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Induction of KLF4 in response to heat stress

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Abstract Krüppel-like factor 4 (KLF4) is an evolutionarily conserved zinc finger–containing transcription factor with diverse regulatory functions in cell growth, proliferation, differentiation, and embryogenesis. However, little is known about the response of KLF4 to heat stress. In this study, Western blot and reverse transcriptase–polymerase chain reaction were performed to determine the changes in KLF4 expression in response to heat stress. The results showed that heat stress up-regulated KLF4 messenger RNA and protein levels in a time-dependent manner in vivo and in 4 cell lines. Moreover, a study with heat shock transcription factor 1 (Hsf1) gene knockout mice indicated that the induction of KLF4 in response to heat stress was mediated by Hsf1. This process occurred rapidly, indicating that KLF4 is an immediate early response gene of heat stress. Next, the roles of KLF4 under heat stress conditions were analyzed for cells overexpressing or deficient in KLF4. The results showed overexpression of KLF4 increased the death rate of C_2C_{12} cells, whereas KLF4 deficiency decreased the injury of C_2C_{12} cells from heat stress conditions, suggesting that KLF4 might play an important role in cell injury induced by heat stress. KLF4 might be an immediate early response gene and could play an important role in cell injury induced by heat stress.

INTRODUCTION

It is well known that heat stress results in finely tuned alterations in gene expression, enabling the cell to react in an appropriate manner via regulation of cell growth and division, differentiation, and metabolism. Heat stimuli can recruit active transcription factor complexes to upstream regulatory sequences of immediate early (IE) genes and initiate transcription by RNA polymerase II (Moggs and Orphanides 2003). IE genes encode a range of proteins that include transcription factors and cytokines, and these in turn regulate secondary transcriptional responses appropriate for the particular stimulus to which a cell is exposed. Cellular responses to stress involve a number of processes aimed at redressing the balance of homeostasis, and a component of this response is mediated via the expression of IE genes. IE genes, such as a family of stress-induced protein genes called heat shock proteins (Hsps), usually result in repair of damaged proteins and survival of the cell, mainly through the chaperone function of Hsps (Pirkkala et al 2001). The inducible synthesis of Hsps is known to be tightly controlled by heat shock transcription factor 1 (Hsf1) in mammalian organisms (McMillan et al 1998; Xiao et al 1999). Heat stress results in the activation of Hsf1 through a process that includes trimerization and nuclear translocation. Activated Hsf1 binds to heat shock elements (HSEs) in Hsp gene promoters and induces expression of Hsps (Wu 1995; Morimoto 1998; Morano and Thiele 1999). Recent studies indicate that in addition to being a transcriptional activator of Hsps, Hsf1 functions as a regulator of a number of other IE genes, including *iNOS* (inducible nitric oxide synthase; Goldring et al 2000), *c-fos* (Chen et al 1997), and *IL-6* (interleukin 6; Inouye et al 2004).

Krüppel-like factor 4 (KLF4), also known as gut-enriched Krüppel-like factor, is a eukaryotic zinc finger protein (Garrett-Sinha et al 1996; Shields et al 1996; Yet et al 1998) that has 3 carboxy-terminal zinc fingers with a high degree of homology to the tissue-specific transcription factors KLF1 (erythroid Krüppel-like factor), KLF2 (lung

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Krüppel-like factor), and KLF3 (basic Krüppel-like factor) (Miller and Bieker 1993; Anderson et al 1995; Crossley et al 1996). The amino terminus of KLF4 possesses multiple proline and serine residues consisting of transcriptional activation domains. KLF4 might play an important role in the regulation of cell growth and differentiation (Shields et al 1996; Shie et al 2000a, 2000b). Whether KLF4 is a potential immediate early response gene under heat stress conditions has not been investigated.

We undertook this study to examine the expression of KLF4 under heat stress conditions in male Kunming mouse tissues and cultured cells. Moreover, Hsf1 gene knockout mice were used to determine whether the induction of KLF4 in response to heat stress was mediated by Hsf1. The effect of KLF4 overexpression or inhibition on proliferation and apoptosis of murine C_2C_{12} cells under heat stress was investigated as well.

MATERIALS AND METHODS

Antibodies

The following antibodies were used: mouse anti-Hsp70 monoclonal antibody (Stressgen, Ann Arbor, MI, USA), rabbit anti-KLF4 polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse anti–glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) monoclonal antibody (Sigma, St. Louis, MO, USA), and peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G (IgG; Boster Biological Technology, Wuhan, China).

Animal and whole-body hyperthermic challenge

Adult male Kunming mice were purchased from the Animal Center of Central South University (Changsha, Hunan, China). Hsf1 gene knockout mice were from Dr Benjamin (the University of Utah, Salt Lake City, Utah, USA). The animals were treated with a sublethal heat shock by increasing the rectal temperature to 42°C for 15 minutes with a 250A infrared heat lamp and then returned to their cages for recovery. Anesthetized control mice were kept under similar conditions without heat shock, and their body temperatures were maintained at 37°C ± 0.5°C. Mice in both experimental groups were administered 0.2 mL of 0.9% sodium chloride intraperitoneally to compensate for water loss during the procedure and recovery periods. All animal procedures were approved by the Animal Care and Use Committee of Central South University.

Cell culture and heat shock treatment

Murine C_2C_{12} myogenic cells, murine RAW264.7 macrophages, human U-251 cells, and rat H_9C_2 cardiac myocytes were routinely grown at 37°C in Dulbeccos modified Eagles medium (DMEM) and 10% fetal calf serum in a humidified atmosphere with 5% CO_2. For heat shock experiments, actively growing cells were fed with medium preincubated at 42°C and transferred to a 42°C preset incubator for 1 hour, whereas control cells were maintained at 37°C.

Western blot analysis

Cells were lysed in B-buffer containing 10 mM Tris-HCl, pH 7.4; 150 mM NaCl; 1 mM ethylenediaminetetraacetic acid, pH 8.0; and 1% sodium dodecyl sulfate (SDS). Proteins were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto Protran nitrocellulose membranes (Schleicher & Schuell). The membranes were blocked overnight in phosphate-buffered saline (PBS), containing 10% nonfat dry milk and 0.5% Tween-20, and incubated with primary antibodies for 2 hours. Horseradish peroxidase–conjugated anti-rabbit or anti-mouse IgG was used as the secondary antibody. The immunoreactive bands were visualized with the use of diaminobenzidine (Boster Biological Technology). Anti-GAPDH antibody was used to normalize for equal amounts of proteins and calculate the relative induction ratio.

