Original article

Pseudophysiological transcomplementary activation of reconstructed oocytes as a highly efficient method used for producing nuclear-transferred pig embryos originating from transgenic foetal fibroblast cells

M. Samiec[1], M. Skrzyszowska[1], D. Lipiński[2]

[1] National Research Institute of Animal Production, Department of Biotechnology of Animal Reproduction,
Krakowska 1, 32-083 Balice n. Kraków, Poland
[2] Poznań University of Life Sciences, Department of Biochemistry and Biotechnology,
Wołyńska 32, 60-637 Poznań, Poland

Abstract

The completely new strategy of pseudophysiological transcomplementary (transcytoplasmic) activation (PP-TCA) of nuclear-transferred oocytes, which had been derived from *pWAPhGH-GFPBsd* transfected foetal fibroblast cells, was recently applied to the somatic cell cloning of pigs. It resulted in the considerable enhancing not only the cleavage activity of cultured cloned embryos, but also their morula and blastocyst formation rates as compared to the use of standard simultaneous fusion and electrical activation of reconstituted oocytes (77% vs. 57%, 63% vs. 46% and 40% vs. 27%, respectively). Altogether, the use of cytosolic components descended from heterologous (rabbit) zygotes as the agents for stimulation of porcine clonal cytoplasmic hybrids (cybrids) turned out to be reliable and feasible strategy for the generation of transgenic blastocysts by somatic cell nuclear transfer (SCNT). Furthermore, to our knowledge, no previous study has reported the preimplantation developmental outcome of transgenic nuclear-transferred pig embryos following the PP-TCA that was developed and optimised in our laboratory.

Key words: pig, reconstructed oocyte, pseudophysiological activation, transcomplementary (transcytoplasmic), nuclear-transferred embryo, preimplantation development

Introduction

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 Sousa et al. 2002, Lee et al. 2003a) or *in vitro*-matured oocytes (Samiec et al. 2003, Lee et al. 2003a, 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

Correspondence to: M. Samiec, e-mail: marcin.samiec@izoo.krakow.pl

activate MII-stage oocytes and to initiate embryo development is essential for successful cloning by SCNT (Samiec 2004, Vajta et al. 2007). 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 optimised (Gil et al. 2010). An optimal time frame to activate gilt or sow oocytes may depend on both the time required for completion of nuclear-cytoplasmic maturation and the time by which aging process of mature oocytes starts. Cytoplasmic maturation is likely to include changes in the properties, size, and density of cytoplasmic Ca^{2+} release channels necessary for the oocyte to elicit an increase in intracellular Ca^{2+} concentration in response to the activating stimuli and subsequent development (De Sousa et al. 2002, Martinez Diaz et al. 2002).

Activation of oocytes, which has been induced either during fertilisation or by artificial agents during the cloning procedure, evokes the cytosolic calcium concentration ($[Ca^{2+}]_c$) oscillations or single $[Ca^{2+}]_c$ transients (Fissore et al. 1999, Machaty et al. 1999, Ozil and Huneau 2001). Despite the uncertainty of how the initial rises in $[Ca^{2+}]_c$ are prompted, it is widely accepted that physiological or artificial activation stimulates the phosphoinositide pathway, with the generation of *myo*-inositol-1,4,5-*tris*phosphate (InsP$_3$) by the enzymatic action of phospholipase C (PLC) and the subsequent release of calcium cations from endoplasmic reticulum (Fissore et al. 1999, Machaty et al. 1999, Wu et al. 2001, Marangos et al. 2003). Further investigation into the role of PLC isoforms (PLC-β, PLC-γ) as the triggers of $[Ca^{2+}]_c$ increases has led to the recent identification of a sperm-specific PLC (PLC-ξ) as the putative sperm-derived oocyte activating factor (Nomikos et al. 2005, Yoneda et al. 2006). It is known that InsP$_3$-mediated calcium signalling pathway is responsible for downregulation of both maturation/meiosis-promoting factor (MPF) and cytostatic factor (CSF), the latter of which is dependent on the complex activity (enzymatic cascade) of mitogen-activated protein kinases, i.e., extracellular-regulated protein kinases (MAPKs/ERKs). In turn, diminishments in the concentrations and activities of MPF and CSF-related MAPKs contribute to such events during oocyte activation as resumption and termination of meiosis, extrusion of the second polar body, pronuclear formation, transition from meiotic to mitotic control of cell cycle and initiation of embryonic cleavage (Prather et al. 1999, Martinez Diaz et al. 2002, Ito et al. 2004).

