Review

Creating genetically modified pigs by using nuclear transfer
Liangxue Lai and Randall S Prather*

Address: Department of Animal Science, University of Missouri-Columbia, Columbia, MO 65211, USA

Email: Liangxue Lai - LaiL@Missouri.edu; Randall S Prather* - PratherR@Missouri.Edu

* Corresponding author

Abstract

Nuclear transfer (NT) is a procedure by which genetically identical individuals can be created. The technology of pig somatic NT, including in vitro maturation of oocytes, isolation and treatment of donor cells, artificial activation of reconstructed oocytes, embryo culture and embryo transfer, has been intensively studied in recent years, resulting in birth of cloned pigs in many labs. While it provides an efficient method for producing transgenic pigs, more importantly, it is the only way to produce gene-targeted pigs. So far pig cloning has been successfully used to produce transgenic pigs expressing the green fluorescence protein, expand transgenic pig groups and create gene targeted pigs which are deficient of alpha-1,3-galactosyltransferase. The production of pigs with genetic modification by NT is now in the transition from investigation to practical use. Although the efficiency of somatic cell NT in pig, when measured as development to term as a proportion of oocytes used, is not high, it is anticipated that the ability of making specific modifications to the swine genome will result in this technology having a large impact not only on medicine but also on agriculture.

Introduction

Pronuclear DNA micro-injection has long been the most reliable method to produce transgenic pigs. Despite the ease with which transgenic pigs can be generated this technique has limitations. The DNA integrates randomly and potentially in multiple copies. In addition, the random site of integration limits the ability to control expression in the desired tissues or at the appropriate level. Moreover, the animal's endogenous genes cannot be specifically altered by using this technique.

Successful nuclear transfer (NT) of cultured cells, which was first demonstrated in cattle [1], has provided an alternative for obtaining genetically modified pigs. McCreath et al. [2] reported the first success of obtaining gene-targeting sheep by using gene-targeted fibroblasts as a source of donor nuclei for NT. Although NT of pigs once lagged behind that in mice, cattle and sheep, since the first piglet

from somatic cell NT was reported in 2000 [3], tremendous progress has been made. Successful production of pigs resulting from random genetic modification in vitro followed by NT [4–6], as well as those with a specific modification (knock out) have been reported [7–10] by several groups in a short period. The production of cloned transgenic pigs is now in the transition from investigation to practical application. In this paper, we discuss the present status of production efficiency of transgenic pigs by NT; as well as the problems and offer a few perspectives.

History of pig cloning

The first successful cloning experiment in pigs was reported as early as in 1989. Prather et al., [11] used blastomeres from 4-cell stage embryos as donor nuclei and in vivo-derived metaphase II oocytes as recipient cytoblasts. A total of 88 NT embryos were transferred to recipient gilts

for continued development. A single piglet was born. Similar success was not reported with embryonic cells until more than 10 years later [12]. With a similar NT technique that produced Dolly – in which a cultured differentiated somatic cell is fused with a mature egg whose genetic material has been removed [13], successful cloning of pigs was not reported until 3 years later [3,14]. Pigs are physiologically similar to humans and so there has been intense interest in using genetically modified pigs as organ donors for transplantation to humans, as well as models of human disease.

In 2000, the first somatic cell cloning success was Polejaeva et al. [3] who announced the birth of five healthy cloned piglets. These animals were produced via a different technology from that generally used for NT. The authors first fused porcine granulosa-derived donor cells to enucleated mature oocytes. After 18 hours, the donor nucleus was removed from the first oocyte and transferred to the cytoplasm of a fertilized egg. The investigators adopted this double NT strategy because they surmised that in the original one-step method, the activation stimulus provided after NT was insufficient to support full-term development of the embryo. This report lead the pig cloners to think that the procedures of pig cloning might be more complicated and difficult than other animals. However, almost at the same time, Onishi et al. [14] reported the birth of a live cloned piglet, by directly injecting porcine fetal fibroblast donor nuclei into enucleated oocytes with piezo-actuated microinjection. The significance of Onishi's success is that they proved that two-step NT is unnecessary to make somatic NT pig. Both groups used mature oocytes collected directly from female pigs rather than culturing immature oocytes in vitro. Matured oocytes are needed in large numbers and in vivo matured oocytes are very expensive to acquire. Thus many have chosen to use in vitro matured oocytes. Immature oocytes are derived from ovaries obtained from the slaughterhouse and subsequently matured in vitro. Betthauser et al. [15] had systematically optimized each step in the NT procedure, including the source of oocytes and their maturation in vitro, the culture of donor cells, the activation of oocytes following NT, and the in vitro culture of embryos and their transfer to recipient gilts. The result is a more reproducible methodology that enables strategies to genetically modify pigs.

