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Recent Progress of Somatic Cell Nuclear Transfer in Pigs

Xu Xiaoming Dou Zhongying

(*Northwest A&F University, Shaanxi Branch of National Stem Cell Engineering and Technology, Yangling 712100)*

Research in the field of somatic ceil nuclear transfer (SCNT) and transgenic cloning in pigs has become a global hotspot, because porcine organs probably can be the first source of donor organs for human xenotransplantation. In recent years, though great progress has been made in porcine SCNT, the efficiency of nuclear transfer remains very low (< 1 %). Thus, it is necessary to improve the procedure of nuclear transfer and to investigate some basic problems further, Recent progress and the related problems of SCNT in pigs are reviewed and analyzed so as to offer some beneficial illumination to researchers.

Key words nuclear transfer, cloning, transgene, pig

1 Introduction

Since the birth of the cloned sheep "Dolly" derived from somatic cells, research on cloned animals became very popular. Onishi et al.[1] obtained the first live cloned piglet in the world in 2000. Xenotransplantation of pig organs is an attractive option because of its compatible size, physiology, and anatomic structure. Nuclear transfer combined with transgenic technique will speed up the clinic applications of xenotransplantation, and bring new hope for human health and longevity. So, these research fields have attracted many researchers' interests and obtained great improvement. However, the efficiency of SCNT has been very low, the success rate is under 1 %, when measured as a proportion of used oocytes which developed to term. A number of variables limit the success of reproduction pigs by somatic cells cloning. It is necessary to improve nuclear transfer procedure and to investigate some basic problems further.

2 Major Progress in Porcine SCNT

In August 2000, Onishi et al.[1] microinjected the nuclei of porcine fetal fibroblasts into enucleated in vivo matured porcine oocytes, after 3—4 h of in vitro culture, and reconstructed embryos were induced by electro-activation and further cultured for 20—40 h. The transfer of 110 reconstructed embryos to four surrogate mothers had only one success which maintained the pregnancy to term and a normal piglet was produced (0. 9%). Almost at the same time, Ploljaeva et al.[2] published their paper in Nature. They used double nuclear transfer procedure instead of premature artifical activation methods after learning some useful lessons from others. When the pronuclear is formed after fusing granulose cells with enucleated M II oocytes, they exchanged the pronuclear between fertilized and reconstructed zygotes. Totally, 401 double nuclear transfer embryos were transferred to 7 recipients. Two recipients became pregnant and one maintained the pregnancy to term with 5 piglets delivered (1. 2%). Then, Batthauser et al.[3] obtained 2 cloned piglets with fetal fibroblasts and another 2 cloned piglets with fetal genital ridge cells. Neither two frontal groups used the simple method of fusing nuclear donor cells into enucleated in vitro matured (IVM) oocytes to produce those clones. Batthauser et al. obtained IVM oocytes at first. An electrical pulse of 1. 9 kv/cm for 45 μ*s* was then used to fuse the membranes of donor cell and oocyte forming a cytoplasmic hybrid. After 4 h of culture, the cytoplasmic hybrid was activated by incubation in 15 μ*M* calcium ionomycin for 20 min followed by incubation with 1.9 mM 6-DMAP for 3—4 h. Reconstructed embryos were cultured for 8—76 h. 902 reconstructed embryos were transferred to 7 recipients (116—164 embryos/recipient), 4 recipients became pregnant, 2 maintained the pregnancy to term, and 2

