Molecular conditions of the cell nucleus remodelling/reprogramming process and nuclear-transferred embryo development in the intraooplasmic karyoplast injection technique: a review

M. Samiec, M. Skrzyszowska

Department of Animal Reproduction Biotechnology, Immuno- and Cytogenetics, National Research Institute of Animal Production, Balice/Kraków, Poland

ABSTRACT: The introduction of cell nuclei into enucleated recipient cells, beyond enucleation, is the most significant stage of somatic cloning procedure. Microsurgical transfer of somatic nuclei can be an alternative method of clonal nuclear-cytoplasmic hybrid reconstruction towards cell fusion induced in the electric field, not only from the aspect of molecular mechanisms of nuclear chromatin rearrangement, advantageously influencing epigenetic reprogramming and structural remodelling of exogenous genetic material, but also because it was proved in recent studies on pig cloning that the effectiveness of piezo-driven microinjection of ear-derived fibroblast karyoplasts measured by the percentage of oocytes preserving vitality after cell nuclei transplantation operation did not differ significantly from the survival rate (viability) of clonal cybrids reconstituted by an electrofusion method. The intraooplasmic injection system of karyoplasts prepared from cells at G0/G1 or G2/M stages of cell cycle could also increase considerably the total efficiency of somatic cloning technique in pigs and other mammal species.

Keywords: somatic cloning; microsurgical nuclear transfer; karyoplast; intraooplasmic injection; chromatin remodelling; epigenetic reprogramming

The basic assumption of somatic cloning is the fact that the donor cell nucleus has to be completely reprogrammed by specific oocytic agents in order to support the development of the reconstituted embryo to term. It is now believed that the chief cause of the low developmental potential of cybridic clonal (nuclear-transferred) zygotes is abnormal adaptation of transplanted somatic nuclei to biochemical conditions of the oocyte cytoplasmic microenvironment (Hill *et al.*, 1999, 2000; Kikyo and Wolffe, 2000; Dean *et al.*, 2001, 2003; Ono *et al.*, 2001; Surani, 2001; Boiani *et al.*, 2002, 2003; Chavatte-Palmer *et al.*, 2002; Renard *et al.*, 2002; Yamazaki *et al.*, 2003; Samiec, 2004). In other words, it is their incomplete and/or defective remodelling/ reprogramming in the cytoplasm of enucleated oocyte (ooplast), that gives rise to problems. Moreover, no consistent definition of cell nuclei reprogramming has been provided so far. It can be assumed, however, that this process comprises all the changes to which cell nuclei are subjected after introduction into ooplasts, and which lead to structural and functional assimilation of these nuclei to zygote pronuclei. The remodelling of introduced cell nuclei would then include consequent transformations, occurring within somatic chromatin, of its spatial conformation collectively defined as denomination of constitutional and metabolic rearrangement of nuclear genetic apparatus (Han *et al.*, 2003; Jouneau and Renard, 2003; Moreira *et al.*, 2003; Reik *et al.*, 2003; Shi *et al.*, 2003; Samiec, 2004). As it has been mentioned,

Supported by the State Committee for Scientific Research as Solicited (Project No. PBZ-KBN-084/P06/2002).

