이학석사학위논문

생쥐 웅성생식세포에서 eIF2α kinases 발현연구

Studies on expression of eIF2α kinases in the mouse male germ cells

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ABSTRACT

Studies on expression of eIF2α kinases in the mouse male germ cells

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The mammalian male germ cells should be maintained below the core body temperature for their successful development. A previous study in my laboratory reported that eIF2α (α subunit of eukaryotic translation initiation factor 2) phosphorylation is one of immediate responses of male germ cells against heat stress. In this work, the expression of eIF2α protein was observed in all stages of male germ cells. However, phospho-eIF2α was detected only at cytoplasm of germ cell stages up to early pachytene spermatocytes by heat treatment. In order to identify a candidate kinase responsible for eIF2α phosphorylation upon heat stress in male germ cells, I examined expression of the four known eIF2α kinases (HRI, PKR, PERK, and GCN2) in mouse testis. The results showed that all four eIF2α kinases are expressed with distinct developmental stage-specific manners. Furthermore, a phosphorylated form of

PERK is detected in the testis from heat-treated mouse. These results suggest that PERK is the candidate kinase that phosphorylates eIF2α by sensing heat stress in the mouse male germ cells.

Key Words: Heat stress, Mouse testis, eIF2α phosphorylation, eIF2α kinase

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INTRODUCTION

Generally, mammalian male germ cells are kept below the core body temperature during development for their optimal function (Huston *et al*., 1997). It has been reported that heat stress on mammalian testis leads to apoptosis of the male germ cells and, therefore, subfertility or infertility is induced (Lue *et al*., 1999; Rockett *et al*., 2001). In artificial cryptorchidism, one or both testes are experimentally forced to stay within the abdomen, and this also induces apoptosis of the male germ cells (Shikone *et al*., 1994; Yin *et al*., 1997). Based on these results, it has been proposed that mammalian male germ cells have a lower temperature threshold (Sarge, 1995; Sarge *et al*., 1995). Accordingly, the suitable temperature regulation in testis is essential for normal germ cell development. However, the precise molecular mechanisms of thermal regulation in male germ cells are not still clear.

The translational control plays a crucial role in cellular gene expression system in response to various environmental stresses. The regulation of translation provides the stressed cells with ways to react immediately through rapid changes in protein level (Holcik and Sonenberg, 2005). Previous investigations reported that the level of translation is reduced in heat stressed male germ cells. Parallel to these findings, it has also been suggested that heat

can decrease the assembly of amino acids into proteins and the formation of polysomes (Nakamura and Hall, 1978; Nakamura *et al*., 1978; Cataldo *et al*., 1997). Furthermore, the reduction of protein synthesis is tightly linked to the attenuated formation of the translation initiation complex (Nakamura and Hall, 1980).

Eukaryotic translation initiation factor 2 (eIF2) is one of the components that constructs eIF2-GTP-Met-$tRNA_i$ ternary complex, and it transports initiator tRNA to 43S ribosome. The typical factor that regulates translational initiation is GTP-bound eIF2, which is essential for the assembly of 43S initiation complex. The replacement of GDP to GTP on eIF2 is mediated by catalytic reaction of eIF2B; however, this eIF2B-mediated GTP substitution is inhibited by the phosphorylation of α-subunit (at Serine-51) of eIF2 (Rowlands *et al*., 1988; Krishnamoorthy *et al*., 2001). Phosphorylation of eIF2α has been observed and can be generated in various types of stressful conditions (e.g. nutritional deprivation, viral infection, and heat etc.), and it is commonly followed by inhibition of global translation (Dever, 2002). The decline of comprehensive protein synthesis under stressful conditions is also known cell survival mechanism. There are experimental data that S51A (Serine substitution of Alanine) double-mutant mice are dead in a few hours after birth (Scheuner *et al*., 2001). In other words, phosphorylation of eIF2α has ability to cope with

cellular stresses by altering translational steps.

There are four eIF2α kinases which have been studied in mammalian cells: (1) HRI (Heme-Regulated Inhibitor) is activated under heme-deprived condition in erythrocyte (Han *et al*., 2001; Lu *et al*., 2001); (2) PKR (Protein Kinase R or double stranded RNA dependent kinase) reacts to viral infection (Barber, 2005); (3) PERK (PKR-like ER Kinase) activation is occurred by the unfolded proteins in ER (ER stress) (Patil and Walter, 2001); and (4) GCN2 (General Control Non-derepressible 2) is activated due to the amino acid deprivation and UV irradiation (Kimball, 2001; Deng *et al*., 2002). These four kinases have a conserved catalytic domain; therefore, they can share a common downstream event in which eIF2α is phosphorylated at Ser-51 (Dever, 2002).

A recent study revealed that eIF2α phosphorylation is rapidly occurred in heat treated mouse testis, even at 37°C, that is similar to the core body temperature (Kim *et al*., 2012). Thus, eIF2α phosphorylation in heat-stressed testis might block the initiation step of translational pathway in male germ cells. This could explain the reduction of polysomes in heat-treated testis (Cataldo *et al*., 1997). Unfortunately, only handful amount of information is known about the heat stress-related eIF2α kinase (Lu *et al*., 2001; Zhan *et al*., 2002), thus, further extensive *in vivo* studies are required to fully understand the precise mechanism underlying male germ cells' response to heat stress.

Here, I investigated the four known eIF2α kinases to find a candidate kinase that responds to heat stress in mouse testis. In order to identify a kinase responsible for phosphorylating eIF2α by heat stress, I examined both expression and phosphorylation of HRI, PKR, PERK, and GCN2 in mouse testis. The results showed that all four eIF2α kinases were expressed with distinct pattern in the mouse testis and, especially, PERK phosphorylation was specifically observed in heat-treated mouse testis.

