pISSN 1225-4991, eISSN 2288-0178
J. Emb. Trans. (2015) Vol. 30, No. 4, pp. 305~313
http://dx.doi.org/10.12750/JET.2015.30.4.305

Production of Transgenic Micro-Pig Expressing Human Heme Oxygenase 1

Ok Jae Koo[1], Hyun Ju Oh[2] and Byeong Chun Lee[2,†]

[1]Laboratory Animal Research Center, Samsung Biomedical Research Institute, Suwon 440-746, Korea
[2]Dept. of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, Seoul 151-742, Korea

ABSTRACT

Xenotransplantation of pig islet regarded as a good alternative to allotransplantation. However, cellular death mediated by hypoxia-reoxygenation injury after transplantation disturb success of this technique. In the present study, we produce transgenic pig expressing human heme oxygenase 1 (HO1) genes to overcome cellular death for improving efficiency of islet xenotransplantation. Particularly, Korean miniature pig breed, Micro-Pig, was used in the present study. Somatic cell nuclear transfer (SCNT) technique was used to produce the HO1 transgenic pig. Six alive transgenic piglets were produced and all the transgenic pigs were founded to have transgene in their genomic DNA and the gene was expressed in all tested organs. Also, *in vitro* cultured fibroblasts derived from the HO1 transgenic pig showed low reactive oxygen species level, improved cell viability and reduced apoptosis level.

(Key words : transgenic pig, somatic cell nuclear transfer, heme oxygenase 1, islet xenotransplantation)

INTRODUCTION

Nearly 200 million patients over the world were suffered from diabetes (Emamaullee *et al.*, 2005). Hopefully, recent clinical outcomes show us that islet transplantation is an alternative and ideal therapeutic approach for treatment of diabetes. However, number of islet donor is too smaller than patients who wait for transplant. And this shortage is the major limiting factor for successful islet allotransplantation. As an alternative of this human to human transplantation, xenotransplantation, using the pig as the source of islets, is regarded as a potential solution to this problem.

Recently, several reports show that more than 6 months survival of pig islets transferred to nonhuman primates (Cardona *et al.*, 2006; Hering *et al.*, 2006). Though, these results were far much better than other xenotransplantation research trying to transplant kidney, heart or any other organs, it is still not sufficient to use this technique in clinical cases especially for young patients who need treatment for their whole lifetime. One of the most biggest obstacles for successful islet transplantation is necrosis and apoptosis of transplanted islets induced by oxidative stress and other insults. It was reported that even in optimal condition, approximately 60% of transplanted islets were undergone apoptosis or necrosis.

Heme oxygenase 1 (HO1) is the rate-limiting enzyme in the heme degradative pathway that catalyzes the oxidation of heme into biliverdin, carbon monoxide (CO), and free iron (Katori *et al.*, 2002; Maines, 1997). Previous reports show that HO1 is induced by cellular stress and shows antioxidant, antiapoptotic, anti-inflammatory, and immune modulating effect (Petrache *et al.*, 2000; Stocker, 1990; Willis *et al.*, 1996). Therefore, overexpression of HO1 may reduces damage of islet cells and improve viability of transplanted islets. It has been shown that overexpression of HO1 improve the viability and function of cells or tissues in *in vitro* culture or *in vivo* transplantation (Chen *et al.*, 2007; Laumonier *et al.*, 2008; Zhen-Wei *et al.*, 2007). Transgenic animals expressing HO1 has been reported in rodents (Braudeau *et al.*, 2003; Maines, 2002) as animal models. Recently, our group reported HO1 expressed White Yucatan miniature pig (Yeom *et al.*, 2012).

This study was financially supported by the Cooperative Research Program for Agriculture Science and Technology Development (no. PJ009802), Rural Development Administration, Korea and Ministry of Trade, Industry and Energy of Korea (#10048948), Korea IPET (#311011-05-5-SB010), Research Institute for Veterinary Science, BK21 Plus. Also we are grateful to Young Hee Nam and staffs from Gyeonggido Veterinary Service for valuable contributions in animal husbandry, and Yo Il Kim and researchers in SNU Theriogenology Lab. for technical assistance.

† Correspondence : bclee@snu.ac.kr

In this study, we produce Korean miniature pigs (Micro-Pig, Medi Kinetics) expressing human heme oxygenase 1 using somatic cell nuclear transfer technique. We used cytomegalovirus (CMV) promoter for overexpression of HO1 gene in whole body, therefore, expression of HO1 in each major organ of transgenic piglets was examined. The function of introduced gene in cultured cells derived from transgenic piglet was also examined as a preliminary study for xenotransplantation.

MATERIALS AND METHODS

1. Chemicals

All chemicals were obtained from Sigma-Aldrich Corp. (St. Louis, MO, USA) unless otherwise stated.

2. Animal Care and Use

For surrogate, mixed breed pigs were used. For collecting donor cells for somatic cell nuclear transfer, Micro-Pig purchased from MediKinetics (Pyeongtaek, Korea) was used. Gyeonggido Veterinary Service was responsible for breeding of the pigs in accordance with the Guide for the Care and Use of Laboratory Animals at Gyeonggido Veterinary Service, Korean Government.