the somatic nuclei which are remodelled after artificial activation of reconstructed oocytes, and arrested at Metaphase II meiotic division block, not only resemble morphologically but also imitate cytophysiologically interphase nuclei which are formed after oocyte fertilization. That is why in the nuclear transfer (NT) embryos at 1-cell stage are very often known as pseudopronuclei or apparent pronuclei as well as spurious pronuclei and presumptive/pretended pronuclei. But, in spite of undergoing the series of ultrastructural and biochemical changes such as nuclear envelope breakdown (NEBD), dispersion of nucleoli, premature chromosome condensation (PCC) before oocyte activation, and also chromosome decondensation, nuclear envelope restoration as well as intensive nucleologenesis and nuclear swelling, after oocyte activation, these pseudopronuclei are not yet fully reprogrammed. They are therefore unable to direct the entire pre- and/or post-implantation development of clonal embryos and foetuses (Kang *et al.*, 2001a,b; Renard *et al.*, 2002; Vignon *et al.*, 2002; Campbell and Alberio, 2003; Cezar *et al.*, 2003; Moreira *et al.*, 2003; Wrenzycki and Niemann, 2003). This is the reason why the functional reprogramming of remodelled somatic nuclei is not a one-step (single-phase) biochemical process, but rather a multi-stage one, and it takes place in the blastomere nuclei in cycles of all preimplantation phases of embryogenesis. At the present stage of investigations it is assumed that a complete and 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 (Kono, 1997; Campbell, 1999b; Daniels *et al.*, 2000; Rideout III *et al.*, 2001; Wrenzycki *et al.*, 2001, 2002; Inoue *et al.*, 2002; Niemann *et al.*, 2002; Bortvin *et al.*, 2003; Mann *et al.*, 2003; Samiec, 2004). These epigenetic modifications such as DNA methylation and histone deacetylation are crucial processes in the regulation of transcription during embryonic development and associated with gene silencing (Kikyo and Wolffe, 2000; Dean *et al.*, 2001; Reik *et al.*, 2001; Rideout III *et al.*, 2001; Boiani *et al.*, 2002, 2003; Vignon *et al.*, 2002; Archer *et al.*, 2003; Bortvin *et al.*, 2003; Cezar *et al.*, 2003). In early stages of clonal mammalian embryo development two-step changes in the somatic tissue-specific pattern of donor genomic DNA methylation occur, which are related to epigenetic nuclear reprogramming (Latham, 1999; Renard *et al.*, 2002; Cezar *et al.*, 2003; Kang *et al.*, 2003; Yamazaki *et al.*, 2003). After reconstruction (by intraooplasmic karyoplast microinjection or nucleus donor cell-ooplast couplet electrofusion) and artificial activation (chemical or physical) of clonal nuclear-ooplasmic hybrids advanced processes of somatic DNA active demethylation (replication independent) and passive demethylation (replication dependent) take place, which persist up to the blastocyst stage. This first phase of genome wide reprogramming in preimplantation cloned embryos may be a prerequisite for removing somatic epigenetic information in order to allow embryonic gene expression and restore totipotency of cell nuclei. In turn, this last phenomenon is essential for the formation of pluripotent stem cells that are important for the later development and differentiation of many somatic cell lines in clonal embryo. After implantation of reconstituted embryo, DNA hypomethylation status subsists in the cells of extraembryonic tissues derived from trophectoderm. In the second cycle of donor genome reprogramming, DNA of epiblast somatic cell lines is largely methylated *de novo*, during gastrulation. After an increase of the overall genomic methylation level, selective demethylation of DNA cytosine residues, characteristic of individual differentiating cell lines, sets in. This process is associated at least partially with subsequent selective gene expression in these cells (Daniels *et al.*, 2000; Kang *et al.*, 2001a,b; Reik *et al.*, 2001; Niemann *et al.*, 2002; Wrenzycki *et al.*, 2001, 2002; Enright *et al.*, 2003; Santos *et al.*, 2003; Wrenzycki and Niemann, 2003).

A significant molecular mechanism of epigenetic reprogramming of donor genomic DNA can also be erasure ("zeroing/nulling") and later reestablishment of genomic parental (gametic) imprinting (uniparental expression) phenomenon in the post-implantation clonal embryos (Inoue *et al.*, 2002; Lucifero *et al.*, 2002, 2004; Dean *et al.*, 2003; Lee *et al.*, 2003a; Ruddock *et al.*, 2004;). Genomic imprinting is an epigenetic system by which alleles of some genes in the mammalian genome are marked to be active or inactive in somatic tissues of the offspring, depending on the parental origin (paternal or maternal). In the first reprogramming cycle, which occurs in the preimplantation reconstructed embryos, progressive reduction of the overall DNA methylation level does not significantly affect the imprinted patterns of the epigenetic gene marking system. This denotes that genes being subjected to