In the swine somatic cell cloning technology, commonly used activating stimuli are physical agents such as electric (DC) pulses (Martinez Diaz et al. 2002, Im et al. 2004, Hölker et al. 2005), or chemical agents such as specific ionophore antibiotics (e.g., calcium ionomycin, Ca^{2+} ionophore A23187/calcimycin) (Boquest et al. 2002, Yin et al. 2002, Hyun et al. 2003) or thimerosal in combination with dithiothreitol (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 optimising technical parameters of electrical field involving strength, duration of DC pulses, number of pulses and time interval between them (Martinez Diaz et al. 2002, 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). This group of repressory agents involves such members as 6-dimethylaminopurine (6-DMAP) (Boquest et al. 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-related p34^{cdc2}/CDK1 kinases and selective inhibitor of MPF-related p34^{cdc2}/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 (Yin et al. 2002, Martinez Diaz et al. 2002, Lee et al. 2003a).

The physicochemical agents such as electric pulses or calcium ionophore antibiotics, which are commonly used for artificial activation of porcine nuclear-transferred (NT) oocytes, can affect detrimentally or cytotoxically the clonal cybrids (nuclear-cytoplasmic hybrids) and thereby inhibit the development or decrease the quality of cloned embryos. Therefore, we have recently developed the novel method of pseudophysiological transcomplementary (transcytoplasmic) activation to stimulate the developmental program of pig oocytes reconstructed by the somatic cell cloning. Our efforts have been focused on the estimation of *in vitro* developmental competences of transgenic cloned pig embryos following pseudophysiological transcytoplasmic activation of oocytes receiving *pWAPhGH-GFPBsd* gene construct-nucleofected foetal fibroblast cell nuclei (Samiec and Skrzyszowska 2010, Samiec et al. 2010).

Materials and Methods

In vitro production of transgenic fibroblast cell-cloned pig embryos

Novel method of pseudophysiological transcomplementary activation of reconstituted oocytes

In the cloning procedure *in vitro*-matured gilt/sow oocytes were used as recipient cells for cell nuclei of positively selected *pWAPhGH-GFPBsd* transgenic fibroblast cells. Their mitotic cycle had been previously synchronised at G1/G0 stages through the contact inhibition of migration and proliferative growth after reaching the total confluency state under *in vitro* culture conditions. Cumulus-denuded *in vitro*-matured oocytes were incubated in the maturation medium supplemented with 0.4 µg mL^{-1} demecolcine (DMCC; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) and 0.05 M sucrose (Sigma-Aldrich) for 1 h. The treated oocytes were subsequently transferred into a micromanipulation chamber filled with Tissue Culture Medium-199 (TCM-199; TC-199 medium, Gibco BRL, Life Technologies Inc., Grand Island, NY, USA) containing 4 mg mL^{-1} bovine serum albumin (fraction V; BSA-V, Sigma-Aldrich) and 5 µg mL^{-1} cytochalasin B (CB; Sigma-Aldrich). Metaphase chromosomes, which had been allocated into the chemically induced protrusion of the plasma membrane, were removed microsurgically. The chemically-assisted enucleation was accomplished by gently aspirating the ooplasmic cone, which contained the condensed chromosome cluster, with the aid of a bevelled micropipette. The reconstruction of the previously enucleated oocytes (i.e., ooplasts) was performed by microinjection of either the somatic cell-derived karyoplasts or intact whole tiny nuclear donor cells, whose diameter ranged between 8 µm and 12 µm, directly into the cytoplasm. The karyoplasts were prepared through mechanically-induced cytolysis. The somatic cell plasma membranes were broken by gentle, repeated aspiration of the fibroblast cells into and out of the pipette whose tip had an external diameter smaller than the diameter of the selected cells. The reconstructed oocytes were incubated in North Carolina State University-23 (NCSU-23) medium supplemented with 0.4% BSA-V for 0.5 to 1 h before their pseudophysiological transcomplementary activation (PP-TCA). The PP-TCA of porcine NT oocytes was achieved by electrofusion of them with the xenogenic cytoplasts isolated from *in vivo*-derived rabbit zygotes (i.e., with the so-called zygoplasts), which led to the formation of triple xenocytoplasmic hybrids (xenocybrids) or diploid multicellular triplets. These latter originated from three sources: 1. whole nuclear donor fibroblast cells or their karyoplasts; 2. allogenic (homologous) enucleated oocytes (ooplasts) and 3. xenogenic (heterologous) zygote-derived cytoplasts (Samiec and Skrzyszowska 2010, Samiec et al. 2010).