RNA extraction, reverse transcriptase–polymerase chain reaction, and Northern blot analyses

Total RNA was extracted with the TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. Dilutions corresponding to 1 μg of total RNA were reverse transcribed (Invitrogen Life Technologies Inc, Carlsbad, CA, USA), and reverse transcriptase–polymerase chain reaction (RT-PCR) was performed with the use of the iCycler Apparatus (Biometra, Goettingen, Germany). For PCR amplification, the following were used: GAPDH forward (5′-AAG CCC ATC ACC ATC TTC CA-3′) and reverse (5′-CCT GCT TCA CCA CCT TCT TG-3′) primers and KLF4 forward (5′-GCG GGA AGG GAG AAG ACA C-3′) and reverse (5′-GGG GAA GAC GAG GAT GAA GC-3′) primers. Total RNA (20 μg/lane) was fractionated on a formaldehyde 1% agarose gel and transferred on Hybond N nylon strips (Amersham Biosciences, Uppsala, Sweden); 18S ribosomal RNA (rRNA) was used as equal loading control.

Generation of constructs

Oligonucleotide primers were designed to amplify the full length of the mouse KLF4 complementary DNA (cDNA) coding sequence, yielding a 1.5-kb PCR product. The KLF4 forward (5′-AGTTGGACCCAGTATACATTCC

GCCACAGCAGCCT-3′) and reverse (5′-TTAAAAGTGC CTCTTCATGTGTAAGGCAAGGTGGT-3′) oligonucleotide primer sequences were used. The cycling condition was 30 seconds at 95°C, 30 seconds at 62°C, and 1 minute at 72 °C for 35 cycles. The PCR product was electrophoresed onto 0.9% agarose, and a 1.5-kb fragment was purified with the purification system (Qiagen, KJVenlo, Netherlands). The fragment was then inserted into the pcDNA3.1 vector (Strategene, La Jolla, CA, USA) at the site and sequenced commercially (TaKaRa, Otsu, Shiga, Japan).

Lipofectamine-mediated gene transfection

Transfection of C_2C_{12} myogenic cells was carried out according the manufacturer's instructions (lipofectamine 2000™, Invitrogen). Briefly, about 5×10^5 cells per bottle containing 5 mL of appropriate complete growth medium were seeded and incubated at 37°C with 5% CO_2 until the cells were 70% to 80% confluent (24 hours). After the cells were rinsed with serum-free and antibiotics-free medium, the cells were transfected separately with 10 μg pcDNA3.1-KLF4 per 20 μL lipofectamine (experimental group) or 10 μg pcDNA3.1 per 20 μL lipofectamine (vector control), followed by incubation at 37°C in a CO_2 incubator for 6 hours. The medium was then replaced with DMEM culture medium containing 20% fetal bovine serum. After 48 hours of recovery, G418 (Gibco/BRL, Gaithersburg, MD, USA) was added at 1,000 μg/mL for 20 days, when colonies were picked and expanded under G418 selection.

Loss of function assay with morpholino oligonucleotides

A KLF4 morpholino antisense oligonucleotide (Liu et al 2003) was designed to target the initiation site for KLF4 translation (KLF4-AS, agactcgccaggtggctgcctcatt) and was synthesized commercially (Invitrogen). Morpholino oligonucleotides were transfected into C_2C_{12} myogenic cells with lipofectamine according to the manufacturer's instructions (Invitrogen) 24 hours after plating. The specificity of the antisense oligonucleotide was validated by employing a control oligonucleotide (KLF4-Ctrl, ttactccg tcggtggaccgctcaga) and a group treated only with lipofectamine (Lipo). Cell samples were collected 48 hours after transfection with Trizol reagent (Invitrogen) according to the manufacturer's instructions.

Detection of cellular viability

Murine C_2C_{12} cells were plated at a density of 10^4 cells/well on 96-well plates in 100 μL of DMEM. Cell viability was determined by the conventional MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) reduction assay. The cells of each microwell were incubated with 20 μL of 0.5% MTT for 2 hours at 37°C, and the reaction was stopped by adding 150 μL of dimethyl sulf oxide. The amount of MTT formazan product was determined by measuring absorbance with a microplate reader (Bio-Rad, Hercules, CA, USA) at a test wavelength of 570 nm and a reference wavelength of 630 nm.

Analysis of apoptosis by flow cytometry

The apoptosis rate was measured by flow cytometry. Briefly, C_2C_{12} cells were washed with PBS (pH 7.4), fixed in cold 70% (v/v) ethanol, and incubated at −20°C for at least 2 hours. The fixed cells were harvested by centrifugation at $250 \times g$ for 5 minutes. The cell pellets were resuspended in 1 mL of PBS at room temperature for 10 minutes, recentrifuged, resuspended in 500 μL of PBS containing 0.2 g/L RNase A, and incubated at 37°C for 30 minutes. After incubation, the cells were stained with 20 g/L propidium iodide at 4°C for 30 minutes. The fluorescence of cells was measured with FACScalibur flow cytometer (BD BioSciences, San Jose, CA, USA). The relative content of DNA indicated the distribution of a population of cells throughout the cell cycle. Apoptotic cells caused the appearance of a subdiploid peak in the cell cycle profile. The percentage of apoptotic cells was determined by BD CellQuest software (BD BioSciences).

Statistical analysis

Data in the figures and text were expressed as the mean ± SEM. Each experiment was performed at least 3 times, and statistical analysis was performed with a 2-tailed Student's *t*-test or Fisher's least significant difference test. Otherwise, representative data were shown. $P < 0.05$ was considered significant.

RESULTS

Expression of inducible Hsp70 protein in mouse tissues under heat stress conditions

To determine whether the hyperthermia treatment induced Hsp70 accumulation, inducible Hsp70 protein was detected in heart, lung, brain, kidney, and skeletal muscle of mice after 12 hours of recovery after the heat treatment (42°C, 15 minutes). As shown in Figure 1, after sublethal hyperthermia, Hsp70 protein expression was significantly up-regulated in all 5 organs compared with controls. These results are consistent with our previous study (Xiao et al 1999). These results indicate that the heat-induced stress response is present in mouse heart, lung, brain, kidney, and skeletal muscle tissues.