expression from either parental genome preserve their methylation status so well that the epigenetically programmed cellular memory of the way in which they have been marked is kept. In this stage of embryogenesis the transcriptional apparatus seems to be insensitive to an imprinted methylation degree of genes, but generally speaking, we have to do with biallelic (biparental) gene expression (Young *et al.*, 2001; Inoue *et al.*, 2002; Jouneau and Renard, 2003; Lee *et al.*, 2003a; Mann *et al.*, 2003; Ogawa *et al.*, 2003; Shi *et al.*, 2003; Ruddock *et al.*, 2004;). In contrast, in the postimplantation cloned embryos the second epigenetic reprogramming cycle of donor genomic DNA leads during gastrulation to intensive changes in the level of imprinted methylation patterns in differentiating themselves in the epiblast cells of germ cell line and somatic cell lines. In primordial germ cells the genome hypomethylation state from the preimplantation phase of embryogenesis is maintained and additionally methylation imprinting the genes undergoing uniparental (monoallelic) expression is obliterated (Reik *et al.*, 2003; Han *et al.*, 2003; Dean *et al.*, 2001, 2003; Ogawa *et al.*, 2003; Lucifero *et al.*, 2002, 2004; Yamazaki *et al.*, 2003). "Vanishing" of sex specific parental imprints in the somatogenic genome of reconstituted embryos consists in gradual removal of epigenetic markers imprinting expressed uniparentally alleles of many genes of paternal or maternal origin, otherwise in reduction of a number of methylated forms of configurational islets/islands (palindromic minisequences) of 5′-cytidine-3′-monophosphate-5′-guanosine-3′ (^{Me}CpG). This process is continued until movement of the specific dynamic equilibrium of epigenetic marking system towards hyperdemethylation. The reversal of original imprinting in either allele, induced by this reaction, makes chromosomes derived from both parents become of equal rank. During almost the entire gametogenesis gene expression is then biallelic. But in the late gametogenesis stages of cloned individuals chromosomes are epigenetically marked *de novo* according to the previous imprinting pattern suitable for a given sex, and the DNA methylation level reaches a high degree in both sexes (Mann *et al.*, 2003; Lee *et al.*, 2003a; Reik *et al.*, 2001, 2003; Enright *et al.*, 2003; Han *et al.*, 2003; Ruddock *et al.*, 2004; Lucifero *et al.*, 2002; Kang *et al.*, 2003; Jouneau and Renard, 2003). In contradistinction to primordial germ cells, in somatic cell lines of postimplantation reconstituted embryo an increase in the overall genome methylation level is observed. However, this phase of epigenetic reprogramming does not involve the CpG islets/islands of donor genomic DNA and non-marked alleles of genes succumbing to the expression from one parental genome. Sex specific parental imprints of genes modified during total genome methylation will be erased selectively depending on the differentiation pathway of somatic cell lines (Latham, 1999; Eggan *et al.*, 2000, 2001; Rideout III *et al.*, 2001; Young *et al.*, 2001; Chavatte-Palmer *et al.*, 2002; Inoue *et al.*, 2002; Lee *et al.*, 2003a; Lucifero *et al.*, 2002, 2004; Mann *et al.*, 2003; Ogawa *et al.*, 2003; ; Reik *et al.*, 2003; Shi *et al.*, 2003; Ruddock *et al.*, 2004).

The remodelling and reprogramming of somatic nuclear apparatus is then a result of the interaction of factors accumulated in the nucleoplasm and attached to chromatin, configured in the form of metaphase plate in consequence of appropriate rearrangement of its spatial structure and nucleosome repression, with protein factors of the recipient cell (oocyte) cytoplasm. It shows that these processes, crucial for mammalian somatic cloning, are not direct effects of conformance of the exogenous genetic material to cytophysiological conditions of Metaphase II ooplast. That is why the nuclei of somatic cells have a tendency to minimize the manifestation degree of their own developmental program after its introduction into foreign cytoplasm of allogenic origin (Campbell, 1999a; Rideout III *et al.*, 2001; Renard *et al.*, 2002; Hiendleder *et al.*, 2004; Samiec, 2004). In turn, the low contribution of realizing the somatic genetic program in the preimplantation development of reconstituted embryos should be revealed in conservation, through the exogenous nuclear apparatus, of the competence for easy adaptation to the program of meiotic, and then mitotic control of restriction points of cell cycle, forced upon it in turn by cytoplasmic microenvironment of oocytes and somato-gametogenic cytosolic environment of dividing cybridic clonal zygotes (Kono, 1997; Campbell, 1999a,b; Fissore *et al.*, 1999; Vignon *et al.*, 2002; Campbell and Alberio, 2003; Jouneau and Renard, 2003; Hiendleder *et al.*, 2004). However, the abilities of transplanted cell nuclei to fully direct the developmental program of reconstructed embryos are most likely the result of correct course of molecular mechanisms accompanying both nuclear chromatin remodelling and reprogramming of somatic cell genome. Proper rearrangement of the exogenous genetic apparatus induces only the

