEM-based non-essential amino acid solution (MEM-NEAA) and 2% MEM-based essential amino acid solution (MEM-EAA). The number of embryos per droplet of culture medium that had been overlaid with light mineral oil ranged from 20 to 30. After 72 to 96 h of *in vitro* culture, the cleaved embryos were transferred into a 50-μL drop of NCSU-23/BSA/MEM-NEAA/MEM-EAA medium enriched with 10%

FBS for an additional 72 h. The reconstructed embryos were incubated at 39ºC, 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), the embryos were evaluated morphologically for morula and blastocyst formation rates (Samiec and Skrzyszowska, 2010 a, 2012 a; Skrzyszowska et al., 2008).

Experimental schedule

In the first series of experiments, the percentage of apoptotic and/or necrotic cells (i.e., apoptotic/necrotic index) in the populations of contact-inhibited or serum-starved adult ear skin-derived and foetal fibroblast cells was determined by their *intra vitam* analysis aimed at the fluorescent tagging with either YO-PRO-1 DNA dye or Annexin V-eGFP conjugate.

In the second series of experiments, the *in vitro* developmental potential was assessed among porcine nuclear-transferred (NT) embryos reconstructed with either contact-inhibited or serum-deprived adult dermal and foetal fibroblast cells that had been analysed for apoptosis through the vital fluorescent staining.

In the third series of experiments, the cleavage activity, morula and blastocyst formation rates were examined among cloned embryos descended from YO-PRO-1-/Annexin V-eGFP-negative and randomly selected adult dermal or foetal fibroblast cells, the mitotic cycle of which had been previously synchronized at the G0/G1 phases by either contact inhibition or serum starvation.

Statistical analysis

The chi-square test was applied to compare the proportions of apoptotic/necrotic cells between the subpopulations of cultured adult cutaneous and foetal fibroblast cells. This test was also used for estimation of the statistical variations in the percentages of successfully reconstructed oocytes, the percentages of cleaved embryos and the percentages of embryos at the morula and blastocyst stages between different experimental groups. The experimental groups included the type of nuclear donor cells, the various methods of cell cycle synchronization and the presence/absence of YO-PRO-1-/Annexin V-eGFP-mediated pre-selection of non-apoptotic and non-necrotic fibroblast cells for the cloning procedure.

Results

The use of live-DNA and plasma membrane fluorescent markers for detection of apoptosis in fibroblast cells prior to somatic cell cloning in pigs

YO-PRO-1-mediated fluorescent analysis of cultured (contact-inhibited or serum-deprived) gilt ear skin-derived and foetal fibroblast cells revealed that a relatively high proportion of nuclear donor cells exhibited ultrastructural proapoptotic changes. Additionally, the morphologic features of these cells suggested that they were undergoing middle-to-late-phase apoptosis and/or necrosis. The percentages of apoptotic and/or necrotic (YO-PRO-1-positive) cells with advanced morphologic

transformations ranged from approximately 20 to 25%, 35 to 40% or 15 to 20%, and 20 to 25% per each of 50 analysed random samples of contact-inhibited adult dermal fibroblasts and foetal fibroblasts or serum-starved adult dermal fibroblasts and foetal fibroblasts, respectively (Table 1). On the contrary, cells that were evaluated as morphologically normal did not emit yellowy-green YO-PRO-1-derived fluorescence. These data suggest that the YO-PRO-1-negative cells are non-apoptotic/non-necrotic, or, alternatively, that the YO-PRO-1 dye may be not able to detect the early phases of apoptosis, which are typically not accompanied by morphologic changes of the somatic cells.

Table 1. Effect of the cell cycle synchronization strategy on the apoptosis/necrosis incidence in different types of fibroblast cells analysed using live-DNA flurochrome YO-PRO-1

Type of nuclear donor cells undergoing YO-PRO-1-based analysis	Percentage of middle- to late-phase apoptotic/necrotic (YO-PRO-1-positive) cells
Cultured (contact-inhibited) adult dermal fibroblast cells	20–25% aA
Cultured (serum-starved) adult dermal fibroblast cells	15–20% bD
Cultured (contact-inhibited) foetal fibroblast cells	35–40% B
Cultured (serum-starved) foetal fibroblast cells	20–25% aC

a, a – values with identical letters within the same column do not vary significantly ($P \geq 0.05$, chi-square test). Number of replicates = 50.

a, b – values with different letters within the same column differ significantly ($P < 0.05$, chi-square test). Number of replicates = 50.