3. Preparation of Porcine Fibroblasts

Ear tissue was obtained from miniature pigs. The tissue was washed three times in Ca^{2+}− and Mg^{2+}− free DPBS (Invitrogen, Carlsbad, CA, USA) and minced with a surgical blade. The minced tissues were dissociated in TrypLE Express (Invitrogen) for 1 h at 37℃. Trypsinized cells were washed once in DPBS by centrifugation at 1,500 RPM for 2 min, and seeded onto 100-mm plastic culture dishes (Becton Dickinson, Lincoln Park, NJ, USA). Subsequently, cells were cultured for 8～10 days in Dulbecco's modified Eagle's /Nutrient Mixture F-12 medium (DMEM/F12; Invitrogen) supplemented with 10% (v/v) FBS (Invitrogen), 1 mM Glutamax I (Invitrogen), 25 mM $NaHCO_3$, 1% (v/v) minimal essential medium (MEM) non-essential amino acid solution (Invitrogen) and 1%(v/v) Antibiotic-Antimycotics at 39℃ in a humidified atmosphere of 5% CO_2 and 95% air. After removal of unattached clumps of cells or explants, attached cells were further cultured to confluence. The cells were subcultured (at intervals of 4～6 days) by trypsinization for 1 min. Trypsinized cells were allocated to three new dishes for further passaging, or stored in liquid nitrogen at −196℃. The freezing medium consisted of 70% (v/v) DMEM/F12, 10% (v/v) dimethylsulfoxide and 20% (v/v) FBS.

4. Establishment of Human HO1 Gene Expressing Porcine Fibroblast

The human HO1 cDNA fragment was amplified from the HO1 cDNA pHHO1 by PCR. The pHHO1 plasmid was supplied from Seoul National University Hospital and the PCR primers were forward; 5'-TTAAAAGCTTATGGAGCGTCC-GCAACCCGA-3' and reverse; 5'-TTAATCTAGAAAGAAG-GCCTTCCACCGG-3'. Human HO1 gene was inserted into Nhe I and EcoR I sites of pcDNA3.1 (Invitrogen), a mammalian expression vector. The plasmid was linearized by Sca I and introduced into primary pig fibroblasts, using Lipofectamin 2000 (Invitrogen). Transfected cells were cultured in the presence of 1 mM G418 for 2 weeks. The colonies isolated by picking were amplified and frozen for somatic cell nuclear transfer.

5. Preparation of Recipient Oocytes

Ovaries were collected from a local slaughterhouse. Cumulus-oocyte complexes (COCs) aspirated from 3 to 6 mm diameter of follicles were cultured in tissue culture medium-199 (TCM-199, Invitrogen, Carlsbad, CA, USA), supplemented with 10 ng/ml EGF, 0.57 mM cysteine, 0.91 mM sodium pyruvate, 5 μg/ml insulin, 1%(v/v) Pen-Strep (Invitrogen), 1 mM of dibutyryl adenosine 3',5'-cyclic monophosphate (dbcAMP), 0.5 μg/ml follicular stimulating hormone (FSH), 0.5 μg/ml lutenizing hormone (LH) and 10% porcine follicular fluid at 39℃ in a humidified atmosphere of 5% CO_2. After culturing for 22 h, COCs were washed then cultured again in medium without dbcAMP, FSH and LH. At 38 to 42 h of maturation culture, oocytes were freed from cumulus cells by repeated pipetting in 0.1% hyaluronidase.

6. Somatic Cell Nuclear Transfer

For somatic cell nuclear transfer (SCNT), a micromanipulation system (NT-88, Nikon-Narishige, Japan) attached to an inverted microscope (TE-2000, Nikon Instrument Korea, Korea) was used. After maturation culture, cumulus-free oocytes were placed in porcine zygote medium (PZM)-5 (Suzuki *et al.*, 2007) supplemented with 2.5 μg/ml cytochalasin D and 25 mM Hepes. The first polar body and adjacent cytoplasm, presumably containing the metaphase-II chromosomes, were removed with beveled micro-pipette. Enucleation was confirmed by stai-

ning with Hoechst 33342 (5 μg/ml) during manipulation. Single fibroblast donor cell with a smooth surface were selected under a microscope and transferred into the perivitelline space of enucleated oocytes. These couplets were placed in a pulsing medium for 1 min and transferred to a chamber consisting of two electrodes overlaid with pulsing medium. The pulsing medium was 0.26 M mannitol solution containing 0.5 mM HEPES, 0.1 mM $CaCl_2$ and 0.1 mM $MgSO_4$. Couplets were fused with a single 1.5 kV/cm DC pulse for 60 μsec using electro cell fusion generator (Nepagene LF101, Ichikawa, Chiba, Japan). Couplets were washed then incubated in PZM-5 for 1 h. Fused couplets were selected under microscope and artificial activation was performed. Activation of fused oocytes was induced by cultured with 0.2 mM thimerosal for 10 min, washed then additional cultured with 8 mM dithiothreitol for 40 min. Thimerosal and dithiothreitol were diluted in PZM-5 medium supplemented with 2.5 μg/ml cytochalasin D and 2 mM 6-dimethylaminopurine.

