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# Nuclear Transfer using Human CD59 and IL-18BP Double Transgenic Fetal Fibroblasts in Miniature Pigs

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## ABSTRACT

Xenotransplantation involves multiple steps of immune rejection. The present study was designed to produce nuclear transfer embryos, prior to the production of transgenic pigs, using fibroblasts carrying transgenes human complement regulatory protein *hCD59* and interleukin-18 binding protein (*hIL-18BP*) to reduce hyperacute rejection (HAR) and cellular rejection in pig-to-human xenotransplantation. In addition to the hCD59-mediated reduction of HAR, hIL-18BP may prevent cellular rejection by inhibiting the activation of natural killer cells, activated T-cell proliferation, and induction of IFN-ɣ. Transgene construct including *hCD59* and *ILI-18BP* was introduced into miniature pig fetal fibroblasts. After antibiotic selection of double transgenic fibroblasts, integration of the transgene was screened by PCR, and the transgene expression was confirmed by RT-PCR. Treatment of human serum did not affect the survival of double-transgenic fibroblasts, whereas the treatment significantly reduced the survival of non-transgenic fibroblasts ($p<0.01$), suggesting alleviation of HAR. Among 337 reconstituted oocytes produced by nuclear transfer using the double transgenic fibroblasts, 28 (15.3%) developed to the blastocyst stage. Analysis of individual embryos indicated that 53.6% (15/28) of embryos contained the transgene. The result of the present study demonstrates the resistance of hCD59 and IL-18BP double-transgenic fibroblasts against HAR, and the usefulness of the transgenic approach may be predicted by RT-PCR and cytolytic assessment prior to actual production of transgenic pigs. Further study on the transfer of these embryos to surrogates may produce transgenic clone miniature pigs expressing hCD59 and hIL-18BP for xenotransplantation.

(Key words: hCD59, hIL-18BP, nuclear transfer, miniature pig, xenotransplantation)

## INTRODUCTION

Xenotransplantation may ease the shortage of human donor organs, and attention has been focused on the pig as an organ donor. Pigs are available in large number, and their organs are anatomically and physiologically similar to those of humans (Yang and Sykes, 2007). However, various types of immunological rejection occur when pig organs are grafted to humans. Hyperacute rejection (HAR) leads to serious damage to the grafted organs and to reject them within minutes following reperfusion (Mollnes and Fiane, 2003).

The feature of hyperacute rejection (HAR) is an activation of complement on recipient endothelial surfaces of graft blood vessels, leading to devastation of the graft within a few minute. In xenotransplantation, the complement cascade activated by xenoreactive natural antibodies (XNA) causes rapid and irreversible destruction of the transplanted organs (Bach, 1998).

Complement regulatory proteins (CRP), such as CD55, CD46, and CD59, downregulate the complement activation cascade to protect cells from damage (Liszewski *et al.*, 1996). Hence, transgenic pigs expressing various CRP could prevent the HAR. The transplantation of organs from these transgenic pigs has resulted in the inhibition of the complement activation cascade and reduction of the HAR (McCurry *et al.* 1995; Chen *et al.*, 1999). In particular, CD59 inhibits the complement activation cascade-mediated membrane attack complex at the final stage of complement activation. Human CD59 (hCD59) has shown to prevent the destruction of transplanted organs (Rollins *et al.*, 1991). Prolonged survival of a grafted heart from an hCD59-transgenic pig into a baboon has been reported (Fordor

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*et al.*, 1994).