A, B; B, C; B, D – values with different letters within the same column vary significantly ($P < 0.001$, chi-square test). Number of replicates = 50.

Table 2. Effect of the cell cycle synchronization strategy on the apoptosis/necrosis incidence in different types of fibroblast cells analysed using live-plasmalemma fluorescent marker Annexin V-eGFP

Type of nuclear donor cells undergoing Annexin V-eGFP-based analysis	Percentage of middle- to late-phase apoptotic/necrotic (Annexin V-eGFP-positive) cells
Cultured (contact-inhibited) adult dermal fibroblast cells	30% a
Cultured (serum-starved) adult dermal fibroblast cells	20% bA
Cultured (contact-inhibited) foetal fibroblast cells	40% cB
Cultured (serum-starved) foetal fibroblast cells	25% abC

a, a; b, b – values with identical letters within the same column do not vary significantly ($P \geq 0.05$, chi-square test). Number of replicates = 50.

a, b; a, c – values with different letters within the same column differ significantly ($P < 0.05$, chi-square test). Number of replicates = 50.

A, B; B, C – values with different letters within the same column vary significantly ($P < 0.001$, chi-square test). Number of replicates = 50.

In turn, fluorescent analysis of both types of Annexin V-eGFP-tagged contact-inhibited or serum-starved fibroblast cells revealed that the proportions of the cells with middle- to late-phase apoptotic and/or necrotic changes are comparable to those of the YO-PRO-1-labelled fibroblast cells. The percentages of middle- to late-phase apoptotic and necrotic cells with advanced biochemical, biophysical, ultrastructural and morphologic transformations did not exceed approximately 30%, 40% or 20% and 25% per each of 50 diagnosed random samples of contact-inhibited adult dermal fibroblasts and foetal fibroblasts or serum-starved adult dermal fibroblasts and foetal fibroblasts, respectively (Table 2). Moreover, the Annexin V-eGFP-based analysis confirmed the extremely low rate (ranging from 0 to 5%) of the cells with normal plasmalemma ultrastructure and morphology that emitted the green eGFP-derived bioluminescence. These findings indicate that such Annexin V-eGFP-positive cells are early- to middle-phase apoptotic, i.e., that apoptosis process in these cells did not pass through the irreversibility check-point at the middle phase.

Generation of porcine nuclear-transferred embryos with the use of non-apoptotic and/or non-necrotic fibroblast cells

In Groups IA and IB (Tables 3, 4 and 5), a total of 256 and 272 enucleated oocytes were reconstituted with non-apoptotic/non-necrotic contact-inhibited or serum-starved adult cutaneous fibroblast cell nuclei, respectively. Out of 256 and 272 simultaneously fused and electroactivated oocytes, 231 (90.2%) and 237 (87.1%) were cultured *in vitro*, respectively. From among 231 and 237 cultured NT embryos, 159 (68.8%) and 132 (55.7%) underwent cleavage divisions, respectively. The frequencies of cloned embryos that developed to the morula and blastocyst stages were 125/231 (54.1%) and 68/231 (29.4%) or 99/237 (41.8%) and 43/237 (18.1%), respectively.

Table 3. Comparison of the *in vitro* developmental competences between porcine nuclear-transferred embryos derived from contact-inhibited fibroblast cells not exhibiting proapoptotic and/or pronecrotic symptoms

Type of non-apoptotic/ non-necrotic nuclear donor cells	Number of oocytes/embryos			Development to	
	reconstructed	cultured (%)	cleaved (%)	morulae (%)	blastocysts (%)
Cultured (contact-inhibited) adult dermal fibroblast cells [Group IA]	256	231/256 (90.2) a	159/231 (68.8) A	125/231 (54.1) C	68/231 (29.4) a
Cultured (contact-inhibited) foetal fibroblast cells [Group IIA]	261	245/261 (93.9) a	197/245 (80.4) B	171/245 (69.8) D	97/245 (39.6) b

a, a – values with identical letters within the same column do not vary significantly ($P \geq 0.05$, chi-square test). Number of replicates = 7.

a, b – values with different letters within the same column differ significantly ($P < 0.05$, chi-square test). Number of replicates = 7.

