Microsurgical nuclear transfer by intraooplasmic karyoplast injection as an alternative embryo reconstruction method in somatic cloning of pigs and other mammal species; application value of the method and its technical advantages: a review

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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 in recent studies on pig cloning it was proved that the effectiveness of piezo-driven microinjection of ear-derived fibroblast karyoplasts measured by the percentage of oocytes preserving vitality after the cell nuclei transplantation operation did not differ significantly from the survival rate (viability) of clonal cybrids reconstituted by an electrofusion method. An 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 in other mammal species.

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

The introduction of cell nuclei into enucleated recipient cells (Metaphase II oocytes), besides enucleation, is the most significant stage of somatic cloning procedure. It is essential that the transplantation of cell nucleus into a new cytoplasmic environment should not cause irreversible damage to the nucleus and to the recipient cell. It is also important that the procedure as a whole should be relatively efficient, otherwise the percentage of blastocysts obtained and offspring born would be further decreased, thereby limiting the number of clones. Many studies investigating the somatic cloning of different farm and laboratory animal species confirmed that the high rate of *in vitro* developmental capacity to the blastocyst stage by reconstructed embryos can only be attained through a right coordination between the nucleus donor cell phenotype and the cell cycle stage as well as an appropriate combination in the exogenous somatic nucleus transplantation procedure of maternal chromosome elimination (recipient oocyte enucleation) method, oocyte reconstruction technique, and clonal cybridic zygote activation system (Galli et al., 1999, 2002; Kato et al., 2000; Polejaeva et al., 2000; Wakayama and Yanagimachi, 2001; Roh and Hwang, 2002; Yin et al., 2002a; Nagashima et al., 2003; Samiec et al., 2003b; Samiec, 2004). At present, the most common embryo reconstruction techniques are nuclear transfer followed by the electric pulse fusion of

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cytoplast (enucleated Metaphase II oocyte) with somatic cell (cattle: Cibelli et al., 1998; Wells et al., 1999; Skrzyszowska et al., 2000; Koto et al., 2000; sheep: Wilmut et al., 1997; McCreath et al., 2000; goats: Baguisi et al., 1999; Keefer et al., 2001; pigs: Koo et al., 2000; Polejaeva et al., 2000; Boquest et al., 2002; Yin et al., 2002a,b,c, 2003; rabbits: Dinnyes et al., 2001; Chesne et al., 2002; Yin et al., 2002b,c) or the use of a suspension of inactivated Sendai virus (HVJ) particles with an accurately defined number of haemagglutination activity units (mice: Kato et al., 1999; Ono et al., 2001; horses: Li et al., 2002). The procedures of microsurgical nuclear transfer, involving both direct karyoplast (cell nucleus with residual, perinuclear protoplasmic "ring", i.e. perikaryon) injection into the cytoplasm of an enucleated oocyte (pigs: Onishi et al., 2000; Lai et al., 2001; Park et al., 2001; Roh and Hwang, 2002; Samiec et al., 2003a,b; Kurome et al., 2003; Kawano et al., 2004; mice: Wakayama et al., 1998; Chung et al., 2002; Kim et al., 2002; horses: Choi et al., 2002; endangered species of free-living animals e.g. mouflon – interspecies cloning by xenonuclear transfer of mouflon somatic cell-derived karyoplasts into ovine oocyte cytoplasm: Loi et al., 2001), and whole cell intraooplasmic microinjection (pigs: Lee et al., 2003) are also used frequently.

A microsurgical technique was used for the first time for transplantation of murine morula, inner cell mass (ICM) and trophectoderm blastocyst cell nuclei into non-enucleated zygotes (Illmensee and Hoppe, 1981; Modliński, 1978, 1980, 1981), and bovine ICM and granulosa cell nuclei enucleated Metaphase II oocytes (Collas and Barnes, 1994). However, the microinjection technique has acquired greater significance only recently thanks to the studies carried out by Wakayama et al. (1998) in mice. What was really groundbreaking in these experiments was the use of the piezoelectric unit of micromanipulator for direct introduction of follicular (cumulus) cell nuclei into the cytoplasm of Metaphase II (Met II) oocytes. The experiments of Wakayama et al. (1998) aroused a general interest in the microinjection technique, vesting it with a broad range of application possibilities, not only in mouse somatic cloning, but above all in pig somatic cloning.

