Augmentation of Thermotolerance in Primary Skin Fibroblasts from a Transgenic Pig Overexpressing the Porcine HSP70.2

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ABSTRACT : A high environmental temperature affects the economic performance of pigs. Heat shock protein 70 (HSP70) has been reported to participate importantly in thermotolerance. This study aims to produce transgenic pigs overexpressing porcine HSP70.2, the highly inducible one of HSP70 members, and to prove the cellular thermotolerance in the primary fibroblasts from the transgenics. A recombinant plasmid in which the sequence that encodes the porcine HSP70.2 gene is fused to green fluorescence protein (GFP) was constructed under the control of cytomegalovirus (CMV) enhancer and promoter. Two transgenic pigs were produced by microinjecting pCMV-HSP70-GFP DNA into the pronucleus of fertilized eggs. Immunoblot assay revealed the varied overexpression level (6.4% and 1.4%) of HSP70-GFP in transgenic pigs. After heating at 45°C for 3 h, the survival rate (78.1%) of the primary fibroblast cells from the highly expressing transgenic pig exceeded that from the non-transgenic pig (62.9%). This result showed that primary fibroblasts overexpressing HSP70-GFP confer cell thermotolerance. We suggest that transgenic pigs overexpressing HSP70 might improve their thermotolerance in summer and therefore reduce the economic loss in animal production. ***(Asian-Aust. J. Anim. Sci. 2005. Vol 18, No. 1 : 107-112)***

Key Words : Heat Shock Protein 70.2, Transgenic Pig, Thermotolerance

INTRODUCTION

The heat shock proteins (HSPs) have been extensively studied, especially with reference to their cellular localization, regulation, and functionality (Lindquist and Craig, 1988; Hightower, 1991; Welch, 1992; Morimoto et al., 1994; Benjamin and McMillan, 1998). Synthesis of the 70 kDa-HSP (HSP70) is increased by multiple stressors including heat treatment. The precise functions of individual member of HSP70 family have not been fully elucidated.

One of the first physiological functions associated with the stress-induced accumulation of the inducible HSP70 is acquired thermotolerance, which is defined as the ability of a cell or organism to become resistant to heat stress after a prior sublethal exposure to heat (Landry et al., 1982; Li and Werb, 1982; Mizzen and Welch, 1988; Moseley, 1997; Kregel et al., 2002). Acquired thermotolerance is transient and depends mainly on the severity of the initial heat stress. Advances in molecular biological techniques have provided researchers with tools to address more directly the causal link between HSP induction and thermotolerance. Cells overexpressing certain HSP70s have been demonstrated to increase their survivability to heat (Landry and Chretien, 1983; Lewis and Pelham, 1985; Johnston and Kucey, 1988; Riabowol et al., 1988). For example, transfection of a plasmid that contains the *Drosophila* HSP70 gene into a monkey fibroblast cell line greatly increased in HSP70 accumulation in these cells and increased tolerance to a heat shock paradigm (Lewis and Pelham, 1985).

Although the detail mechanisms for improving cellular thermotolerance in association with an increase in HSP levels have not been described, proteins in the HSP70 family can be postulated to help to prevent protein denaturation and/or the processing of denatured proteins and protein fragments that are produced by stressors such as hyperthermia (Hightower 1991; Li et al., 1991). The synthesis of HSP70 in mice liver was promoted by hyperthermia preconditioning more than in the control animals. Porcine HSP70.2, the highly inducible member of HSP70 gene family, has been well documented (Schwerin et al., 1999; Schwerin et al., 2001; Chen et al., 2003). Its function has not been reported. Moreover, thermotolerance in large transgenic animals such as swine has not been investigated. This study reports that transgenic pigs carrying porcine HSP70.2 were generated and their primary fibroblasts confer cell thermotolerance.