A, B – values with different letters within the same column vary significantly ($P < 0.01$, chi-square test). Number of replicates = 7.

C, D – values with different letters within the same column differ significantly ($P < 0.001$, chi-square test). Number of replicates = 7.

In Groups IIA and IIB (Tables 3, 4 and 6), a total of 261 and 253 enucleated oocytes were subjected to reconstruction with non-apoptotic/non-necrotic contact-inhibited or serum-starved foetal fibroblast cell nuclei, respectively. Among 261 and 253 simultaneously fused and electrically activated oocytes originating from Group IIA and Group IIB, 245 (93.9%) and 227 (89.7%) were intended to be *in vitro* cultured, respectively. Out of 245 and 227 cultured NT embryos, 197 (80.4%) and 151 (66.5%) were able to divide, respectively. The proportions of cloned embryos that completed their development to the morula and blastocyst stages were 171/245 (69.8%) and 97/245 (39.6%) or 132/227 (58.1%) and 63/227 (27.8%), respectively.

Table 4. Comparison of the *in vitro* developmental competences between porcine nuclear-transferred embryos derived from serum-starved fibroblast cells not exhibiting proapoptotic and/or pronecrotic symptoms

Type of non-apoptotic/ non-necrotic nuclear donor cells	Number of oocytes/embryos			Development to	
	reconstructed	cultured (%)	cleaved (%)	morulae (%)	blastocysts (%)
Cultured (serum-starved) adult dermal fibroblast cells [Group IB]	272	237/272 (87.1) a	132/237 (55.7) A	99/237 (41.8) C	43/237 (18.1) a
Cultured (serum-starved) foetal fibroblast cells [Group IIB]	253	227/253 (89.7) a	151/227 (66.5) B	132/227 (58.1) D	63/227 (27.8) b

a, a – values with identical letters within the same column do not vary significantly (P≥0.05, chi-square test). Number of replicates = 7.

a, b – values with different letters within the same column differ significantly (P<0.05, chi-square test). Number of replicates = 7.

A, B – values with different letters within the same column vary significantly (P<0.01, chi-square test). Number of replicates = 7.

C, D – values with different letters within the same column differ significantly (P<0.001, chi-square test). Number of replicates = 7.

Table 5. Effect of the cell cycle synchronization approach on the *in vitro* developmental potential of porcine cloned embryos reconstructed with non-apoptotic/non-necrotic adult dermal fibroblast cell nuclei

Strategy of mitotic cycle synchronization	Number of oocytes/embryos			Development to	
	enucleated	fused (%)	cleaved (%)	morulae (%)	blastocysts (%)
Contact inhibition [Group IA]	256	231/256 (90.2) a	159/231 (68.8) A	125/231 (54.1) C	68/231 (29.4) C
Serum starvation [Group IB]	272	237/272 (87.1) a	132/237 (55.7) B	99/237 (41.8) D	43/237 (18.1) D

a, a – values with identical letters within the same column do not vary significantly (P≥0.05, chi-square test). Number of replicates = 7.

A, B – values with different letters within the same column differ significantly (P<0.001, chi-square test). Number of replicates = 7.

C, D – values with different letters within the same column vary significantly (P<0.01, chi-square test). Number of replicates = 7.

Table 6. Effect of the cell cycle synchronization approach on the *in vitro* developmental potential of porcine cloned embryos reconstructed with non-apoptotic/non-necrotic foetal fibroblast cell nuclei

Strategy of mitotic cycle synchronization	Number of oocytes/embryos			Development to	
	enucleated	fused (%)	cleaved (%)	morulae (%)	blastocysts (%)
Contact inhibition [Group IIA]	261	245/261 (93.9) a	197/245 (80.4) A	171/245 (69.8) C	97/245 (39.6) C
Serum starvation [Group IIB]	253	227/253 (89.7) a	151/227 (66.5) B	132/227 (58.1) D	63/227 (27.8) D

a, a – values with identical letters within the same column do not vary significantly (P≥0.05, chi-square test). Number of replicates = 7.

A, B – values with different letters within the same column differ significantly (P<0.001, chi-square test). Number of replicates = 7.

C, D – values with different letters within the same column vary significantly (P<0.01, chi-square test). Number of replicates = 7.