piglets were obtained from 2 recipients respectively (0.4%). Park et al.[4] reported for the first time that they obtained the transgenic piglets derived from somatic cells with enhanced green fluorescent protein (EGFP). After transfer of nuclei from a modified fibroblast cell line into IVM porcine oocytes, fusion (2 DC pulses of 1.2 kv/cm for 30 μs), 308 reconstructed embryos were transferred, together with parthenogenetic embryos, into 3 recipients. One of them maintained pregnancy to term, and produced 5 cloned piglets expressing EGFP. Subsequently, they obtained 4 cloned piglets expressing EGFP derived from ear epithelial cells. Lai et al.[5] reported the production of 7 pigs with 4 live pigs of which one allele of the α-1, 3-galactosyltransferase locus was knocked out. These pigs were produced by nuclear transfer technology; clonal fetal fibroblast cell lines were used as nuclear donors for embryos reconstruction and reconstructed embryos were transferred to a mated surrogate. De Sousa et al.[6] transferred fetal fibroblasts into enucleated IVM oocytes with 2 h delay between electrical fusion and activation. The reconstructed embryos were transferred to 8 recipients, 3 of which maintained the pregnancy (37.5%) and a live cloned piglet was produced from a pregnancy. Yin et al.[7] produced 8 cloned pigs from adult heart cells of a female pig by chemically assisted removal of IVM maternal chromosomes. 1439 reconstructed embryos cultured in vitro for 24—48 h were transferred to 6 recipients, 3 of which became pregnant (50%) and give a production of 8 cloned pigs (0.5%). Lee et al.[8], using adult skin fibroblasts as donor cells and enucleated IVM oocytes as recipients, conducted a whole-cell injection technique for nuclear transfer, activated 3 h after injection, cultured them for 20—24 h. Then a total of 685 whole-cell injected oocytes were transferred to 9 recipients, 6 of which were confirmed pregnant (66.7%). Finally, 4 live piglets were born from three recipients (0.5%). Using fetal fibroblasts as donor cells and IVM enucleated oocytes as recipients, Phelps et al.[9] transferred reconstructed embryos to 16 recipients. 10 of the recipients became pregnant (62.5%) with 2 of them maintained the pregnancy to term, and 4 healthy α-1, 3-galactosyltransferase-deficient gene-knockout (two alletes were knocked out) female piglets were produced. Kolber-Simonds et al.[10] also obtained a healthy α-1, 3-galactosyltransferase double-knockout piglet. The piglet carrying a point mutation in the α-1, 3-galactosyltransferase gene hold significant value, as they will allow production of α-1, 3-galactosyltransferase-deficient pigs free of antiboiotic-resistance genes and patients will not response to hyperacute rejection (HAR) of porcine organs, which have the potential to make a safer production for human use. Prof. Li Ning of China Agricultural University claimed that they had produced a live cloned piglet for the first time in China lately. SCNT in pigs with different methods is shown in table 1.

Table 1 Somatic cells nuclear transfer in pigs with different methods

Don or cells	In vivo or in vitro-matured oocytes	Method of injection donor nucleus	Time of cloned embryos culture	No. of live piglet	Transgenic pigs	References
GC	In vivo	Serial NT	20 h	5	no	Polejaeva, et al. Nature, 2000
FF	In vivo	IC	20—40 h	1	no	Onishi, et al. Science, 2000
FF	In vitro	IP	8—76 h	4	no	Bettauser, et al. Nat Biotechnol, 2000
ASF	In vivo	IP	24—32 h	2	yes	Bondiolik, et al. Mol Reprod Dev, 2001
FF	In vitro	IP	18—22 h	5	yes	Park et al. Animal Biotech. 2001
FF	In vivo	IP	5—36 h	4	yes	Lai, et al. Science, 2002
AHC	In vitro	IP	24—48 h	8	no	Yin, et al. Biol Reprod, 2002
FF	In vivo	IP	12—36 h	2	no	Boquest, et al. Biol Reprod, 2002
FF	In vitro	IP	1 h	1	no	De Sousa, et al. Biol Reprod, 2002
FF	In vitro	IP	12 h	1	yes	Lai, et al. Mol Reprod Dev, 2002
FF	In vitro	IP	12—36 h	28	no	Walker et al. Clonig Stem Cell, 2002
EP	In vitro	IP	18—22 h	4	yes	Park, et al. Biol Reprod. 2002
FF	In vitro	IP	12—48 h	6	yes	Dai, et al. Nat Biotechnol, 2002
FF	In vitro	IP	8—24 h	3	1 yes; 2 no	Hyun, et al. Biol Reprod, 2003
ASF	In vitro	IC	20—24 h	4	no	Lee, et al. Biol Reprod, 2003
FF	In vitro	IP	12—48 h	4	yes	Phelps, et al. Science, 2003

* FF (fetal fibroblasts); GC (granulose cells); ASF (adult skin fibroblasts); AHC (adult heart cells); EP (ear epithelial cells); IC (injection of donor nucleus into the cytoplasm); IP (injection of donor cells into perivitelline space). A total of 82 live cloned piglets, of which 27 piglets are transgenic.