MATERIALS AND METHODS

Generation of transgenic pigs

The procedures for generating transgenic pigs were those described in a previous report (Tu et al., 1999). Transgenic pigs were generated using a chimeric transgene pCMV-HSP70-GFP that consisted of a porcine HSP70.2 gene (Genbank accession no. AY466608) inserted into the

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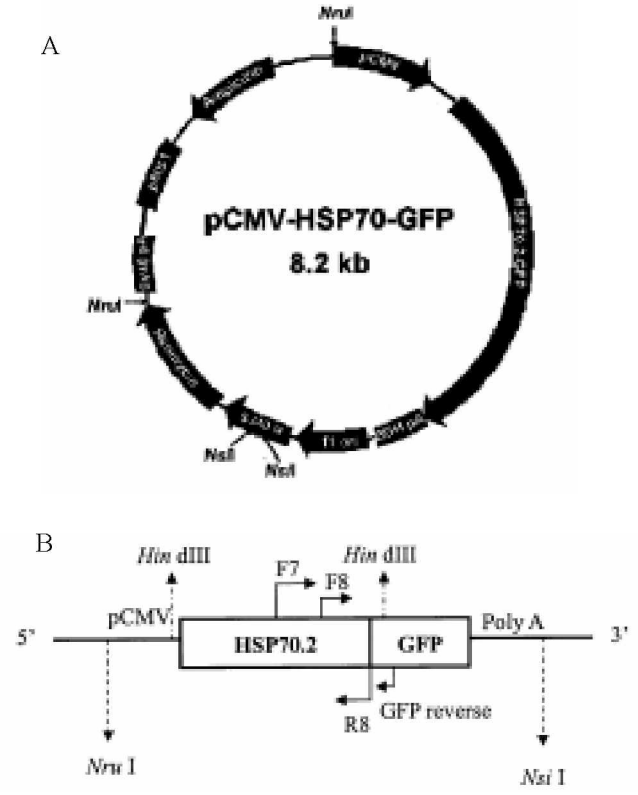

Figure 1. Physical map of the pCMV-HSP70-GFP transgene. A. The coding region of the porcine inducible HSP70.2 gene and GFP gene is under the control of the human CMV immediate-early enhancer and promoter (pCMV). The chimeric gene is followed by the SV40 polyadenylation signal. The *Nru*I to *Nsi*I fragment was cutting the coding region used to generate transgenic pigs. B. For Southern blot analysis, *Hin*dIII enzyme was used to derive a 2.1 kb fragment. The primer pair F7-R8 was used to obtained a 0.9 kb fragment served as the probe. For indentification of transgenic pigs, primer pair F8-GFP reverse was used to detect a 0.6 kb fragment as the transgene-specific sequence.

vector pcDNA3.1/CT-GFP (Invitrogen, Carlsbad, CA). The pcDNA3.1/CT-GFP construct places the porcine HSP70.2 gene under the control of the human cytomegalovirus promoter (pCMV) and a reporter GFP gene in carboxyl-terminus (Figure 1). The 4.2 kb-fragment containing transgene HSP70-GFP was cut from the plasmid by *Nru*I and *Nsi*I digestion, purified and used to generate transgenic pigs. The linear DNA preparation was diluted in TE buffer (10 mM Tris-HCl, 0.1 mM EDTA, pH 7.4) to 2 ng/μl. DNA fragments were microinjected into pronuclei of fertilized eggs, producing transgenic pigs. Prepubertal pure breed Duroc and Landrace gilts were used as embryo donors and recipients, respectively. The genomic DNA of transgenic positive pigs was screened by PCR with primers F8: 5'-GAC GCC AAC GGC ATC CTG AAC-3' and GFP-reverse: 5'-TAG AAG GCA CAG TCG AGG-3'. The PCR reaction was conducted as follow: 95°C, 1 min; 58°C, 1 min; 72°C, 1 min, for 40 cycles.