MATERIALS AND METHODS

Animals and heat treatment

The experimental procedure using mice was granted by Institutional Animal Care and Use Committee at Seoul National University (SNU-110726-3). The testes were obtained from FVB mice on postnatal day-7, -17, and -42. The mice were used for heat-treatment experiment. The anesthetized male mice were placed in a water bath and heat treated at up to 42°C for 20 minutes. After the treatment, the mice were sacrificed and their testes were isolated.

Reverse transcription and quantitative real-time PCR

Total RNA was extracted from mouse testes with using Trizol reagent (Invitrogen) according to the manufacturer's recommendations. 1 μg of RNA was used as a template for reverse transcription. Samples containing 1 μg of RNA were heat-denatured for 10 minutes at 70°C. After chilling the mixture on ice, the RNA was incubated at 37°C for 1.5 hour in a total volume of 20 μl that contained 5 μM random hexamer, AMV reverse transcriptase, 0.5 mM dNTP mixture, RNasin, 10 mM DTT, and RT buffer. The reaction was terminated by heating at 70°C for 15 minutes. Then, cDNAs were stored at -20 °C until use.

Real-time PCR was carried out with the Applied Biosystems 7300 Real

Time PCR System by using SYBR Green with high ROX (Enzynomics) with gene-specific primers as listed in Table 1. Real-time PCR was performed in triplicate and the experiment was repeated twice. Relative gene expressions were calculated using the comparative Ct method and were normalized to GAPDH cDNA. Standard errors were determined using SigmaPlot 10.0.

Antibodies

Antibodies against eIF2α (sc-11386), PKR (sc-708), and phospho-PERK (sc-32577) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against PERK (#3192) and GCN2 (#3302) were purchased from Cell Signaling Technology (Beverly, MA, USA). Antibodies to HRI (#07-728), phospho-PKR (#44668G), phospho-GCN2 (ab75836), and phospho-eIF2α (KAP-CP131) were purchased from Millipore (Bedford, MA, USA), Invitrogen Life Technology (Carlsbad, CA, USA), Abcam (Cambridge, MA, UK), and Stressgen (San Diego, CA, USA), respectively.

Immunohistochemistry

Mouse testes were fixed overnight at 4°C in Bouin's solution (Sigma-Aldrich) and embedded in paraffin. The paraffin-embedded testes were sectioned at 5μm by microtome. Then, the sectioned testes were deparaffinzed and hydrated. 10mM

Tris-HCl (pH 9.0) or 1mM EDTA (pH 8.0) was used for antigen retrieval. The sectioned tissues were blocked with PBST (phosphate-buffered saline with 0.1% Triton X-100) containing 3% BSA (bovine serum albumin) for 30 min. Then, the sectioned testes incubated at 4°C for overnight with primary antibodies which were diluted in 3% BSA in PBST. Immunostaining with primary antibodies were performed in accordance with the manufacturer's instructions. Next day, after washing with PBST, the sectioned testes were incubated with either fluorophore-conjugated (Invitrogen) or biotinylated (Vector) secondary antibodies for 30 min at room temperature. For DAB staining, the biotinylated antibody was then incubated with avidin-biotin peroxidase complex (Vector) for 30 min. Subsequently, the color was developed with 3, 3'-diaminobenzidine tetrachloride (DAB) (Sigma-Aldrich). DAPI and hematoxylin were used for counterstaining for fluorescence and DAB staining, respectively.

Immunoblot analysis

Mouse testes were decapsulated in ice-cold PBS, followed by homogenization in RIPA buffer [150mM Sodium Chloride, 1.0% NP-40, 0.5% Sodium Deoxycholate, 0.1% SDS, 50mM Tris (pH 8.0), 10mM Sodium Fluoride, 1mM Sodium Orthovanadate, 1mM EDTA (pH 8.0), 1mM EGTA (pH 8.0), and Protease inhibitor cocktail (Sigma)]. After ice incubation for 10 min, samples were

centrifuged at 12000 rpm for 10 min, and the supernatant was collected. Then, lysates were stored at -80 °C until use. Proteins were loaded in SDS-polyacrylamide gels and then, resolved by electrophoresis. The resolved proteins were transferred to nitrocellulose membranes. The membranes were blocked in 5% BSA or 5% skim milk in TBST (20mM Tris, 150mM NaCl, 0.1% Tween 20) Immunoblot analyses with primary antibodies were performed according to the manufacturer's instructions. After washing the primary antibodies with TBST, the membranes were incubated with peroxidase-conjugated secondary antibodies for 30 min at room temperature. The membranes further washed with TBST, the protein bands were detected by peroxidase activity using an ECL reagent.

For measurement of each eIF2α kinase levels, the relative band intensity measurement was performed using Image J software (NIH). The signal intensity of expression level of each kinase was normalized to GAPDH expression.

RESULTS

Developmental stage-specific phosphorylation of eIF2α by heat stress in male mouse germ cells

A previous study has shown that heat stress phosphorylates eIF2α in mouse testes (Kim *et al*., 2012). I performed immunoblot analyses with phospho-eIF2α antibody to make sure that a mild heat indeed induces eIF2α phosphorylation. The result confirmed that heat treatment at 37˚C and 42˚C for 20 min increases the phosphorylated form of eIF2α in mouse testes (Figure 1A). Then, I investigated the expression of eIF2α in mouse male germ cells. To test this, mouse testes were obtained from mice on postnatal days 7, 17, and 42 (adult). In mouse testis, spermatogenesis is initiated shortly after birth. Therefore, the 7-day-old mouse testis consists mostly of spermatogonia, whereas 17-day-old mouse testis consists of spermatogonia down to pachytene spermatocytes. In the case of adult mouse testis, it consists of all stages of germ cells (Bellve *et al*., 1977). The result implies that the eIF2α is expressed in both immature and mature male germ cells under normal condition, confirming eIF2α expression in male germ cells of all stages of development (Figure 1B).