program of active action of donor genomic DNA on the hybridic clonal embryo cytoplasm and on the mitochondrial DNA (mtDNA) molecules of heteroplasmic origin and from ooplasmic (maternal) inheritance (Cummins 2001a,b; Garesse and Vallejo, 2001; Brüggerhoff *et al.*, 2002; Dean *et al.*, 2003; Shi *et al.*, 2003; Hiendleder *et al.*, 2004; Samiec, 2004). Nucleoplasmic (karyolymphatic) factors of somatic cell, which are engaged directly or indirectly in its structural and functional differentiation, e.g. transcriptional factors, histones, non-histone HMG (high mobility group) proteins, interacting with transcriptionally active chromatin, nuclear lamins, polysubunitary protein complexes responsible for remodelling of spatial conformation of chromatin structures and for DNA topology changes (among others nucleosome remodelling factor; NURF, or *brahma* family proteins: BRG1 and BRM, homological with yeast factors SWI2/SNF2; switch of mating type/sucrose non-fermenting) are associated with nuclear chromatin, and their qualitative and quantitative composition undergoes changes together with progressing cytodifferentiation state. When the whole donor cell is fused with enucleated oocyte, then those specific factors of somatic cell are also transferred into the cytoplasm of recipient oocyte and may block an ability of endogenous oocytic factors for appropriate remodelling and reprogramming of foreign (allogenic) cell nucleus (Campbell, 1999b; De Sousa *et al.*, 1999; Loi *et al.*, 2001; Rideout III *et al.*, 2001; Renard *et al.*, 2002; Vignon *et al.*, 2002; Campbell and Alberio, 2003). Exogenous cytoplasmic factors of donor cell are incorporated together with own proteins and maternal transcripts (mRNA molecules) of oocyte into the remodelled somatic cell nucleus (pseudopronucleus), after its formation in consequence of reconstructed (NT) oocyte activation. In turn, a surplus of these hypothetical foreign agents in the ooplasm causes a considerable dilution of specific internal oocyte factors, owing to mutual mingling in the hybridic cytoplasmic environment, diminishing simultaneously the probability of complete donor nucleus reprogramming (Campbell, 1999a; Fissore *et al.*, 1999; Prather, 2000; Vignon *et al.*, 2002; Campbell and Alberio, 2003; Santos *et al.*, 2003; Hiendleder *et al.*, 2004). The chief purpose of somatic nucleus intraooplasmic microinjection procedure is to avoid all the above-mentioned problems related to the processes of molecular nature. Introduction of practically only the donor cell nucleus into the cytoplasm of enucleated oocyte increases many times the likelihood of proper action of specific cytosolic oocyte agents on the processes of foreign nuclear chromatin remodelling and genome reprogramming, because in this case the only source of exogenous proteins and mRNA transcripts is the nucleoplasm of transplanted karyoplast. Insignificant numbers of perinuclear cytoplasm (perikaryon) likely remain without a greater effect on the further embryonic development of mammalian clonal zygotes (Prather, 2000; Lacham-Kaplan *et al.*, 2000; Galli *et al.*, 2002; Roh and Hwang, 2002; Lee *et al.*, 2003b; Samiec *et al.*, 2003b; Hiendleder *et al.*, 2004; Samiec, 2004). Moreover, reducing the volume of allogenic somatic cytoplasm, transplanted into the cytosolic ooplast microenvironment, allows for complete avoidance of considerable limitation of the hybridization possibility of heteroplasmic sources of mitochondrial DNA and messenger RNA (including also polycistronic mitochondrial mRNA), originating from the somatic donor-cell of nuclear genetic material and from recipient-cytoplast (ooplast). The lack of the impurities in the form of somatic mtDNA in the cytoplasmic environment of reconstructed oocyte, or the lack of the so-called mtDNA heteroplasmy brings about a consequent decrease in the frequency of the disorders in the epigenetic reprogramming of nuclear DNA and mitochondrial DNA (in consequence of hypermethylation or excessive demethylation of DNA cytosine residues; Garesse and Vallejo, 2001; Reik *et al.*, 2001; Surani, 2001; Roh and Hwang, 2002; Gomez *et al.*, 2003; Wrenzycki and Niemann, 2003; Hiendleder *et al.*, 2004). Hence all disturbances in dynamic homeostasis of epigenetic modifications of somatic cell genome may result from asynchronous structural remodelling of nuclear chromatin (non-coordinated deacetylation/acetylation of histones and elevation of the nucleosomal repression level through a decrease of SWI2/SNF2 protein complex activity) as well as asynchronous changes of spatial configuration of regulatory D-loop of "naked", circular mtDNA molecules of nuclear-transferred embryos. The maintenance of correct DNA methylation pattern in the nuclei of all descendant blastomeres of preimplantational clonal embryos favours also the preservation in the intact form of the mechanisms responsible for parental genome imprinting (uniparental/monoallelic gene expression). In turn, this is reflected in flawless rearrangement of exogenous chromatin as well as reprogramming of nuclear and mitochondrial genetic apparatus,