Southern blot analysis

Genomic DNA was isolated from porcine tail and digested with *Hin*dIII enzyme. Restriction digestions were carried out for 4 h in 50 μl containing 5 μg of genomic DNA and 40 units of enzymes in the recommended buffer supplemented with 4 mM spermidine. The fragments were separated on 0.7% agarose gels and were then transferred to a nylon membrane. The nylon membrane was baked at 80°C under vacuum for 2 h before hybridization. DNA probe was synthesized by PCR reaction with primers F7: 5'-GCG ACG CGA AGC TGG ACA AG-3' and R8: 5'-CCA CCT CCT CGA TGG GG-3' and then was radiolabeled to a specific activity of $2\text{-}3\times10^8$ cpm/μg with [^{32}P]deoxy-CTP by the random primer procedure. Nylon membrane were prehybridized for 1.5 h at 68°C in 5×SSC, 5×Denhardt's reagent, and 250 μg/ml denatured salmon DNA and the for 3 h in 5×SSC, 5×Denhardt's reagent, 10% dextran sulfate, 250 μg/ml denatured salmon DNA, and 0.1% SDS. ^{32}P-Labeled probe at 3×10^6 cpm/ml was added, and the incubation was continued overnight. At the end of hybridization, the nylon membrane was washed four times with 50 mM Tris, 1 mM EDTA, 1×Denhardt's solution, 0.1% SDS, and 0.1% sodium pyrophosphate (pH 8.0) over a 1 h period. The nylon membrane was dried briefly in a vacuum oven and exposed to X-ray film. Hybridization signals were detected on a Typhoon 9410 Variable Mode Imager (Amersham, Piscataway, NJ) and quantified with a Molecular Dynamics PhosphorImager. The transgene copy number was determined by comparison with amount of the restriction enzyme fragment derived from endogenous HSP70.2.

Expression of porcine HSP70 in transgenic pigs

Sample preparation used in gel electrophoresis and immunoblot analysis was conducted as described previously (Lee et al., 1996). Tails from 3 d-old piglets were thawed and sliced into pieces, each weighing around 0.2 g. In each preparation, 1 ml of homogenization buffer containing protease inhibitors (20 μg pepstatin, 20 μg leupeptin, 40 μg Pefabloc; Boehringer Mannheim, Indianapolis, Ind.), 10% sucrose, and 50 mM Tris-HCl, pH 7.5, was added to the tubes (15×100 mm) that contained a tissue sample. The tissues were homogenized using a polytron (Kinematica, Littau, Switzerland) at 20,000 rpm for 3 min at on ice. The crude homogenates were centrifuged at 12,000 rpm for 5 min at 4°C. The supernatant was lysed in a sample buffer (62.6 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.002% bromophenol blue). The concentration of protein was determined by the Lowry method (1951) using BSA as a standard.