It was reported that phospho-eIF2α is located at the cytoplasm in cells under stress (Kedersha *et al*., 2002). Hence, I examined the phosphorylation

pattern of eIF2α in both heat-stressed and control adult mouse testes by immunofluorescence histochemistry. Because the unique developmental progression of spermatogenesis in the adult mouse testes has been established well (Oakberg, 1956), the pattern of eIF2α phosphorylation was analyzed in adult mouse testes. By contrast to control, phospho-eIF2α signal was detected in the cytoplasm by heat treatment. Surprisingly, it was also observed that the patterns of eIF2α phosphorylation in mouse testes may have cell type specificity (Figure 1C). To test this possibility, I investigated whether the phosphorylation of eIF2α by heat stress really occurs in a cell type-specific manner in mouse male germ cells. To visualize eIF2α phosphorylation patterns clearly, DAB staining were conducted. In normal condition, phospho-eIF2α was detected in the nuclei of germ cells. In heat-stressed condition, however, phospho-eIF2α was observed in the cytoplasm of spermatogonia, leptotene spermatocytes, and early pachytene spermatocytes, but not in late pachytene spermatocytes and post-meiotic spermatids (Figure 2A, B). This result indicates that the phosphorylation of eIF2α in heat-stressed male germ cells is developmental stage-specific, and its phosphorylation is reduced in male germ cells before the onset of meiosis.

Expression levels of eIF2α kinases in mouse testis

Because the pattern of eIF2α phosphorylation had developmental stage-specificity, I expected that its upstream kinase would also have developmental stage-specific expression. In order to identify a kinase responsible for eIF2α phosphorylation by heat stress in male germ cells, I investigated the expression levels of the four known eIF2α kinases (HRI, PKR, PERK, and GCN2) in mouse testes. There were a few studies on the expression of eIF2α kinases in mouse testis (Shi *et al*., 1998; Ladiges *et al*., 2000; Zhang *et al*., 2002); however, no study focused on their expression in mouse male germ cells. Therefore, I examined the RNA and protein expression levels of the four eIF2α kinases in the mouse testes. To verify the expression levels of these kinases, I firstly performed quantitative real-time PCR and determined the mRNA levels of eIF2α kinases in mouse testes of postnatal days 7, 17, and 42 (adult). The reason why I collected these samples is because the constitution of testicular germ cells is different as mice get older (Barakat *et al*., 2008) (Figure 3A). *PRM1* used as a control gene which is functioning for sperm head condensation in developing spermatids (Wykes *et al*., 1995). I confirmed that *PRM1* mRNA expression is detected only in the adult mouse testes. The mRNA expression level of *HRI* was increased with testes maturation. *PKR* had distinctive mRNA expression in mouse testes because the expression level was declined in the 17-day-old mouse testes compared to the 7-day-old and adult mouse testes. The

expression level of *PERK* mRNA was decreased as testes maturation. In the case of *GCN2* mRNA level, the peak level was observed in the 17-day-old mouse testes. Collectively, the results of qRT-PCR showed that all four eIF2α kinases are expressed in the mouse male germ cells. Also, the result implies there seems to be developmental stage-specificity in mRNA expression of each eIF2α kinase.

To examine the correlation between mRNA and protein expression, I carried out immunoblot analyses with the antibodies of each eIF2α kinase. The immunoblot data show the protein expression levels of each eIF2α kinase in mouse testes (Figure 4A). The result represented that HRI expression level was raised as germ cell development. PKR expression level was slightly decreased in the 17-day-old mouse testes comparing the 7-day-old and adult mouse testes. In the case of PERK, the sudden decline of protein expression was observed in the adult mouse testes. The expression of GCN2 protein, the highest record of its level was detected at the 17-day-old mouse testes. The result indicates that there is also developmental stage-specific protein expression of each eIF2α kinase. The correlation between the expression of mRNA and protein is confirmed as well.

Developmental stage-specificity of eIF2α kinases expression in male mouse

germ cells

Continually, I performed immunohistochemistry to observe the localization pattern of each eIF2α kinase in the mouse testis. Adult mouse testes were immunostained with the antibodies specific to each eIF2α kinase for conducting this experiment. Then, I could observe that developmental stage-specific localization of the eIF2α kinases during spermatogenesis (Figure 5A). HRI was detected in the nuclei of all stages of male germ cells. PKR was observed in the nuclei and cytoplasm of spermatogonia and weakly observed in the cytoplasm of spermatocytes. It seemed that PKR signal was also feebly detected in the nuclei of round spermatids. PERK expression was detected in the nuclei and its level was declined as male germ cells develop up to pre-meiotic germ cells. The GCN2-positive signal was mainly observed in the both cytoplasm and nuclei of spermatogonia, leptotene spermatocytes, and early pachytene spermatocytes. GCN2 was also observed weakly in the nuclei of late pachytene spermatocytes and round spermatids (Figure 5B). This result represents that the expression of each eIF2α kinase has a developmental stage-specific pattern in mouse male germ cells. Also, the immunostaining analyses indicated consistent results with the qRT-PCR and immunoblot results (Figure3, 4). Accordingly, this can explain the results of investigating each eIF2α kinase expression level of mRNA and protein in the immature and mature mouse testes. From the results

so far, the expression features of each eIF2α kinase in mouse testes were characterized. However, by comparing the pattern of eIF2α phosphorylation and each kinase expression, I could not deduce which kinase is responsible for eIF2α phosphorylation by heat stress in mouse testis.

