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Production of Wuzhishan Miniature Pigs Expressing High-Level Human CD46

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Abstract: The development of *α-1,3-galactosyltransferase* gene knockout pigs along with overexpression of human Complement Regulatory Proteins (hCRPs) has been an important step in overcoming hyperacute rejection when pig organs are transplanted into nonhuman primates. Most previous studies have used commercial pigs as organ donors. However, little has been reported about the use of inbred miniature pigs as genetic donors expressing hCRPs. As expression of hCRP on the surface of pig cells, especially human membrane cofactor protein (hCD46) could effectively protect them against human complement-mediated rejection, researchers constructed an engineered hCD46 minigene expression vector and generated transgenic Wuzhishan miniature Pigs (WZSP) expressing hCD46. Expression of hCD46 with a distinct tissue-specific pattern similar to the human one was observed using immunohistochemistry. Various tissues revealed strong immunostaining in most epithelial tissues and vascular endothelium. Flow cytometry analysis of hCD46 porcine AECs showed expression levels similar to human AECs. Complement-mediated cytolysis of transgenic porcine Aortic Endothelial Cells (AECs) with human serum showed significant protection. Distinct differences between ABO blood groups were also observed with much less cytolysis of porcine AECs treated with B blood group serum than with other serum types. In conclusion, transgenic WZSPs with sufficient expression of hCD46 have been established successfully and will facilitate further xenotransplantation research.

Key words: hCD46, Wuzhishan miniature pigs, xenotransplantation, aortic endothelial cells, complement-mediated cytolysis, ABO blood groups

INTRODUCTION

Xenotransplantation is now not substantially limited by Hyperacute Rejection (HAR) which has been reduced by knockout of the heterogenic antigen α-1,3-Galactosyltransferase (αGal) (Phelps *et al*., 2003; Fujimura *et al*., 2008; Ko *et al*., 2013) and also when donor organs overexpress human Complement Regulatory Proteins (hCRPs) such as CD46, CD55 or CD59 (Diamond *et al*., 1996; Chen *et al*., 1999). Among hCRPs, the human membrane cofactor protein hCD46 plays an effective role in inhibiting complement-mediated rejection by both the classical and alternative pathways (Loveland *et al*., 1993; Christiansen *et al*., 1996). The entire genomic CD46 region encodes several isoforms on nucleated human cell surfaces (Liszewski and Atkinson, 1992) of which the BC1 isoform plays the most important role as a regulator of complement activation (Purcell *et al*., 1991; Loveland *et al*., 1993). It binds to C3b and C4b to allow their cleavage by the serine protease Factor I there by protecting host membrane cells from complement attack (Johnstone *et al*., 1993; McKenzie *et al*., 2003; Loveland *et al*., 2004; Geis *et al*., 2010).

Several research groups have made efforts to produce transgenic animals expressing high levels of human CD46 (hCD46) for xenotransplantation studies (Diamond *et al*., 2001; Loveland *et al*., 2004; Hara *et al*., 2008). Earlier studies had difficulty achieving high-level, widespread expression by cDNA driven by heterologous promoters (Mora *et al*., 1996; Mulder *et al*., 1997). High expression was observed using a large genomic construct that encompasses the 60 kb human *CD46* gene as various isoforms produced via alternative splicing might be too fragile for transferring *in vitro* (Diamond *et al*., 2001). The hCD46 minigene encoding the BC1 isoform was used to generate hCD46-expressing transgenic mice by pronuclear injection with a level ~75% of that observed

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in human cells (Thorley *et al*., 1997). High expression of hCD46 in transgenic pigs was achieved with another engineered CD46 minigene including an optimized promoter (Loveland *et al*., 2004). However, little has been published regarding the use of inbred miniature pigs as genetic donors expressing.

The Wuzhishan miniature Pig (WZSP) is a native miniature pig breed that has been inbred continuously on Hainan Island off the South of China for thousands of years. As a consequence, pigs of this breed have attained highly stable genetic backgrounds and integrity (Yao *et al*., 2006) with an adult body weight of ~35 kg. Moreover, the natural lack of the Porcine Endogenous Retrovirus (PERV) C isoform in the WZSP breed, together with the small amount of replication-competent PERV in its genome (Fang *et al*., 2012) is favorable for safe applications in pig to human xenotransplantation.