Table 7. Effect of YO-PRO-1- and Annexin V-eGFP-mediated pre-selection of contact-inhibited adult dermal fibroblast cells on the *in vitro* developmental outcomes of porcine nuclear-transferred embryos

YO-PRO-1- and Annexin V-eGFP-based pre-selection of non-apoptotic and non-necrotic nuclear donor cells	Number of oocytes/embryos			Development to	
	enucleated	fused (%)	cleaved (%)	morulae (%)	blastocysts (%)
+	256	231/256 (90.2) a	159/231 (68.8) a	125/231 (54.1) a	68/231 (29.4) A
−	211	195/211 (92.4) a	114/195 (58.5) b	90/195 (46.2) b	34/195 (17.4) B

a, a – values with identical letters within the same column do not vary significantly (P≥0.05, chi-square test). Number of replicates ≥6.

a, b – values with different letters within the same column differ significantly (P<0.05, chi-square test). Number of replicates ≥6.

A, B – values with different letters within the same column vary significantly (P<0.01, chi-square test). Number of replicates ≥6.

In the next step, we evaluated the electrofusion rates of somatic cell-ooplast couplets and the *in vitro* developmental capacities of porcine nuclear-transferred embryos reconstituted with YO-PRO-1-/Annexin V-eGFP-negative and randomly selected adult dermal or foetal fibroblast cells, the mitotic cycle of which had been previously synchronized at the G0/G1 phases by contact inhibition (Tables 7 and 8). We additionally compared the electrofusion rates and developmental competences between NT embryos descended from YO-PRO-1-/Annexin V-eGFP-negative and randomly selected adult dermal or foetal fibroblast cells that had been subjected to serum starvation under *in vitro* culture conditions (Tables 9 and 10). The percent-

ages of enucleated oocytes that were successfully fused with contact-inhibited and serum-starved adult cutaneous or foetal fibroblast cells did not vary significantly whether YO-PRO-1-/Annexin V-eGFP-mediated pre-selection was accomplished or not. However, not only for contact-inhibited adult cutaneous fibroblast cells and foetal fibroblast cells, but also for their serum-deprived cell counterparts, the cleavage activities and morula/blastocyst formation rates tended to be significantly higher among the nuclear-transferred embryos reconstructed with YO-PRO-1- and Annexin V-eGFP-negative cells as compared to those derived from the cells that did not undergo YO-PRO-1- and Annexin V-eGFP-based pre-selection.

Table 8. Effect of YO-PRO-1- and Annexin V-eGFP-mediated pre-selection of contact-inhibited foetal fibroblast cells on the *in vitro* developmental outcomes of porcine nuclear-transferred embryos

YO-PRO-1- and Annexin V-eGFP-based pre-selection of non-apoptotic and non-necrotic nuclear donor cells	Number of oocytes/embryos			Development to	
	enucleated	fused (%)	cleaved (%)	morulae (%)	blastocysts (%)
+	261	245/261 (93.9) a	197/245 (80.4) A	171/245 (69.8) A	97/245 (39.6) A
–	223	210/223 (94.2) a	143/210 (68.1) B	123/210 (58.6) B	59/210 (28.1) B

a, a – values with identical letters within the same column do not vary significantly (P≥0.05, chi-square test). Number of replicates ≥6.

A, B – values with different letters within the same column differ significantly (P<0.01, chi-square test). Number of replicates ≥6.

Table 9. Effect of YO-PRO-1- and Annexin V-eGFP-mediated pre-selection of serum-starved adult dermal fibroblast cells on the *in vitro* developmental outcomes of porcine nuclear-transferred embryos

YO-PRO-1- and Annexin V-eGFP-based pre-selection of non-apoptotic and non-necrotic nuclear donor cells	Number of oocytes/embryos			Development to	
	enucleated	fused (%)	cleaved (%)	morulae (%)	blastocysts (%)
+	272	237/272 (87.1) a	132/237 (55.7) A	99/237 (41.8) a	43/237 (18.1) a
–	225	199/225 (88.4) a	88/199 (44.2) B	67/199 (33.7) b	19/199 (9.5) b

a, a – values with identical letters within the same column do not vary significantly (P≥0.05, chi-square test). Number of replicates ≥6.

a, b – values with different letters within the same column differ significantly (P<0.05, chi-square test). Number of replicates ≥6.

A, B – values with different letters within the same column vary significantly (P<0.01, chi-square test). Number of replicates ≥6.