Phosphorylation of PERK in heat-stressed mouse testis

According to the previous reports, the four eIF2α kinases (HRI, PKR, PERK, and GCN2) can be autophosphorylated by sensing the stressful conditions. HRI is autophosphorylated in response to heme dissociation (Chen *et al*., 1995). The autophosphorylation of PKR is occurred by binding to viral double-stranded RNA (Manche *et al*., 1992). PERK is characterized as an endoplasmic reticulum-resident transmembrane protein. It also can be autophosphorylated by ER stress (Harding *et al*., 1999). Another kinase of eIF2α, GCN2, is activated by autophosphorylation under the amino acid deficient condition (Wek *et al*., 1995). To identify the candidate kinase that responds to heat stress in the testes, I examined whether the specific eIF2α kinase is phosphorylated in heat-stressed mouse testes. Therefore, I investigated the phosphorylation levels of each eIF2α kinase in heat-treated mouse testes by immunoblot analyses.

To determine the phosphorylation status of each eIF2α kinase, the heat-treated (42°C for 20min) and untreated testes were obtained from 7-, 17-day-old,

and adult mice. Then, immunoblot analyses were carried out with the phospho-antibodies specific to each eIF2α kinase, except for HRI. Detection of HRI phosphorylation was seen by its upshift. Interestingly, the result showed that PERK is phosphorylated in heat-stressed mouse testes (Figure 6). The phosphorylation level of PERK was increased in heat-treated mouse testes compared to the control. In particular, its phosphorylation was confirmed in the 17-day-old and adult mouse testes, but not in the 7-day-old mouse testes. In order to exclude the possible effect of changes in the expression level, I examined the protein expression of the four eIF2α kinases. I confirmed that there were no changes in their expression levels upon heat stress (Figure 6). From these results, I showed that PERK is activated by heat-treated mouse testis, and thereby, activated PERK can contribute to eIF2α phosphorylation. Nonetheless, the other potential mechanisms that phosphorylate eIF2α in heat-stressed male germ cells could be considered since the PERK phosphorylation was not observed in heat-stressed 7-day-old mouse testes.

ER stress induction by heat stress in mouse testis

PERK is an ER transmembrane protein kinase, and it can be phosphorylated by ER stress (Harding *et al*., 1999). So I wanted to determine whether heat stress can induce ER stress in the mouse testis. To assess the ER stress in heat-stressed

mouse testis, the expression levels of ER stress markers BiP/GRP78 (Munro *et al*., 1986) and CHOP (Wang *et al*., 1996) were examined. For this experiment, the lower bodies of the adult mice were heat-treated at 42°C for 20 minutes and recover at room temperature for 0, 2, 4, 6, or 8 hours. Then, immunoblot analyses were conducted with anti-BiP/GRP78 and anti-CHOP antibodies (Figure 7). BiP/GRP78 expression was increased when heat-treated mouse testes were recovered for 4-6 hr. The protein expression CHOP was also raised during recovery from heat stress. This result indicates that ER stress is induced by heat stress in the mouse testis.

Figure 1. eIF2α phosphorylation in heat-stressed mouse testis.

(A) Adult mice were placed on water bath for heat treatment at 37 or 42°C for 20 min. The testicular lysates were subjected to immunoblot analyses with the antibodies specific to phospho-eIF2α, eIF2α and α-tubulin. (B) Immunoblot analyses of eIF2α were performed with testes from postnatal day 7-, day 17-, and day 42-old mice. (C) Testes from control and heat-treated (42°C for 20 min) mice were subjected to immunohistochemical analyses with phospho-eIF2α antibody (red). DNA was stained with DAPI (blue). Scale bar, 10μm.