and, in extreme cases, even in partial remodelling of chromatin structures, and it enables to avoid the inhibition of transcriptional activity of a larger part of embryonic genome in the early stages of embryogenesis (Latham, 1999; Daniels *et al.*, 2000; Smith *et al.*, 2000; Cummins, 2001a,b; Dean *et al.*, 2001; Niemann *et al.*, 2002; Renard *et al.*, 2002; Kang *et al.*, 2003; Mann *et al.*, 2003; Hiendleder *et al.*, 2004).

A completely different approach to this problem is the microinjection into enucleated Metaphase II oocyte (cytoplast) of only the metaphase chromosomes of donor cell instead of whole somatic cell or whole cell nucleus (with intact integrity of nuclear envelope), whose cytosolic bearer is karyoplast, microsurgically released from disrupted somatic cell. This strategy, first used in porcine somatic cloning by Lai *et al.* (2001), should prevent the transfer of the overwhelming majority of specific donor cell factors (cytosolic and nucleoplasmic), which may have an effect on better nucleus remodelling and reprogramming. In contradistinction to microinjection of complete interphase donor nuclei, as a result of microsurgical transfer of only metaphase plates, formed in the activated oocytes, in the remodelling (nuclear envelope reconstitution/restoration and swelling) process, spurious/pretended pronuclei would accumulate in the nuclear matrix only the endogenous ooplasmic proteins in the form associated with chromatin. Donor genome reprogramming as a result of complete synchronization of cell cycle phases of karyoplasts and cytoplasts would proceed without any disturbances. The technique of direct intraooplasmic microinjection of the metaphase chromosomes ultimately allows for complete elimination of the negative effects of action on the introduced genetic material of the surplus of exogenous (heteroplasmic) protein agents, disturbing the interchange and cooperation (synergism) between the nuclear and cytosolic factors of gametogenic (oocytic) origin, chiefly those from the group of enzymes belonging to the cyclin-dependent kinase (CDK) family, phosphatases, CDK inhibitors, and also diverse transcriptional factors. Another advantage of the microinjection method is that it prevents against disturbances in the specific dynamic balance of the entire cascade of processes catalyzed by different enzymes regulating transition from meiotic to mitotic control of reconstructed oocyte cell cycle, and also enables the correct functioning of the various regulatory proteins (both from the group of stimulators and inhibitors) in the cleaved clonal (NT) embryos. The transplantation of interphase cell nuclei or metaphase plates by a microsurgical method has another fundamental advantage over a cell electrofusion technique. It also allows to avoid the detrimental effect of surplus protein factors, often of antagonistic action, and the deficit of protein agents reacting synergistically in consequence of conjunction and mingling (hybridization) of two different cytoplasmic environments of donor cell and ooplast and formation of cybridic clonal zygote (clonal nuclear-cytoplasmic hybrid; Lai *et al.*, 2001, 2002b; Samiec, 2004).