The samples for one-dimensional SDS-PAGE were

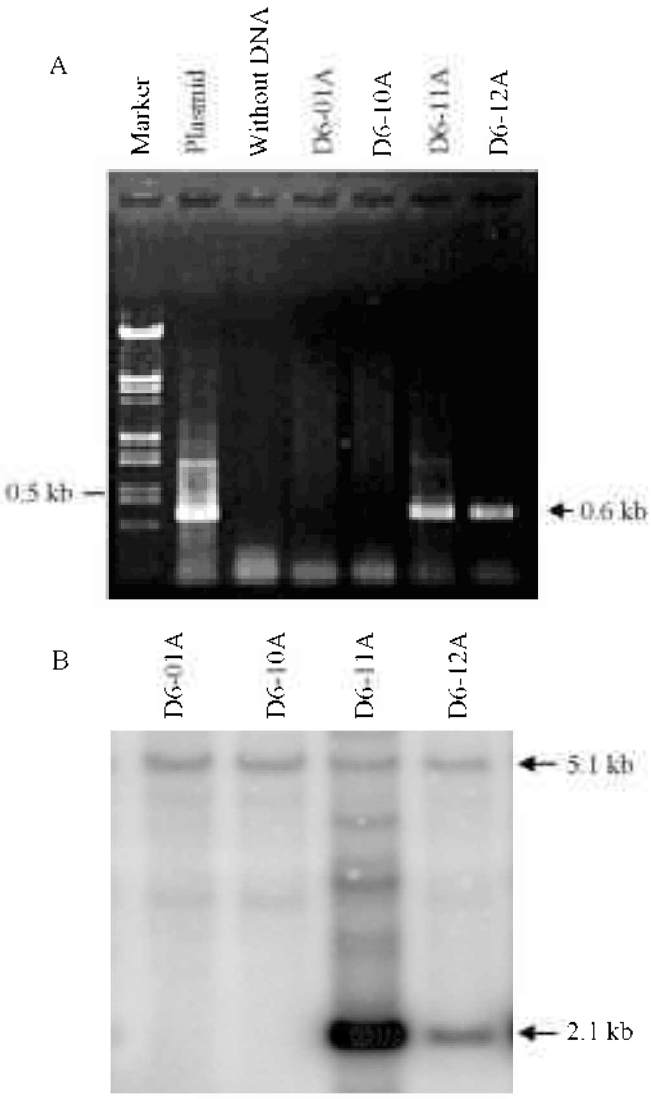

Figure 2. A: PCR analysis of genomic DNA for identifying in transgenic pigs. B: A representative Southern blot for determining the transgene copy number. Porcine genomic DNA was digested with *Hin*dIII enzyme from four founder transgenic pigs. The probe used in this study encodes the porcine HSP70.2 (shown also in Figure 1B). When hybridized with porcine genomic DNA at high stringency, the probe detected DNA fragments from endogenous (5.1 kb) and the integrated HSP70.2 (2.1 kb).

heated in boiling water for 5 min and then microfuged for 3 min before loading. 100 μg of proteins were applied to 9% SDS-polyacrylamide gel. After electrophoresis, the gel was soaked in transfer buffer (50 mM Tris-borate, pH 8.3, 1 mM EDTA) for 10 min. Resolved proteins were then electro-transferred onto a nitrocellulose membrane by a semi-dry method. The membrane was incubated for 1 h with 3% gelatin in Tween 20 that contained Tris-buffered saline (TTBS: 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 0.05% Tween 20) and then rinsed with TTBS. Subsequently, the membrane was incubated with anti-HSP70 polyclonal antibody (SPA-812, Stressgene; diluted 1:2,000 in TTBS that contained 1% gelatin) or with anti-GFP monoclonal antibody (Invitrogen; diluted 1:2,000) at room temperature for 1 h. After three washes with TTBS, the membranes were reacted with goat anti-rabbit antibody conjugated with alkaline phosphatase (Sigma; diluted 1:2,000 in TTBS containing 1% gelatin) at room temperature for 30 min. The membrane was then rinsed three times with TTBS and developed at room temperature in a developing buffer (15 mg of nitro blue tetrazolium, 0.7% N, N-dimethylformamide, 30 mg of 5-bromo-4-chloro-3-indolyl phosphate per 100 ml, 1 mM $MgCl_2$, and 100 mM $NaHCO_3$, pH 9.8). Quantitation of the bands on the membrane recognized by antibodies was performed (Huang et al., 1999).

Expression of porcine HSP70.2 in primary fibroblast cells confers thermal resistance

Primary fibroblasts isolated from the ears of the 7 mo-old transgenic pigs were cultured in Dulbecco's modified medium for the assay of thermotolerance. Briefly, after dispase treatment at 37°C for 2 h, the ear tissues were then immersed in 0.1% collagenase at 37°C for another 2 h. The cells released to the suspension were collected by centrifuging at 800 rpm for 10 min and were subsequently cultured on 25 cm^2 flask.

The primary fibroblast cells were grown to 90% confluent and then treated with a lethal dose of heat shock at 45°C for 3 h in a water bath. Thereafter, the media were removed to a centrifuge tube and the cells released to the media were collected by centrifuging at 800 rpm for 10 min. The cells still attached on the tissue culture plates were combined with those released to media as described above. The cell viability was assessed by trypan blue exclusion. Data were analyzed according to analysis of variance (ANOVA) procedure and means were compared using Tukey's Studentized Range (HSD) Test (SAS Institute, 1989). A value of $p<0.05$ was considered statistically significant different.