Table 10. Effect of YO-PRO-1- and Annexin V-eGFP-mediated pre-selection of serum-starved foetal fibroblast cells on the *in vitro* developmental outcomes of porcine nuclear-transferred embryos

YO-PRO-1- and Annexin V-eGFP-based pre-selection of non-apoptotic and non-necrotic nuclear donor cells	Number of oocytes/embryos			Development to	
	enucleated	fused (%)	cleaved (%)	morulae (%)	blastocysts (%)
+	253	227/253 (89.7) a	151/227 (66.5) a	132/227 (58.1) A	63/227 (27.8) A
–	214	193/214 (90.2) a	110/193 (57.0) b	89/193 (46.1) B	31/193 (16.1) B

a, a – values with identical letters within the same column do not vary significantly ($P \geq 0.05$, chi-square test). Number of replicates ≥6.

a, b – values with different letters within the same column differ significantly ($P < 0.05$, chi-square test). Number of replicates ≥6.

A, B – values with different letters within the same column vary significantly ($P < 0.01$, chi-square test). Number of replicates ≥6.

Discussion

The use of live-DNA and plasma membrane fluorescent markers for detection of apoptosis in fibroblast cells prior to somatic cell nuclear transfer in pigs

The current investigation has indicated that the morphologic criteria, commonly applied to assessment of cell viability, seem to be insufficient selection factors for the qualitative and quantitative evaluation of nuclear donor cells before their use for the somatic cell cloning. This key assumption relies on the *intra vitam* diagnostics of adult cutaneous or foetal fibroblast cells with the use of fluorescent markers such as YO-PRO-1 and Annexin V-eGFP that, in general, did not reveal the presence of the early-apoptotic symptoms in the subpopulations of morphologically normal cells. These cells usually did not emit the bright green bioluminescence after the excitation of the YO-PRO-1-nuclear DNA or Annexin V-eGFP-PS residue complexes with blue light. Nonetheless, there was identified a negligible percentage (not exceeding 5%) of the early- to middle-phase apoptotic cells that displayed the ability to be tagged with the Annexin V-eGFP conjugate and simultaneously their plasma membranes did not undergo the ultrastructural and morphologic transformations. Furthermore, using either YO-PRO-1-/Annexin V-eGFP-negative adult cutaneous fibroblast cells (Tables 7 and 9) or foetal fibroblast cells (Tables 8 and 10) to reconstruct the enucleated oocytes, we observed significantly higher frequencies of cloned pig embryos that were able to reach the morula and blastocyst stages under *in vitro* culture (morula, 42% to 54% or 58% to 70%; blastocyst, 18% to 29% or 28% to 40%, respectively) than those noticed for nuclear-transferred embryos derived from randomly selected adult dermal or foetal fibroblast cells (morula, 34% to 46% or 46% to 59%; blastocyst, 10% to 17% or 16% to 28%, respectively). This finding was demonstrated among both embryos reconstituted with contact-inhibited fibroblast cell nuclei

(Tables 7 and 8) and embryos descended from serum-starved fibroblast cell nuclei (Tables 9 and 10).

In contrast to the non-vital methods for detection of apoptotic symptoms, which require either the fixation, permeabilization or preparation of cytosolic/nuclear extracts or homogenates resulting in irreversible deteriorating changes or direct damage of plasma membrane of analysed cells, live-DNA or plasmalemma diagnostics for apoptosis could provide a practical means of the biochemical, biophysical and ultrastructural evaluation of nuclear donor cells prior to their utilization in the somatic cell cloning. Moreover, these methods of apoptosis detection could be supplementary to morphologic evaluation of somatic cells, because the vitally diagnosed cells can be used directly for the nuclear transfer procedure (Samiec and Skrzyszowska, 2012 a; Skrzyszowska et al., 2006).