7. Embryo Transfer and Pregnancy Diagnosis

SCNT embryos at 1 to 4 cell stages were loaded into a sterilized 0.25 ml straw (Minitüb, Germany) in a laboratory and kept in a portable incubator (Minitüb) during transportation and taken out just before transfer into recipient. An estrous-synchronized recipient was anesthesized and one oviduct was exposed by laparotomy. The embryo-loaded straw was put directly into the oviduct of the recipient and embryos were injected. Recipients were checked for pregnancy by transabdominal ultrasound examination on Day 30 after embryo transfer.

8. Polymerase Chain Reaction (PCR) and Reverse Transcription-PCR

Total genomic DNA was extracted using G-spin Genomic DNA Extraction Kit (iNtRON Biotechnology, Korea) follow supplier's instruction. Total mRNA was extracted using easy-spin Total RNA Extraction Kit (iNtRON), and cDNA was synthesized using Maxime RT Premix (iNtRON) follow supplier's instruction. The PCR amplification was carried out using Maxime PCR PreMix (i-StarTaq). Primer sets and annealing temperature used in this study was summarized in Table 1. Amplified PCR product was visualized under UV light on 1.5% agarose gel in 1 × TAE buffer (iNtRON) containing 1 μg/ml ethidium bromide. The intensity of each bad was scanned and quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Expression level of mRNA was represented as the ratio of gene to β-actin.

9. Treatment of Cell Inducing Reactive Oxygen Species or Apoptosis

To induce reactive oxygen species, fibroblasts were cultured with 0.01% hydrogen peroxide for 30 min. To induce apoptosis fibroblasts were cultured with 10 μg/ml cycloheximide for 15 h.

10. Measurement of Reactive Oxygen Species

The level of reactive oxygen species (ROS) was examined using dichlorohydrofluorescein diacetate (DCHFDA). After treatment of hydrogen peroxide, fibroblasts were cultured with 10 μM DCHFDA for 30 min. After incubation, single cells were harvested using TrypLE Express (Invitrogen). Level of ROS was analyzed by Flow Cytometry (FACSCalibur with Cell Quest software, Becton Dickinson, Rutherford, NJ) at 485 nm. For each sample, 10,000 events were recorded. In histogram, region was devided into 2 groups, R1 & R2, based on the highest peak value obtained from HO1 transgenic group (Fig. 4). Histogram plots were created and proportion of cells in each experimental regions was calculated using WinMDI software (Version 2.5, Joseph Trotter).

11. Measurement of Cell Viability

Table 1. Primer sets used for PCR and RT-PCR

Gene	Primer sequence (5'-3')	Annealing temperature (℃)	PCR fragment size (bp)	NCBI accession number
HO1[1]	F:ATGGAGCGTCCGCAACCCGACAG R:TCACATGGCATAAAGCCCTACAG	62	867	X06985
ACTB[2]	F:CATCACCATCGGCAACGA R:GTTGGCGTAGAGGTCCTTCCT	62	147	U07786

[1] Homo sapiens heme oxygenase 1, [2] *Sus scrofa* actin beta.

(a)

(b)

Fig. 1. Photograph of cloned transgenic piglet expressing human heme oxygenase 1. (a) born from surrogate 55-1, (b) born from surrogate 44-2.

After treatment of hydrogen peroxide, fibroblasts were additionally cultured for 3 h then harvested using TrypLE Express (Invitrogen). Harvested cell were resuspended in DPBS then mixed with same amount of 0.4% tryphan blue. Total cell number and blue-stained cell (dead cells) number were counted using a hemocytometer (Marienfeld GmbH, Marienfeld, Germany) and viability of cells was calculated.

12. Measurement of Apoptosis

Apoptosis were analyzed using Vybrant Apoptosis Assay Kit #2 (Molecular Probes, Eugene, OR, USA) follow supplier's instruction. Briefly, harvested cells including floating cells were stained with Alexa Fluor 488-annexin V conjugate then analyzed with flow cytometry (FACSCalibur with Cell Quest software, Becton Dickinson, Rutherford, NJ) at 488 nm. Proportion of apoptotic cells were calculated using WinMDI software (Version 2.5, Joseph Trotter).

13. Statistical Analysis

All data were subjected to one-way ANOVA followed by Tukey's test using Prism software (Version 5.0, GraphPad Software, San Diego, CA, USA) to determine differences among experimental groups. Statistical significance was determined when the P value was less than 0.05.