If HAR was inhibited, then cellular rejection of xenograft should be resolved. Cellular rejection is characterized by interstitial infiltrattion of T cells, macrophages, and natural killer (NK) cells (Satyananda *et al.*, 2013). Various cytokines, such as IL-1, IL-2, IL-4, IL-10, IL-12p40, IFN-ɣ, and TNF-ɣ are important in cellular rejection of xenografts. Interleukin-18 (IL-18) might also be involved in such rejection during xenotransplantation because IL-18 has been known to induce IFN-ɣ production by T helper 1 (Th1) cells. Moreover, IL-18 has a variety of biological functions in immune response, including the stimulation and proliferation of T cells, the enhancement of the NK cell activation, the induction of granulocyte macrophage colony-stimulating factor by activated T-cells, and the stimulation of FasL-mediated cytotoxicity by NK and T cells (Muneta *et al.*, 2002). Interestingly, IL-18 and IL-12 have a synergistic effect on the stimulation of IFN-ɣ production by T cells (Nakanishi *et al.*, 2001). Interleukin-18 binding protein (IL-18BP) that specifically binds to IL-18 reduces various biological functions of IL-18. Hence, IL-18BP may be beneficial to reduce cellular rejection in xenotransplantation.

The present study was conducted to investigate reduction of HAR by overexpression of hCD56 and hIL-18BP in porcine fibroblasts, and to produce double transgenic nuclear transfer (NT) embryos to overcome both HAR and cellular rejection in xenotransplantation. The results of this study may contribute to the producing transgenic pigs for xenotransplantation by providing double transgenic NT embryos in an effort of overcoming xenograft rejections.

## MATERIALS AND METHODS

### 1. Animal Ethics

All procedures in this study were carried out in accordance with the Code of Practice for the Care and Use of Animals for Scientific Purposes and approved by the Institutional Animal Care and Use Committee, Dankook University.

### 2. Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise stated.

### 3. Culture of Miniature Pig Fibroblasts

Miniature pig fibroblasts were prepared as previously described in Ahn *et al.* (2011). Briefly, pig fetuses at 28～39 days of gestation were obtained from Minnesota miniature pigs maintained in specific pathogen-free (SPF) conditions at Seoul National University. The head, dorsal spine of the medial section, and tail were removed before collection of embryonic fibroblasts. Small pieces of remaining tissues were washed in Dulbecco's PBS (DPBS; Invitrogen, Carlsbad, CA, USA) and minced with a surgical blade in a 100-mm petridish. Cells were then dissociated from the tissues in 0.25% Trypsin-EDTA (Invitrogen) for 10 min at 39℃. After centrifuging the cell suspension three times at 800 × g for 5 min, pellets were re-suspended and seeded on 100-mm culture dishes and cultured for 6 to 8 days in Dulbecco's modified Eagle medium (DMEM; Invitrogen) supplemented with 10% (v/v) fetal bovine serum (FBS; Hyclone, Logan, UT, USA), 1 mM L-glutamine, 100 units/ml penicillin, and 0.5 mg/ml streptomycin in a humidified atmosphere of 5% $CO_2$ in 95% air. After removal of unattached clumps of cells by washing culture plates with DMEM, attached cells were further cultured until confluent and subcultured at intervals of 5 to 7 days by trypsinization until used for transfection.

### 4. Transgene Constructs

The coding region of *hCD59* and *hIL-18BP* was obtained by reverse transcription-polymerase chain reaction (RT-PCR) from total mRNA of human fibroblasts, as described previously (Lee *et al.*, 2006). Human CD59 cDNA was inserted into the pSELECT-puro/mcs vector (InvivoGen, San Diego, CA, USA) containing the EF1α/HTLV promoter and a selectable puromycin cassette. A 3.8-kb transgene construct containing the genes encoding hCD59 and $Puro^r$ under the regulation of separate promoters is represented in Fig. 1. pCAGGS/Neo-hIL-18BP vector was a kind gift of Dr. Soohyun Kim (Konkuk University, Seoul, Korea). The subunit A of human IL-18BP cDNA was inserted into the pCAGGS/Neo vector containing the chicken beta-actin promoter and a selectable neomycin cassette. A 6.7-kb transgene construct containing the genes encoding IL18-BP and $Neo^r$ under the regulation of separate promoters is shown in Fig. 1.