Onishi et al. (2000), creators of Xena gilt, showed that it was possible to produce somatic clones also in pigs through direct exogenous cell nuclear microinjection into the cytoplasm of enucleated Metaphase II oocytes. However, they conceded that they did not know all factors that could contribute to the first major success in porcine somatic cloning. The authors suggested that it could be caused at least partially by the rate and accuracy of micromanipulations accomplished through the piezoelectric control of enucleation and injection pipette motion, which limited to the minimum the extent of losses related to diminution of oocyte viability as a result of extensive damage to plasmolemma or drastic dislocations of cytoskeleton and membrane skeleton elements. The method of cell nucleus microsurgical transfer, with the use of piezoelectric manipulator, was adapted to the studies on pig somatic cloning for many reasons (Roh and Hwang, 2002). It has a multitude of technical advantages. In contradistinction to microinjection carried out manually, it guarantees the completely straight-line progressive motion of injection pipette, without any side deflections from its balance point, which could disrupt the integrity of the delicate oolemma ultrastructure. Moreover, precise adjustment of the micromanipulator piezoelectric unit parameters to current requirements, dependent mainly on the mature porcine oocyte diameter that ranges from 110 to 125 μm, allows for the very accurate regulation of penetration depth and velocity of the microinjection pipette. The immersion of piezo-actuated injection micropipette in oocyte cytosol should not only exceed half the length of oocyte diameter but also it can reach three-fourths of diameter length. The cell nucleus (or to be exact, small karyoplast), which has not been deposited deep enough into the ooplasm, can be pushed out under the zona pellucida as a result of violently withdrawing the injection pipette (Loi et al., 2001; Wakayama and Yanagimachi, 2001; Rybouchkin et al., 2002). Excessively shallow deposition of the karyoplast inside the oocyte cytoplasm can thus be a reason for a considerable percentage of technical failures of microinjection operation and cytoplast degeneration (Galli et al., 1999; Ogura et al., 2000). Onishi et al. (2000) and Prather (2000), the precursor of pig cloning studies, also observed that when the manipulator piezoelectric unit is used for a microinjection operation, the vibratory nature of the pipette motion allows for much more rapid disruption of the nucleus donor cell plasmolemma continuity, minimizing the probability of damage to the cell nucleus itself. In addition to the slower course of the entire microoperation and less accurate estimation of karyoplast deposition site within the ooplasm, the manual microsurgical transfer technique differs little from the experimental protocol of Wakayama

et al. (1998) and Onishi et al. (2000) microinjection carried out with the use of piezoelectric manipulator (Galli et al., 1999, 2002; Loi et al., 2001; Samiec et al., 2003b; Kawano et al., 2004).

Taking into account all the widely discussed aspects of cell nucleus microsurgical transfer technique, we decided on the use of the manual variant of this strategy in our own studies (Samiec et al., 2003a,b; Skrzyszowska et al., 2003). Observations made during our own experiments consistently point to the smaller amplitude of deflections of microinjection pipette from the straight-line motion path during a microinjection operation that is accomplished quickly and efficiently. Together with the increased rapidity of injection micropipette progressive motion, a greater dependence was observed between the location of a slit made in the zona pellucida and the position of a microhole "drilled" in the cytoplast oolemma. The quicker the pipette overcomes both these barriers (zona and oolemma), the points marked by both microperforations lie on the same line that is more or less straight as measured with the use of micrometric scale (micrometer dial), showing lower fluctuations of deflections from the pipette balance point (Samiec et al., 2003b; Samiec, data unpublished).