RESULTS

1 Production of Human Heme Oxygenase 1 Transgenic Piglets

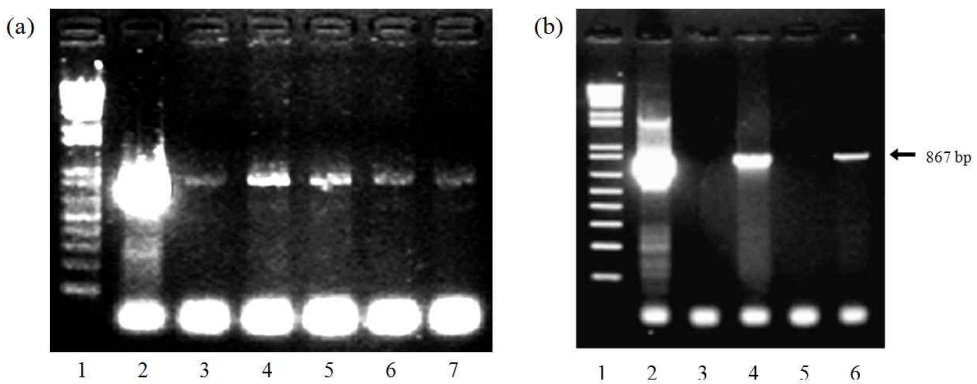

Fig. 2. PCR analysis of human heme oxygenase 1 transgene integration in transgenic piglets. (a) genomic DNA analysis of transgenic piglets born from surrogate 55-1. Lane 1, 1kb marker, Lane 2; hHO1 expression vector, Lane 3-7; skin tissues from transgenic piglets; (b) genomic DNA analysis of transgenic piglets born from surrogate 44-2. Lane 1, 1kb marker; Lane 2, hHO1 expression vector; Lane 3, wild type pig skin tissue; Lane 4, transgenic pig skin tissue; Lane 5, wild type pig fibroblast; Lane 6, transgenic fibroblast (donor cells) used for pig cloning.

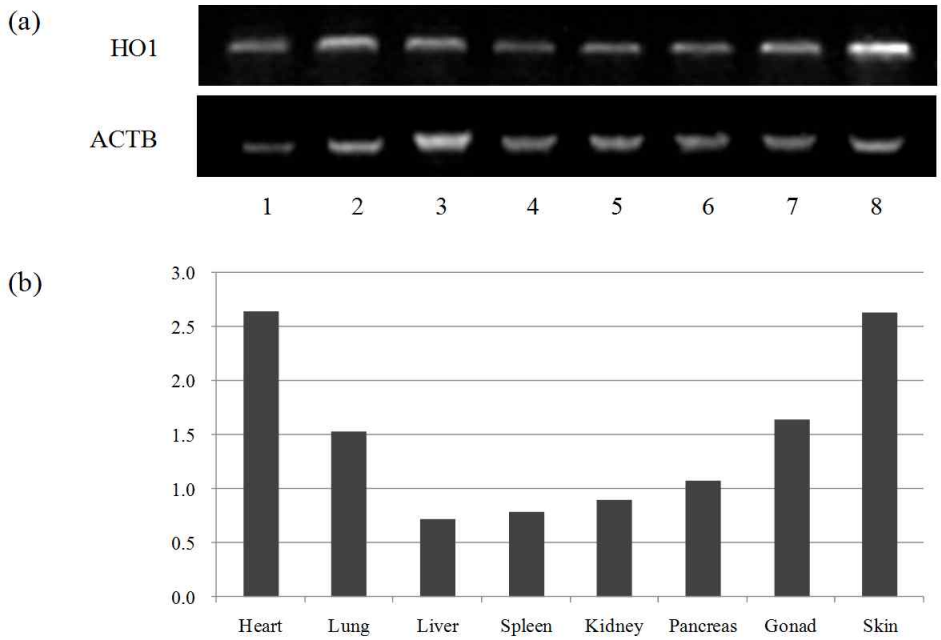

Fig. 3. Expression of the human heme oxygenase 1 (HO1) transgene in various organs from transgenic cloned piglet (surrogate: 44-2). (a) Representative gel photograph after RT-PCR amplification. Lane 1, Heart; Lane 2, Lung; Lane3, Liver; Lane 4, Spleen; Lane 5, Kidney; Lane 6, Pancreas; Lane 7, Gonad (Testis); Lane 8, Skin. (b) Expression level of the transgene in each organs normalized against actin beta (ACTB) expression.

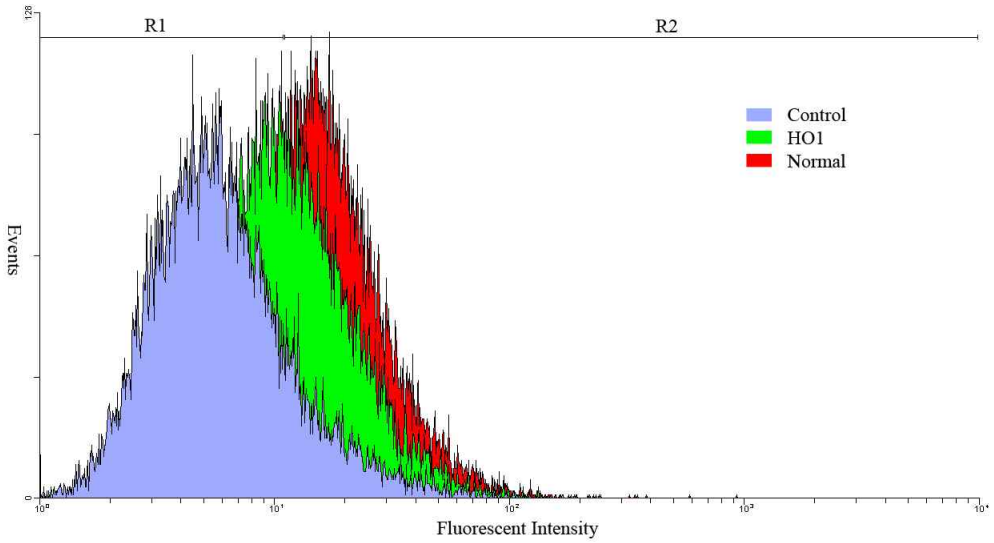

Fig. 4. Flow cytometry histogram of induced reactive oxygen species (ROS) after treatment of hydrogen peroxide. R1, R2: Divided regions used for ROS analysis (See also Table 3) 2 regions were divided based on the highest peak value of HO1 group; Control: no treatment of hydrogen peroxide; HO1: human heme oxygenase 1 expressing fibroblast derived from transgenic piglet (surrogate: 55-1); Normal: fibroblast derived from wild type piglet.