### 5. Transfection of Miniature Pig Fetal Fibroblasts

Miniature pig fetal fibroblasts were transfected with the transgene constructs (1.5 μg of the linearized transgene construct for transfection of $5 \times 10^5$ cells) using Amaxa Nucleofection

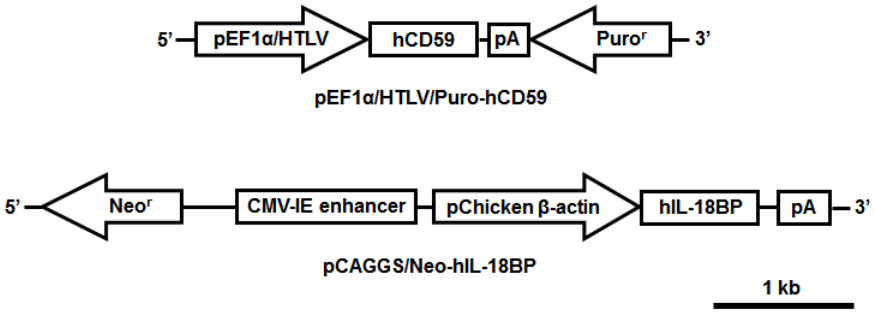


Fig. 1. A diagram of the hCD59 and hIL-18BP transgene construct.

System (Lonza, Basel, Switzerland) with the program #U-023 and Primary Fibroblast Kit (Lonza) according to the manufacturer's protocol. Starting from 24 h after the transfection, transfected cells were selected with medium containing 10 μg/ml either G418 (Invitrogen) for 14 days or 3 μg/ml puromycin for 7 days depending on the constructs. Antibiotic-resistant colonies were isolated and continuously cultured at 39℃ in a humidified atmosphere of 5% $CO_2$ and 95% air.

### 6. Detection of Transgene

Transgenesis was confirmed by the amplification of *hCD59* and hIL-18BP gene from genomic DNA samples of transfected fibroblasts and blastocyst stage NT embryos. Genomic DNA from antibiotic-resistant fibroblasts was prepared using the DNeasy Tissue Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. For NT embryos, blastocysts were washed twice in phosphate buffered saline (PBS), and individual blastocysts were separately transferred into 0.2-ml PCR tubes, each containing 5 μl of embryo lysis solution (pH 8.6) composed of 20 mM Tris-Cl (pH 8.5), 0.9% Tween 20, 0.9% Nonidet P-40, and 0.4 mg/ml protease K. Prior to PCR, embryos were incubated at 55℃ for 30 min, and then the protease was inactivated by a 15-min incubation period at 94 ℃. For all samples, PCR was performed using AccuPower PCR Premix (Bioneer, Daejeon, Korea). Thirty-five cycles of PCR amplification were performed as follows: denaturation at 94℃ for 1 min, annealing at 54℃ for 1 min, and extension at 72℃ for 1 min. Sequences of the upstream and downstream primer pairs and lengths of PCR products were as follows: *hCD59* (5'-CCAAGGAGGGT-CTGTCCTGT-3' and 5`-AATTGACGGCTGTTTTGCAG-3`, 122 bp) and *hIL-18BP* (5`-CTG- CCACTGCCTCAGTTAGA-3` and 5`-TCCACGAGCACACAGGAGA-3`, 346 bp).

### 7. Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA was prepared from transfected miniature pig fibroblasts using the Easy-spin Total RNA Extraction Kit (Intron Biotechnology, Seongnam, Korea), and aliquots of 5 μg of total RNA were used for cDNA synthesis using the SuperScript III First-Strand Synthesis System (Invitrogen) according to the manufacturer's protocol. Synthesized cDNA was amplified using AccuPower PCR Premix. Thirty-five cycles of PCR amplification were performed as stated above. For endogenous housekeeping standard, GAPDH was amplified using primer sets (5`-TCAT-CATCTCTGCCCCTTCT-3` and 5`-GTCATGAGTCCCTCAC-GAT-3`) that give rise to 172-bp PCR product.

