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High developmental capability of porcine cloned embryos following trichostatin A-dependent epigenomic transformation during *in vitro* maturation of oocytes pre-exposed to *R*-roscovitine*

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The study was carried out in order to evolve, adopt and optimize the new system for preparation of nuclear recipient cells at different stages preceding the somatic cell nuclear transfer (SCNT) in pigs, including *in vitro* maturation (IVM) of oocytes. The system was applied to facilitate and accelerate the epigenomic reprogrammability for gene expression of donor cell nuclei that had been transplanted into cytoplasmic microenvironment of recipient oocytes and subsequently underwent the dedifferentiating and re-establishing the totipotent epigenetically conditioned status of their transcriptional activity during the preimplantation development of cloned embryos. The use of trichostatin A (TSA)-mediated epigenetic modulation of *in vitro*-maturing porcine nuclear recipient oocytes that had been pre-treated with *R*-roscovitine (*R*-RSCV) resulted in significantly increased blastocyst formation rate among the cloned embryos compared to the *R*-RSCV- and TSA-unexposed group (almost 44% *vs.* 26%).

KEY WORDS: pig / oocyte / *in vitro* maturation / *R*-roscovitine / trichostatin A / epigenomic maturity / somatic cell nuclear transfer / cloned embryo / epigenetic reprogramming

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The propagation of mammalian specimens by somatic cell nuclear transfer (SCNT) has important economical implications in biotechnology and biomedicine as so far has been demonstrated by generation of transgenic cloned animals with either the ability to produce valuable recombinant human proteins in their body fluids (milk, urine, seminal plasma, blood) or the resistance to interspecies transmissible and heritable diseases. Pig embryo engineering (transgenesis combined with somatic cell cloning) is also a particularly important research field of assisted reproductive technologies (ARTs) due to increasing role of the porcine organs in xenotransplantology or creation of animal bioreactors providing with biopharmaceuticals [Kind and Schnieke 2008, Samiec and Skrzyszowska 2011ab]. Nonetheless, the efficiency of SCNT technology in mammals remains unsatisfactory. In pigs, especially when compared to other farm livestock species, the percentage of somatic cell clones produced is significantly lower. As a rule, in relation to the number of the reconstructed oocytes, it does not exceed an average of 0.5 to 1.0% [De Sousa *et al.* 2002, Hyun *et al.* 2003, Samiec 2004]. Despite tremendous progress in 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 appear still often in this species [Samiec 2004, Samiec and Skrzyszowska 2011b]. 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 dysfunctions) leading to high pregnancy losses and neonatal deaths, may be an incomplete epigenomically-conditioned reprogramming of transcriptional activity for donor cell-descended genes [Mann *et al.* 2003, Santos and Dean 2004, Shi *et al.* 2004, Bonk *et al.* 2008].

Transcriptional activity of donor cell 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 [Santos and Dean 2004, Samiec 2005a, Zhao *et al*. 2009]. The remodelling/reprogramming processes 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 histones H3 and H4 [Samiec and Skrzyszowska 2005, Yamanaka *et al.* 2009, Zhao *et al*. 2010]. 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, Samiec 2005b].

The remodelling and reprogramming of somatic cell-derived nuclear apparatus in cloned embryos is a result of interaction of protein factors accumulated in the nucleoplasm and attached to the chromatin, configured in the form of metaphase plate in consequence of appropriate rearrangement of its spatial structure and nucleosome