Park et al. [4] reported the first pigs produced by genetically modifying the fibroblast cells prior to NT. This was followed by Lai et al. [7] showing the first pigs with a gene knocked out. Homologous recombination was used to disrupt the sequence of the α-1, 3-galactosyltransferase (GGTA1) gene in mini-pig fibroblasts. The targeted fibroblast nuclei were transferred into enucleated in vitro matured oocytes. Four live GGTA1 knockout pigs were successfully produced. An important practical significance of this experiment is to facilitate studies in xenotransplantation of pig organs to humans, as elimination of terminal α-1, 3-galactosyl epitopes from the pig is expected to be a solution to the problem of hyperacute and delayed vascular rejection.

Unique aspects of pig cloning technology

The efficiency of somatic cell NT, when measured as development to term as a proportion of oocytes used, has been very low (1–2%). A number of variables influence the ability to reproduce a specific genotype by cloning. These include species, source of recipient ova, cell type of nuclei donor, treatment of donor cells prior to NT, the method of artificial oocyte activation, embryo culture, possible loss of somatic imprinting in the nuclei of reconstructed embryos, failure of reprogramming the transplanted nucleus adequately, and the techniques employed for NT. In the pig, there is an additional difficulty that at least 4 good embryos are required to induce and maintain pregnancy. Therefore, to increase the chance of producing offspring efforts to minimize the inefficiencies at each step of NT procedure must be made. Some of the variables are discussed below.

Selection of cell types used to produce cloned pigs

In mice, at least eight types of fetal and adult origin from males and females and different genetic backgrounds have been tested. Live offspring were obtained with similar efficiency only with fibroblast, undefined fetal gonad and cumulus cells [16]. Many cell types (macrophages, spleen, brain and mature Sertoli cells) repeatedly failed to develop after implantation. In cattle, at least fifteen somatic cell donors of fetal, newborn and adult origin from males and female and different genetic backgrounds were tested [17] and all supported development in vitro, and live offspring were obtained from cumulus, oviduct, skin and liver cells. Ten different somatic cell types have proven complete cloning-competence so far. In pigs, fibroblast and cumulus cells have been clonable. The use of isolated precursor cells of adipocytes from the subcutaneous adipose tissue of adult pigs for NT [18] have also has been documented.

Treatment of donor cells

The use of cultured cell populations for production of animals by NT is now well documented in a number of species. Analysis of these cells has shown considerable variation in development between individual cell populations and at present has provided no definitive method for identification of cell populations that are best suited for NT. Factors that are thought to influence the suitability include the effects of oxidative damage associated with metabolism, genome instability and chromosomal pathologies. All of these factors may be influenced by the

method of isolation and culture, and the number of population doublings in culture. Even different subclones of fibroblasts derived from same fetus and cultured in same conditions to same generation lead to different in vitro developmental potential of reconstructed embryos [19]. Another factor affecting the efficiency of NT is the cell cycle phase of donor cells, which is still a topic being debated in the NT field. Wilmut et al. [13] stated that the donor cells for NT must be in G0 of the cell cycle (quiescent phase), while Cibelli et al. [20] showed that cycling cells, which contain cells in different cycle stages, could be successfully used for NT in cattle. Cells in G2/M stage of the cell cycle may be another option as these have been used as nuclear donors to produce cloned mice [21], a pig [6], and sheep [22].

Possible donor cells for genetic modification
Cells to be used as a source of donor nuclei for the production of genetically modified pigs must meet two criteria: 1) they must be able to direct term development, and 2) they should possess a proliferative ability such that correct DNA modification can be selected. Fetal-derived fibroblasts are a popular choice of cells to begin studies as they are capable of extensive proliferation.

The problem with all somatic cells is that they tend to become senescent before sufficient rounds of gene transfer and/or targeting and selection can be performed. This problem may be overcome by isolation of readily transfectable and selectable cells with high proliferative potential and long-term karyotypical normalcy, similar to murine ES and EG cell lines. Primordial germ cell-derived lines have been isolated from pig fetuses, and transfected lines have been shown to contribute to chimera formation when injected into pig blastocysts [23,24], but in no case has germ line transmission been demonstrated. Thus further development is needed to create cells that are developmentally competent and able to proliferate indefinitely in vitro.