Here, researchers reconstructed the engineered hCD46 minigene and tried to generate transgenic WZSPs with a high level of hCD46 expression. Flow cytometry analysis of the acquired hCD46 transgenic porcine AECs showed expression levels similar to human AECs. Researchers observed that the hCD46 transgene pigs showed an expression pattern similar to endogenous human CD46. An *in vitro* study showed a protective effect of hCD46 on porcine Aortic Endothelial Cells (AECs) against human complement-mediated cytolysis.

MATERIALS AND METHODS

Animals and materials: All research involving animals was conducted according to the Regulations for the Administration of Affairs Concerning Experimental Animals (Ministry of Science and Technology, China, revised in June 2004) and approved by the Institutional Animal Care and Use Committee in the College of Animal Science and Technology, Sichuan Agricultural University, Sichuan, China under permit No. DKY-S20112728.

Four commercial breed pigs (large white) were used as surrogates in this experiment. Established WZSP ear fibroblasts were used as donor cells to generate transgenic cloned pigs. Porcine AECs were generated as described (Pan *et al*., 2010). Human AECs were purchased from ScienCell (San Diego, CA, USA) and cultured in medium with endothelial cell growth supplementadded. Serum samples were obtained from 15 healthy volunteers and each ABO blood type used was a mixture from at least three donors. A human CD46-expressing Bacterial Artificial Chromosome (BAC; PR11-454L1) was purchased from the Children's Hospital Oakland Research Institute, (CA, USA). Reagents used for construction of the vector were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Construction of the hCD46 minigene vector: Researchers reconstructed an hCD46 minigene vector of 9.5 kb encoding the BC1 isoform (Fig. 1a) very similar to that used previously (Loveland *et al*., 2004). Briefly by using the Gap-Repair Method mediated by a red homologous recombination system in *Escherichia coli*, 6.2 kb homologous sequences from a human CD46 BAC were cloned and inserted into the pBR322 vector. Homologous sequences including the promoter sequence (636 bp upstream of the major transcriptional initiation site) through to intron 2 (Cui *et al*., 1993) were inserted into the pBluescript vector containing exons 3-14 as a cDNA fragment (exon 7 excluded) with a shortened SV40 polyA sequence. The expression construct was ~9.5 kb in length.

Transfection and selection of hCD46 colonies for SCNT: Porcine primary ear fibroblasts established from 1 year old male inbred WZSPs were used for transfection. A total of 2×10^6 cells were trypsinized (EDTA-Trypsin; Sigma-Aldrich, St. Louis, MO, USA) washed twice with Phosphate-Buffered Saline (PBS; Gibco BRL, Gaithersburg, MD, USA); finally, aliquots of 1×10^6 cells were suspended in 100 μL electroporation buffer (LonzaInc., Walkersville, MD, USA) containing 6 μg DNA of linearized plasmid. Cells were electroporated using an AMAXA Nucleofector (Lonza Inc.) with program T-016. Then, cells were plated on two 25 cm^2 dishes and cultured for 2 days. After that cells were transferred to twenty 100 cm^2 dishes for hCD46 cell selection with puromycin (2.5 μg mL^{-1}) for 15 days until cell colonies had grown. Surviving colonies were stained with mouse monoclonal anti-CD46 antibody conjugated with fluorescein isothiocyanate (FITC; Sigma-Aldrich, SAB4700431) (Fig. 1b) for flow cytometry analysis of Mean Fluorescence Intensity (MFI). Colonies staining strongly were used for Somatic Cell Nuclear Transfer (SCNT). SCNT was performed with micromanipulation equipment (NIKON TE2000-U, Japan) as described by Pan *et al*. (2010).

Production of transgenic pigs expressing hCD46: Tissues from live-born piglets were screened for the minigene by Polymerase Chain Reaction (PCR) using human CD46-specific primers. Reverse Transcription (RT)-PCR of transgenic piglet ear tip tissue was also performed for the analysis of primary mRNA expression. Transgenic offspring were tested similarly to establish heritability of the transgene.

Immunohistochemistry of transgenic tissues: Fresh tissue samples (heart, spleen, kidney, pancreas and lung)