Generation of porcine nuclear-transferred embryos with the use of non-apoptotic and/or non-necrotic fibroblast cells

The results of our study have confirmed that the methods applied to the synchronization of the cell cycle of nuclear donor cells affect the *in vitro* developmental abilities of porcine NT embryos according to the type of fibroblast cells used for the reconstruction of enucleated oocytes. The YO-PRO-1- and Annexin V-eGFP-negative foetal fibroblasts that had undergone the contact inhibition under the conditions of a total confluence displayed a higher cloning competence than the non-apoptotic and/or non-necrotic adult cutaneous fibroblast cells treated in the same way. Analogously, the cell nuclei of the serum-deprived foetal fibroblast cells that were not tagged with YO-PRO-1 dye and eGFP bioluminescent fluorochromoprotein conjugated with Annexin V were characterized by a higher remodelling/reprogramming ability retained in cloned embryos than serum-deprived adult dermal fibroblast cell nuclei. Therefore, nuclear transfer-derived embryos that had been reconstructed with non-apoptotic and/or non-necrotic contact-inhibited foetal fibroblast cells exhibited significantly higher cleavage activity and developmental capacity to morula/blastocyst stages than NT embryos reconstructed with contact-inhibited adult dermal fibroblast cells. Furthermore, both the cleavage activity and developmental potential to morula/blastocyst stages of cloned embryos that had been reconstituted with cell nuclei of YO-PRO-1-/Annexin V-eGFP-negative and serum-deprived adult ear skin-derived fibroblasts were considerably lower than the morphokinetic parameters of preimplantation development dynamics among NT embryos reconstituted with serum-deprived foetal fibroblasts. Similarly to the results of our study, Lee et al. (2003 a) showed that a higher epigenomic propensity/susceptibility for cloning in pigs (i.e., enhanced epigenetic reprogrammability of nuclear genome), as measured with blastocyst yield, was characteristic of the serum-starved foetal fibroblasts compared to the adult dermal fibroblasts (approximately 16% vs. 8%). In our experiments, NT embryos reconstituted with contact-inhibited foetal or adult ear skin-derived fibroblast cells that were identified as non-apoptotic and/or non-necrotic displayed significantly higher blastocyst formation rates than those for cloned embryos descended from serum-starved foetal or adult cutaneous fibroblasts. Nevertheless, the porcine cloned embryos reconstructed with cell nuclei of contact-inhibited foetal fibroblasts

developed *in vitro* to blastocyst stage at an almost two-fold higher rate (nearly 40% vs. 23%) than those in the study by Boquest et al. (2002). The cavitation rate of pig embryos that had been developed from enucleated oocytes receiving the cell nuclei of YO-PRO-1-/Annexin V-eGFP-negative and contact-inhibited adult dermal fibroblasts was also significantly (about five-fold) higher in our study (approximately 29.5% vs. 6%) than the cavitation rate indicated in other studies (Miyoshi et al., 2002; Roh and Hwang, 2002). But, in these studies, the nuclear donor cells were not diagnosed *intra vitam* for apoptosis.

Additionally, the *ex vivo* developmental outcomes of porcine cloned embryos derived from foetal and adult cutaneous fibroblast cells that had been previously classified as both YO-PRO-1- and Annexin V-eGFP-negative were significantly better in our current investigation (40% vs. 30.5% and 29.5% vs. 21% blastocysts, respectively) when compared to those of our previous study (Samiec and Skrzyszowska, 2012 a), in which NT oocytes reconstituted with only YO-PRO-1-negative fibroblast cells were also stimulated using the strategy of simultaneous fusion and electrical activation. Moreover, our present and previous (Samiec and Skrzyszowska, 2012 a) studies have shown that compared to the genomic DNA descended from contact-inhibited foetal fibroblast cells, the use of nuclear genome originating from contact-inhibited adult cutaneous fibroblast cells for reconstruction of enucleated *in vitro*-matured oocytes resulted in the nearly one-and-a-half times diminished percentages of the NT blastocysts generated. It 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 dermo-integumentary tissue. That is why, the capability of nuclear DNA inherited 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 cutaneous and subcutaneous tissues (Samiec, 2004, 2005 a, b; Samiec and Skrzyszowska, 2005 b, 2011 a, b).

In conclusion, the *in vitro* developmental competences of porcine nuclear-transferred embryos varied significantly according to not only the method of somatic cell cycle synchronization, but also the type of YO-PRO-1-/Annexin V-eGFP-negative fibroblast cells used for the reconstruction of enucleated oocytes. Nevertheless, regardless of the origin/provenance of nuclear donor fibroblast cells (derivation from adult animals or foetuses), the morula and blastocyst yields for cloned embryos reconstructed with the cell nuclei of non-apoptotic and/or non-necrotic contact-inhibited cells were higher than those for embryos reconstructed with the cell nuclei of non-apoptotic/non-necrotic serum-deprived cells. To our knowledge, the generation of NT pig embryos with the use of *in vitro*-matured oocytes receiving the cell nuclei descended from somatic cells that had been previously analysed for the lack of proapoptotic symptoms by both vital DNA and plasma membrane staining has not yet been reported. It is also the first report, in which the impact of different methods of artificially synchronizing the mitotic cycle of porcine somatic cells at the G0/G1 phases on the preimplantation development of cloned embryos, was examined

depending on the type of YO-PRO-1- and Annexin V-eGFP-negative nuclear donor fibroblast cells used for SCNT procedure.