Moreover, Onishi et al. (2000) assumed that the success of porcine somatic cloning was largely limited by the oversensitiveness of ooplast intracellular environment to the contamination of nucleus donor cell with cytoplasm. This would favour the application of microinjection technique in the transplantation of cell nuclei as an alternative method for electrofusion, a procedure that is commonly used in the somatic cloning of other livestock species. In contradistinction to cell fusion, microinjection allows for selective removal of a large part of the exogenous nucleus donor cell cytoplasm, thereby enabling relative dilution of components of donor cytoplasm remainders in the inner microenvironment of ooplast and early stage embryo. The direct result is to avoid the disadvantageous influence of karyoplast cytoplasmic components on the remodelling and reprogramming of transferred somatic cell nucleus, and consequently on the correctness of reconstituted zygote embryonic development (Do et al., 2001, 2002; Rybouchkin et al., 2002; Ikumi et al., 2003; Kurome et al., 2003; Kawano et al., 2004). On the other hand, Park et al. (2001) noted significant advantages of the microinjection method for the transplantation of small-sized somatic cell nuclei because a distinctly higher *in vitro* developmental potential was observed among porcine embryos reconstructed by the microsurgical transfer technique of small donor cells in relation to the group of clonal zygotes produced by electrofusion of small donor cells and recipient oocytes. The small (compared with blastomeres) diameter (12–20 μm) of fibroblast or follicular cells used as a source of donor nuclei is the reason for a considerable reduction of the contact surface between plasma membranes of ooplast and karyoplast, which consequently leads to decreased percentage of successfully fused cell complexes. Removing a part of oocyte cytoplasm (usually up to 10 to 30% of total volume) during the enucleation microoperation brings about an increment in the perivitelline space volume, which cannot be compensated by the small size of somatic cell, even if the latter were wedged between zona pellucida and oocyte plasmolemma after the injection (Tao et al., 1999; Ogura et al., 2000; Galli et al., 2002). Direct injection of small karyoplast into the cytoplasm of oocyte therefore allows to avoid all the technical problems resulting from the failure to ensure a very close contact between plasma membranes, which limits the effectiveness of electrofusion to the highest degree. Another advantage of the cell nucleus microsurgical transfer strategy is omitting the second stage of somatic cloning procedure that is necessary with the standard electrofusion technique, namely the introduction of donor cell beneath the zona pellucida of an enucleated oocyte (cytoplast). Assuming the manual abilities of the operator, this allows for the entire cell nucleus transplantation (nuclear transfer) procedure to be slightly shorter (Galli et al., 1999; Loi et al., 2001; Wakayama and Yanagimachi, 2001).

In the microsurgical method nucleus donor cells are aspirated into the micropipette the diameter of which is about half the size of cell diameter. The nucleus donor cell size thus determines the diameter of pipette to be used for transplantation. The smaller the cell, the shorter the diameter of injection micropipette and the smaller the damage to the plasma membrane (Rybouchkin et al., 2002; Nagashima et al., 2003). When a source of donor nuclei is porcine *in vitro* cultured foetal fibroblasts, the sharp and bevelled tip of injection pipette should have an external diameter range of 7 to 10 μm (Tao et al., 1999; Onishi et al., 2000). In our own studies (Samiec et al., 2003a,b; Skrzyszowska et al., 2003), in which a source of donor nuclei was fresh cumulus cells derived from expanded cell layers surrounding *in vitro* matured oocytes, the best re-

sults were obtained when the diameter of injection pipette tip was 7 to 8 μm like in the experiments of Uhm et al. (2000). However, in the case of *in vitro* cultured mural granulosa cells of porcine ovarian folliculi, the tip diameter of micropipette has to be approximately 10 μm (Park et al., 2001). Such parameters of the diameter size of microinjection pipette end pieces are absolutely essential for adequate hypotension/negative pressure disrupting the plasma membranes of somatic cells. Delicate, alternating aspiration of the sucked donor cell inwards and pushing outside or towards the bevelled outlet of pipette tip inevitably leads to disruption of plasmolemma integrity and removal of the majority of cytoplasmic material which is a needless burden on the delicate cytosolic microenvironment of oocyte and decreases viability of reconstructed embryo. A cell nucleus, released from the donor cell by mechanically induced disintegration (cytolysis), is surrounded from this moment only with a very thin layer of residual cytoplasm. It is important that the release of cell nucleus during cytolysis should occur inside the pipette to minimize its contact with harmful extracytoplasmic environment. Aspirating the surplus volume of manipulation medium into a micropipette makes it blend with the residual perinuclear cytoplasm, which can adversely affect further functionality (metabolism) of cell nucleus (Wakayama et al., 1998; Ogura et al., 2000). Porcine oocytes, similar to murine ones, tolerate only small amounts of exogenous fluids (up to 3–4% of oocyte volume), deposited with their cytosol during direct microinjection. When a larger volume is introduced, the fluid flows out (into the perivitelline space) together with the oocyte cytoplasm, which inevitably induces oocyte shrinkage stresses and cracks, and consequently oocyte degeneration (Wakayama and Yanagimachi, 2001; Roh and Hwang, 2002; Rybouchkin et al., 2002).