3 The Problems and Way out

3.1 Selection of donor cell

At present, successfully cloned piglets derived from somatic cells mostly derived from fetal fibroblasts. Lee et al.[11] demonstrated that the type of donor somatic cells was important for improving preimplantation development after porcine SCNT. Fetal fibroblasts were the most effective donor cell among fetal fibroblasts, adult fibroblasts, cumulus cells and oviduct epithelial cells. In contrast to the results of SCNT, the results from embryonic cells or embryonic stem cells are more efficient. Because embryonic cells or embryonic stem cells require less reprogramming of their genome, the genes necessary for early embryonic development are already active. The lower the degree of differentiated cells which requires less reprogramming is, the higher its development potential is. Establishment of porcine ES cell lines can constantly provide donor cells for nuclear transfer. However, so far porcine ES cell lines have not been established. Therefore, using adult stem cells as donor cells in nuclear transfer may improve cloning efficiency and have promising applications because adult stem cells also remain an undifferentiated state.

3.2 Selection of recipient oocytes

IVM oocyte systems produce abundant oocytes which can be obtained from slaughtered animals in comparison to the expensive in vivo matured oocytes. So IVM oocytes become the mainstream in the research of nuclear transfer. But the system of porcine IVM is not perfect yet, such as unsynchronized maturation of nuclear and cytoplasm, and longtime of IVM. How to improve the conditions of porcine oocytes IVM and obtain best matured oocytes are imperative questions to be solved. A number of reports show that oocytes matured in vivo have better developmental competence than those matured in vitro, resulting in improved morula compaction and blastocyst formation[1,2]. However, De Sousa et al.[6] reported that developmental capacity was not different when in vivo-matured and in vitro-matured oocytes were used as enucleated recipient oocytes in porcine SCNT. Lee et al.[11] also demonstrated in parthenogenetic development that the rate of development to blastocysts was higher in in vivo maturation oocytes than IVM ones, but there was no significant difference in the rate of development to blastocysts in porcine SCNT.

In recent years, oocytes derived from sow were found to be more suitable to porcine SCNT. Hyun et al.[12] compared the influence of oocytes source on the development ability of SCNT embryos, showing that SCNT embryos derived from sow oocytes were more meiotically competent and yielded higher development ability and total cell numbers more than those derived from gilt oocytes when transferred to recipient.

3.3 Procedure of nuclear transfer

Enucleation is a very important step in SCNT since whether it is successful or not directly influence the efficiency of nuclear transfer. At present, "blind enucleation" method is commonly used in enucleation of porcine SCNT. Although "blind enucleation" helping with Hoechest 33342 staining can increase enucleation efficiency, some researchers still do not approve this method.[8] Because the condensed chromosome is always located in the cytoplasm underneath the first polar body, we used "blind enucleation" method, confirmed by Hoechest 33342 staining. The rate of enucleation is 85% (unpublished data). Enucleation under spindle-view system can enhance the efficiency of enucleation and reduce the loss of cytoplasm as little as possible. But porcine oocytes cytoplasm contains a lot of fat-drop, it is impossible to discern the spindle under the spindle-view system. So, it is necessary to explore some feasible methods of enucleation. Yin et al.[7] demonstrated that brief treatment of IVM porcine oocytes with demecolcine resulted in a membrane protrusion that contains a condensed chromosome mass which can be easily removed by aspiration. This chemically assisted enucleation method is characterized by simplicity, less cytoplasm loss, high enucleation rate and insignificant difference in development rate to blastocysts comparing to other methods. They produced 8 healthy cloned piglets in this way. Zona Pellucida (ZP) free nuclear transfer method does not require expensive micromanipulation and complex manipulation and fits to production application. Its procedure is as the following: bisection of oocyte, discard of one half containing DNA, fusion donor cell with two half parts which did not contain DNA. The quality of blastocysts produced by this method is not affected by ZP. But this method wastes half of oocytes and increases mitochondrial heterogeneity because mitochondria of re-

constructed embryos have three different origins. Lately, Sullivan et al.[13] developed a novel system for remodeling mammalian somatic nuclei in vitro prior to cloning by nuclear transfer. The system involves permeabilization of the donor cells using SLO and chromatin condensation in a mitotic cell extract to promote removal of nuclear factors solubilized during chromosome condensation. $CaCl_2$ were added for membrane resealing. Resealed cells were fused with enucleated oocytes. Five healthy calves were produced by chromatin transfer. They demonstrated that this method could improve viability of cloned calves. Additionally, manipulation of this system might lead to further improvements in the efficiency of mammalian cloning.