RESULTS

By pronuclear microinjection, 188 fertilized eggs containing the restriction fragment (4.2 kb) derived from pCMV-HSP70-GFP were impregnated to 8 foster sows. To these transgene recipients of porcine HSP70.2, 4 sows were delivered and a total of 12 piglets obtained. Analysis of DNA prepared from tail biopsy specimens showed that 2 generation zero (F0, founder) pigs had integrated the transgene. The integration of genomic HSP70.2 was evident by PCR product of 0.6 kb fragment (Figure 1B and 2A). The percentage of pronuclear injected embryos that developed into transgenic animals is 1.1% and the transgenic rate is 16.7%.

Table 1. Copy number of HSP70-GFP transgene and cell survivability of skin fibroblasts in four founder pigs after heat shock

Founder pigs	D6-01A	D6-10A	D6-11A	D6-12A
Copy number	0	0	60	12
Cell survivability (%)	62.9±4.5[b]	ND	78.1±1.9[a]	60.9±1.5[b]

[a, b] Means within the same row with different superscript differ significantly ($p<0.05$). ND: not determined.

Figure 3. Western blot analysis for verifying transgene's expression. Four protein samples prepared from tail clips of the animals, which separated by SDS-PAGE and transferred to nitrocellulose membrane. Subsequently, the membrane was incubated with anti-HSP70 (A) or anti-GFP (B) antibody and then the membrane were reacted with secondary antibody conjugated with alkaline phosphatase and developed.

Copy number for the transgene integrated into the animal's genome was determined by Southern blot analysis (Figure 2B). The restriction enzyme *Hin*dIII is selected according to a restriction map of a sequence from Genbank (accession no. AL773725). It has a 100% sequence identity to accession no. AY466608. Cleavage of genomic DNA with *Hin*dIII released two internal fragments of 5.1 and 2.1 kb for endogenous and transgene HSP70-GFP, respectively. They were revealed with a probe specific for the HSP70.2 structure gene (Figure 1B), and thus both genes were detected simultaneously. By comparing DNA band intensities from tail biopsy specimens with known copies of endogenous HSP70.2, a varied copy number of 12 and 60 from the transgene was detected in the two lines (Figure 2B and Table 1).

Immunoblotting was performed using the antibodies against HSP70 or GFP which detected the fused 97 kD HSP70-GFP protein in the tail as shown in Figure 3. When compared to the endogenous HSP70 (inducible form of HSP70), expression levels of the HSP70-GFP was 1.4 and 6.4% in the transgenic lines D6-11A and D6-12A, respectively. There might be an association between transgene copy number and the expressed protein level, both of which in D6-11A are five times than that in D6-12A.

Figure 4 shows the survival rate of primary fibroblasts under heat stress at 45°C for 3 h. Up to 78.1±1.9% of cells from transgenic animal D6-11A that overexpresses HSP70-GFP survive after heat stress, whereas 62.9±4.5% and 60.9±1.5% of cells from non-transgenic animal D6-01A and from transgenic D6-12A, respectively, survive after treatment ($p<0.05$). This result presents clear evidence that