In the manual microinjection method, a pipette together with the inside end-piece somatic cell nucleus is cautiously forced in the first place into the volumetrically enlarged perivitelline space of enucleated oocyte (cytoplast), through its zona pellucida. The tip of injection pipette is fitted with a special, thermically pulled out, bottom elongated pointed edge known as "spike" to make the penetration of zona pellucida and oolemma easier. During this microoperation the same perforation in the zona is usually used that was previously hollowed during piercing performed with the "spike" of enucleation pipette (Tao et al., 1999; Kühholzer et al., 2000; Ogura et al., 2000; Loi et al., 2001; Do et al., 2002). The next microinjection pipette is introduced mechanically through a heavily indented surface fragment of plasmolemma into the cytoplasm of the ooplast incubated previously in micromanipulation medium containing cytochalasin B (CB) or cytochalasin D (CD). The cell nucleus is very gently released from the pipette top into ooplasm, and the pipette has to be withdrawn with great caution outside the recipient cell. Invagination of oolemma, induced by the injection pipette, declines gradually after clonal nuclear-cytoplasmic hybrid reconstruction microoperation (Galli et al., 1999, 2002; Wakayama and Yanagimachi, 2001; Rybouchkin et al., 2002; Samiec et al., 2003a,b; Skrzyszowska et al., 2003).

The microinjection technique is a very demanding method, requiring enormous experience in the way of microsurgery. The most critical moment, apart from obtaining the isolated karyoplast in the end-piece of micropipette, is its introduction and withdrawal from the oocyte to prevent its destruction. The objective is to make the least possible damage to the oocyte plasma membrane in order that the oolemma will not lose its own integrity (Wakayama et al., 1998; Galli et al., 1999; Ikumi et al., 2003). A great convenience used in our own studies (Samiec et al., 2003a,b; Skrzyszowska et al., 2003), allowing to avoid a number of technical problems of microinjection procedure or possibly to reduce detrimental effects of methodological imperfections, is the addition of cytochalasin B (in the amount of about 5–7.5 μg/ml) to the micromanipulation medium and its continuous presence in this medium not only during the enucleation operation but also in the course of microinjection of cell nuclei (karyoplasts). A decrease in the surface tension generated by the CB medium as well as shrinkage stresses, and the increased elastic potential of oolemma in the place of its perforation with injection micropipette prevent disruption of the stretched plasma membrane indented into ooplast cytosol. Progressive stabilization of oocytic cytoskeleton by CB induces slackness of mechanical strains in the network of microfilaments all along the flit of microinjection pipette in the ooplasm. In turn, the cytoplast stress decrease facilitates deep penetration of recipient cell and ensures free and easy expulsion of karyoplast as well as integration in the facultative capacity point of enucleated oocyte cytoplasm. Cytochalasin B as an actin microfilament polymerization reversible inhibitor enables also the instantaneous cicatrizing of the microslot formed

in the oolemma after karyoplast microinjection. Moreover, by levelling down the stress pattern and stress intensity in the ooplasm as well as by decreasing the strain energy of oocyte distortion and strain energy of oocyte volume change, CB allows for rapid elastic restoration of the normal (original) spherical form to the oocyte heavily strained (deformed) after micropipette pressure, through an increase of the relaxation degree of its cytoskeleton and membrane skeleton (Galli et al., 1999; Loi et al., 2001; De Sousa et al., 2002; Rybouchkin et al., 2002).

The efficiency of the cell nucleus microinjection method in pigs and other mammal species oscillates within rather broad bounds, from approximately 40% to even 80% and depends on both the subjective factors and the objective ones. The subjective factor is undoubtedly the experience and skills of the operator in the way of microsurgery. The objective factors are origin of recipient oocytes and size of nucleus donor cells (Kühholzer et al., 2000; Lacham-Kaplan et al., 2000; Ogura et al., 2000; Lai et al., 2001; Roh and Hwang, 2002; Nagashima et al., 2003). Porcine or murine as well as horse oocytes are much more sensitive to damage of their plasma membrane than ovine, caprine, bovine and rabbit oocytes (Galli et al., 1999; Kato et al., 1999, 2000; McCreath et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000; Keefer et al., 2001; Loi et al., 2001; Ono et al., 2001; Choi et al., 2002; Chung et al., 2002; De Sousa et al., 2002; Li et al., 2002; Rybouchkin et al., 2002; Yin et al., 2002a,b,c). Oocytes of postpubertal gilts and sows as well as *in vivo* mature (freshly ovulated) oocytes, independently of their origin, female hormonal stimulation (superovulated or non-stimulated gilts and sows) or female postnatal ontogeny age/puberty timing (pre- and postpubertal gilts or sows), are much more resistant to an increase in plasma membrane forces, elastic strains, shrinkage stresses and strain cracks by accomplishing both the subzonal donor cell insertion (before electrofusion) and rather invasive karyoplast intracytoplasmic microinjection procedure than oocytes derived from still prepubertal gilts and abattoir-derived, *in vitro* matured oocytes obtained from both gilts and sows. This phenomenon seems to be caused by differences in the surface ultrastructure of plasmolemma between both oocyte groups. *In vivo* or *in vitro* mature sow oocytes and freshly ovulated gilt oocytes have a more homogeneous surface of plasma membrane and much more stable and elastic membrane skeleton than *in vivo* or *in vitro* mature gilt oocytes and *in vitro* matured sow oocytes. That is why, because of higher susceptibility to strains induced by somatic cell nuclear transfer microoperations, the percentage of permanent mechanical damage among oocytes derived from sows as well as among *in vivo* mature oocytes retrieved from pre- and postpubertal porcine ovaries is distinctly lower (Betthauser et al., 2000; Onishi et al., 2000; Kühholzer et al., 2001; Boquest et al., 2002; De Sousa et al., 2002; Hyun et al., 2003; Ramsoondar et al., 2003).