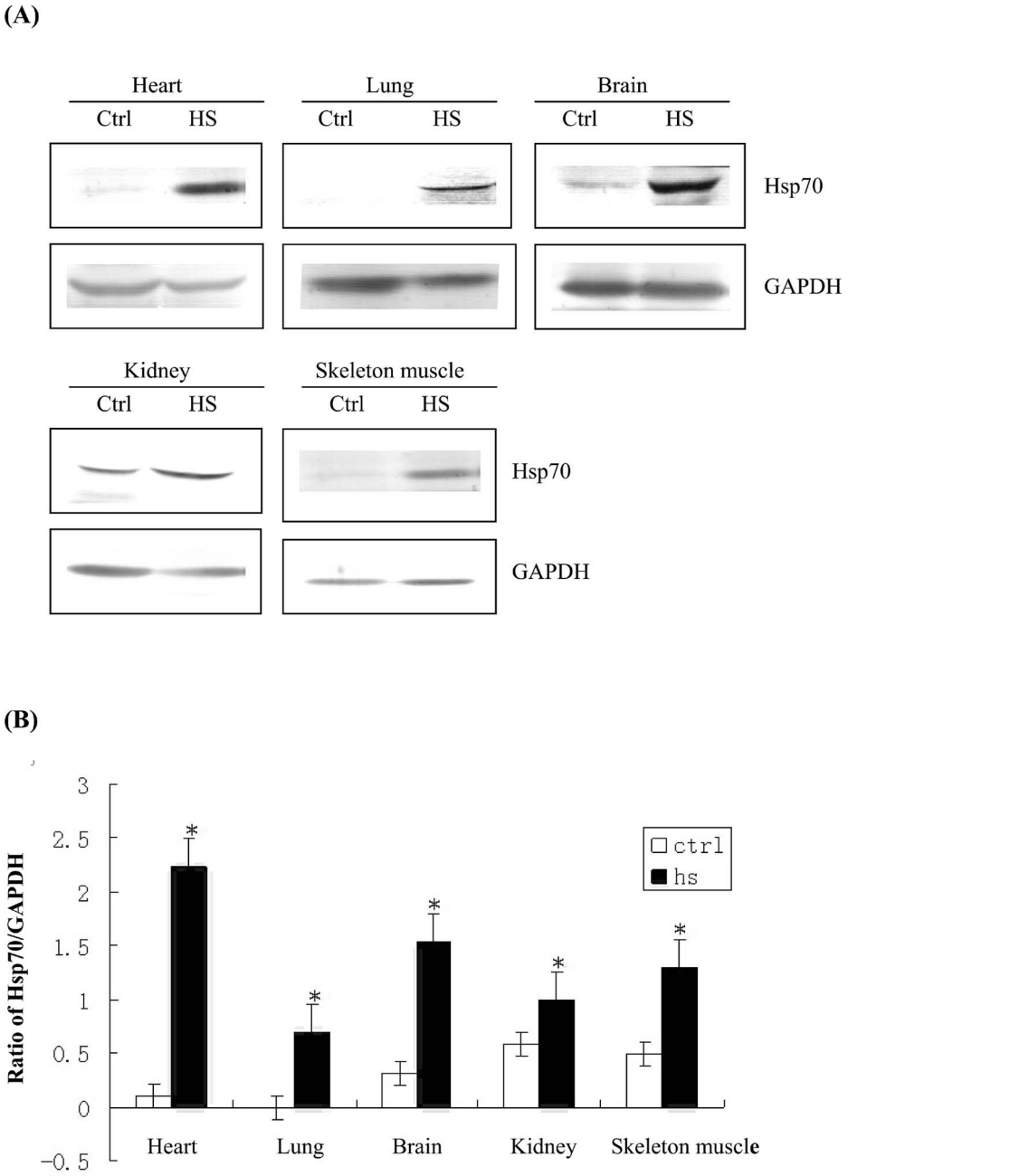

Fig 1. Effects of heat stress on the expression of Hsp70 protein in mouse tissues. (A) Kunming mice were treated with a sublethal heat stress (rectal temperature at 42°C for 15 minutes) followed by 3 hours of recovery. Next, total protein samples of heart, lung, brain, kidney, and skeletal muscle were extracted. The expression of Hsp70 protein was determined by Western blot. GAPDH was used as an internal control. Blot shown is representative of 3 experiments with similar results. (B) Shown in the histogram are the mean ± SEM of 3 independent experiments. * $P < 0.05$ compared with the control group (Ctrl).

Expression of KLF4 protein in mouse tissues under heat stress conditions

To determine whether KLF4 protein expression increased in response to the hyperthermia treatment, Western blot analysis was used to detect the level of KLF4 protein in several organs of adult mice under normal conditions and when exposed to heat stress (Fig 2). When stimulated by heat stress (42°C, 15 minutes), a significant increase in KLF4 protein expression occurred in all 5 organs examined (Fig 2A) compared with controls. A low level of KLF4 protein was mea-