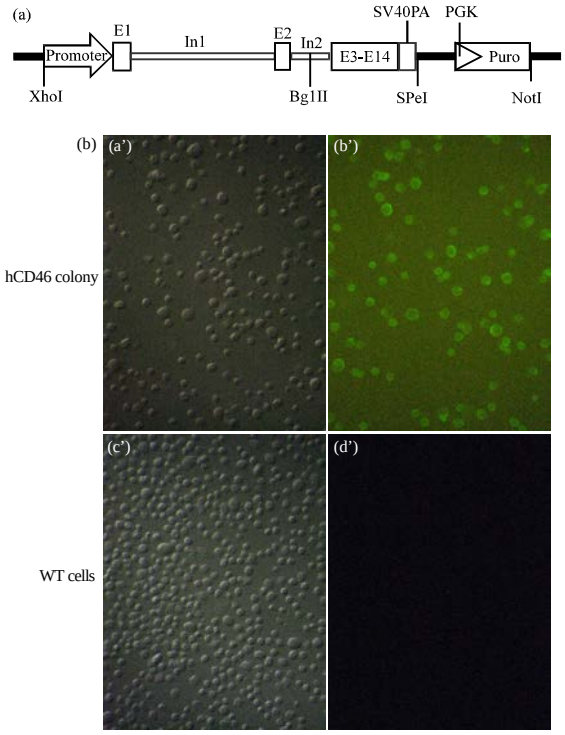

Fig. 1: A human CD46 minigene was constructed and used to express the hCD46 protein. a) A 9.5 kb fragment was used as an expression construct and inserted into a pBluescript vector to express hCD46; b) hCD46+colony immunostained with a fluorescein isothiocyanate-conjugated anti-hCD46 antibody. a' and b' represent hCD46 expressing cells, c' and d' represent WT cells, b' and d' was under the excitation wavelength 340 nm while a and c' was not excited

were fixed in neutral buffered formalin and then embedded in paraffin wax before being sectioned. Tissue sections (5 μm) were cut and air-dried before dewaxing and antigen retrieval. The sections were stained with primary rabbit antibodies against human CD46 (Abcam, Cambridge, MA, USA; #AB108307) and horseradish peroxidase conjugated goat anti-rabbit IgG according to the manufacturer's instructions.

Flow cytometryof hCD46 on surface of AECs: Porcine AECs from generated hCD46-expressing transgenic pigs were stained with optimal dilutions of the anti-CD46-FITC antibody according to the manufacturer's instructions (Sigma-Aldrich). Surface expression of the hCD46 protein on human AECs was used as a positive control. The stained cells were analyzed using a BD™ LSR II flow cytometer (Beckton Dickinson, Franklin Lakes, NJ, USA) as described by Hara *et al.* (2011).

Assay for complement-mediated cytolysis of hCD46 porcine AECs: AECS plated into 24 well plates at 80% confluency were incubated with human serum samples of different ABO blood groups. The serums were diluted 20% into Dulbecco's modified Eagle's medium and added to triplicate wells at 250 μL/well. After the lytic reactions had stopped, independent observers scored the proportions of lysed cells. All assays were performed in triplicate at least twice. The data from cytolysis assays were analyzed statistically using two-way Analysis of Variance (ANOVA) using SPSS (Version 19.0) for IBM Software (IBM Corp, Armonk, NY, USA).

RESULTS

Generation of transgenic piglets: Researchers designed the 9.5 kb hCD46 minigene construct for transfection and generated hCD46-expressing transgenic WZSPs. Three colonies with high MFI values were selected for SCNT. Four surrogate mothe rs became pregnant and gave birth successfully to 15 transgenic piglets; 13 derived from the B37 colony and two from the D7 colony. One of the transgenic piglets was stillborn and another died within 2 days for unknown reasons.

Expression of hCD46 in the tissues of transgenic pigs: Distinct tissue-specific patterns of staining were observed by immunohistochemistry in transgenic tissues whereas there was no immunostaining for hCD46 in control tissues (Fig. 2). In the kidney, the renal tubules and glomeruli showed stronger staining. In the heart, intense staining was found in the endothelium of blood vessels whereas cardiac muscle showed moderate staining. In the lung, intense staining was observed in the epithelial cells lining bronchi and alveolar septa. In the spleen and pancreas, disperse uniform staining was observed in the parenchyma with relatively strong staining in epithelial cells lining exocrine ducts and glands. In summary, tubule and epithelium-specific expression patterns were observed, similar to human tissues.

Flow cytometry of hCD46 on the surface of porcine cells: Expression of hCD46 transgenic porcine cells was confirmed by flow cytometry (Fig. 3). The single-isoform heterozygote Endothelial Cells (pAECs) of transgenic pigs (28 MFI) had ~75% of the level of cell surface expression of CD46 compared with human positive control cells as multi-isoform homozygotes. Incubation of nontransgenic endothelial cells with the antibody gave a similar background.