In total 893 SCNT embryos were transferred to 10 surrogate pigs. Among them 4 surrogates were detected as pregnancy. One pregnant (3-1) was terminated by cesarean section at 35 d of gestation to obtain transgenic fetal fibroblast for further study. Among the other 3 pregnant, 2 surrogates (55-1 and 44-2) delivered 6 live and 6 dead piglets, respectively (Fig. 1).

Detailed embryo transfer records were shown in Table 2.

2. Heme Oxygenase 1 Expression in Transgenic Piglets

All lively born transgenic piglets has HO1 gene in their genomic DNA (Fig. 2). Detailed HO1 expression levels in heart, lung, liver, spleen, kidney, pancreas, gonad, and skin were examined with tissues obtained from autopsied piglet. HO1 was expressed in all organ examined (Fig. 3a). Especially, expression was well founded in tissues from heart and skin (Fig. 3b).

3. Antioxidant Effect of HO1 in Fibroblast derived from Transgenic Piglet

Treatment of hydrogen peroxide induces significantly higher level of ROS both in normal and HO1 transgenic fibroblasts compared to untreated control. However, level of ROS in normal fibroblast shows significantly higher level. Detailed events recorded in each region were founded in Table 3. Cell viability after treatment of hydrogen peroxide treatment was also significantly reduced in both normal and HO1 transgenic fibroblast compared to untreated control group (Fig. 5, 86.2±1.0). Between normal and HO1 transgenic fibroblasts groups, HO1 groups show significantly higher viability than normal fibroblast group (71.7±1.1% vs. 65.7±1.6%, respectively).

Table 2. *In vivo* development of cloned HO1 transgenic embryos transferred to surrogates

Surrogate	Embryos (n)	Pregnancy	Delivery (day of gestation)
3-1	120	Yes	4 fetuses were retrieved (35 d)
55-1	93	Yes	5 live & 3 dead piglets (117 d)
4-1	105	No	-
25-4	86	Yes	Abortion (75 d)
10-4	95	No	-
13-3	89 + 28 parthenote	No	-
44-2	58 + 20 parthenote	Yes	1 live & 3 dead piglets (112 d)
30-2	73	No	-
25-1	96	No	-
2-2	78	No	-

Table 3. Level of reactive oxygen species induced by H_2O_2 exposure in fibroblast derived from normal or heme oxygenase 1 transgenic piglets (surrogate: 55-1)

	Regions in Histogram (Also see Fig. 4)	
	R1	R2
Control	81.6±1.0[a]	18.4±1.0[a]
Normal fibroblast	49.3±2.5[b]	50.7±2.5[b]
HO1 transgenic	34.9±2.1[c]	65.1±2.1[c]

[a~c] different superscript represent significantly differences.

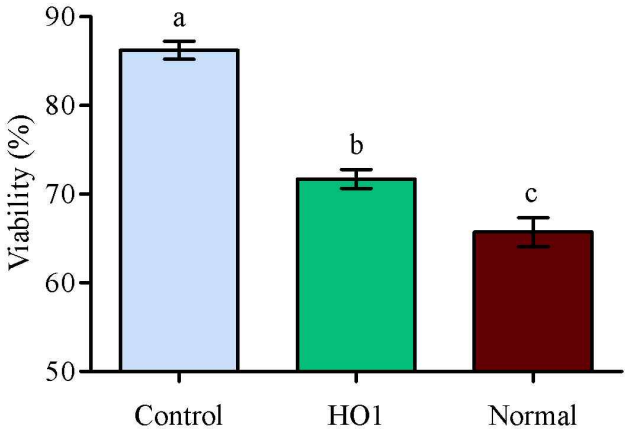

Fig. 5. Cell viability of fibroblasts derived from normal or heme oxygenase 1 transgenic piglet after treatment of hydrogen peroxide. Control: no treatment of hydrogen peroxide; HO1: human heme oxygenase 1 expressing fibroblast derived from transgenic piglet (surrogate: 55-1); Normal: fibroblast derived from wild type piglet. [a~c] Different superscripts marked on top of bars in graph means significantly differences.

4. Antiapoptotic Effect of HO1 in Fibroblast derived from Transgenic Piglet

Fig. 6 shows that treatment of cycloheximide induces significantly higher level of apoptosis in normal fibroblasts compared to untreated control cells (10.0±0.5% vs. 4.5±0.2%). However, there is no significantly difference of proportion of apoptotic cells was found between untreated control cells and cycloheximide treated HO1 transgenic cells (4.3±0.6%).