### 8. Human Serum-Mediated Cytolytic Assay

The viability of trangenic and non-transgenic fibroblasts after human serum treatment was measured by Premix WST-1 Cell Proliferation Assay System (Clonetech, Mountain View, CA, USA) using the manufacturer's protocol. Briefly, fibroblasts were plated at $10^4$ cells per well on gelatin-coated 96-well plates 24 h prior to the performance of the assay. Cells were then washed with serum-free DMEM and exposed to increasing concentrations of AB-type normal human serum for 4 h at 39 ℃. Cell viability was measured based on the cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells, relative to the no serum added condition, using colorimetric detection of optical absorbance at 450 nm with SpectraMax multi-well spectrophotometer (Molecular Devices, Sunnyvale, CA, USA).

### 9. Somatic Cell Nuclear Transfer

The *in vitro* maturation (IVM) of oocytes and nuclear transfer were performed as described previously (Ahn *et al.*, 2011). Briefly, 42 h after the onset of IVM, oocytes were enucleated with a 20-μm (internal diameter) glass pipette by aspiration of the first polar body and the second metaphase plate with a

small volume of surrounding cytoplasm in HEPES-buffered TCM-199 supplemented with 0.3% bovine serum albumin (BSA) and 5 μg/ml cytochalasin B. After the enucleation, oocytes were stained with 5 μg/ml bisbenzimide (Hoechst 33342) for 5 min and observed under a Nikon TE-300 inverted microscope equipped with epifluorescence. Oocytes containing DNA materials were excluded from the subsequent experiments. Fibroblasts were trypsinized into single cells and transferred into the perivitelline space of enucleated oocytes. The resulting couplets were equilibrated for 1 min in 0.3 M mannitol solution containing 0.5 mM HEPES, 0.05 mM $CaCl_2$, and 0.1 mM $MgCl_2$ in a chamber containing two electrodes. Using a BTX Electro-Cell Manipulator 2001 (Harvard Apparatus, Holliston, MA, USA), couplets were fused with a double DC pulse of 1.5 KV/cm for 45 μs. Following the electrical stimulation, reconstructed oocytes were cultured in porcine zygote medium-3 (PZM-3) supplemented with 3 mg/ml fatty acid-free BSA and 5 μg/ml cytochalasin B for 3 h in order to suppress extrusion of the second polar body. Oocytes were then cultured for 4 days in PZM-3 containing 4 mg/ml fatty acid-free BSA and transferred to PZM-3 containing 10% FBS and cultured for another 3 days. All NT embryos were cultured at 39℃ in a humidified atmosphere with 5% $CO_2$ and 95% air.

### 10. Statistical Analysis

Statistical analyses were carried out using SPSS version 11.0 for Windows. At least three replicates were conducted for each experiment, and data were presented as means±SEM. All data were analyzed by one-way ANOVA. A value of $p<0.05$ was considered significant.

## RESULTS

### 1. Transgenesis of Miniature Pig Fetal Fibroblasts

The vectors containing *hCD59* and *hIL-18BP* were introduced into miniature pig fetal fibroblasts. Surviving colonies of fibroblasts after antibiotic selection were analyzed by PCR. As shown in Fig. 2, four fibroblast clones (no. 1~4) with antibiotic resistance were hCD59 and hIL-18BP double transgenic. In RT-PCR analysis, one of the PCR-positive clones chosen for subculture expressed both hCD59 and hIL-18BP (Fig. 3).

### 2. Human Serum-Mediated Cytolytic Assay

To test the resistance of hCD59- and hIL-18BP-transgenic

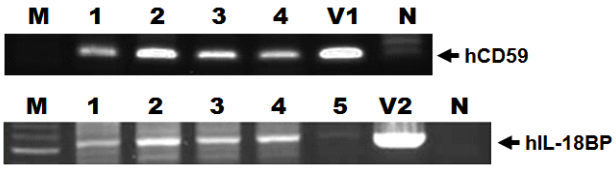


Fig. 2. PCR screening of antibiotic-resistant colonies obtained from porcine fibroblasts transfected with vectors containing hCD59 and hIL-18BP. M: size marker, 1-5: transfected fibroblast clones (the same numbers represent identical clones), V1: vector containing hCD59, V2: vector containing hIL-18BP, N: non-transfected fibroblasts.