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Uzyskiwanie klonalnych zarodków świni z wykorzystaniem komórek fibroblastycznych poddanych inhibicji kontaktowej lub deprywacji troficznej oraz analizowanych przyżyciowo w kierunku apoptozy

STRESZCZENIE

Efektywność klonowania somatycznego determinuje wiele czynników. Jednym z nich jest strukturalno-funkcjonalna jakość komórek-dawców jąder. Kryteria morfologiczne stosowane dotychczas do oceny jakościowej komórek somatycznych mogą być niedostateczne w odniesieniu do procedury klonowania. Zmiany biochemiczne i biofizyczne, które są jednymi z najwcześniejszych symptomów transdukcji sygnału apoptotycznego, mogą nie znajdować odzwierciedlenia w zmianach morfologicznych komórek somatycznych. Dlatego też, w naszych eksperymentach, fibroblasty tkanki skórnej dorosłych osobników lub fibroblasty płodowe, które stanowiły źródło genomowego DNA w procedurze klonowania, analizowano przyżyciowo w kierunku wykrywania biochemicznych i biofizycznych transformacji proapoptotycznych przy wykorzystaniu markerów fluorescencyjnych wiążących się z DNA (YO-PRO-1) lub błoną plazmatyczną (Aneksyna V-eGFP). W grupach IA i IB, odsetek zarodków klonalnych uzyskanych z nieapoptotycznych i/lub nienekrotycznych fibroblastów tkanki skórnej dorosłych osobników, hodowanych *in vitro* w warunkach inhibicji kontaktowej lub głodzonych, które rozwinęły się do stadium moruli

i blastocysty, wynosił, odpowiednio, 125/231 (54,1%) i 68/231 (29,4%) oraz 99/237 (41,8%) i 43/237 (18,1%). Z kolei w grupach IIA i IIB odsetek zarodków zrekonstruowanych z jąder nieapoptotycznych i/lub nienekrotycznych fibroblastów płodowych, hodowanych w warunkach inhibicji kontaktowej lub głodzonych, które osiągnęły stadium moruli i blastocysty, utrzymywał się na poziomie, odpowiednio, 171/245 (69,8%) i 97/245 (39,6%) oraz 132/227 (58,1%) i 63/227 (27,8%). Podsumowując, inhibicja kontaktowa migracji i aktywności proliferacyjnej zarówno fibroblastów tkanki skórnej dorosłych osobników, jak i fibroblastów płodowych skutkowała znacznie wyższymi kompetencjami ich jąder komórkowych do pokierowania rozwojem *in vitro* klonalnych zarodków świni niż deprywacja troficzna obu rodzajów komórek fibroblastycznych. Ponadto, niezależnie od metod zastosowanych do sztucznej synchronizacji cyklu mitotycznego komórek-dawców jąder w fazach G0/G1, istotnie wyższe zdolności rozwojowe *in vitro* do osiągnięcia stadium moruli i blastocysty stwierdzono w odniesieniu do zarodków zrekonstruowanych z jąder nieapoptotycznych i/lub nienekrotycznych fibroblastów płodowych, w porównaniu z subpopulacją zarodków zrekonstruowanych z jąder nieapoptotycznych i/lub nienekrotycznych fibroblastów tkanki skórnej dorosłych osobników. Zgodnie z naszą wiedzą jest to pierwsza praca naukowa dotycząca uzyskiwania klonalnych zarodków świni z oocytów pochodzenia poubojowego, rekonstytuowanych z jąder hodowanych w warunkach inhibicji kontaktowej lub głodzonych komórek somatycznych, które poddano wszechstronnej, przyżyciowej diagnostyce potwierdzającej brak występowania biochemicznych i biofizycznych zmian proapoptotycznych w ich plazmolemmie.