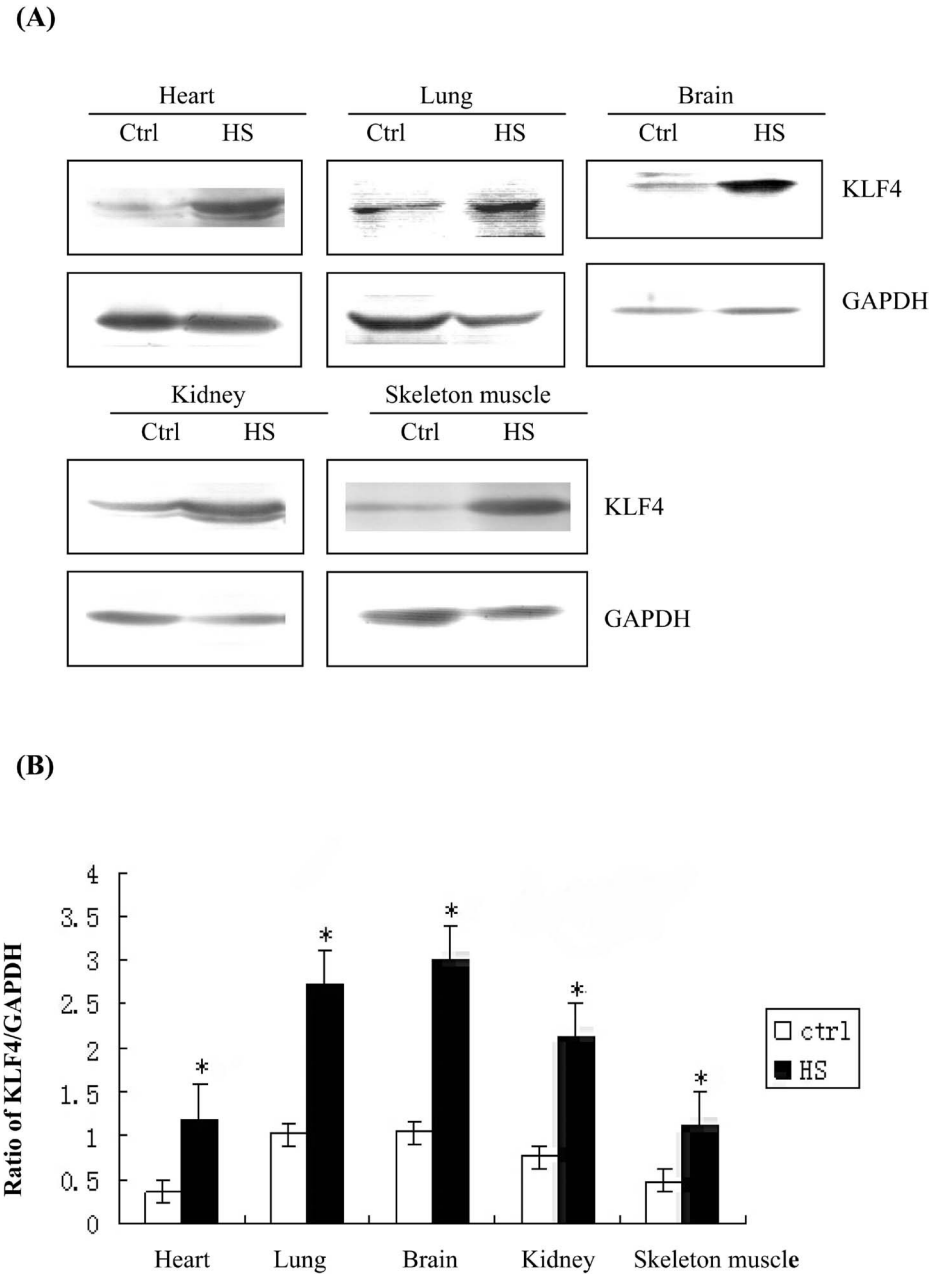

Fig 2. Effects of heat stress on the expression of KLF4 protein in mouse tissues. (A) Kunming mice were treated with a sublethal heat stress (rectal temperature at 42°C for 15 minutes) followed by 3 hours of recovery. Total protein samples of heart, lung, brain, kidney, and skeletal muscle were extracted. The expression of KLF4 protein was determined by Western blot. GAPDH was used as an internal control. Blot shown is representative of 3 experiments with similar results. (B) Shown in the histogram are the mean ± SEM of 3 independent experiments. * $P < 0.05$ compared with the control group.

sured in the heart, lung, brain, kidney, and skeletal muscle under normal conditions. The increase of KLF4 protein was most significant in the brain, and its level was increased about 3-fold compared with controls. Skeletal muscle had the lowest increase of KLF4 protein, with only about a 2-fold increase in expression relative to controls (Fig 2B).

Up-regulation of KLF4 protein in cultured cells under heat stress conditions

In this study, murine Raw264.7 macrophages, murine C_2C_{12} myocytes from skeletal muscle, rat H_9C_2 cardiac myocytes, and human U-251 astrocytic tumor cells were challenged with heat stress (43°C, 1 hour) then analyzed by Western blot to measure KLF4 protein levels (Fig 3). The results