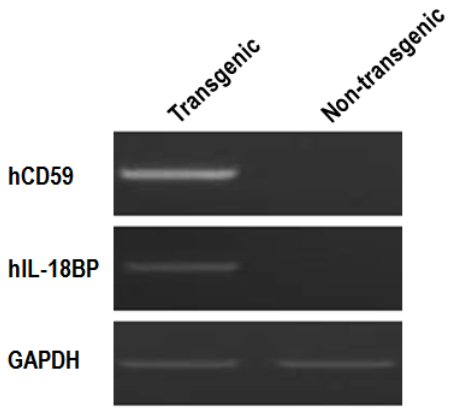


Fig. 3. RT-PCR analysis for hCD59 and hIL-18 expression in transgenic and non-transgenic fibroblasts.

cells against HAR mediated by human XNA and complement, transgenic and non-transgenic fibroblasts were treated with human serum. Cell viability, as assessed by the WST-1 assay, is shown in Fig. 4. Double transgenic fibroblasts carrying the transgenes hCD59 and hIL-18BP had much higher mitochondrial activity than their non-transgenic counterparts after treatment with human serum, indicating the protection from complement-mediated cytolysis by expression of hCD59 in transgenic cells.

### 3. *In Vitro* Development and Transgene Detection in NT Embryos

Double transgenic miniature pig fibroblasts, capable of resistance against the HAR (Fig. 4) and presumably cellular rejection in xenotransplantation, were used as nuclear donors for subsequent transfer of the nucleus into enucleated oocytes. Among 337 reconstituted oocytes, 28 (15.3%) developed to the blastocyst stage as represented in Table 1. Analysis of individual NT embryos by PCR indicated that 53.6% (15/28) of the embryos

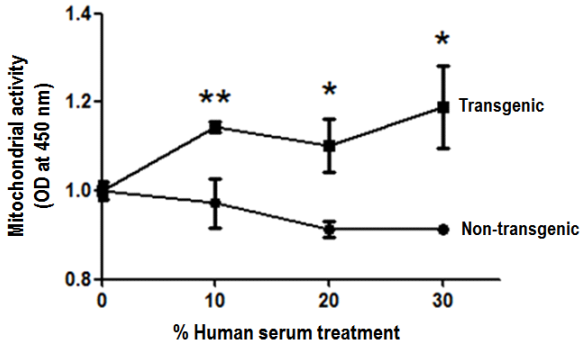


Fig. 4. Human serum-mediated cytolytic assay. Mitochondrial activity of hCD59- and hIL-18BP-double transgenic and non-transgenic miniature pig fibroblasts represented by optical absorbance after treatment with normal human serum. * $P$<0.05, ** $P$<0.01.

Table 1. *In vitro* development of nuclear transfer embryos

| No. of reconstructed oocytes | No. (%)* of fused oocytes | No. (%)** of embryos developed to blastocysts | No. (%)*** of transgenic embryos |
|---|---|---|---|
| 337 | 183(54.3) | 28(15.3) | 15(53.6) |

* Calculated from the number of reconstructed oocytes.
** Calculated from the number of fused oocytes.
*** Calculated from the number of blastocysts.

contained both transgenes *hCD59* and *hIL-18BP*. Part of the PCR analysis to identify double transgenic embryos was shown in Fig. 5.

## DISCUSSION

Transgenic animals can be produced by somatic cell NT. Selection of transgenic nuclear donor cells prior to NT may raise the efficiency in the production of transgenic animals (Niemann, 2001; Macháty *et al.*, 2002).