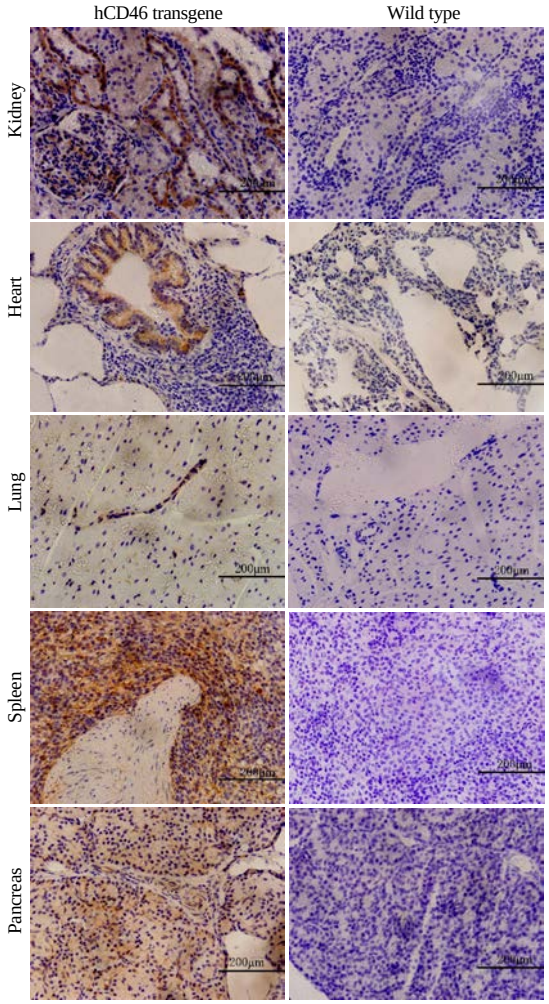

Fig. 2: Immunohistochemistry of hCD46 expression in different tissues

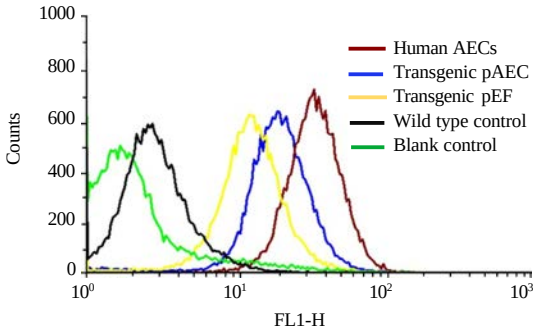

Fig. 3: Flow cytometry of acquired porcine cells

Protection of hCD46 host cells against complement-mediated cytolysis: The ABO blood group serum samples were diluted to 1/5 for cytolysis of hCD46 host cells to assay the regulatory effects of human complement. Cytolysis of hCD46-expressing endothelial

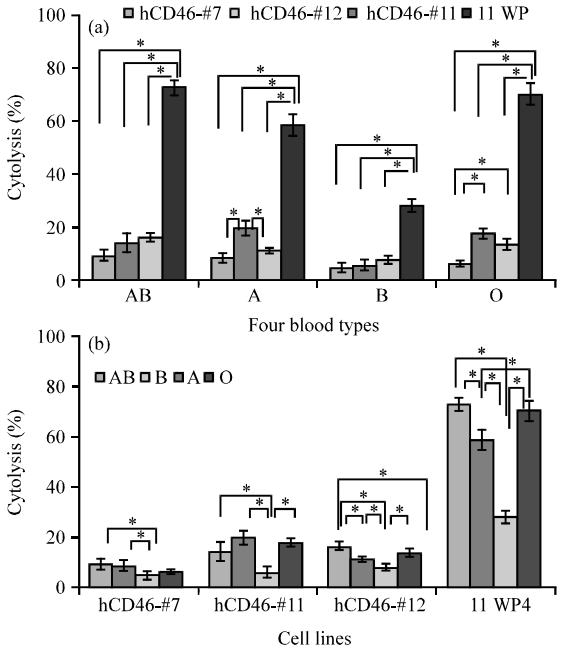

Fig. 4: hCD46 protein expression on host cells inhibited human complement-mediated lysis. a) All the hCD46 host cells were significantly protected from lysis compared with cells from WT animals; b) the percentage of lysis in the four cell lines exposed to different blood types showed significant differences. In particular, the cytolysis of cells exposed to blood type B was significantly lower than among those exposed to the other blood types for each cell line (*p<0.05)

Table 1: Two-factor ANOVA of the impact on cytolysis by cell lines and blood groups