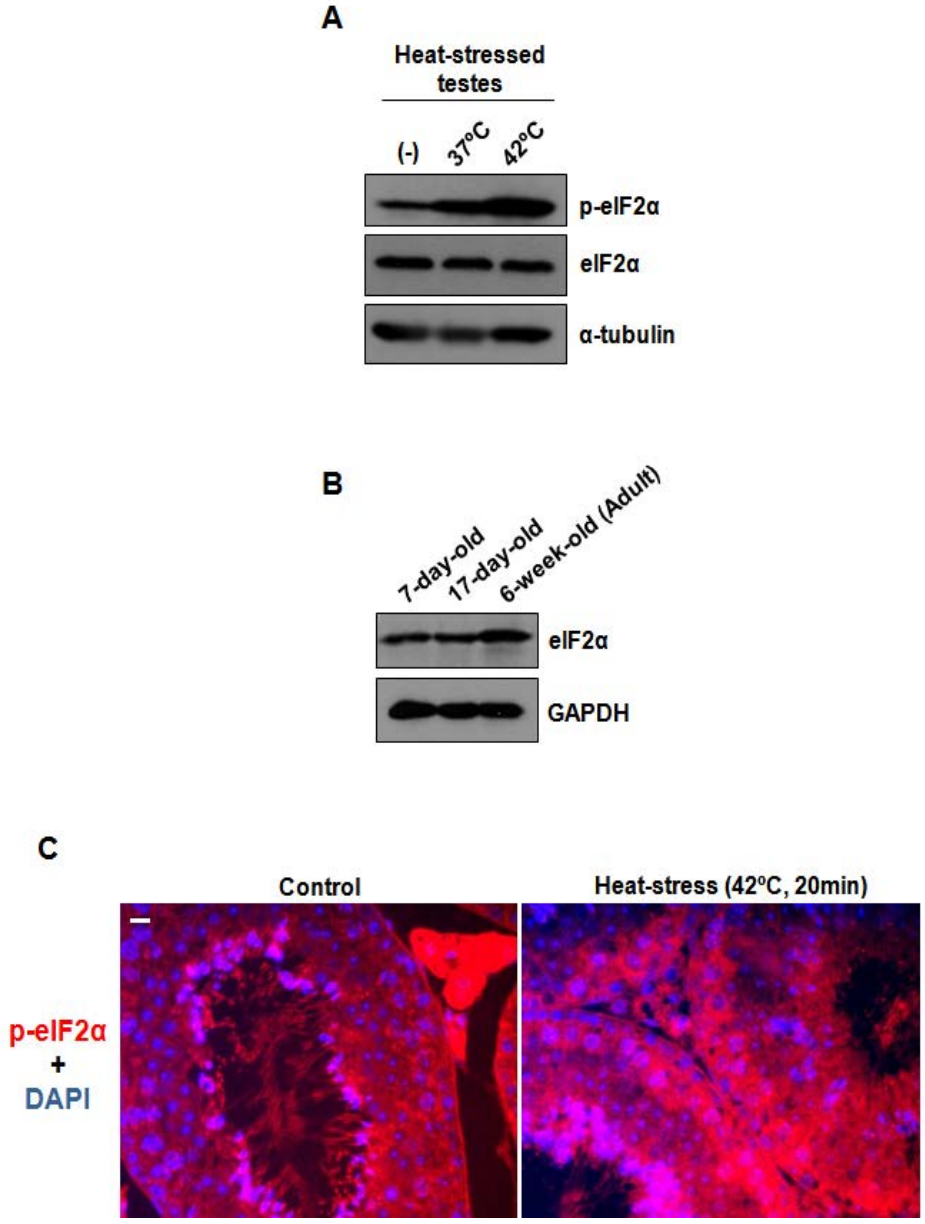
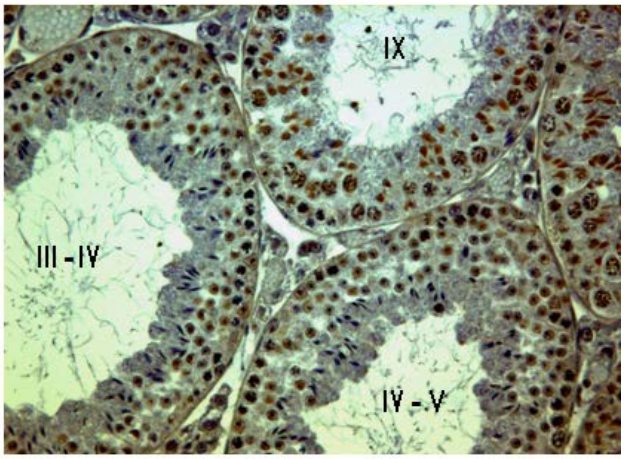

A
Heat-stressed testes
(-)
37°C
42°C
p-eIF2α
eIF2α
α-tubulin
B
7-day-old
17-day-old
6-week-old (Adult)
eIF2α
GAPDH
C
Control
Heat-stress (42°C, 20min)
p-eIF2α
+
DAPI

Figure 2. Immunohistochemical analysis of eIF2α phosphorylation in heat-treated testis.

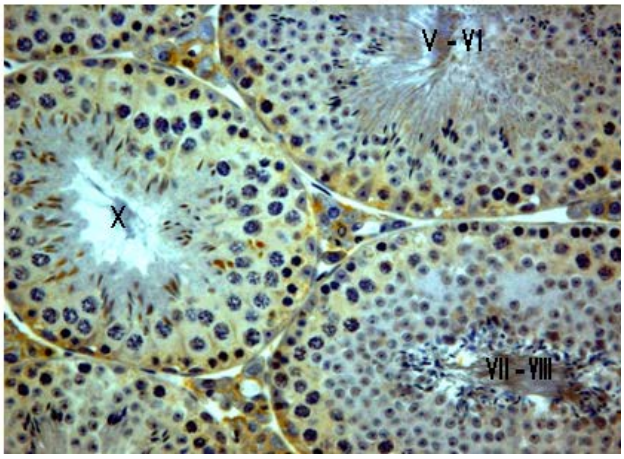
(A) Testes from control and heat-treated (42°C for 20 min) adult mice were subjected to immunohistochemical analyses with phospho-eIF2α antibody. The positive signals were stained brown with DAB. The cycle of the seminiferous epithelium tubules were determined in accordance with Russell *et al*. (1990). (B) Magnified images of A. Representative immunotaining signals of phospho-eIF2α in mouse male germ cells were shown. Spg, spermatogonium; L, leptotene; EP, early pachytene; LP, late pachytene; Rspd, round spermatids.

A

Control



Heat-stress (42°C, 20min)