In the present study, transgene constructs separately including the human complement regulatory protein *hCD59* and hIL-18BP gene as represented in Fig. 1 was introduced into fetal fibroblasts. After antibiotic selection of transfected cells, the transgenes were detected from the fetal fibroblasts as verified by PCR (Fig. 2). The expression of hCD59 and hIL-18BP driven by EF1α/HTLV and CAGGS promoter, respectively, was analyzed by RT-PCR. As shown in Fig. 3, both hCD59 and hIL-18BP were expressed in transfected cells, whereas no expression was detected from non-transfected cells. Use of these cells for subsequent NT and production of cloned miniature pigs would ensure high expression of transgenes, presumably resulting in efficient reduction of HAR and cellular rejection in xenotransplantation.

Production of transgenic pigs is laborious, time-consuming, and costly. Moreover, whether produced transgenic pigs are clinically applicable is unpredictable. Hence, the *in vitro* assessment of HAR to predict what extent the transgenic approach is effective would be important. The effect of transgene expression on the reduction of HAR can be assessed indirectly by the treatment of cells with human serum containing XNA and complement. The inhibition of human serum-mediated cytolysis in porcine embryonic fibroblasts expressing various CRP has been reported (Lee *et al.*, 2006; Won *et al.*, 2009). In the present study, the resistance of hCD59 and hIL-18BP double transgenic cells against HAR was tested by complement-mediated cytotoxicity using WST-1 assay. After treatment with human serum containing XNA and complement, the mitochondrial activity of transgenic cells was significantly higher ($p$<0.01 in 10% and $p$<0.05 in 20～30% human serum) compared with the non-transgenic control (Fig. 4). The mitochondrial activity of transgenic cells after treatment with human serum reflects the survival of these cells under the conditions of HAR. These

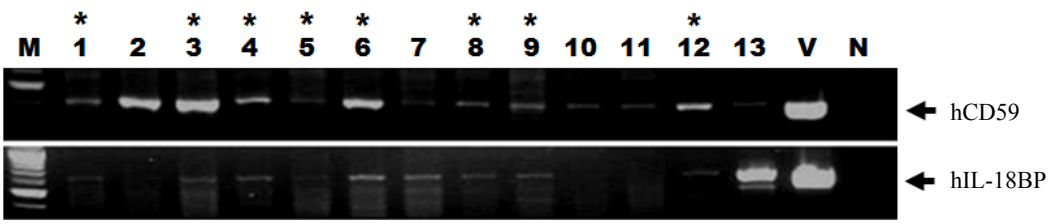


Fig. 5. PCR screening of transgene hCD59 and hIL-18BP in nuclear transfer embryos. M: size marker, 1～13: nuclear transfer blastocysts from transfected fibroblast cells, V: vector containing hCD59 or hIL-18BP, N: nuclear transfer blastocysts from non-transfected fibroblast cells. The asterisk (*) indicates hCD59 and hIL-18BP double transgenic blastocysts.

transgenic cells, presumably capable of overcoming the HAR, were used as nuclear donors to produce transgenic NT embryos.

In PCR analyses of individual cloned embryos for transgenes as shown in Table 1 and in part in Fig. 6, 53.6% (15/28) of embryos were identified as double transgenesic. This might be due to insufficient selection pressure of antibiotics resulting in heterogeneous population of transgenic and non-tranasgenic fibroblasts.

Successful acceptance of xenografts occurs after overcoming a series of obstacles, including hyperacute, acute vascular, cellular, and chronic rejection (Yang and Sykes, 2007). Hence, production of pigs with multiple transgenes may be necessary to eliminate such hurdles (Piedrahita and Mir, 2004). In the present study, the fibroblasts overexpressing transgenes hCD59 and hIL-18BP were produced. The cytolytic analysis demonstrated a reduction of the HAR in hCD59 and hIL-18BP double transgenic cells, and the subsequent transfer of these cells into enucleated oocytes successfully produced double transgenic clone embryos. This would benefit further studies to produce double transgenic clone pigs with assured expression of transgenes for xenotransplantation by transferring NT embryos to surrogates.

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