3.4 Effect of electro-fusion and intracytoplasmic nuclear injection methods on the efficiency of cloning

At present, the electro-fusion method seems to be more common because of a larger number of cloned pigs produced (Table 1). Nagashima et al.[14] verified that the rates of normal cleavage and blastocysts formation were significantly higher in fusion (45.6% versus 32. 1 %, 19.2% versus 5.4%) than intracytoplasmic injection. Futhermore, it was confirmed that the nuclear transferred embryo by fusion is capable of developing to a normal cloned fetus. From various aspects, however, advantages and disadvantages of both methods can be summarized as the following. Intracytoplasmic nuclear injection causes no activation of recipient oocytes. The injection method is technologically difficult but it has fewer steps in micro-manipulation. Nuclear transfer efficiency is independent of the state of donor cell membrane. On the other hand, the fusion method uses widespread techniques, and its reliability is supported by successful results of cloning. Electro-fusion may cause activation of recipient oocytes (at least partially) simultaneously with nuclear transfer and nuclear transfer efficiency is related to the state of donor cell membrane.

3.5 Activation method

Most researchers used electrical activation method to successfully produce cloned pigs[1,4-6,12]. Hyun et al.[12] demonstrated that a simultaneous electrical fusion/activation protocol is simple and efficient for producing porcine SCNT embryos indicating that no further chemical activation such as ionomycin and 6-DMAP·is necessary for postactivation. However, Betthauser et al.[3] reported that activation with ionomycin and 6-DMAP is better than electrical activation protocols in the rate of blastocysts formation, total cell numbers of blastocysts and the rate of pregnancy. But negative effects of chemical material on the development of embryos remain to be further examined. Although artificial activation methods were commonly used in nuclear transfer, the cytoplasm setting of enucleated fertilized zygotes, because of its natural activation, was considered to be better than MII oocytes as recipients. Polejaeva et al.[2] adopted double nuclear transfer procedure and successfully produced viable cloned piglets to minimize the potential inefficiencies of artificial activation. They first fused donor cell with nucleated MII oocyte. Pseudopronucleus was then transferred into enucleated, in vivo produced zygote. This latter procedure allows the formation of a final reconstructed one-cell embryo whose membrane has been activated during fertilization.

3. 6 In vitro culture of reconstructed embryos and maintenance of pregnancy

Recently, evidence for the inadequacy of in vitro culture systems for porcine embryos is the paucity of reports about the birth of piglets from IVF blastocysts. Addition of serum to culture medium may affect the abnormal expression of imprint gene in the early stage of embryo development in vitro culture. The small cell numbers of cultured NT, IVF, and parthenogenetic blastocysts compared to those of in vivo blastocysts probably reflects these inadequacies (66 cells for NT and IVF in vitro blastocysts and 49 cells for parthenogenetic blastocysts versus 200—300 cells for in vivo blastocysts)[3]. Reconstructed embryos that successfully developed to produce cloned piglets were cultured in vitro less than 78 h (Table 1) in all reports. In pigs, one method can avoid this inefficiency of culture system, i.e., we can transfer reconstructed embryos into oviduct of pseudopregnant recipients so as to develop in vivo for several days and then collect embryos by reverse flush of oviducts.