repression, with protein factors of recipient oocyte cytoplasm (*i.e.*, host ooplasm). Both former and latter protein factors, whose concentration and activity at the high levels are the prerequisites for establishment of the state of cytoplasmic, nuclear and epigenomic maturity of enucleated host oocyte (ooplast/cytoplast) for somatic cell-inherited nuclear genome, involve many pathways of intracellular enzymatic machinery. The most important protein members of this machinery are cyclin-dependent kinases (CDKs). At the metaphase II (MII) stage of the meiotic cell cycle, they include, among others, maturation/meiosis-promoting factor (MPF) and cascade of mitogen-activated protein kinases (C-MAPKs) related to the activity of cytostatic factor (CSF). MPF is a heterodimeric enzyme complex that consists of the catalytic subunit ($p34^{cdc2}$/CDK1; 34-kDa cell division control protein kinase 2/cyclin-dependent protein kinase 1) and the regulatory subunit (cyclin B). Furthermore, at the anaphase II (AII) stage, the protein factors regulating the oocyte meiotic division cycle include, e.g., polysubunitary protein complex of ubiquitin ligase, which was named anaphase-promoting complex or cyclosome (APC/C) [Prather *et al.* 1999, Lee and Campbell 2006, Samiec and Skrzyszowska 2009]. Besides determination of cytoplasmic and nuclear (meiotic) maturity of recipient oocytes, the above-mentioned CDKs influence on the architectural remodelling of both oocyte-descended microtubule organizing centres (MTOCs), in other words, its acentriolar astrosphere and nuclear donor somatic cell-inherited chromatin as well as MTOCs (*i.e.*, dicentriolar centrosomes or tetracentriolar diplosomes), which have been transplanted into host ooplasm [Fissore *et al.* 1999, Campbell and Alberio 2003, Ito *et al.* 2004]. In turn, the basic agents, the presence of which is required for acquisition of epigenomic maturity of MII-staged oocytes, are: 1) competitive inhibitors of DNA methyltransferases/methylases (inhibitors of DNMTs; iDNMTs) such as isosteric blockers of the DNMT1o and DNMT3a/3b isoenzymes; 2) repressors of activity of methyl-CpG-binding proteins (MeCPs; proteins binding methylated 5′-cytidine-3′-monophosphate-5′-guanosine-3′/CpG dinucleotides/motifs), and 3) H3 and H4 histone acetyltransferases/acetylases (HATs) [Beaujean *et al.* 2004, Santos and Dean, 2004, Bonk *et al.* 2008, Das *et al.* 2010]. These include also: 4) isosteric inhibitors of histone deacetylases (HDACs); 5) supressory proteins of histone methyltransferases (HMTs); 6) histone demethylases/deiminases (HDMs), as well as 7) polysubunitary protein complexes with the activity of ATPases such as chromatin remodelling complexes (ChRs) [Kishigami *et al.* 2006, Bui *et al.* 2007, Yamanaka *et al.* 2009, Zhao *et al.* 2009, 2010].

The level of progression for the processes of genome-wide epigenetic alterations that are mediated by DNMTs 1o and 3a/3b and HDACs can be modulated (*i.e.*, reversed) *via* exogenous inhibitors of these enzymes throughout oocyte *in vitro* maturation (IVM). Moreover, the use of the artificial modifiers of epigenomically-conditioned gene expression, including the subclass of ectopic HDAC inhibitors, whose representative member is trichostatin A (TSA) [Costa-Borges *et al.* 2010, Martinez-Diaz *et al.* 2010, Bo *et al.* 2011], leads to the suppression of transcriptional silencing the genomic DNA of *ex vivo* cultured oocytes that provide a source of