The rabbit zygotes were flushed post-mortem from the separated oviducts of superovulated post-pubertal female donors 18 to 20 h after human chorionic gonadotropin (hCG) administering and copulation. Superovulation of postpubertal New Zealand White rabbit does had been induced by intramuscular injection of 100 IU of equine chorionic gonadotropin (eCG; Serogonadotropin, Biowet, Drwalew, Poland). After 72 h the females had been intravenously injected with 100 IU of hCG (Biogonadyl, Biomed, Lublin, Poland) and mated with fertile rabbit bucks.

Before isolation of zygoplasts in the glass micromanipulation chamber filled with TC-199 medium supplemented with 4 mg mL^{-1} BSA-V and 5 µg mL^{-1} CB, the rabbit zygotes were incubated in the TCM-199/BSA enriched with 7.5 µg mL^{-1} CB for 15-20 min. The zygote-descended cytoplasts (i.e., zygoplasts) were prepared through gentle aspiration of the small amounts of cytoplasm under the plasmalemma of rabbit zygotes (apronuclear live-membrane structures with 10-20% of the volume of the zygote cytoplasm) into the micromanipulation pipette whose tip had an external diameter about 15-20 µm (the same as used for enucleation of oocytes). Single rabbit zygoplasts were inserted into the perivitelline space of previously reconstructed pig oocytes. The resulting zygoplast-NT oocyte couplets underwent subsequently fusion which was induced by generation of two successive DC pulses of 1.2 kV cm^{-1} for 60 µs. The electrofusion of rabbit zygoplast and porcine reconstructed oocyte (clonal cybrid) plasma membranes occurred in an isotonic dielectric solution deprived of Ca^{2+} ions which consisted of 0.3 M mannitol supplemented with 0.1 mM MgSO$_4$ (Sigma-Aldrich) and 0.2 mg mL^{-1} fatty acid-free bovine serum albumin (FAF-BSA; Sigma-Aldrich). The successful fusion process of the zygoplast with the SCNT unit terminated usually after 30 to 50 min. The transcytoplasmically-activated xenocybrids were cultured *in vitro* in NCSU-23 medium supplemented with 4 mg mL^{-1} BSA-V for 72-96 h. Afterwards, cleaved embryos were cultured in NCSU-23/BSA medium supplemented with 10% foetal bovine serum (FBS; Sigma-Aldrich) for an additional 72 h up to morula and blastocyst stages (Skrzyszowska and Samiec 2009, Samiec et al. 2010).

Table 1. Effect of oocyte activation strategies on the *in vitro* developmental competences of *pWAPhGH-GFPBsd* transgenic NT porcine embryos.

Activation method	No. of oocytes/embryos		Development to		
	Enucleated	Fused (%)	Cleaved (%)	Morulae (%)	Blastocysts (%)
PP-TCA (novel)	216	195/216 (90.3)[a]	151/195 (77.4)[A]	122/195 (62.6)[A]	77/195 (39.5)[A]
SF-EA (standard)	276	234/276 (84.8)[a]	133.234 (56.8)[B]	107/234 (45.7)[B]	63/234 (26.9)[B]

[a,a] Values with identical letters within the same column do not vary significantly ($P>0.05$, χ^2 test).
[A,B] Values with different letters within the same column differ significantly ($P\leq0.001$, χ^2 test). No. of replicates ≥ 6.

Standard method of simultaneous fusion and electrical activation of reconstituted oocytes

A control group of SCNT-derived oocytes was artificially stimulated using the protocol of simultaneous fusion and electrical activation (SF-EA). In the SF-EA protocol, electric pulses that induced a fusion of cytoplast-somatic cell couplets were simultaneously the stimuli initiating the activation of reconstructed oocytes. The complexes of enucleated oocytes and transfected foetal fibroblast cells were subjected to plasma membrane electroporation by application of two successive DC pulses of 1.2 kV cm^{-1} for 60 µs. The electropermeabilisation of cell plasma membranes was performed in an isotonic dielectric solution with concentration of calcium cations increased up to 1.0 mM L^{-1} (Skrzyszowska et al. 2008, Samiec and Skrzyszowska 2010). After SF-EA, cloned embryos were cultured *in vitro* in NCSU-23/BSA/FBS medium for 6-7 days up to morula/blastocyst stages.

Statistical analysis

The χ^2 test was applied in order to compare the number of succesfully reconstituted and activated oocytes, the number of cloned embryos undergoing cleavage divisions and the number of morulae and blastocysts generated *in vitro* between the groups that are related to the PP-TCA and SF-EA. The effect of methods applied to activate NT pig oocytes was regarded as: significant at the level of random error probability value $0.01<P\leq0.05$, highly significant at the level of $0.001<P\leq0.01$ and very highly significant at the level of $P\leq0.001$, respectively.