The efficiency of nuclear transfer is not high in any species, especially in pigs. One of the specific problems is that, in pig, several good quality embryos are required to be present in the uterus to establish and maintain pregnancy. This is most critical around day 11 or 12 of gestation when conceptus-derived estrogen is responsible for maternal recognition of preg-

nancy. During this stage, a minimum of four or five viable embryos is required for pregnancy maintenance. After day 14, however, the number of embryos becomes less critical. Since embryo quality is compromised after nuclear transfer and a significant percentage of the blastocytes produced are not able to develop to term, a very large number of embryos have to be transferred. If enough cloned embryos are not available for a transfer, the following three strategies can be attempted to induce and maintain pregnancy. Firstly, NT embryos can be co-transferred with 30—40 parthenogenetic embryos of the same development stage, which are thought to enhance the signal for maternal recognition of pregnancy but would degenerate later in gestation because of genomic imprinting. Secondly, estradiol cypionate, which is supposed to be a signal to stimulate the development of the uterus for accepting attachment of the embryos, is injected on day 12 after embryos transfer. Lastly, reconstructed embryos can be transferred into a naturally bred gilt on the day when the surrogate is fertilized[15].

3.7 Problems of genetic manipulations and human xenotransplantation

A major barrier to progress in pig-to-primate organ transplantation is the presence of terminal α-1, 3-galactosyl epitopes on the surface of pig cells. Humans and Old World monkeys have lost the corresponding galactosyltransferase activity in the course of evolution and therefore produced natural antibodies to the epitope which are responsible to HAR of porcine organs. The α-1, 3-galactosyltransferase gene can be effectively knocked out in pig by nuclear transfer[5,9]. However, to solve this problem of xenotransplantation thoroughly, not only the knockout of α-1, 3-galactosyltransferase gene is necessary, but also the anti-protein and vascular cell adhesion molecular are important to avoid subsequent acute or chronic tissue damage. So, it will be essential to develop strategies to eliminate the risk that endogenous retroviruses produced by porcine cell lines might infect human cells.

3.8 Dedifferentiation and reprogramming

Inadequate reprogramming of the donor nucleus is thought to be the major reason for the developmental losses of most clones. Unlike the normal zygote, the donor nucleus can not perform genomic modification to an embryonic pattern of expression completely. The process of nuclear reprogramming is to reactivate some silent genes in donor nucleus which needed to be expressed in normal embryonic development. Dedifferentiation follows epigenetic reprogramming at the molecular level, including some protein correlated with chromatin (histone and transcription factors) genomic methylation, histone modification, and the overall chromatin structure, which needs to be reprogrammed to be in a compatible state during embryonic development. Any aberrant gene expression in this process can cause abnormal development of cloned embryos and clones. SCNT needs the remodel of chromatin structure. Some proteins of donor nucleus must be lost and other proteins of oocytes must enter the donor nucleus[11,13]. Nuclear reprogramming is related to genomic methylation/demethylation and histone acetylation. In mammalian, the interaction between DNA and protein can be altered through the genome methylation, providing some non-encoding sequences (including introns, repeating sequences and some potentially active transposons) and genetic mechanism of some heritable silent genes correlated with development. Nuclear reprogramming needs to break the silence state of the genes and to reactivate them. At present, the molecular mechanism of dedifferentiation and reprogramming is unknown. So, further study of these problems will help to understand the mechanism of nuclear transfer and improve the efficiency of cloning.

3.9 The problem of mitochondrial heteroplasmy

Recently, electrical-fusion was used commonly in porcine SCNT. This method inevitably combined with two different original mitochondria derived from donor cells and oocytes. The replication of both mitochondria will cause mitochondrial heteroplasmy. But, if one of them is selectively replicated, the other will be selectively destroyed. Therefore, the mitochondria of clones may either derive from donor cell, or from oocyte, or from both. How can the donor nucleus support and harmonize mitochondria from donor cells and oocytes? How does mitochondrial DNA derived from donor cells or from oocytes reprogram? What are the effects of inadequate reprogramming of mitochondria nucleus on the expression of donor nucleus? All of those questions remain unclear and need to be further studied.

4 Prospect

Great progress has been made in porcine SCNT whose combination with thransgenic technology will realize the dream of transfer of porcine organs into human body. However, the efficiency of SCNT is quite low and its procedures and protocols need to be improved. Molecular mechanisms of oocytes IVM, embryonic development and nuclear reprogramming must be further studied. And much attention should be paid to the issue of mitochondrial heteroplasmy. We believe that porcine SCNT technology will become perfect and can serve people better by endeavor of all researchers over the world.

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