Construction of nuclear transfer embryos
Oocytes must be enucleated for use in NT. One of the common chemicals used to facilitate enucleation by permitting visualization of the chromosomes is bisbenzimide. Unfortunately, exposure to this compound has deleterious effects on the development of pig oocytes to the blastocyst stage. Since the condensed chromosomes are always located in cytoplasm underneath the first polar body, enucleation of in vitro matured metaphase II oocytes can be performed by aspirating the first polar body and adjacent cytoplasm without staining the chromatin. By using this "blind enucleation" method the enucleation rate varies between 85% and 90% [25].

There are two approaches to put the donor nuclei into the cytoplasts; one is direct injection of donor cells into enucleated oocytes; while the second approach is to inject the intact donor cell into the perivitelline space and subsequently fuse the donor cell with the recipient oocyte by electrical pulses. With direct microinjection, the plasma membrane and much of the cytoplasmic material of the donor cell is not transferred. In contrast, with cell fusion, all of the components of the donor cell (nuclear, cytoplasmic and plasma membrane) merge with the enucleated oocyte.

After the donor nuclei are transferred into the enucleated oocytes, the reconstructed embryos must be activated to initiate subsequent development. Activation of oocytes can be induced artificially by a variety of physical and chemical agents. Miyoshi et al. [26] found that a delay of 3 hours between fusion and activation improved the rate of blastocyst formation. The rate of development to the blastocyst stage after NT has been as low as 3%. When presumptive G0 fibroblast cells were transferred to oocytes and electrically activated, only 7% formed blastocysts. Betthauser et al. [15] reported 4–8% blastocysts resulting from NT that were activated by ionophore followed by 6-dimethylaminourine. Recently, Kühholzer et al. [19] reported almost 20% blastocysts from electrically activated oocytes. Combined thimerosal/DTT treatment of the oocytes also could effectively activate porcine oocytes and 42.0% of the oocytes developed to the compact morula or blastocyst stage in vitro culture [27].

Embryo transfer
In the pig pregnancy recognition by the surrogate requires a signal from four or more embryos around day 12 of gestation [28]. To minimize any adverse effect on the in vitro conditions on the development of NT embryos transfer to the surrogate is generally at a very early stage. Since the NT embryos are generally of a low quality a large number are transferred into a single surrogate.

If not enough NT embryos are available for transfer, then two different strategies might be employed. The first is to co-transfer "helper embryos" as an aid to inducing and maintaining pregnancy. These "helper embryos" maybe parthenogenetic embryos that are capable of establishing a pregnancy but degenerate by day 30 of gestation [6,29] because of genomic imprinting. Alternatively the helper embryos might be derived from a normal mating [7]. Finally, administration of estradiol, the normal signal for maternal recognition of pregnancy, on day 12 can maintain the pregnancy of small litters [6,11]. Although it is difficult to document that any of these strategies were beneficial, they did not appear to hinder development.

Applications of pig cloning in genetic modification

Genetic modification in swine could have many agricultural and medical applications. In the agricultural field, modification of the genome could (1) alter the carcass composition such that it is a healthier product, (2) produce pork faster or more efficiently, (3) create animals that are resistant to specific diseases, (4) reduce the major losses normally observed during the first month of swine embryogenesis, and (5) create animals that are more environmentally friendly.

In the medical field, making specific genetic modifications in the pig provides the possibility of producing recombinant products in animals for biomedical or nutraceutical uses and the possibility of producing models of human genetic disease for research and drug development. Somatic NT could play important roles in genetic modification by the following three methods.

Improving generation of transgenic pigs

Transgenic technology developed to add genes has been widely applied to livestock species because it is technically simple, but inefficient. Not all injected eggs will develop into transgenic pigs, and then not all transgenic pigs will express the transgene in the desired manner. Somatic NT would allow more efficient generation of transgenic animals. Foreign DNA could be introduced into cell lines in culture, and cells containing the transgene in the right configuration could be grown up. The expression level can be detected in individual cells, which could be determined by the addition of a reporter gene with a target gene. The cells with high level foreign gene expression would be selected and used as a source of nuclei for transfer, ensuring that all offspring are transgenic and have high level expression. We added a gene for the enhanced green fluorescent protein (EGFP) to a fetal-derived cell line by using a replication-defective retrovirus [4]. These cells were then used as donors in a NT scheme that used oocytes that had been matured in a defined system. The genetically marked tissues (EGFP) from the pigs produced from these cells will likely be very useful for basic research where such marked cells are required. Our laboratory is looking forward to the possibility of EGFP expressing sperm, oocytes and embryos for our studies on fertilization and embryo development.

