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Progress in Transgenic Cloned Pig for Xenotransplantation

Kwang-Wook PARK

MGenbio, Inc. Woore Building 3F, 684-2 Deungchon 3-dong, Seoul 157-033, Korea

ABSTRACT

Pig organ is thought to be the most suitable nonhuman organ for xenotransplanstation. However, one of the major constraints to using pig organs for xenotransplantation is human natural antibody-mediated hyperacute rejection (HAR). Elimination of a(1,3) galactosyltransferase (GGTA1) from the pig is expected to be a solution to the problem of hyperacute rejection. Many efforts have made characterization of GGTA1 in structure and function, improvement in the technique of DNA transfection of somatic cells and advancement of the pig NT, a specific modification has been made to one copy of the GGTA1 gene by Missouri group in 2002. To date because homozygousity of the genetic modification has been achieved in this gene, the role of gala(1,3) gal specific natural antibody in HAR and the efficacy of xenotransplantation in a nonhuman primate model will be addressed. If other genes are found to be involved in rejection of pig donors by primates, the technology will be available to modify those genes so that rejection can be overcome.

INTRODUCTION

Direct microinjection of DNA into the zygote pronuclei has been used to produce transgenic livestock,since the method was established by Hammer *et al.* (1). However, the integration rate of transgene DNA into the genome is low (5 to 15%) (2). Because the integration of foreign DNA is random, transgene expression is unpredictable and often insufficient. In

addition, one-third of transgenic founder animals produced by microinjection are mosaic i.e., an integrated gene is not present in all cells and the transgene transmission rate from founder to offspring is low (2). This inefficiency of microinjection absorbs much labor and cost during the production of transgenic animals.

Nuclear transfer (NT) would provide solutions to the disadvantages of traditional microinjection. Since the birth of the first cloned animal "Dolly" (3), derived from an adult somatic cell, progress has been made in cloning animals by using somatic cell NT.Animals produced by NT with genetically modified cells are mainly transgenic, which greatly improves the efficiency of transgenic animal production (4-9). In pigs, the first cloned piglets were reported from *in vivo*-derived oocytes (10, 11) and then from an *in vitro* system (12). By using genetically manipulated donor cells, transgenic piglets have also been produced (13).

Pig organ is thought to be the most suitable nonhuman organ for xenotransplanstation. Organ transplantation has become a very successful treatment for many diseases associated with terminal organ failure, but there are not enough donations to meet the need. However, one of the major constraints to using pig organs for xenotransplantation is human natural antibody-mediated hyperacute rejection (HAR). Elimination of a(1,3) galactosyltransferase (GGTA1) from the pig is expected to be a solution to the problem of hyperacute rejection. Recently, two groups reported the production of pigs in which one allele of the a(1,3) galactosyltransferase locus has been knocked out (14, 15). GGTA1 null (homozygous knockout) piglets have been then produced in a short period (16; Dr. Prather group, 2003; Infigen 2003).

Production of Nuclear Transferred-Derived Swine that Express the Enhanced Green Fluorescent Protein

While the simple addition of a gene is important, it is a necessary prerequisite to site-specific genetic modifications. Such specific genetic modifications will be necessary to provide a better possibility of using swine organs for xenotransplantation (17). In addition, knocking-out other genes that may be responsible for single gene traits, such as the chloride channel to attempt to manifest the cystic fibrosis phenotype (18), would

have utility as disease models for humans. Finally, knockouts of genes such as myostatin (19) may have significant impacts on agricultural productivity. The enhanced green fluorescent protein (EGFP) gene was transfected to fetal-derived cell line by using a replication-defective retroviral vector. These cells were then used as donors in a nuclear transfer scheme that used oocytes that had been matured in a defined system. After cell fusion-mediated nuclear transfer, the nuclear transfer embryos were transferred to surrogate gilts that had shown signs of estrus, but had not yet ovulated. Five live cloned piglets were delivered by cesarean section and four visibly express the EGFP (13).