As a result of the transplantation procedure of *in vitro* cultured somatic cell nuclei over 90 piglets have been obtained so far (Betthauser *et al.*, 2000; Onishi *et al.*, 2000; Polejaeva *et al.*, 2000; Bondioli *et al.*, 2001; Park *et al.*, 2002b; Boquest *et al.*, 2002; Dai *et al.*, 2002; De Sousa *et al.*, 2002; Lai *et al.*, 2002a,b; Walker *et al.*, 2002; Yin *et al.*, 2002, 2003a,b; Hyun *et al.*, 2003; Lee *et al.*, 2003b; Phelps *et al.*, 2003; Ramsoondar *et al.*, 2003). The system of intraooplasmic microinjection of karyoplasts prepared from the cells at G0/G1 or G2/M stages of division cell cycle could increase considerably the overall effectiveness of somatic cloning technique in pigs, and also in other mammal species. However, the preservation of correct ploidy through porcine embryos reconstructed with the nuclei of cells at G2/M phase of mitotic cycle will be possible only after expulsion by them of additional polar body-like structures (the so-called pseudopolocytes), in consequence of completion of the second pseudomeiotic division after artificial NT oocyte activation (Lai *et al.*, 2001).

Microsurgical transfer of somatic cell nuclei can be an alternative method for clonal nuclear-cytoplasmic hybrid reconstruction (Tao *et al.*, 1999; Kühholzer *et al.*, 2000; Onishi *et al.*, 2000; Uhm *et al.*, 2000; Lai *et al.*, 2001; Park *et al.*, 2001a; Nagashima *et al.*, 2002; Samiec *et al.*, 2003a,b; Skrzyszowska *et al.*, 2003; Kawano *et al.*, 2004) prior to cell fusion induced in the electric field. In terms of the molecular mechanisms of nuclear chromatin rearrangement, this has beneficial influences on epigenetic reprogramming and structural remodelling of exogenous genetic material. Recent studies on pig cloning (Roh and Hwang, 2002) also proved the effectiveness of piezo-driven microinjection of ear-derived fibroblast karyoplasts. In this study the percentage of viable oocytes after the cell nuclei transplantation procedure (76.5%) did

not differ significantly from that of clonal cybrids reconstituted by the electrofusion method (74.6%). In addition, there is no significant difference in pseudo-pronuclear formation rate, cleavage activity, blastocyst formation rate or cell numbers in blastocysts between groups of porcine clonal cybrids reconstituted with ear skin-derived fibroblast nuclei by electrofusion and piezo-driven nuclear injection (Roh and Hwang, 2002; Kawano *et al.*, 2004). On the other hand, in the studies carried out by Nagashima *et al.* (2003) and Kurome *et al.* (2003), using foetal fibroblast cell nuclei simultaneous comparison between two nuclear transfer methods (ooplast-donor cell complex electrofusion and intracytoplasmic piezo-electric microinjection into enucleated oocytes) revealed clear differences in the pattern of nuclear remodelling and developmental potential of embryos to blastocyst stage as well as in the morphological quality of the clonal blastocysts as measured by total cell number. However, considering the results of the majority of the studies for nuclear transfer in farmed livestock and laboratory animal species (Lacham-Kaplan *et al.*, 2000; Ogura *et al.*, 2000; Park *et al.*, 2001a; Wakayama and Yanagimachi, 2001; Choi *et al.*, 2002; Galli *et al.*, 2002), it can be concluded that intraooplasmic injection of karyoplasts prepared from cells at G0/G1 or G2/M stages of cell cycle could also increase considerably the total efficiency of somatic cloning in pigs and other mammalian species.