The efficiency of cell nucleus microinjection method measured by the percentage of oocytes surviving the operation and selected to a post-activation procedure, and followed to *in vitro* culture, can therefore be different, nonetheless it does not exceed 85% to 90% at best. Tao et al. (1999) showed that after a microinjection operation of cell nuclei of small-sized foetal fibroblasts (15 μm diameter), derived from confluent *in vitro* cultures, the percentage of porcine oocytes having the intact plasmolemma structure and qualified for *in vitro* culture was 42.2%. In contrast, Kühholzer et al. (2000) proved that it was possible to obtain an almost twice higher percentage (at a rate of 78%) of favourably reconstructed porcine oocytes by the use of a similar, manual microinjection technique of karyoplasts prepared from small (10 to 12 μm of diameter), transfected, serum-starved (deprived) foetal fibroblasts. In our own studies (Samiec et al., 2003a,b; Skrzyszowska et al., 2003), the survival (viability) rate of embryos after a microsurgical transfer operation of cell nuclei of cumulus cells of diameter oscillating in the range of 10 to 15 μm, was maintained at an approximate level (71.5% to 82.2%) comparable to results obtained by Kühholzer et al. (2000). In turn, the studies by Lai et al. (2001) confirmed that considerably higher was the viability potential of porcine oocytes reconstructed with cell nuclei of small-sized (10 to 12 μm), serum-starved foetal fibroblasts being at a borderline of G0/G1 stages of cell cycle (69.5%) compared to the survival rate of oocytes reconstituted with cell nuclei of much larger (20 to 25 μm), *in vitro* cultured foetal fibroblasts, synchronized at G2/M stages of mitotic cycle (47.5%). Based on the results of our own studies (Samiec et al., 2003b; unpublished data), it appears that in this last case the viability of reconstituted porcine oocytes could be greatly limited not only by the large cytoplasm volume of nucleus donor cells at G2/M stage, but above all by using cell nuclei in the transplantation, the injection micropipettes of excessive terminal external diameter, oscillating in the range of 14 to

17 μm. Such a broad pipette could cause both vast damage to plasma membrane and drastic dislocations of cytoskeleton microfilaments, and both of these defects together led to rapid ooplasm fragmentation followed by nucleus recipient-oocyte degeneration (Lai et al., 2001).

Another advantage of direct microinjection of somatic cell nuclei into the oocyte cytoplasm is that it is the "cleanest" method of nuclear transfer (NT), requiring no application of any physicochemical mediators which often bring about negative effects that decrease the *in vitro* developmental potential of reconstructed embryos of pigs and other mammal species. In contradistinction to the cell electrofusion technique, in which all components of donor cell (both nuclear and cytoplasmic – organelles and cytoskeleton elements) become an integral part of oocyte, in the case of nucleus microsurgical transfer, as already mentioned, plasmolemma and a vast majority of cytoplasmic material of nucleus donor cell are rejected after cell lysis (Lacham-Kaplan et al., 2000; Prather, 2000; Galli et al., 2002; Roh and Hwang, 2002; Rybouchkin et al., 2002; Ikumi et al., 2003). That is why only trace amounts of residual cytoplasm in the form of a narrow edge of plasma membranized protoplasm (protoplasmic "ring/circlet" or "border/areola") around the cell nucleus (i.e. the so-called perikaryon) are introduced in the form of small karyoplast into an enucleated oocyte. This is of significant importance in some researches on nuclear-cytoplasmic interactions in the hybridic clonal (NT) zygotes of pigs and other mammal species (Galli et al., 1999; Onishi et al., 2000; Nagashima et al., 2003).

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