Transgenic Pig Expressing the EGFP Protein Produced by NT using G2/M Stage Donor cells

Successful somatic cell nuclear transfer provides a promising method to produce transgenic animals. This concept was strongly supported by generation of transgenic animals derived from nuclear transfer approaches by using transfected somatic cells. For pigs, somatic cell nuclear transfer has another special significance, as is itwould allow the use of genetic modification procedures to produce tissues and organs from cloned pigs with reduced immunogeniticity for use in xenotransplantation. However, there are at least two obstacles for using nuclear transfer approaches to produce transgenic animals. First, senescence of primary somatic cells in livestock species is generally seen following approximately 30 populations doubling ex vivo in non clonal cultures. Second, the efficiency of nuclear transfer in all species is very low. Oneof the many factors affecting the efficiency of nuclear transfer is the cell cycle phase of donor cells. Wilmut *et al.* (3) using sheep mammary cells, stated that the donor cells for nuclear transfer must be in G0 of the cell cycle (quiescent phase). But Cibelli *et al.* (4) showed that cycling cells, which contain cells in different cycle stages, could be successfully used for nuclear transfer in cattle. In contrast, G2/M stage synchronization donor nuclei might represent an advantage, compared to other cell cycle-stages used in nuclear transfer (20), when considering nuclear-cytoplasmic synchronization of karyoplasts and cytoplasts. This argument was supported by some early studies on nuclear transfer using G2/M stage blastomeres as nuclear do-

nors to producecloned mice (21) and sheep (22) and recent successes of using G2/M stage ES cells as donor to produced cloned mice (23, 24).

Lai *et al.* (25) used a replication defective vector to transduce fetal fibroblasts, followed by treatment of colchicines, which theoretically would synchronize the cells into G2/M cell cycle stage, and subsequently used these cells as donor nuclei to conduct nuclear transfer, producing a transgenic cloned pig expressing the enhanced green fluorescent protein.

Mosaic Gene Expression in Nuclear Transfer-Derived embryos and the Production of Cloned Transgenic Pigs from Ear-derived Fibroblasts

Genetically modified domestic animals have many potential applications ranging from basic research to production agriculture. One of the goals intransgenic animal production schemes is to reliably predict the expression pattern of the foreign gene. Establishing a screening method of genetically modified embryos for transgene expression prior to transfer to surrogates may improve the likelihood of producing offspring with the desired expression pattern. In order to determine how transgene expression might be regulated in the early embryo, porcine embryos from two distinct genetically modified cell lines were generated by using the nuclear transfer (NT) technique. Both cell lines expressed the enhanced Green Fluorescent Protein (eGFP), the first was a fibroblast cell line derived from the skin of a newborn pig that expressed eGFP (26), while the second was a fetal derived fibroblast cell line into which the eGFP gene was introduced by a retroviral vector (13, 27). The reconstructed embryos were activated by electrical pulses and cultured in NCSU23. While the *in vitro* developmental ability of each group of NT embryos was not different, the eGFP expression pattern was different. All embryos produced from the transduced fetal cell line fluoresced, while only 26% of the embryos generated from the newborn cell line fluoresced, and among those that did express eGFP, more than half had a mosaic expression pattern. This was unexpected since the fetal cell line was not clonally selected and each cell potentially had different sites of integration. Embryos generated from the newborn cell line were surgically transferred to 5 surrogate gilts. One gilt delivered 4 female piglets, all of which expressed eGFP and all had microsatellites identical to the donor. Park *et al.* (28) demonstratedthat transgene expression in all the

blastomeres of an NT embryo is not uniform. Additionally, transgene expression in a genetically manipulated embryo may not be an accurate indicator of expression in the resulting offspring.

Production of Transgenic Cloned Pigs Expressing Green Fluorescent Protein in Muscle Tissues