recipient cells in the technology of mammalian SCNT [Cervera *et al.* 2009, Das *et al.* 2010]. The onset of gene transcriptional activity is evoked by non-specific/non-selective and reversible blocking the biocatalytic activity (*i.e*., transient inactivation) of histone deacetylases (HDACs) by the TSA [Zhang *et al.* 2007, Costa-Borges *et al.* 2010, Kim *et al.* 2011]. TSA-induced non-specific hyperacetylation of lysine residues on nucleosomal core-related histones H3 and H4 can play a role of epigenetic recognition coding system for the increased recruitment of histone acetyltransferases (HATs) and the enhanced association of double bromodomain-containing chromatin adaptor protein-4 (BRD4) molecules to acetylated histones of meiotic condensed chromosomes in the *in vitro*-maturing oocytes. It has been shown that the preferential binding of BRD4 proteins to lysine moieties of hyperacetylated core histones forming the octomeric nucleosome contributes to the rhythmic conversion of transcriptionally repressive chromatin (heterochromatin) to the transcriptionally permissive chromatin (euchromatin) [Rybouchkin *et al.* 2006, Nagashima *et al.* 2007, Martinez-Diaz *et al.* 2010]. As a result, after replacement of metaphase II chromosomes in the oocytes with the somatic cell-inherited chromatin, inhibition of global histone deacetylation through TSA-mediated down-regulation of HDAC activity can facilitate and accelerate the architectural remodelling and epigenetic reprogramming processes of nuclear donor cell-descended chromatin within the preimplanted cloned embryos. It is, therefore, conceivable that the erasing of epigenomic memory can occur in the somatic cell nuclei after their introduction into the cytoplasm of enucleated oocytes [Das *et al.* 2010, Kim *et al*. 2011]. On one hand, this can thereby give rise to the remarkable transformation of cytosine residue methylation marking of nuclear donor DNA from epigenetic pattern of differentiated cells into the totipotent dedifferentiated status of embryonic (*i.e*., zygotic) cells. On the other hand, the establishment of a transcriptionally permissive chromatin state within the rearranged donor cell nuclei *via* inducible active acetylation of histones H3 and H4 followed by indirect genome-wide demethylation of DNA cytosine residues can cause the silencing of gene expression to cease in the cells of nuclear-transferred (NT) embryos developing to the morula and blastocyst stages [Rybouchkin *et al.* 2006, Nagashima *et al.* 2007, Bo *et al.* 2011].

We have recently examined whether the inducible epigenetic modification, which was triggered *via* TSA (*i.e*., non-specific inhibitor of HDACs) during IVM of pig oocytes pre-treated with *R*-roscovitine (*R*-RSCV; specific inhibitor of MPF-related $p34^{cdc2}$/CDK1 kinase), affects the preimplantation developmental capacity of cloned embryos.

Material and methods

Abattoir-derived 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. 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) with the addition of 4 mg/mL bovine serum albumin (fraction V; BSA-V). The COCs were selected for *in vitro* culture under atmospheric conditions and subsequently were pre-matured with 50 μM of the *R*-enantiomer of roscovitine (*R*-RSCV) for 22 h. Fifty to sixty COCs were then incubated for a further 20 h in 500 μL of 25 mM HEPES- and 26.2 mM sodium bicarbonate ($NaHCO_3$)-buffered TC 199 medium supplemented with 0.1 IU/mL human menopausal gonadotropin (hMG), 10% porcine follicular fluid (pFF), 0.6 mM *L*-cysteine and 10 ng/mL recombinant human epidermal growth factor (rhEGF), followed by their continuous IVM for 22 to 24 h in the same medium enriched with 80 nM TSA [Samiec and Skrzyszowska 2010ab]. The IVM of recipient oocytes occurred at 39°C in a 100% water-saturated atmosphere of 5% CO_2 and 95% air.

In a control group, the maturation medium comprised HEPES- and $NaHCO_3$-buffered TC 199 medium supplemented with 0.1 IU/mL hMG, 10% pFF, 0.6 mM *L*-cysteine and 10 ng/mL rhEGF. A population of COCs ranging from 50 to 60 was cultured in the maturation medium for 42 to 44 h at 39°C in humidified air with 5% CO_2 [Samiec and Skrzyszowska 2010ab].

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 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.

In the somatic cell cloning procedure, enucleated *in vitro*-matured oocytes were reconstructed with the genomic DNA of foetal fibroblast cells. Then, nuclear transfer-derived oocytes were artificially stimulated with the use of simultaneous fusion and electrical activation (SF-EA). In the SF-EA protocol, the complexes of enucleated oocytes and fibroblast cells underwent plasma membrane electroporation by application of two successive DC pulses of 1.2 kV/cm for 60 μs [Skrzyszowska *et al.* 2008, Samiec and Skrzyszowska 2010cd]. After activation treatment, cloned embryos were cultured in 50-μL droplets of the North Carolina State University (NCSU)-23 medium supplemented with 4 mg/mL BSA-V that had been overlaid with light mineral oil. The number of embryos per droplet of culture medium ranged from 20 to 25. After 72 to 96 h of *in vitro* culture, dividing embryos were transferred into a 50-μL drop of NCSU-23/BSA-V medium supplemented with 10% foetal bovine serum (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 [Samiec *et al.* 2003, Skrzyszowska *et al.* 2008].

