Production of Cloned Korean Native Pig by Somatic Cell Nuclear Transfer

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ABSTRACT: The Korean native pig (KNP) have been considered as animal models for animal biotechnology research because of their relatively small body size and their presumably highly inbred status due to the closed breeding program. However, little is reported about the use of KNP for animal biotechnology researches. This study was performed to establish the somatic cell nuclear transfer (SCNT) protocol for the production of swine leukocyte antigens (SLA) homotype-defined SCNT KNP. The ear fibroblast cells originated from KNP were cultured and used as donor cell. After thawing, the donor cells were cultured for 1 hour with 15 µM roscovitine prior to the nuclear transfer. The numbers of reconstructed and parthenogenetic embryos transferred were 98 ± 35.2 and 145 ± 11.2, respectively. The pregnancy and delivery rate were 3/5 (60%) and 2/5 (40%). One healthy SLA homotype-defined SCNT KNP was successfully generated. The recipient-based individual cloning efficiency ranged from 0.65 to 1.08%. Taken together, it can be postulated that the methodological establishment of the production of SLA homotype-defined cloned KNP can be applied to the generation of transgenic cloned KNP as model animals for human disease and xenotransplantation researches.

Key words: Korean native pig, Swine leukocyte antigens, Somatic cell nuclear transfer, Animal model, Cloning efficiency

INTRODUCTION

Somatic cell nuclear transfer (SCNT) can potentially be used to produce various species such as sheep (Wilmut et al., 1997), cattle (Kato et al., 1998), goat (Baguish et al., 1999), and pig (Bethauser et al., 2000; Onishi et al., 2000; Polejaeva et al., 2000) or unlimited animal clones that naturally exhibit high-performance or other desirable traits. Because of their similar anatomy and physiology, pig have become a progressively more important large animal model for organ transplantation research relating to, for example, immunological rejection, effectiveness of immunosuppressive drugs on tolerance, and development of induction protocols for long-term tolerance (Dehoux & Gianello, 2007; Zhao et al., 2009; Ahn et al., 2011; Hanekamp et al., 2011).

Especially, miniature pig also shares many physiological similarities with humans and offers several breeding, such as Yucatan, Hanford, Sinclair, and Massachusetts General Hospital (MGH), and handling advantages, making it an optimal species for preclinical and biomedical experimentation (Yeom et al., 2012; Kim et al., 2013). The Korean native pig (KNP) have been considered as animal models for...
transplantation and xenotransplantation research because of their relatively small body size (less than 70 kg at maturity) and their presumably highly inbred status due to the closed breeding program since 1986 (Kim et al., 2002).

Swine leukocyte antigens (SLAs) are the major histocompatibility complex (MHC) molecules of swine, consist of a group of genes designated as MHC systems. SLAs have been repeatedly implicated in swine disease resistance and vaccine response and play very important roles in the adaptive immune system (Chardon et al., 2000; Kelley et al., 2005). In using pigs as large animal models for human transplantation and as potential xenograft donors, the SLA antigens are important targets for allo- and xeno-immunological recognition and can be directly recognized by various human immune cell subsets (Shishido et al., 1997; Kwiatkowski et al., 1999).

In KNP, the three functional SLA class I loci, SLA-1, SLA-2 and SLA-3, have been reported (Woo et al., 2005, 2006; Kim et al., 2006). After that, two important SLA class II genes, DQB1 and DRB1, were additionally identified in KNP (Woo et al., 2007). Based on the analysis systems, we have established homotype-defined 5 SLAs (DQB1, DRB1, SLA-1, SLA-2, and SLA-3) KNP herd (Cho et al., 2010). This study was performed to establish the SCNT protocol for the production of swine leukocyte antigens (SLA) homotype-defined SCNT KNP.

**MATERIALS & METHODS**

1. General information

Our study protocol and standard operating procedures for the treatments of the pigs used were reviewed and approved by the Institutional Animal Care and Use Committee of the National Institute of Animal Science, RDA (Approval number: NIAS2015-670, D-grade).

2. Ear fibroblast cell culture

Ear tissue originated from homotype-defined SLA (DQB1, DRB1, SLA1, SLA2, and SLA3) Korean native pig (KNP, Subtropical Livestock Research Institute, NIAS, Jeju) (unpublished data). Ear fibroblasts obtained from the KNP were cultured in Dulbecco’s modified Eagle medium (WelGene Inc., Korea) containing 20% FBS, 1 mM of non-essential amino acid (GIBCO), 1 mM of Sodium Pyruvate (WelGene, Korea), 55 μM of β-mercaptoethanol (GIBCO), and 1% antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin; GIBCO) at 37°C in an atmosphere of 5% CO₂ in air (Fig. 1). When the cells were confluent in the culture dish, the cells were detached and aliquoted (5 × 10⁷/mL/tube) for nuclear transfer.