B

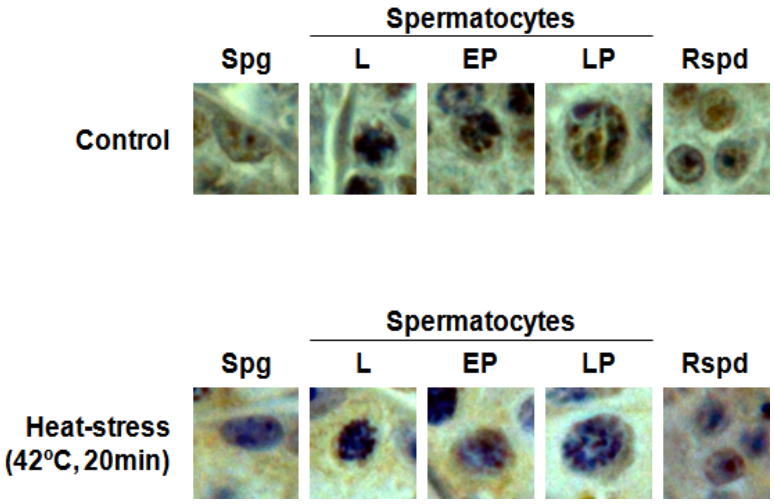
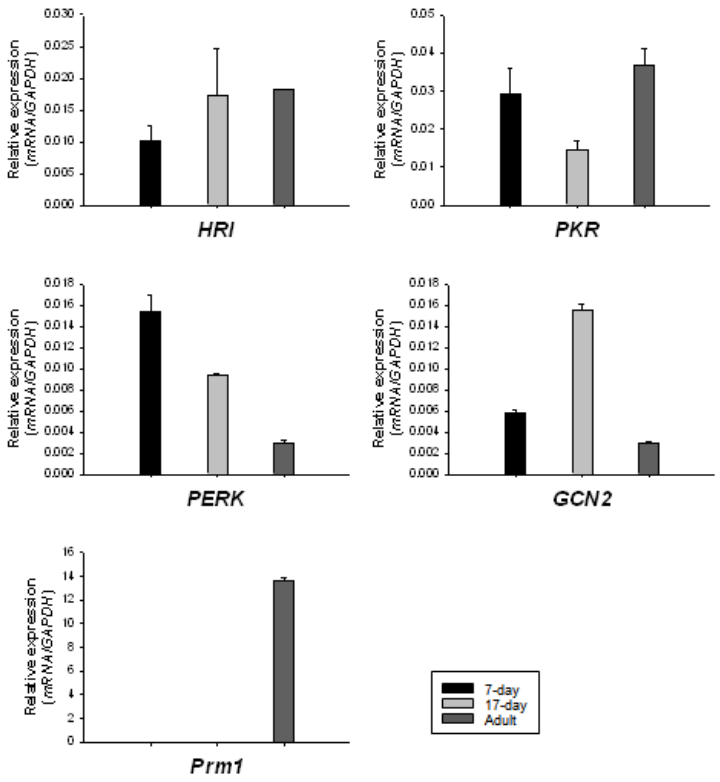

Spermatocytes
Spg
L
EP
LP
Rspd
Control
Spermatocytes
Spg
L
EP
LP
Rspd
Heat-stress
(42°C, 20min)

Figure 3. Quantitative RT-PCR analyses of eIF2α kinases expression in the mouse testes.

(A) Total RNA were prepared from testes of immature (7- and 17-day-old) and adult (42-day-old) mice and subjected to quantitative RT-PCR for eIF2α kinase genes (HRI, PKR, PERK and GCN2). Testicular protamine-1 mRNA level (PRM1) was determined as a control. Experiments were repeated twice. Values are means and standard errors. (B) Summary table for eIF2α kinases expression in the mouse testis at RNA levels. Relative expression levels were presented as +++, ++, and + from the highest.

A



B

	7-day	17-day	42-day (Adult)
HRI	+	++(+)	+++
PKR	+++	++	+++
PERK	+++	++	+
GCN2	+	+++	+

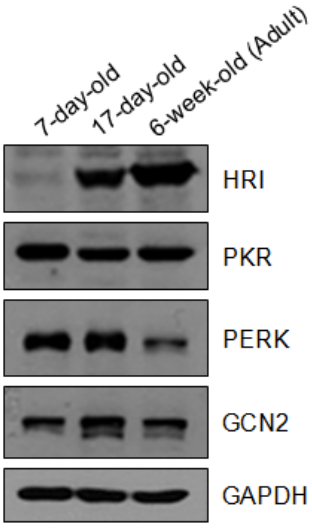
Table 1. PCR primer sets for quantitative RT-PCR analyses for eIF2α kinases expression.

Gene	Upstream primer	Downstream primer	Nucleotides product size	GenBank Accession no.
HRI/Eif2ak1	5'-gtgctacgggaagtgaaggt-3'	5'-aatcggaactctgtcttgtgg-3'	114 bp	NM_013557
PKR/Eif2ak2	5'-ccgaacaaggagaacaggaa-3'	5'-cccaaagcaaagatgtccac-3'	93 bp	NM_011163
PERK/Eif2ak3	5'-ctcctgtcttggttgggtct-3'	5'-gtgctccgcttattcctttc-3'	160 bp	NM_010121
GCN2/Eif2ak4	5'-cctcctgctgttgtctcta-3'	5'-gggctccatctttccttgtc-3'	135 bp	BC023958.1
PRM1	5'-cgccgctcatacaccataag-3'	5'-ggtggcattgttccttagcag-3'	166 bp	NM_013637.4
GAPDH	5'-tcaagaaggtggtgaagcag-3'	5'-aggtggaagagtgggagttg-3'	111 bp	NM_008084

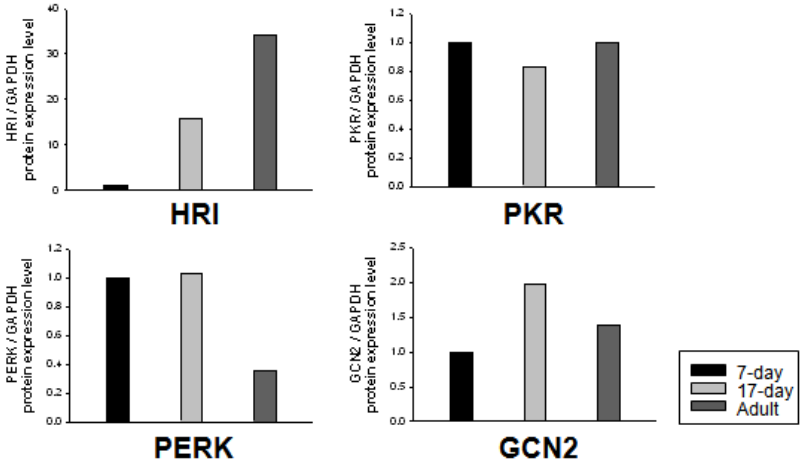
Figure 4. Immunoblot analyses of eIF2α kinases expression in the mouse testes.