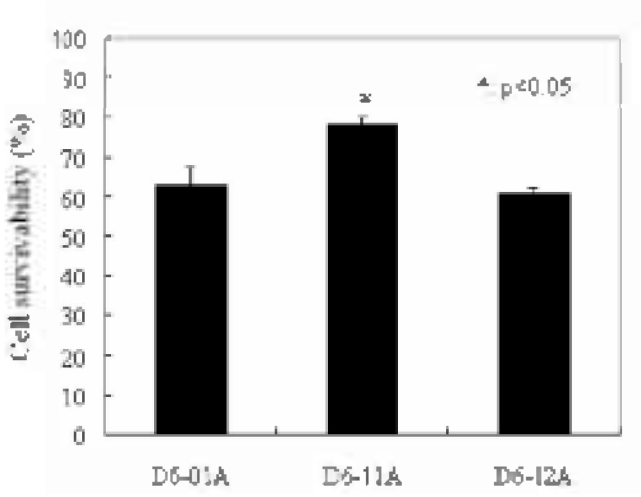

Figure 4. Cell survival after heat stress. Primary fibroblasts isolated from the ears of transgenic pigs were cultured in Dulbecco's modified medium for the assay of thermotolerance. The cell viability was assessed by trypan blue exclusion after heat treatment at 45°C for 3 h. Data were analyzed according to ANOVA procedure and means were compared using Tukey's Studentized Range (HSD) Test. A value of $p<0.05$ was considered statistically significant different.

the overexpressed HSP70-GFP protein (expression level not shown) protect of cells from a lethal heat treatment *in vitro*.

DISCUSSION

According to many studies, the percentage of pronuclear injected embryos that can develop into transgenic animals varied from 0.3% to 4.0% for pigs (Lee and Piedrahita, 2003). In this study, the percentage of successful rate of transgenesis for embryo is 1.1% and it suggested that the microinjection itself maybe cause some degree of embryo lethality or the insertional inactivation and/or positional effects of the transgene.

HSPs are present in both prokaryotic and eukaryotic cells, and their high degree of conservation suggests that they participate importantly in fundamental cell processes. However, their tendency to be conserved across species, coupled with their importance in cell survival under various conditions, implies that these HSPs are essential in both normal cellular function and survival following stress. A plenty of studies have documented thermotolerance in cultured cells and in animals other than mammals (Laszlo and Li, 1985; Mizzen and Welch, 1988; Ulmasov et al., 1992; Kampinga, 1993; Mosely, 1997; Theodorakis et al., 1999). Presently, few studies revealed that thermotolerance in mice can be induced by whole body hyperthermia (Kapp and Lord, 1983; Weshler et al., 1984; King et al., 2002). Moreover, the correlation between myocardial injury due to ischemia and the expression of inducible HSP70 under the

control of β-actin promoter in transgenic mice has been reported (Marber et al., 1995; Hutter et al., 1996; Trost et al., 1998). Mirkes et al. (1999) showed that protection of embryo-lethal from heat was associated with HSP70 level. In this study, we have showed that expression of HSP70 in transgenic pig protects primary fibroblast from hyperthermia, as evidenced by the observation of increase in viability among transgenic cells (Figure 4).

The expression of the transfected gene was transient and may not yield an accurate picture of HSP70 under an equilibrium condition (Munro and Pelham, 1984; Milarski and Morimoto, 1989). However, stably transfected cell lines that express human HSP70 yield a steady-state condition for studying cellular targeting and physiological functions of HSP70 at 37°C, following heat shock or during the development of thermotolerance. Primary fibroblasts from transgenic pigs can express the HSP70 transgene constitutively, and survival rate following hyperthermia was considerably higher than that of the non-transgenic animal. The result suggested that the primary fibroblasts might be a material to detect thermotolerant pigs rapidly.

We have previously showed that synthesis of HSP70 in liver induced by whole body hyperthermia is associated with the survival in mice and the survival rate (88.2%) of animals subjected to preconditioning markedly exceeded that of the control group (37.5%) (King et al., 2002). Because of failure to express detectable level of HSP70, we could not demonstrate the protein function in HSP70 transgenic mice driven by CMV promoter (the same construct used in this study). However, in another study, this porcine HSP70.2 transgene conferred an anti-heat stroke function to the transgenic mice whose gene expression was driven by a β-actin promoter (Lee et al., submitted). This species-specificity on promoter function had been documented in literatures (Aiba-Masago et al., 1999; Grzimek et al., 1999; Xu et al., 2001). After established a protocol for assessing thermosensitivity of livestock animals, we might have the opportunity to present data to support the thermotolerant phenotype of this transgenic pig. The finding could have significant agricultural implications, suggesting that transgenic pigs overexpressing HSP70 might improve thermotolerance and therefore reduce the economic lost especially in tropical area.

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