Results and discussion

The pre-treatment with *R*-RSCV prior to two-grade IVM in the TSA-depleted, and subsequently TSA-enriched medium led to the acquisition of meiotic maturity by almost all the pig oocytes selected for IVM as compared to the control (*i.e.*, *R*-RSCV- and TSA-untreated) group (141/142, 99.3% *vs.* 103/126, 81.7%; P<0.001, chi-square test). As shown in Table 1, the rates of cleaved embryos, morulae and blastocysts (Fig. 1) developing from NT oocytes that had been previously exposed to *R*-RSCV and TSA were significantly higher (P<0.001, chi-square test) than those in the *R*-RSCV- and TSA-untreated group (Fig. 2).

Table 1. Effect of TSA-mediated epigenetic modulation of *ex vivo*-maturing oocytes pre-exposed to *R*-RSCV on the *in vitro* developmental potential of cloned pig embryos

Sequential treatment of oocytes with *R*-RSCV and TSA	Number of oocytes/embryos			Development to	
	enucleated	fused (%)	cleaved (%)	morulae (%)	blastocysts (%)
+	141	133/141 (94.3)	124/133 (93.2)^{A}	94/133 (70.7)^{A}	58/133 (43.6)^{A}
–	103	92/103 (89.3)	61/92 (66.3)^{B}	45/92 (48.9)^{B}	24/92 (26.1)^{B}

^{AB}Values with different superscripts within the same column differ significantly (P<0.001, chi-square test). Number of replicates ≥3.

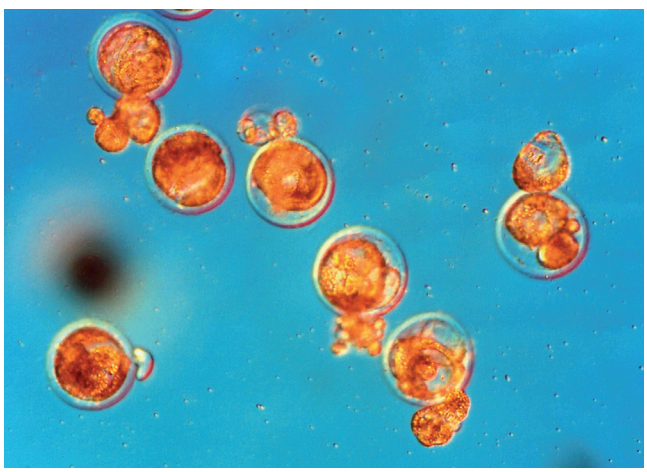

Fig. 1. Expanded and hatching blastocysts developed from porcine cloned embryos reconstructed with oocytes pre-treated with *R*-RSCV followed by TSA-mediated epigenetic modulation throughout *in vitro* maturation. Original magnification 100×.

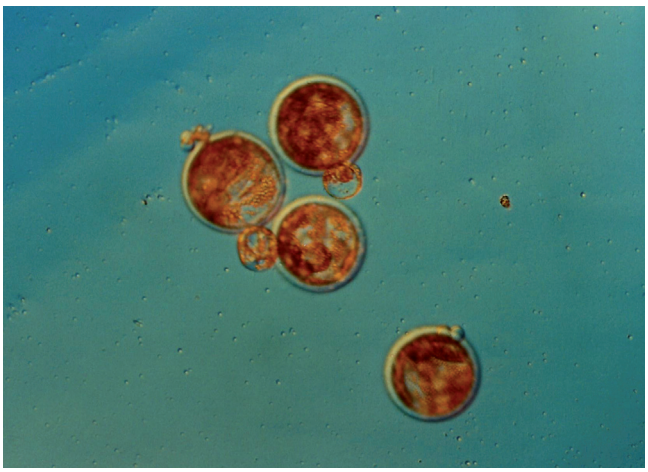

Fig. 2. Expanded and hatching blastocysts developed from porcine cloned embryos reconstructed with control oocytes not exposed to *R*-RSCV and TSA during *in vitro* maturation. Original magnification 100×.