(A) Testicular lysates were prepared from immature (7- and 17-day-old) and adult (42-day-old) mice. Immunoblot analyses were performed with the antibodies specific to each eIF2α kinases (HRI, PKR, PERK, and GCN2) and GAPDH. (B) Immunoblot band intensities of the eIF2α kinases were measured, normalized with GAPDH band intensities and shown as graphs. (C) Summary table for eIF2α kinases expression in the mouse testis at protein levels. Relative expression levels were presented as +++, ++, and + from the highest.

A



B



C

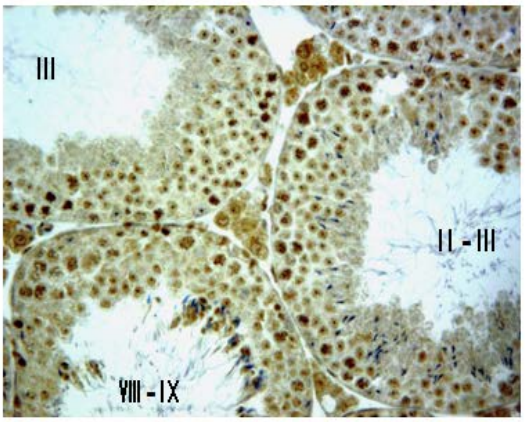
	7-day	17-day	42-day (Adult)
HRI	+	++	+++
PKR	+++	++	+++
PERK	+++	+++	+
GCN2	++	+++	++

Figure 5. Localization of each eIF2α kinase in the mouse male germ cells.

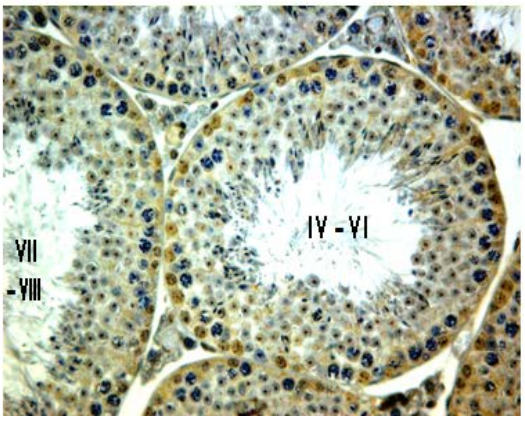
(A) The normal testes were collected from adult mice. Tissue sections were subjected to immunohistochemical analyses with the antibodies against each eIF2α kinase (HRI, PKR, PERK, and GCN2). The positive signals were stained brown with DAB. (B) Magnified images of A. Representative immunostaining signals of each eIF2α kinase at specific developmental stages in mouse male germ cells were shown. Spg, spermatogonium; L, leptotene; EP, early pachytene; LP, late pachytene; Rspd, round spermatids.

A

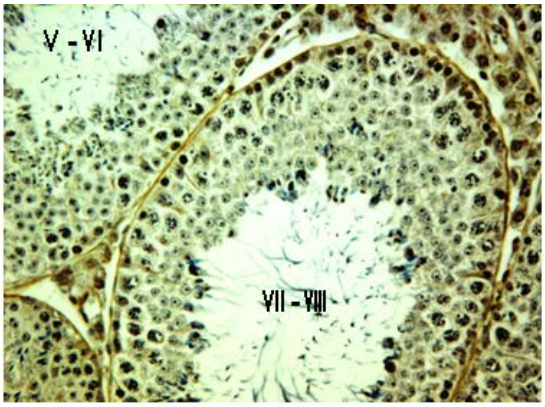
HRI



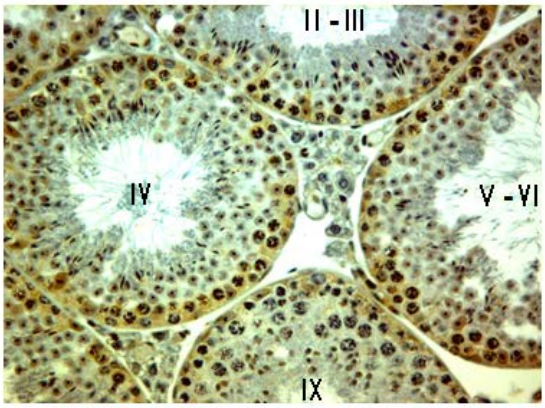
PKR



PERK



GCN2



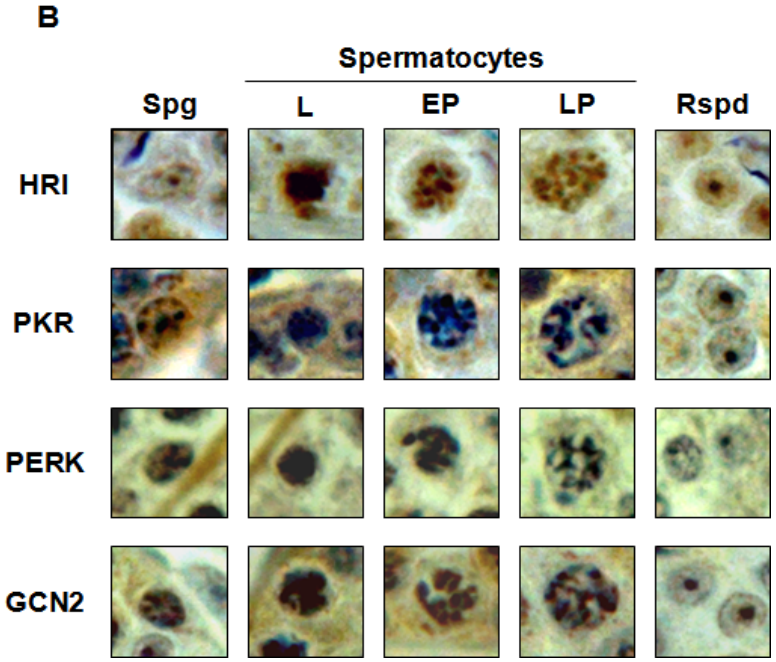

B
Spermatocytes
Spg
L
EP
LP
Rspd
HRI
PKR
PERK
GCN2

Figure 6. PERK is phosphorylated by heat stress in the mouse testis.