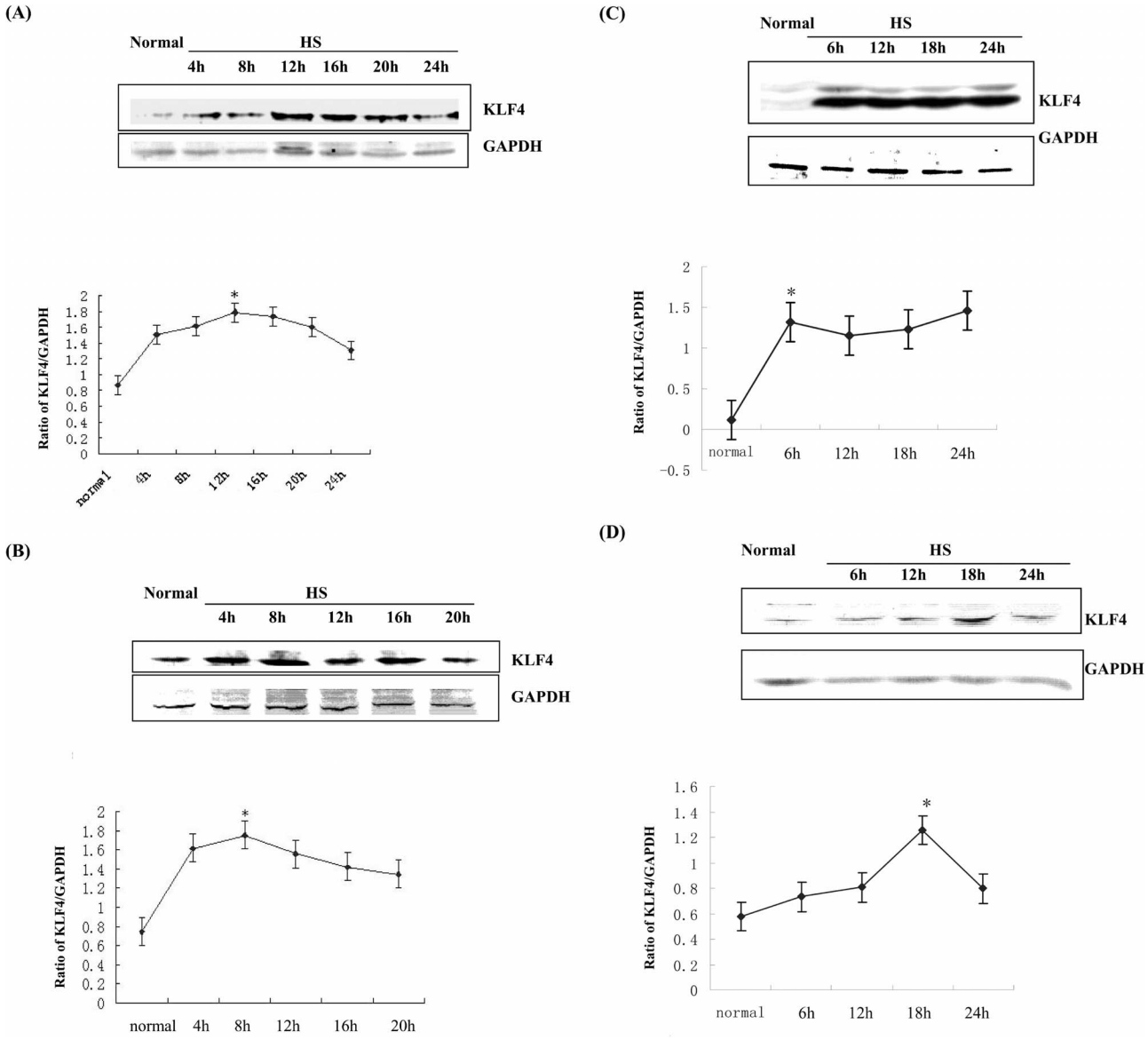

Fig 3. Kinetics of KLF4 protein accumulation in cultured cells challenged by heat stress. Cultured cells were challenged by a sublethal heat stress (43°C for 30 minutes), and samples were obtained for a time course. The expression of KLF4 protein was determined by Western blot. GAPDH was used as an internal control. Blot shown is representative of 3 experiments with similar results. Shown in the top panel is a representative Western blot, and shown in the bottom panel are the mean ± SEM of 3 independent experiments. * $P < 0.05$ compared with the normal group. (A) Changes of KLF4 protein expression in murine Raw264.7 cells after heat stress. (B) Changes of KLF4 protein expression in murine C_2C_{12} myogenic cells from skeletal muscle after heat stress. (C) Changes of KLF4 protein expression in rat H_9C_2 cardiac myocytes after heat stress. (D) Changes of KLF4 protein expression in human U-251 astrocytic tumor cells after heat stress. HS, heat shock; h, hours for recovery after heat stress.

show up-regulation of KLF4 protein levels in all 4 cell lines examined. Under normal conditions, little KLF4 protein was expressed. When challenged with heat stress, the KLF4 protein increased >2-fold in all 4 cell lines. The KLF4 protein levels attained peak values in the different cell lines at different times after hyperthermia treatment. The H_9C_2 cells had the fastest rate of increase in KLF4 protein expression because the peak of expression appeared at 6 hours of recovery after heat stress (Fig 3C). The U-251 cells had the slowest rate of increase; the peak value appeared at 18 hours of recovery after heat stress (Fig 3D).

Induction of KLF4 messenger RNA in cultured cells under heat stress conditions

To further evaluate the change in KLF4 messenger RNA (mRNA) expression in response to heat stress in cultured cells, total RNA was extracted and RT-PCR was per-

formed to detect the levels of KLF4 mRNA expression in the Raw264.7 macrophages and C_2C_{12} myocytes, as well as the control group, at 1 hour and 3, 6, and 9 hours of recovery after the hyperthermia treatment (Fig 4). As expected, the expression of KLF4 mRNA significantly increased in the 2 cell lines challenged with heat stress. The highest level of KLF4 mRNA was detected at 1 hour of recovery in the Raw264.7 macrophages (Fig 4A). In contrast, the increase in KLF4 mRNA levels peaked at 3 hours of recovery in the C_2C_{12} myocytes (Fig 4B). Therefore, similar to protein induction, KLF4 mRNA was also up-regulated in cultured cells under heat stress conditions.

Expression of KLF4 mRNA in Hsf1 gene knockout mice

In vertebrates, Hsf1 has been identified as the heat shock factor that mediates stress-induced heat shock gene expression on the basis of the ability of Hsf1 to display inducible DNA binding activity, oligomerization, and nuclear localization in response to environmental stressors (Wu 1995; Morimoto 1998; Morano and Thiele 1999). Our previous work (Liu et al 2004) has shown that the expression of KLF4 mRNA was down-regulated 0.305-fold in the myocardium of Hsf1 gene knockout mice compared with the wild-type mice in response to heat shock by cDNA microarray assay. Then, HSEs (agttTTCTTCgG AAtatttgtcatcctGAAttTTCcacTTCtg) were found in the promoter region of the KLF4 gene by bioinformatics software. To further confirm whether Hsf1 gene deficiency inhibits KLF4 mRNA expression, we performed RT-PCR and Northern blot to measure the amount of KLF4 mRNA in myocardium and lung tissue of wild-type and homozygous null mice. As Figure 5A shows, under normal conditions, little KLF4 mRNA was expressed in cardiac muscle of the wild type or the homozygous mutants. With recovery for 3 hours after hyperthermic challenge, the significant increase of KLF4 mRNA appeared in wild-type mice, and the increase of KLF4 mRNA in homozygous mutant mice was much less. As expressed in a similar fashion in myocardium, the basal level of KLF4 mRNA expression in lung tissue significantly increased after hyperthermic challenge in wild-type mice but increased little in homozygous mutant mice (Fig 5B). These results suggested that Hsf1 might be a transactivator of KLF4 mRNA expression under heat stress conditions.

Effect of KLF4 overexpression on C_2C_{12} cells viability and apoptosis

To investigate the function of KLF4 in response to heat stress, pcDNA3.1-KLF4 constructs were stably transfected into murine C_2C_{12} myogenic cells (Fig 6A). The effects