In conclusion, mammalian cloning by somatic cell nuclear transfer has been successfully achieved by both fusing of a donor cell with and injecting an isolated donor cell nucleus (karyoplast) into an enucleated oocyte (Wilmut *et al.*, 1997; Kato *et al.*, 2000; Ogura *et al.*, 2000; Onishi *et al.*, 2000; Keefer *et al.*, 2001; Loi *et al.*, 2001; Galli *et al.*, 2002; Lai *et al.*, 2002a,b). Both these methods of oocyte reconstruction, however, involve prolonged manipulation of either the oocytes (electrofusion) or somatic cells (karyoplast preparation). Additionally, these micromanipulation procedures are not only labour intensive (i.e. require specialized equipment for embryo engineering e.g. piezo-actuated micromanipulator or electro cell manipulator) but also they can reduce the overall cloning efficiency due to the low fusion rate or damage to the isolated cell nucleus. Therefore, Lee *et al.* (2003b) recently explored a new method for the generation of clonal cybrids (reconstituted embryos) with the aim of increasing the nuclear transfer effectiveness and simplifying the somatic cloning technique. The alternative strategy of nuclear-cytoplasmic hybrid creation, which has been proposed by Lee *et al.* (2003b), involves intraooplasmic microinjection of a whole somatic cell bypassing both the donor cell-cytoplast couplet fusion and karyoplast preparation steps. Conducting the new protocol of whole cell injection with the use of skin fibroblasts derived from the ear of a sow transgenic for two genes, which encode the porcine lactoferrin and the human clotting factor IX, Lee *et al.* (2003b) produced four healthy cloned genetically engineered piglets. The technique of whole cell microinjection for nuclear transfer has not been attempted so far because of concerns that the plasma membrane of the donor cell may persist in the cytoplasm of recipient ooplasts resulting in a failure to release the somatic cell nucleus. To test the feasibility of cloning by whole cell intraooplasmic injection, Lee *et al.* (2003b) investigated whether, and when, the enucleated oocytes induced the breakdown (dissolution) process of donor cell plasmolemma, the nuclear swelling (enlargement) and forming the pseudopronuclei from interphase (G1/G0) chromatin of the introduced whole cells. To assess successful injection of somatic (fibroblast) cell into a recipient cytoplasm, the plasma membrane was stained with a live plasmolemma fluorescent dye (PKH67 green fluorescent cell liner kit). Immediately after oocyte reconstruction (nuclear transfer), the plasma membrane of the injected fibroblast cells which survived (preserved viability) was clearly intact and emitted bright green fluorescence. The membrane of the majority of the donor cells underwent disintegration relatively rapidly, within an hour of injection. However, the green fluorescence was visible in some nuclear-cytoplasmic hybrids up to 6 hours before becoming undetectable suggesting the plasmolemma can persist in recipient oocytes for relatively long periods of time. At this time, when the plasma membrane of the fibroblast cell was no longer visible, the donor nucleus stained blue with Hoechst 33342 (bisbenzimide) was observed. Full nuclear swelling was detected 12 hours after artificial activation of the reconstituted oocyte.

It is unlikely that the disappearance of the fluorescence, which was emitted by labelled donor cell plasmolemma, resulted from PKH67 dye diffusion because this fluorochrome persisted more than 24 hours in stained somatic cells left in the culture medium. But the exact mechanism for dis-

solution of the donor cell plasma membrane in the cytoplasmic microenvironment of the enucleated oocyte is unclear. Two possible mechanisms can potentially explain the dissolution process. First, the oocyte might have actively recognized the plasmolemma of nuclear donor cell or its specific surface antigen proteins as belonging to a foreign (allogeneic) cell, not to the cytosol of the oocyte. This recognition (immunological response) in the ooplasm would then lead to actively degrade the plasma membrane of somatic cell or transport it to the cell surface of the oocyte and subsequent rejection (removal) through exocytosis of plasmolemma-derived vesicles (the so-called plasma membrane recycling hypothesis). Afterwards, this active dissolution would release the cell nucleus for remodelling/reprogramming process in the reconstituted embryo. The second possible mechanism for donor cell plasma membrane dissolution may be that the somatic cell plasmolemma was damaged during whole cell microinjection and the leaky plasma membrane, which was not repaired in the cytoplasm of recipient oocyte, released then the cell nucleus. This resulted in its chromatin rearrangement, because of lack of the oocyte ability to recognition of injected whole cell as being ectopic. Regardless of the mechanism for donor plasma membrane dissolution, the injected whole cell was competent to support embryo development to the stage of hatched blastocyst under *in vitro* culture conditions. Lee *et al.* (2003b) reported that 37% of nuclear-transferred embryos developed into blastocysts and 0.4% of them developed into live piglets. Because the chromosomes of nuclear donor cells are not directly exposed to the micromanipulation medium in this method of oocyte reconstruction, a high potential for clonal cybrids to reach the blastocyst stage might be expected. However, further studies are required to confirm the reliability and feasibility of the whole cell intracytoplasmic injection technique for efficient production of cloned mammalian embryos and neonates.

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Received: 04–08–11
Accepted after corrections: 05–01–26

Corresponding Author

MSc. Marcin Samiec, National Research Institute of Animal Production, Department of Animal Reproduction, Biotechnology, Immuno- and Cytogenetics, 32-083 Balice/Kraków, Poland
E-mail: klon502@wp.pl