Results

The preimplantation developmental outcomes of porcine transgenic cloned embryos such as cleavage activity, morula and blastocyst formation rates were compared between two groups which differed in oocyte activation method (Table 1). The experimental group included the NT oocytes that were stimulated via pseudophysiological transcomplementary activation (PP-TCA). In the control group the reconstituted oocytes underwent simultaneous fusion and electrical activation (SF-EA). The effect of the activation protocols on the frequencies of cleavage divisions of NT embryos derived from the *pWAPhGH-GFPBsd* fusion gene-transfected foetal fibroblast cells was statistically very highly significant. The cleavage activity of transgenic cloned embryos that originated from the oocytes undergoing PP-TCA was significantly higher than that for NT embryos derived from the oocytes subjected to SF-EA ($P\leq0.001$). There were also very highly significant differences in the developmental capabilities of genetically-transformed SCNT-derived embryos to reach the morula and blastocyst stages between the experimental and control groups. Both the morula and blastocyst formation rates were significantly higher following the PP-TCA compared to those after the SF-EA ($P\leq0.001$).

Discussion

The pseudophysiological transcomplementary (transcytoplasmic) activation of reconstituted pig oocytes was used in the somatic cell cloning of mammals for the first time. The results of our study have demonstrated that this method of stimulating the porcine transgenic clonal cybrids is more efficient than the simultaneous fusion and electrical activation of

NT-derived oocytes as has been confirmed by the very high morula and blastocyst formation rates under *in vitro* culture conditions (approximately 63% vs. 46% and 40% vs. 27%, respectively).

The meaning or etymology of the scientific term "transcomplementary" originates from the fusion or combination of the two key words: transfer and complementary. We have named our method of activation in such manner – transcomplementary or transcytoplasmic, because the mechanism underlying this original technique of activation is the transcytoplasmic influx of sperm-derived proteins, i.e., complementary factors that trigger the intracellular calcium oscillations. The members of this protein family are, among others, isoform ζ of phospholipase C (PLC-ζ) and glucosamine-6-phosphate isomerase or deaminase (GNPI or GNPDA) which are defined as oscillin or oscillogen (Jellerette et al. 2006, Yoneda et al. 2006). These complementary protein agents are accumulated in the cytoplasm and nucleoplasm of fertilised metaphase II-stage oocytes and remain active post resumption of II meiotic cell division. Their calcium-mediated oscillogenic activities are maintained not only for meiotic-to-mitotic transition of cell cycle control, but also throughout all the first cleavage mitotic cycle of zygotes. That is why the oscillatory changes in the cytoplasmic concentration of free calcium cations $[Ca^{2+}]_c$ persist up to the interphase of second cleavage mitotic cycle, before they cease in the early 2-blastomere embryos following gradual ubiquitin-dependent proteasome biodegradation of all the proteins evoking the intracellular calcium waves. The biodestruction of proteins that are able to propagate the transient rises in $[Ca^{2+}]_c$ begins at anaphase of first cell cycle of zygotes (Kono et al. 1995, Żernicka-Goetz et al. 1995, Marangos et al. 2003). The transmission, i.e., transfer together with cytoplasmic "carrier", of these complementary oscillogenic proteins is mediated via heterologous (rabbit) zygote-descended cytoplasts (apronuclear live-membrane structures) into ooplasm of porcine SCNT oocytes. Furthermore, the modified strategy of pseudophysiological (transcytoplasmic) activation has recently been applied to the successful stimulation of embryonic developmental program of nuclear-transferred goat oocytes under *in vitro* culture conditions (Samiec and Skrzyszowska 2008). However, this latter strategy was based on the electrofusion of caprine reconstituted oocytes with the allogenic cytoplasts isolated from *in vitro*-fertilised goat ova (zygotes) which led to the formation of triple allocytoplasmic hybrids (allocybrids). In consequence, these allocybrids were derived from three sources, such as: 1. whole nuclear donor fibroblast cells or their karyoplasts; 2. allogenic (homologous) enucleated doe oocytes (ooplasts) and

3. allogenic zygote-inherited cytoplasts, i.e., zygoplasts (Samiec and Skrzyszowska 2008, Skrzyszowska and Samiec 2010). Adaptation of the optimised PP-TCA method for use in the somatic cell cloning of goats resulted in the relatively high morula and blastocyst yields that were achieved to a comparable extent by the embryos reconstructed with either foetal fibroblast cell nuclei (39.0% and 20.3%, respectively) (Samiec and Skrzyszowska 2008) or adult cutaneous fibroblast cell nuclei (39.6% and 20.8%, respectively) (Skrzyszowska and Samiec 2010).