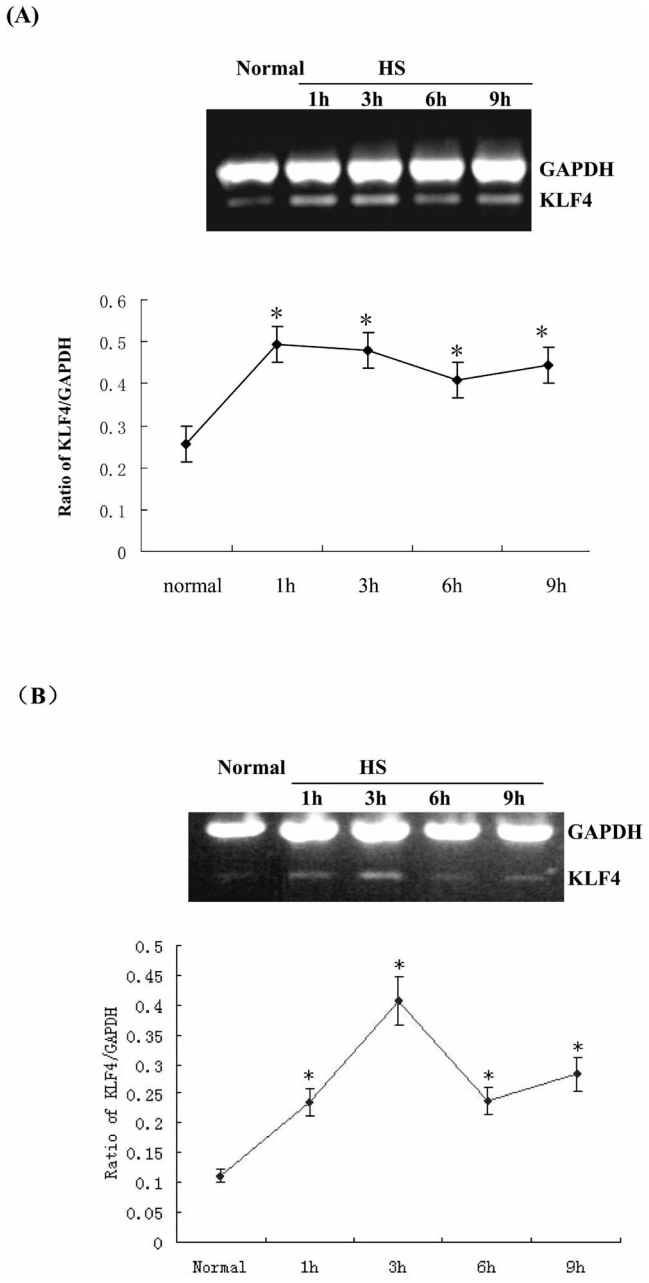

Fig 4. Kinetics of KLF4 mRNA expression in cultured cells challenged by heat stress. Cultured cells were challenged by a sublethal heat stress (43°C for 30 minutes), and samples were obtained for a time course. The expression of KLF4 mRNA was determined by RT-PCR. GAPDH was used as an internal control. Shown in the top panel is a representative result, and shown in the bottom panel are the mean ± SEM of 3 independent experiments. * $P < 0.05$ compared with the normal group. (A) Changes of KLF4 mRNA expression in murine Raw264.7 cells after heat stress. (B) Changes of KLF4 mRNA expression in murine C_2C_{12} myogenic cells from skeletal muscle after heat stress.

of KLF4 overexpression on C_2C_{12} cytotoxicity are shown in Figure 5B,C. KLF4 overexpression increased the death of C_2C_{12} myogenic cells under both normal and heat stress conditions (43°C for 1 hour followed by 12 hours of recovery), as reflected by a decreased MTT reduction (Fig 6B). As analyzed quantitatively by flow cytometry, the apoptosis rates increased from 3.42% ± 1.4% in control cells to 12.7% ± 2.1% in KLF4-overexpressing cells. With hyperthermic challenge, the apoptotic rates increased from 6.31% ± 1.5% to 16.9% ± 1.98% ($P < 0.05$ compared with control; Fig 6C). These results indicated that KLF4 overexpression has negative effects on the viability of C_2C_{12} cells.

Effect of KLF4 deficiency on viability and apoptosis in C_2C_{12} cells

When C_2C_{12} cells were transfected with morpholino antisense oligonucleotides for 48 hours (Fig 7A), the survival rate of cells increased from 100% ± 8.6% to 134% ± 7.1% compared with the control oligonucleotides (Ctrl) under the normal conditions (Fig 7B). Under heat stress conditions (43°C for 1 hour and recovery for 12 hours), the survival rate of cells was down to 72% ± 6.7% in the control, but 101% ± 9.2% in KLF4-deficient cells ($P < 0.05$ compared with control). In addition, the apoptosis rate of KLF4-deficient cells decreased from about 20.4% ± 3.4% to 9.33% ± 2.8% of the control group under normal conditions (Fig 7C). When treated with hyperthermia (43°C for 1 hour and recovery for 12 hours), the apoptosis rate was up to 46.2% ± 3.1% in the control group but 29.7% ± 2.9% in KLF4-deficient cells and was significantly lower than the control group. These results further suggested that KLF4 was a potential factor responsible for cell injury.

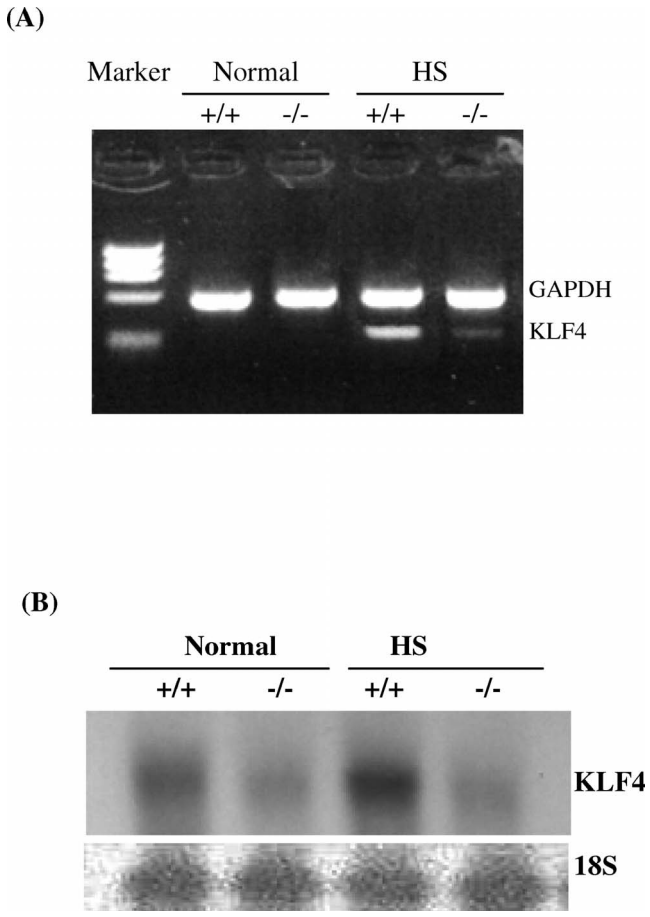

Fig 5. Effect of Hsf1 gene knockout on the expression of KLF4 mRNA. (A) Hsf1 knockout mice were treated at 42°C for 15 minutes and recovered for 3 hours, then KLF4 mRNA level was detected in the myocardium of Hsf1 knockout mice (−/−) compared with the wild types (+/+) by RT-PCR. (B) Hsf1 knockout mice were treated at 42°C for 15 minutes and recovered for 3 hours, and KLF4 mRNA level was detected in the lung tissue of Hsf1 knockout mice (−/−) compared with the wild-type mice (+/+) by Northern blot. Normal, group untreated with heat stress; HS, group treated with heat stress.