Variances	SS	df	MS	F-values	p-values
Cell line	19630.056	3	6543.352	1024.580	0.000
Blood group	2111.035	3	703.678	110.184	0.000
Cell line x blood group	2231.756	9	247.973	38.828	0.000
Error	204.364	32	6.386	-	-
Total	24177.211	47	-	-	-

cells was significantly lower than in the WT samples, indicating an obvious protection of host cells by hCD46 (Fig. 4a). Interestingly, significant differences in cytolysis between blood groups was observed in the transgenic pigs and the WT animals (Fig. 4b). The cytolysis of cells exposed to blood group B was significantly lower than the other blood groups. Moreover, two-factor ANOVA results showed that overall differences in cell lines ($F = 1024.580$, $p<0.001$) contributed more to cytolysis than did the effects of individual blood groups ($F = 110.184$, $p<0.001$) and there was a significant interaction between them ($p<0.001$; Table 1). Thus, there was a significant effect of specific blood groups in mediating complement-mediated cytolysis.

DISCUSSION

As porcine CRPs cannot effectively regulate human complement because of homologous restriction (Morgan *et al*., 2005) porcine organs are extremely vulnerable against human complement-mediated rejection. Expression of human CRPs in transgenic pig grafts to nonhuman primates (Mora *et al*., 1996) especially high-level expression of CD46 can overcome HAR effectively (Loveland *et al*., 2001, 2004). Researchers reconstructed the hCD46 minigene and generated transgenic pigs on a native WZSP background.

Immunochemistry analysis of the transgenic tissues demonstrated distinct tissue-specific distributions of hCD46 on epithelial tissues and vascular endothelium. These locations are essential if an engraft is to survive the injury induced by periods of ischemia, reperfusion, exposure to foreign platelets and antibodies and complement deposition (Loveland *et al*., 2004). The results of the present study indicated that the hCD46 minigene construct could simulate the expression of CD46 in the human body which might be explained by the inclusion of the human genomic CD46 promoter (inclusion of the first two introns). These results were consistent with previous studies (Thorley *et al*., 1997; Loveland *et al*., 2004) indicating that researchers had performed a correct analysis. In addition, the applicability of WZSPs for porcine pancreatic islet isolation and purification has been investigated (Jiang *et al*., 2012) showing that the pancreas from the WZSP breed produced a significantly higher functional islet yield than did that of large commercial breeds of pigs. Thus, the WZSP breed might provide suitable pancreatic islets for xenotransplantation research.

It has been reported several times that porcine cells expressing hCD46 show a significant ability to resist lysis mediated by human serum (Loveland *et al*., 1993; Loveland *et al*., 2004; Hara *et al*., 2011). However, most of the previous studies ignored the effect of different blood groups: mixing the ABO blood group serum samples together (Hara *et al*., 2008) or using a single blood serum (Loveland *et al*., 2004). In this study, researchers optimized the experiment by using separate ABO blood group serum samples as complement sources for lysis reactions in both transgenic and WT cell lines. The results were consistent with a previous study showing that perfusion of porcine hearts with human ABO blood plasma can affect their survival (Manji *et al*., 2003). As has been discussed because the B blood group antigen of human is similar to the αGal antigen in terms of molecular structure, anti-A antibodies can weakly bind to αGal and act as competitive inhibitors to the binding of anti-αGal antibodies in blood-group B serum: this protection was not available for blood group AB serum (Manji *et al*., 2003). This could help in interpreting the result of the experiment. While the transgenic porcine cells were exposed to B blood group serum, the anti-A antibodies reduced the damage mediated by αGal. As for the A and O blood groups existing anti-B antibodies might interact with αGal, analogous to an antigen-antibody interaction resulting in elevated cytolysis. Thus, the study of complement-mediated lysis mediated by blood-group B serum demonstrated that hCD46 might have protected cells by an alternative pathway.

It was difficult for researchers to define the expression level of the hCD46 minigene precisely as it just encodes the BC1 isoform whereas the human genomic *CD46* gene encodes multiple isoforms. Flow cytometry showed that the single-isoform expression of hCD46 in porcine AECs was half the multi-isoform expression in human AECs. Nonetheless, transgenic porcine AECs expressing hCD46 still showed significant protection against complement-mediated cytolysis. These results indicated that researchers had acquired superior hCD46 expression despite the transgenic pigs being hemizygotes. Researchers are optimistic that researchers can obtain homozygotes by breeding and get better CD46 expression.

CONCLUSION

Researchers have obtained hCD46-expressing transgenic pigs on an inbred Wuzhishan miniature pig background. These animals will provide valuable donors for preclinical studies in xenotransplantation of pig tissues to nonhuman primate.

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