Genetically modified domestic animals will provide considerable benefits not just to agriculture but also to medicine. Somatic cell nuclear transfer (NT) technology has improved the transgenic efficiency. In this study, we evaluated the developmental ability of porcine nuclear transfer embryos using transgenic cells, and examined expression of a transgene in cloned pigs. Porcine fetal fibroblasts were transfected with enhanced green fluorescent protein (GFP) gene to generate two clonal transgenic cell lines (PCX8, PCX25). We transferred nuclei from one of these cell lines to enucleated oocytes. The blastocyst formation percentage was 12.9% and the mean cell number of blastocystswas 23.82.0. Reconstructed embryos were transferredto 18 surrogate sows, and eleven healthy female piglets and one stillbirth were delivered from three surrogate sows. PCR and microsatellite analyses showed that all piglets were identical and transgenic. FISH analysis showed that all piglets carried a chromosome 7 (C7) with GFP gene integration identical to that found in metaphase spreads derived from the donor cell line. The GFP gene was located close to the telomere of C7. The green fluorescencewas expressed in donor cells, embryos, and all piglets. Green fluorescence was also detected mostly in muscle related tissues of piglets (hoof, nose, heart, tongue, skin, ear, skeletalmuscle, and intestine) but could not be detected in most of the internal organs and bones. The present study demonstrated that pigs expressing green fluorescence in muscle tissues can be generated from somatic clonal cells transfected with the GFP gene. This technique would accelerate the utility of transgenic pigs in the pharmaceutical or xenotransplantation fields (29).

Production of (1, 3) galactosyltransferase Knockout Pigs by Nuclear Transfer Cloning

Clinical transplantation has become one of the major treatments

for end stage organ failure since the introduction of chronic immunosuppressive drugs in the mid 1980s. One of the major approaches to dealing with the limited supply of human organs is the utilization of alternative species as a source of organs (xenotransplantation). The pig is considered the primary alternative species due to ethical considerations, breeding characteristics, infectious disease concerns and its compatible size and physiology (30).

A major barrier to progress in pig-to-primate organ transplantation is the presence of terminal a(1,3) galactosyl (gal) 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 produce preformed natural antibodies against the epitope that are responsible for hyperacute rejection of porcine organs. The temporary removal of recipient anti-gal antibodies through affinity adsorption and expression of complement regulators in transgenic pigs has allowed survival of pig organs beyond the hyperacute stage. However, returning antibody and residual complement activity are believed to be responsible for the acute and delayed damage that severely limit organ survival even in the presence of high levels of immunosuppressive drugs and other clinical intervention (31). Competitive inhibition of galtransferase in H-transferase transgenic pigs has resulted in only partial reduction in epitope numbers (32). Similarly, attemptsto block expression of gal epitopes in N-acetylglucosaminyltransferase III transgenic pigs also resulted in partial reduction of gal epitopes number, but failed to significantly extend graft survival in primate recipients (33). Given the large number of gal epitopes present on pig cells, it seems unlikely that any dominant transgenic approach of this nature can provide sufficient protection from anti-gal mediated damage. In contrast, a genetic knockout of the a(1,3) galactosyltransferase (GGTA1) locus in pigs would provide permanent and complete protection.

Viable a(1,3) galactosyltransferase knockout mice have been produced by ES cell technology (34). Development of nuclear transfer technology has provided a means for locus-specific modification of large animals, as demonstrated by the production of viable sheep using *in vitro*targeted somatic cells (35). Successful cloning (10-12) and production of transgenic pigs by nuclear transfer of genetically modified somatic cells (13) have been reported. Attempts at targeting the GGTA1 locus

in sheep (36) have been reported also, but failed to result in live birth of animals with the desired modification. In both cases, difficulties in obtaining viable targeted donor cell clones were encountered.

The presence of galactose a(1,3) galactose residues on the surface of pig cells is a major obstacle to successful xenotransplantation. Lai *et al.* (14) reported the production of four live pigs in which one allele of the a(1,3) galactosyltransferase locus has been knocked out. These pigs, the first with a targeted modification, were produced by nuclear transfer technology based on clonal fetal fibroblast cell lines as nuclear donors for embryos reconstructed with enucleated pig oocytes.

CONCLUSION

After the report of the knockout pigs (14), GGTA1 targeted pigs were produced by other groups (15, 37, 38). GGTA1 null (homozygous knockout) piglets have been then reported (16; Dr. Prather group, 2003; Infigen, 2003). In the next step, researchers will concentrate efforts to address the role of gala(1,3) galspecific natural antibody in HAR and acute vascular rejection to examine the efficiency of xenotransplantation in a nonhuman primate. In addition, many researches will proceed to find other genes that are involved in rejection. This technology will be available to modify those genes so that clinical application of pig organ comes earlier.

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