DISCUSSION

Organisms from prokaryotes to humans respond to a variety of stressful environmental stimuli by rapid expression of Hsps. In addition to the classic description of their induction in *Drosophila* subjected to hyperthermia (hence the term Hsp), a variety of mammalian cells have been shown to express Hsps in response to heavy metals, ischemia-reperfusion, oxidants, and endotoxin (Pirkkala et al 2001; Moggs and Orphanides 2003). Hsps, especially Hsp70, can also serve as markers of the stress response. To determine whether a heat stimulus was sufficient to simulate the stress response in our study, we detected the change in Hsp70 protein expression in response to hyperthermia treatment in several murine organs. As expected, Hsp70 protein significantly increased after heat shock in a time-dependent manner (Fig 1). These results are consistent with those reported by other laboratories (Pirkkala et al 2001; Moggs and Orphanides 2003). Therefore, the hyperthermia treatment in our work successfully induced immediate early response genes to complete the stress response.

KLF4 is an epithelial cell–enriched, zinc finger–containing transcription factor that has been widely investigated in both normal development and carcinogenesis (Bieker 2001; Ghaleb et al 2005). In normal conditions, the expression of KLF4 mRNA is most abundant in the colon and skin of mice, but expression of the KLF4 gene is decreased in intestinal adenomas of mice with multiple intestinal neoplasia and in colonic adenomas of patients with familial adenomatous polyposis. In recent years, it has been reported that KLF4 can be induced by H_2O_2, interferon (IFN)-γ, lipopolysaccharide (LPS), and tumor necrosis factor–α (Chen et al 2000, 2002; Nickenig et al 2002; Feinberg et al 2005), thus playing a critical role in

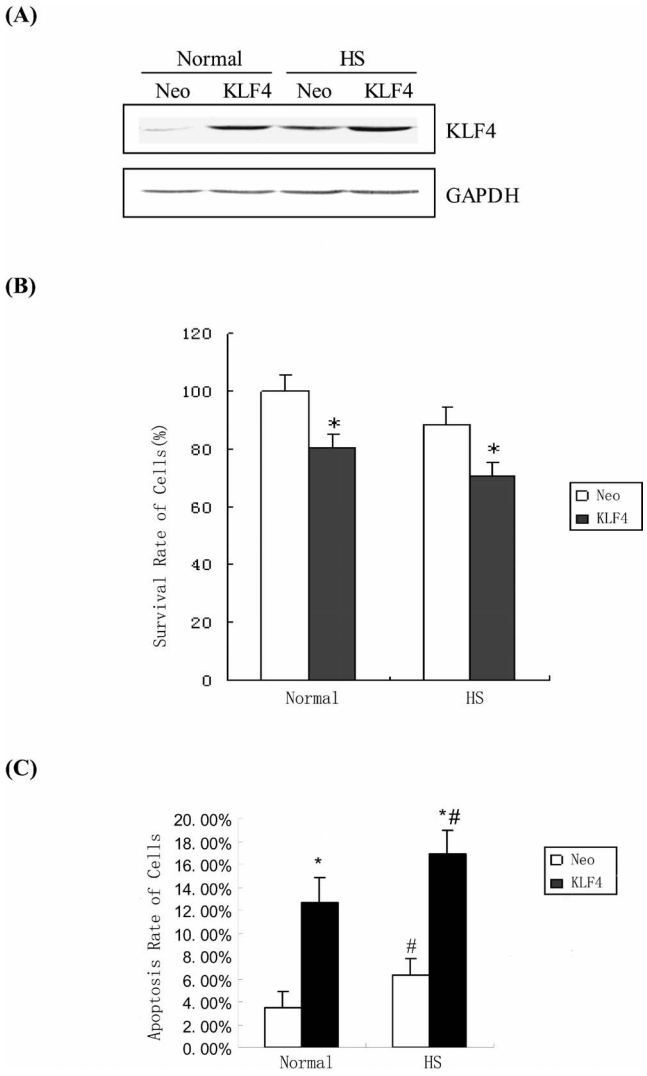

Fig 6. Effect of KLF4 overexpression on the cell viability and apoptosis of C_2C_{12} cells. C_2C_{12} cells were stably transfected with pcDNA3.1-KLF4 plasmid, and expression of KLF4 protein was detected by Western blot compared with the vector control (A); GAPDH was used as an internal control. Cell viability was evaluated by MTT reduction (B). Cell apoptosis was evaluated by flow cytometry (C). Values are mean ± SEM of 3 experiments in duplicate. * Statistically significant compared with the vector control group (Neo) at $P < 0.05$; # statistically significant compared with the normal group at $P < 0.05$.