The results of the present investigation indicate that the abundance in a formation of morulae and blastocysts suggests the improved reprogrammability of epigenetic memory and thereby transcriptional activity for foetal fibroblast cell-inherited nuclear DNA in an epigenomically-matured cytoplasm of recipient oocytes undergoing sequential exposure to *R*-roscovitine and trichostatin A. This finding appears to confirm that the methods applied to the preparation of nuclear recipient cells affect, to a high degree, the *in vitro* developmental capability of cloned pig embryos. The use of two-grade treatment of *ex vivo*-maturing oocytes with *R*-RSCV and TSA in the present study contributed to the achievement of much higher blastocyst yield (approximately 44%) than in the study by Yin *et al.* [2002] who reported that nuclear-transferred embryos derived from the oocytes that had not undergone exposure to *R*-RSCV and TSA throughout IVM, followed by fusion with foetal fibroblast cells and delayed electrical activation were able to reach the blastocyst stage at the rate of 9%. Similarly, after SF-EA, the porcine transgenic NT embryos reconstituted with foetal fibroblast cells and oocytes that were neither pre-matured in the presence of *R*-RSCV, nor modulated epigenomically with TSA displayed two-fold lower blastocyst formation rate (18% to 26%) - Hyun *et al.* [2003] than that obtained in the current investigation for non-transgenic NT embryos descended from *in vitro*-maturing oocytes treated with *R*-RSCV prior to TSA-mediated epigenetic transformation. Lee *et al.* [2003] also demonstrated that cloned pig embryos originating from *ex vivo*-matured oocytes that had not been sequentially incubated with *R*-RSCV and TSA, followed by simultaneous fusion with foetal fibroblast cells and electrical activation exhibited an *in vitro* developmental competence to blastocyst stage (16%) nearly three-fold lower than that for NT embryos generated in this study using pre-maturation of oocytes with *R*-RSCV and their further exposure to TSA (44%).

Altogether, the use of ectopic repressory proteins for specific CDK inhibition (e.g., selective blocker of MPF-linked CDK1, *R*-RSCV) and/or exogenous epigenetic modifiers (e.g., non-specific inhibitors of DNMTs or inhibitors of HDACs) throughout *in vitro* maturation of mammalian dictyotene-/germinal vesicle (GV)-staged oocytes can affect, on the one hand, the ability of the oocytes that reached MII stage to acquire the cytoplasmic and epigenomic maturity state (*i.e*., before or simultaneously with acquisition of the nuclear/meiotic maturity by them) - Coy *et al.* [2005], Bonk *et al.* [2008], Bo *et al.* [2011], Kim *et al.* [2011]. On the other hand, both the timing and incidence (frequency of occurrence) of the cytoplasmic and epigenomic maturation as well as the degree and rapidity of synchronization between cytoplasmic, epigenomic and nuclear (meiotic) maturation can be affected by the treatment of immature (GV-staged) oocytes with above-mentioned agents. Moreover, the developmental capacity of somatic cell-cloned embryos derived from nuclear recipient oocytes that have been matured *in vitro* under such conditions can also be influenced, to a high extent, by the modulators of cytoplasmic and epigenomic *ex vivo* maturation [Schoevers *et al.* 2005, Bui *et al.* 2007, Yamanaka *et al.* 2009, Zhao *et al.* 2010].

Cumulatively, to our knowledge, this is the first report, in which the effect of two-step treatment with *R*-roscovitine and trichostatin A on the *in vitro* maturation of porcine nuclear recipient oocytes and development of nuclear-transferred embryos descended from foetal fibroblast cells was determined. Nonetheless, future studies, which are based on real-time DNA sequenation (*i.e*., pirosequenation), are required to assess the levels of alterations in the methylation status of donor nuclear genome within the blastomeres of preimplanted SCNT-derived pig embryos originating from oocytes that had been pre-matured with *R*-roscovitine and subsequently subjected to IVM in the presence of trichostatin A. Further research is also necessary to explore whether the TSA-mediated epigenomic modulation of nuclear recipient oocytes pre-exposed to *R*-RSCV can result in the generating viable cloned piglets.

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