The host cytoplasm of the non-activated oocyte arrested at metaphase II stage which requires the exposure to exogenous (i.e., artificial) inductors of intracellular calcium oscillations to trigger resumption (and completion) of meiosis and entry into the first cleavage mitotic division cycle, can be competent for architectural remodelling and epigenetic reprogramming of somatic cell nuclei (Bui et al. 2007). However, because artificially-stimulated oscillatory (pulsatile) transient increase of free Ca^{2+} concentration in the ooplasm differs often considerably from the physiological pattern of activation evoked through monospermic fertilisation, it can cause incorrect donor nuclear chromatin rearrangements. In turn, the latter processes may evoke delayed or improper onset of somatic genome transcriptional activity as a result of DNA partial (incomplete) demethylation wave in preimplanted nuclear-transferred pig embryos (Kang et al. 2001, Samiec 2005, Zhang et al. 2007). On the contrary, our hypothesis is that in the cytoplasmic microenvironment of porcine reconstituted oocytes undergoing the pseudophysiological transcomplementary activation, donor cell nuclei appear to be more susceptible to initiation of proper molecular mechanism for structural remodelling of transcriptionally-repressive chromatin (i.e., constitutive and facultative heterochromatin). It involves the cessation of nucleosomal suppression and faithful recapitulation of embryonic nuclear totipotency developmental program-linked epigenetic alterations relative to selective acetylation/hyperacetylation connected with progressive demethylation/hypomethylation of histone lysines (Santos et al. 2003, Kourmouli et al. 2004, Seki et al. 2005). This right restoration of embryonic epigenetic memory in somatic chromatin may be induced by a complete as well as non-cytotoxic and non-genotoxic transduction of repetitive (pulsatory) Ca^{2+} transient-mediated signalling. This latter is maintained in dividing fertilised ova (zygotes) up to late pronuclear stage (Kono et al. 1995, Marangos et al. 2003) or even up to early 2-cell embryonic stage (Nakada et al. 1995, Żernicka-Goetz et al. 1995) as a result of high cytosolic concentration of sperm-transmitted proteins triggering acute oscillatory release of free Ca^{2+}

cations from calcium intracellular deposits (accumulated mainly in the agranular endoplasmic reticulum) (Kimura et al. 1998, Fissore et al. 1999, Saunders et al. 2002). The dramatic covalent changes in the acetylation/methylation level of histone-derived lysine amino acyl residues which take place between zygotic stage and 32-cell stage of embryogenesis (Liu et al. 2004, Sarmento et al. 2004, Zhang et al. 2007), seem to be an epigenomic prelude or prerequisite to further transcriptional reprogramming of DNA cytosine methylation modifications. The dynamics of these modifications increases significantly in the late 4-cell porcine SCNT embryos through somatic genome intensive demethylation wave which persists after maternal to embryonic transition (MET) of gene expression control (Viuff et al. 2002, Reik et al. 2003, Samiec 2004). The correct reprogramming process would affect epigenetic modifications of the somatic genome leading to frequency changes in the degree of expression of several embryonic genes as a result of silencing (repression) or enhancing (stimulation) of their transcriptional activity (Kang et al. 2003, Kourmouli et al. 2004, Liu et al. 2004). These epigenetic rearrangements such as methylation (or rather hemimethylation) of DNA cytosine residues and, associated with the donor cell-derived nuclear DNA epigenomic changes, covalent modifications of H3 and H4 histone amino acyl residues, involving deacetylation and methylation, are crucial processes in the regulation of transcription during embryonic development and are related to gene silencing (Santos et al. 2003, Liu et al., 2004, Sarmento et al. 2004, Samiec and Skrzyszowska 2005).

In conclusion, the inventive pseudophysiological transcomplementary (transcytoplasmic) activation of porcine nuclear transfer-derived oocytes, which has been developed in our laboratory lately and has been utilised in the somatic cell cloning of pigs for the first time, has been found to be relatively highly efficient method of stimulating the preimplantation development of transgenic clonal cybrids. Moreover, it has been demonstrated that the PP-TCA of porcine oocytes reconstructed with *pWAPhGH-GFPBsd* fusion gene-nucleofected foetal fibroblast cell nuclei contributes to achievement of both significantly higher percentage of dividing embryos and almost one-and-a-half times higher morula and blastocyst yields than the standard strategy of SF-EA.

Acknowledgements

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