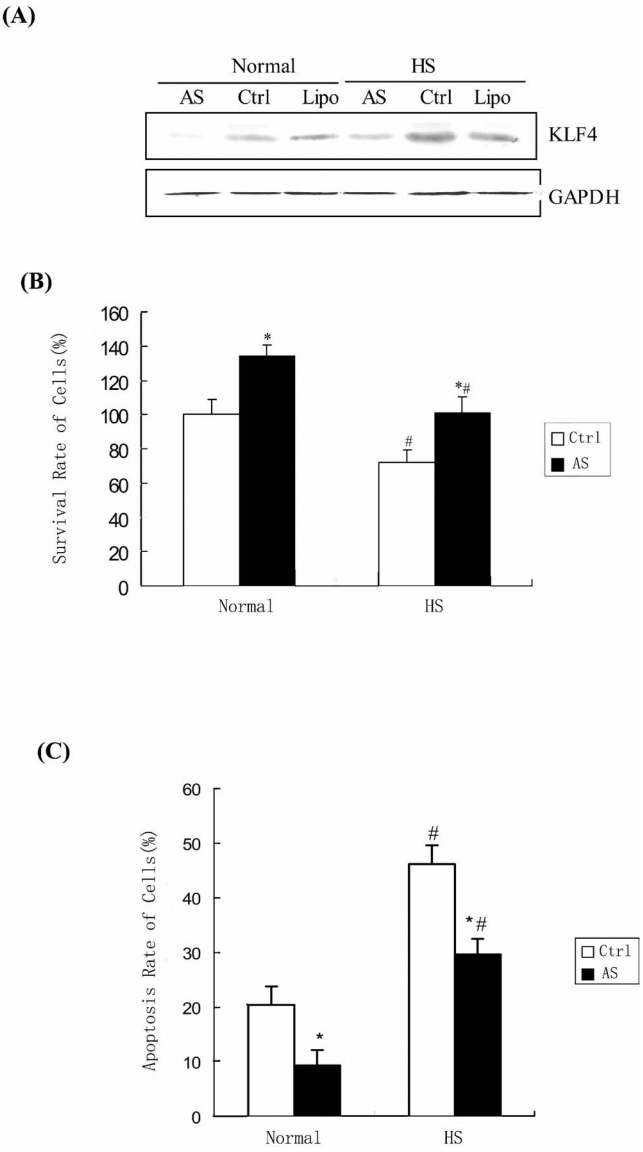

Fig 7. Effect of KLF4 deficiency on the cell viability and apoptosis of C_2C_{12} cells. C_2C_{12} cells were transient transfected with a KLF4 morpholino antisense oligonucleotide, and expression of KLF4 protein was detected by Western blot (A) and compared with the lipofectamine-treated group (Lipo) and the oligonucleotide control (Ctrl); GAPDH was used as an internal control. Cell viability was evaluated by MTT reduction (B). Cell apoptosis was evaluated by flow cytometry (C). Values are mean ± SEM of 3 experiments in duplicate. * Statistically significant compared with the oligonucleotide control at $P < 0.05$; # statistically significant compared with the normal group at $P < 0.05$.

mediating cell response to the stimuli. As well as the other reports, in our previous study (Yuan et al 2001), KLF4 mRNA was up-regulated >10-fold in adult mice lung tissue after endotoxin stimuli, as shown with the Atlas™ mouse cDNA array. In this study, we showed for the first time that both KLF4 mRNA and protein can be induced by heat stress. Furthermore, high expression of KLF4 protein occurred in various tissues, including heart, lung, kidney, brain, and skeletal muscle of mice after exposure to heat stress. In cultured cells, the expression of KLF4 increased in murine skeletal myogenic cells and macrophages, and also in rat cardiac myocytes and human astrocytic tumor cells after the cells were exposed to heat stress. Together with studies from other laboratories and our previous work, the up-regulation of KLF4 during the stress response is considered a common phenomenon in organisms. This phenomenon has little specificity to genera or tissue. According to these results, we consider

KLF4 a potential immediate early response gene to stress stimuli.

Hsf1 is an important regulator of heat shock response (Xiao et al 1999). Our previous work has shown by cDNA microarray assay (Liu et al 2004) that KLF4 mRNA is down-regulated in the cardiac muscles of Hsf1 gene knockout mice compared with wild-type mice during the heat shock response. It is likely that Hsf1 can regulate the expression of KLF4. In this study, Hsf1 gene knockout mice were used to further confirm whether Hsf1 gene deficiency inhibits KLF4 mRNA expression. As expected, the level of KLF4 mRNA in the myocardium and lung tissue was significantly down-regulated in the homozygous null mice compared with wild-type mice under heat stress conditions. The result indicated that Hsf1 increased the expression of KLF4 mRNA in the cardiac muscles and lung tissues of mice in response to heat stress. Therefore, KLF4 might be a downstream target of Hsf1. We also found a series of HSEs in the promoter region of the murine KLF4 gene by bioinformatics methods. As we know, Hsf1 binds to the HSEs in the promoter regions of its downstream genes and regulates their transcription. Together with our results, it is indicated that KLF4 might be directly regulated by Hsf1 in response to heat stress.

As noted, the stress response can stimulate the expression of multiple proteins, and these proteins can function as modulators of the stress response. One of the hottest areas of current research involves the Hsps. Researchers have subsequently demonstrated that most Hsps have strong cytoprotective effects. They are involved in many regulatory pathways and behave as molecular chaperones for other cellular proteins (Pirkkala et al 2001; Moggs and Orphanides 2003). On the other hand, induction of some other proteins during the stress response results in cell injury. For example, E-64 and pepstatin-A, 2 protease inhibitors, exhibit a promotive effect on the changes of cell cycle induced by heat stimuli (Zhu et al 1996). Germ cell apoptosis immediately after heat exposure is mediated by the Fas-FasL system, as proposed for apoptosis caused by androgen withdrawal (Hikim et al 2003). Furthermore, in BC-8 tumor cells, heat shock–induced CD95 gene expression and the expression of CD95 antigen might be involved in triggering apoptosis in these cells upon heat shock (Sreedhar et al 2000). But the functional roles of KLF4, a potential immediate early response gene, in response to the stress stimuli are not well understood. It has been documented both in vitro and in vivo that heat stress is capable of inducing growth arrest, differentiation, and apoptosis in a variety of cells. In this study, we showed that heat stress treatment in murine C_2C_{12} skeletal myocytes results in cell injury and apoptosis. These results are consistent with those reported by other laboratories (Zhu et al 1996; Sreedhar et al 2000; Hikim et al 2003). In vitro, the level of KLF4 mRNA correlated with the proliferative state of cells in a manner similar to that seen in vivo; forced expression of KLF4 by transfection of proliferating cells resulted in an inhibition of DNA synthesis (Shields et al 1996). In addition, overexpression of KLF4 was shown to inhibit cell growth and induce apoptosis in bladder cancer cells (Ohnishi et al 2003) and T-cell leukemia cells (ATL; Yasunaga et al 2004). Also, KLF4 is a downstream target of IFN-γ that mediates its unique functions, including growth arrest and apoptosis in colon cancer cells (Chen et al 2000). Finally, induction of KLF4 in cultures of bitransgenic primary keratinocytes resulted in death of the vast majority of cells (Foster et al 2005). These results indicate that KLF4 and heat stress have similar physiologic properties. In this study, we showed that heat stress stimulated KLF4 expression in a time-dependent manner. To further examine the function of KLF4 under heat stress conditions, cell viability and apoptosis were measured in C_2C_{12} cells overexpressing or deficient in KLF4. As expected, our results showed that KLF4 overexpression decreased the survival rate and increased the apoptosis rate of C_2C_{12} cells under normal conditions. Furthermore, our study also showed that KLF4 promoted hyperthermia-induced cell death in C_2C_{12} cells, suggesting a potential harmful role of KLF4 in the heat stress response. Together, these studies indicate that KLF4 might function as an immediate early response gene of stress that increases cell damage, growth arrest, and apoptosis.

Our study demonstrates for the first time that KLF4 might be a potential immediate early response gene and might play a critical role in the heat stress response. Further studies are needed to illustrate the detailed mechanism.

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