DISCUSSION

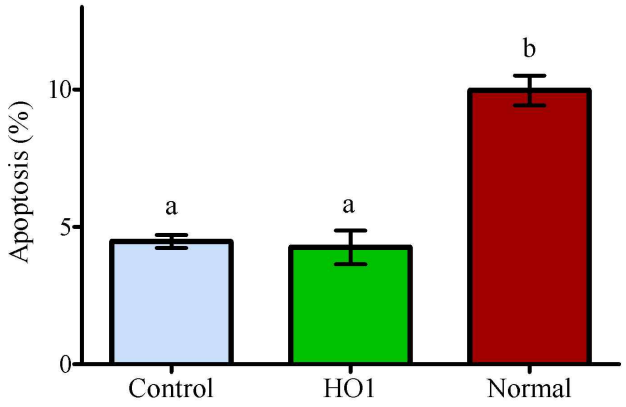

Fig. 6. Proportion of apoptotic cells after treatment of cycloheximide (10 μg/ml for 15 h) in fibroblasts derived from normal or heme oxygenase 1 transgenic piglet. Control: no treatment of hydrogen peroxide; HO1: human heme oxygenase 1 expressing fibroblast derived from transgenic piglet (surrogate: 55-1); Normal: fibroblast derived from wild type piglet. [a,b] Different superscripts marked on top of bars in graph means significantly differences.

Within the pancreas, islets are richly oxygenated by a glomerulus-like microvasculature that forms an intra-islet portal system. However, during islet isolation, this vasculature is completely disrupted and hypoxia was occur in islets. This hypoxic state is continues during *in vitro* culture and even after transplantation, accordingly, oxygen tension and blood perfusion remain lower for at least 1 month.

Hypoxia-reoxygenation is a complex process that generates a high level of ROS. ROS can alter most type of cellular molecules and also damaging cell membranes. Especially, islets appear to be more vulnerable to this oxidative injury than other tissues because they express low level of antioxidant enzymes (Tiedge *et al.*, 1997). In addition, it was well known that ROS were also activate a number of signaling pathways involved in apoptosis. Therefore, we hypothesized that transgenic expression of antioxidative gene or antiapoptotic gene will reduce islets damage from ROS induced by hypoxia-reperfusion during transplantation. Several studies that improve islet graft function by overexpression of antioxidant gene (Lepore *et al.*, 2004; Mysore *et al.*, 2005), or antiapoptotic gene (Emamaullee *et al.*, 2005), encourage us to produce transgenic animals for xenotransplantation of islets.

HO1 is known as ubiquitous stress protein shows both antioxidant and antiapoptotic effect. Initially, HO1 degrade heme into biliverdin, CO and iron. Biliverdin is subsequently reduced into bilirubin, a powerful antioxidant. Also, iron upregulates the expression of ferritin which also protect cells from ROS mediated injuries (Balla *et al.*, 1992). On the other hand, production of CO, an anti-inflammatory molecule, reduces apoptosis by blocking pro-inflammatory cytokines (Pileggi *et al.*, 2001). Therefore, we choose HO1 as an ideal molecule for xenotransplantation of islets.

To produce genetic modified pigs, we used transfection, somatic cell nuclear transfer and embryo transfer technique. As reviewed previously, this protocol is more rapid and relatively inexpensive procedure compared to traditional method for transgenic animals using microinjection and breeding. In present study, we produce 6 live piglets from 786 cloned embryos reconstructed with porcine fibroblast transfected with human HO1 gene. In PCR result, all piglets have human HO1 gene in their own genome. Piglet delivered from surrogate 44-2 was dead 10 days after born due to bacterial and fungal infection. However, no congenital disorder was detected in gross findings during autopsy and micro-pathological findings. Tissue samples from major organs of transgenic pig were obtained during autopsy and further used for RT-PCR analysis. In RT-PCR result, human HO1 gene was well expressed in all tested organs. Especially, expression of HO1 is relatively high in heart and skin. In pancreas, HO1 gene was also well expressed, therefore, we hope that our transgenic pigs improve efficiency of islet xenotransplantation.

Function of human HO1 gene in transgenic piglets was also examined. As a preliminary study for islet transplantation, we analyzed the effect of human HO1 gene in fibroblasts derived from transgenic piglets. As we hypothesized, to improve islet xenotransplantation, antioxidant effect and/or antiapoptotic effect of transfected human HO1 should be confirmed in transgenic piglets. To examine antioxidative effect of HO1, we treat hydrogen peroxide, a powerful member of ROS family, on fibroblasts derived from both normal pig and human HO1 transgenic pig. As expected, in HO1 expressing cells, level of induced ROS in cells were significantly lower than normal cell group (Table 3 and Fig. 4). Accumulation of ROS induces both necrosis and apoptosis. Consequently, high level of ROS in normal cells group resulted in low viability compared to HO1 transgenic cells group after treatment of hydrogen peroxide (Fig. 5). Therefore, expression of HO1 in transgenic pig will protect cellular injuries mediated by oxidative damage in xenotransplantation.