3. *In vitro* maturation

*In vitro* maturation of porcine immature follicular oocytes was performed as follows with slightly modifications (Kim et al., 2005). Porcine ovaries were obtained from a local slaughterhouse (Nonghyup Moguchon, Gimje, South Korea) and transported to the laboratory at 30–35°C. Cumulus-oocyte complexes (COCs) were collected and washed in Tyrode's lactate-Hepes containing 0.1% (w/v) polyvinyl...
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alcohol (PVA). Oocytes with several layers of cumulus cells were selected and washed three times in TCM-199 (GIBCO) supplemented with 0.1% PVA (w/v), 3.05 mM D-glucose, 0.91 mM sodium pyruvate, 0.57 mM cysteine, 0.5 µg/mL luteinizing hormone, 0.5 µg/mL follicle stimulating hormone, 10 ng/mL epidermal growth factor, 10% porcine follicular fluid (pFF), 75 µg/mL penicillin G, and 50 µg/mL streptomycin (maturation medium). For in vitro maturation, 50 COCs were transferred into 500 µL of maturation medium in a four-well dish (Nunc, Roskilde, Denmark). The oocytes were matured for 40 h at 38.5°C under 5% CO₂ in air.

4. Nuclear transfer procedure

Somatic cell nuclear transfer was performed as follows (Hwang et al., 2013). Briefly, matured oocytes were enucleated by aspirating the first polar body and metaphase II chromosomes and a small amount of surrounding cytoplasm in manipulation medium supplemented with 5 µg/mL cytochalasin B. The freshly-thawed donor cells treated with Roscovitine were inserted into perivitelline space. The karyoplast-cytoplast complexes were placed into 0.2-mm diameter wire electrodes (1 mm apart) of a fusion chamber covered with 0.3 M mannitol solution containing 0.1 mM MgSO₄, 1.0 mM CaCl₂ and 0.5 mM Hepes. For reconstructions, two DC pulses (1-sec interval) of 1.5 kV/cm were applied for 30 µs using an Electro-Cell fusion (Fujihira Industry, Japan).

5. Embryo transfer and parturition

Immediately after confirmation of fusion using a stereoscope, the reconstructed embryos were transferred into both oviducts of the surrogate (Landrace) on the same day or 1 day after the onset of estrus. Pregnancy was diagnosed on day 28 after ET and then was checked regularly every week by ultrasound examination. The TG cloned piglets were delivered by natural parturition or Caesarean section.

RESULTS

A summary of the pregnancy and delivery rates of KNP is shown in Table 1. The mean number of the transferred embryos was 243±25.4 per surrogate. Among them, the number of the embryos transferred was 98±35.2 in cloned embryos and 145±11.2 in parthenogenetic embryos, respectively. Among 5 surrogates, three were pregnant (3/5) and two were ongoing to full-term (2/5).

Table 2 shows the individual productivity of homotype-defined SLA cloned KNP. The total number of delivered cloned pigs was 3 (1 alive, 1 mummy, and 1 died after birth) (Fig. 2). The recipient-based individual cloning efficiency was ranged from 0.65% to 1.08%.

DISCUSSION

Cloned embryos have shown high embryonic mortality than normal embryos because of complex procedure of somatic cell nuclear transfer (Lai et al., 2002). Such a high embryonic mortality is mainly due to the asynchronous development of embryos in the maternal uterus and inappropriate interactions between the maternal uterus and developing embryos during implantation and placentation (Pope, 1994). In pig, the blastocysts undergo dramatic morphological changes, developing from 11 to 50 mm

| Table 1. Pregnancy and delivery rate |
|-------------------------------------|
| No. of embryos transferred | NT | Parthenotes | Pregnancy* | Delivery** |
| No. of surrogates             |    |             |            |            |
| 97.6±35.2                     | 145±11.2 | 3/5          | 2/5        |

* Pregnancy was confirmed by gestation sac formation using ultrasound examination at 28 days after embryo transfer. Data were expressed as mean±SD.
** No. of delivered / No. of pregnancy (including the piglets died within 1 day)
The five functional SLAs (DQB1, DRB1, SLA-1, SLA-2, and SLA-3) homotype-defined cloned KNP was delivered by Caesarean section (C-sec) at 117 days after embryo transfer.

These critical changes coincide with the synthesis and release of fetal-maternal recognition signals (estrogen) and cytokines required for the establishment of pregnancy (Bowen & Burghardt, 2000; Spencer & Bazer, 2004; Bazer et al., 2009). To improve the cloning efficiency, we transferred the reconstructed embryos into the oviduct of the surrogates with parthenogenetic embryos simultaneously. Co-transfer of cloned embryos with parthenogenetic embryos with may be one of the methods to enhance the signal for maternal recognition, because fetal development and implantation of parthenotes can be happened like normal fetuses until gestation days 31 and then die shortly afterwards in pigs (Zhu et al., 2000, 2003; Han et al., 2013). Normal fetal development of parthenogenetic fetus originated from parthenogenetic embryos may affect positively to the uterine environment of surrogates and help to implantation of cloned fetuses during early pregnancy period. Like the previous results, we had transferred parthenogenetic embryos and retrieved the normal morphology of parthenogenetic conspectuses at GD 26 (unpublished data).

Because of rare report, it is difficult to compare the cloning efficiency of cloned KNP. The recipient-based cloning efficiency (No. of newborn piglets/No. of transferred cloned embryos) was 0.2–2.54% in MGH minipigs (Lai et al., 2002; Zhao et al., 2009; Hwang et al., 2013). In the present study, the cloning efficiency was ranged from 0.65 to 1.08% in KNP. It can be postulated that the recipient-based cloning efficiency was similar to other minipig species.

Taken together, it can be postulated that the methodological establishment of the production of SLA homotype-defined cloned KNP can be applied to the generation of transgenic cloned KNP as model animals for human disease and xenotransplantation.

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