Improving propagation of transgenic pigs

Nuclear transfer would speed up the expansion of a successful transgenic line by using skin cells of the transgenic pigs to make more clones. Ear skin fibroblasts from a transgenic pig produced by oocyte transduction [30] and expressing eGFP were isolated and used as donor nuclei for NT. Four live cloned pigs were born. As in the nuclear donor pig, all of the offspring expressed the eGFP in sim-

ilar tissues [31]. Bondioli et al. [32] (2001) produced cloned pigs from cultured skin fibroblasts derived from an H-transferase transgenic boar. The cells used in these studies were subjected to an extensive culture time, unsuccessful transformation, freezing and thawing, and clonal expansion from single cells prior to NT. One 90 day fetus and two healthy piglets resulted from NT by fusion of these fibroblasts with enucleated oocytes.

Generating Targeted Gene Alterations

The most powerful technology for genetic manipulation in mammals – gene targeting – was developed in mice, and depends on the ability of mammalian DNA, when added to cells in culture, to recombine homologously with nearly identical DNA sequences in the genome and replace it. In mice, specific mutations can be generated in cultured embryonic stem (ES) cells. For more than a decade, researchers have searched for ES cells from livestock, including pigs. But although some success has been reported, none has passed the crucial test of contributing to the germ cells. Nuclear transfer from non-pluripotent cell lines provides an alternative to the ES cell route for introduction of targeted gene alterations into the germ line. The first such example is that of knocking out a gene that is responsible for hyperacute rejection (HAR) when organs from swine are transferred to primates.

As described above, pig somatic NT has been established well enough to make live cloned pigs. To generate targeted gene alterations in pigs, creation of gene targeted somatic cell lines becomes another critical issue to be addressed.

Generally, fibroblasts cannot proliferate indefinitely in vitro. Senescence of primary fibroblasts in livestock is generally seen following approximately 30 populations doubling ex vivo in non-clonal cultures. In pig fetal fibroblasts, clonal isolates are generally lost at passage following 24–28 population doublings ex vivo. This presents a technical hurdle as compared to mouse ES cells. However, this problem can be overcome to some extent by using a gene trap strategy which may result in a higher targeting rate [7]. To target the GGTA1 locus a long region of homology to the GGTA1 locus was used in a vector constructed from the same inbred line of miniature pig from which the fetal fibroblasts were derived, thus providing for isogeneticity to the target locus. A stop codon and selection cassette was inserted into exon 9, upstream of the catalytic domain of the protein. The selection cassette contained no promoter and was preceded by an internal ribosome entry site. Gene trap designs of this nature have been shown to result in relatively high targeting rates, as the vast majority of non-targeted recombination events do not result in transcription. Additionally, transient expression of the selection cassette cannot occur.

In addition, transfecting the cells as early as possible after isolation (passage 2, or about 6 population doublings) proved to be another helpful measure to overcome the hurdle [7]. RT-PCR can be performed on crude cell lysates the day following transfection to quickly identify potentially targeted clones. In our experiments, an absolute targeting of 8×10^{-7} was obtained, which is similar to that in mouse ES targeting (1×10^{-6}) [7], producing enough cells to be used as donor nuclei. However, it is unlikely we could achieve two targeting events in the one primary cell line and still maintain enough cells for NT. Rather, removal of the second allele would be accomplished by breeding or producing a cloned fetus, or piglet, thus providing cells which can be used for a second round of targeting and NT. To produce homozygous GGTA1 knockout piglets by natural breeding, assuming both male and female heterozygous knockout pigs are available at the same time and are fertile, is feasible but takes up to 12 months. However, by using a second-round knockout and cloning strategy, we could save up to 6 months and all cloned piglets would be GGTA1 double knockout (DKO). One group has chosen to use second-round knockout and cloning strategy to produce homozygous GGTA1 knockout piglets and successfully obtained five galactosyltransferase-deficient pigs [33]. Our group has also produced a GGTA1 DKO by selecting cells that do not express GGTA1 originally isolated from a heterozygous knockout pig (manuscript in preparation).

In conclusion, the techniques of NT in pigs, while developed, are not efficient. Nevertheless, the possibility of making specific genetic modifications to pigs offers great potential to both medicine and agriculture.

Acknowledgements

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