In addition to oxidative damage, apoptotic cellular death after

transplantation is also mediated by inflammation induced cytokines. Therefore, antiapoptotic effect of HO1 transgenic cells also evaluated. In present study, we treat cycloheximide on porcine fibroblasts to induce apoptosis. Cycloheximide is known as to induce overexpression of several pro-apoptotic genes including c-myc, c-fos, c-jun, and p53. These pro-apoptotic genes contribute significantly in transducing the apoptotic signal. After 15 h treatment of cycloheximide, proportion of apoptotic cells was significantly higher compared to non-treated control. However, in HO1 transegnic cells, proportion of apoptosis after treatment of cycloheximide is similar to untreated-control cells (Fig. 6). Therefore, present study shows that expression of HO1 is effectively reduce apoptosis signal in transgenic animal. We expect that this effect also very helpful for xenotransplantation.

One of superior properties of xenotransplantation over allotransplantation is simplicity for genetic modification. Benefits from HO1 transgenic pigs, such as antioxidant and antiapoptic effect as shown above, is hardly obtained in allotransplantation. We planned further *in vivo* xenotransplantation study using our transgenic pigs and hope that our pigs also show same antioxidant and antiapoptotic effect after transplantation. However, one of possible problems is expression level of HO1 in islet cells. Fig. 3 show that mRNA expression level of HO1 in pancreas was less than half of HO1 expression in skin. Fibroblast, used in present study, was originated from skin, therefore, effect of transgenic HO1 possibly reduced in islet cells in pancreas. After few months, our transgenic piglets grow up to proper age, we can clarify this by analyze pancreatic tissues obtained from the pigs.

In conclusion, 6 healthy Micro-Pig miniature pigs expressing human HO1 was born. Transgenes were well expressed in all organs tested. Fibroblasts obtained from transgenic piglets show antioxidant and antiapoptotic effect *in vitro*.

DEFERENCES

Alessenko AV, Boikov P, Filippova GN, Khrenov AV, Loginov AS and Makarieva ED. 1997. Mechanisms of cycloheximide-induced apoptosis in liver cells. FEBS. Lett. 416:113-116.

Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW and Vercellotti GM. 1992. Ferritin: A cytoprotective antioxidant strategem of endothelium. J. Biol. Chem. 267:18148-18153.

Biarnes M, Montolio M, Nacher V, Raurell M, Soler J and Montanya E. 2002. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes. 51:66-72.

Bonner-Weir S and Orci L. 1982. New perspectives on the microvasculature of the islets of Langerhans in the rat. Diabetes. 31:883-889.

Braudeau C, Bouchet D, Toquet C, Tesson L, Menoret S, Iyer S, Laboisse C, Willis D, Jarry A, Buelow R, Anegon I and Chauveau C. 2003. Generation of heme oxygenase-1-transgenic rats. Exp. Biol. Med. (Maywood) 228:466-471.

Cardona K, Korbutt GS, Milas Z, Lyon J, Cano J, Jiang W, Bello-Laborn H, Hacquoil B, Strobert E, Gangappa S, Weber CJ, Pearson TC, Rajotte RV and Larsen CP. 2006. Long-term survival of neonatal porcine islets in nonhuman primates by targeting costimulation pathways. Nat. Med. 12:304-306.

Carlsson PO, Liss P, Andersson A and Jansson L. 1998. Measurements of oxygen tension in native and transplanted rat pancreatic islets. Diabetes. 47:1027-1032.

Chen XB, Li YX, Jiao Y, Dong WP, Li G, Chen J and Tan JM. 2007. Influence of heme oxygenase-1 gene transfer on the viability and function of rat islets in *in vitro* culture. World J. Gastroenterol. 13:1053-1059.

Cooper DK, Gollackner B, Knosalla C and Teranishi K. 2002. Xenotransplantation-how far have we come? Transpl. Immunol. 9:251-256.

d'Apice AJ and Cowan PJ. 2008a. Gene-modified pigs. Xenotransplantation 15:87-90.

d'Apice AJ and Cowan PJ. 2008b. Xenotransplantation: The next generation of engineered animals. Transpl. Immunol. 21:111-5.

Emamaullee J, Liston P, Korneluk RG, Shapiro AM and Elliott JF. 2005. XIAP overexpression in islet beta-cells enhances engraftment and minimizes hypoxia-reperfusion injury. Am. J. Transplant. 5:1297-1305.

Evans JL, Goldfine ID, Maddux BA and Grodsky GM. 2003. Are oxidative stress-activated signaling pathways mediators of insulin resistance and beta-cell dysfunction? Diabetes. 52:1-8.

Gardner AM, Xu FH, Fady C, Jacoby FJ, Duffey DC, Tu Y and Lichtenstein A. 1997. Apoptotic vs. nonapoptotic cytotoxicity induced by hydrogen peroxide. Free. Radic. Biol. Med. 22:73-83.

Gossett R, Kier AB, Schroeder F, McConkey D, Fadok V and

Amoss MS, Jr. 1996. Cycloheximide-induced apoptosis in melanoma cells derived from regressing cutaneous tumours of Sinclair swine. J. Comp. Pathol. 115:353-372.

Hering BJ, Wijkstrom M, Graham ml, Hardstedt M, Aasheim TC, Jie T, Ansite JD, Nakano M, Cheng J, Li W, Moran K, Christians U, Finnegan C, Mills CD, Sutherland DE, Bansal-Pakala P, Murtaugh MP, Kirchhof N and Schuurman HJ. 2006. Prolonged diabetes reversal after intraportal xenotransplantation of wild-type porcine islets in immunosuppressed nonhuman primates. Nat. Med. 12:301-303.

Katori M, Busuttil RW and Kupiec-Weglinski JW. 2002. Heme oxygenase-1 system in organ transplantation. Transplantation 74:905-912.

Koo OJ, Jang G, Kwon DK, Kang JT, Kwon OS, Park HJ, Kang SK and Lee BC. 2008. Electrical activation induces reactive oxygen species in porcine embryos. Theriogenology 70:1111-1118.

Laumonier T, Yang S, Konig S, Chauveau C, Anegon I, Hoffmeyer P and Menetrey J. 2008. Lentivirus mediated HO-1 gene transfer enhances myogenic precursor cell survival after autologous transplantation in pig. Mol. Ther. 16:404-410.

Lepore DA, Shinkel TA, Fisicaro N, Mysore TB, Johnson LE, d'Apice AJ and Cowan PJ. 2004. Enhanced expression of glutathione peroxidase protects islet beta cells from hypoxia-reoxygenation. Xenotransplantation 11:53-59.

Li C and Jackson RM. 2002. Reactive species mechanisms of cellular hypoxia-reoxygenation injury. Am. J. Physiol. Cell. Physiol. 282:C227-241.

Li YX, Li G, Dong WP, Lu DR and Tan JM. 2006. Protection of human islets from induction of apoptosis and improved islet function with HO-1 gene transduction. Chin. Med. J. (Engl) 119:1639-1645.

Maines MD. 1997. The heme oxygenase system: a regulator of second messenger gases. Annu. Rev. Pharmacol. Toxicol. 37:517-554.

Maines MD. 2002. Heme oxygenase 1 transgenic mice as a model to study neuroprotection. Methods Enzymol. 353: 374-388.

Mysore TB, Shinkel TA, Collins J, Salvaris EJ, Fisicaro N, Murray-Segal LJ, Johnson LE, Lepore DA, Walters SN, Stokes R, Chandra AP, O'Connell PJ, d'Apice AJ and Cowan PJ. 2005. Overexpression of glutathione peroxidase with two isoforms of superoxide dismutase protects mouse islets from oxidative injury and improves islet graft function. Diabetes. 54:2109-2116.

Parr EL, Bowen KM and Lafferty KJ. 1980. Cellular changes in cultured mouse thyroid glands and islets of Langerhans. Transplantation 30:135-141.

Petrache I, Otterbein LE, Alam J, Wiegand GW and Choi AM. 2000. Heme oxygenase-1 inhibits TNF-alpha-induced apoptosis in cultured fibroblasts. Am. J. Physiol. Lung. Cell Mol. Physiol. 278:L312-319.

Pileggi A, Molano RD, Berney T, Cattan P, Vizzardelli C, Oliver R, Fraker C, Ricordi C, Pastori RL, Bach FH and Inverardi L. 2001. Heme oxygenase-1 induction in islet cells results in protection from apoptosis and improved *in vivo* function after transplantation. Diabetes. 50:1983-1991.

Rood PP, Buhler LH, Bottino R, Trucco M and Cooper DK. 2006. Pig-to-nonhuman primate islet xenotransplantation: A review of current problems. Cell Transplant. 15:89-104.

Stocker R. 1990. Induction of haem oxygenase as a defence against oxidative stress. Free. Radic. Res. Commun. 9:101-112.

Stocker R, Yamamoto Y, McDonagh AF, Glazer AN and Ames BN. 1987. Bilirubin is an antioxidant of possible physiological importance. Science 235:1043-1046.

Suzuki C, Yoshioka K, Sakatani M and Takahashi M. 2007. Glutamine and hypotaurine improves intracellular oxidative status and *in vitro* development of porcine preimplantation embryos. Zygote 15:317-324.

Tiedge M, Lortz S, Drinkgern J and Lenzen S. 1997. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. Diabetes. 46: 1733-1742.

Willis D, Moore AR, Frederick R and Willoughby DA. 1996. Heme oxygenase: A novel target for the modulation of the inflammatory response. Nat. Med. 2:87-90.

Yeom HJ, Koo OJ, Yang J, Cho B, Hwang JI, Park SJ, Hurh S, Kim H, Lee EM, Ro H, Kang JT, Kim SJ, Won JK, O'Connell PJ, Kim H, Surh CD, Lee BC and Ahn C. 2012. Generation and characterization of human heme oxygenase-1 transgenic pigs. PLoS. One 7:e46646.

Zhen-Wei X, Jian-Le S, Qi Q, Wen-Wei Z, Xue-Hong Z and Zi-Li Z. 2007. Heme oxygenase-1 improves the survival of discordant cardiac xenograft through its anti-inflammatory and anti-apoptotic effects. Pediatr. Transplant. 11:850-859.

Received December 21, 2015, Revised December 24, 2015, Accepted December 29, 2015