Testicular lysates were collected from heat-treated (42°C for 20min) and untreated testes of immature (7- and 17-day-old) and adult (42-day-old) mice. Immunoblot analyses were carried out with the antibodies specific to each target as indicated (HRI, PKR, phospho-PKR, PERK, phospho-PERK, GCN2, phospho-GCN2, eIF2α, phospho-eIF2α, GAPDH).

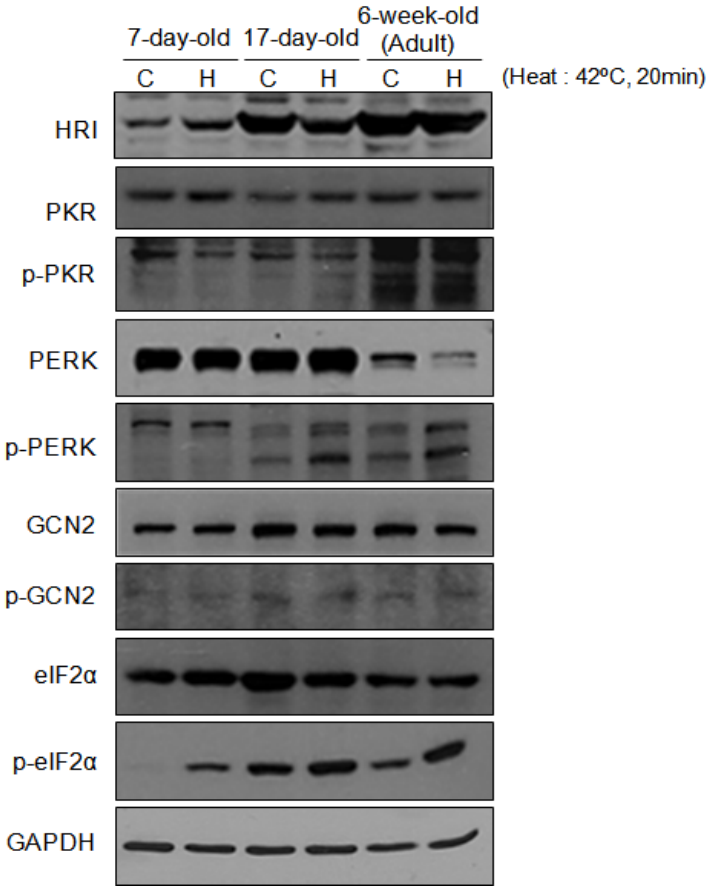
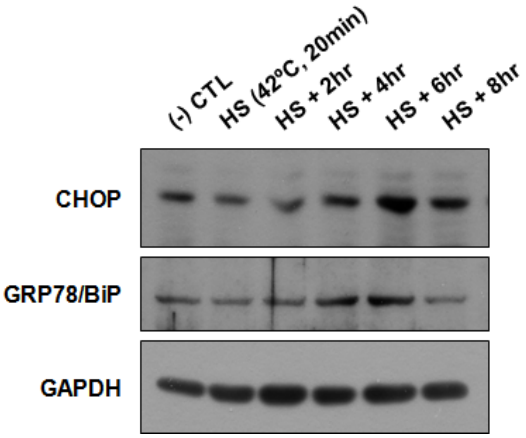

7-day-old
17-day-old
6-week-old
(Adult)
C
H
C
H
C
H
(Heat : 42ºC, 20min)
HRI
PKR
p-PKR
PERK
p-PERK
GCN2
p-GCN2
eIF2α
p-eIF2α
GAPDH

Figure 7. ER stress induction by heat stress in the mouse testis.

Adult mouse testes were treated with heat stress at 42°C for 20 min and were recovered at room temperature for 0, 2, 4, 6, or 8 hr. Testicular lysates were immunoblotted with the antibodies to CHOP, GRP78/BiP, and GAPDH.


(-) CTL
HS (42°C, 20min)
HS + 2hr
HS + 4hr
HS + 6hr
HS + 8hr
CHOP
GRP78/BiP
GAPDH

DISCUSSION

As reported here, PERK is the candidate kinase that is responsible for eIF2α phosphorylation against heat stress in mouse testis. The other eIF2α kinases, HRI, PKR, and GCN2 may not be related to eIF2α phosphorylation in heat-stressed mouse testis since their phosphorylation was not observed (Figure 6). Nevertheless, it is hard to conclude that PERK is the only kinase that phosphorylates eIF2α in heat-treated mouse testes. In this experiment, PERK phosphorylation was observed in heat-stressed 17-day-old and adult mouse testes. However, in the case of 7-day-old mouse testes, eIF2α phosphorylation level was increased independent of PERK activation (Figure 6). Taken together, there seems be other mechanisms that phosphorylate eIF2α in heat stressed male germ cells. Alternatively, there might be another unknown kinase besides the four known kinases. The other possibility is that PERK and unknown protein molecules may work cooperatively to phosphorylate eIF2α. In this work, I could not observe the activation of HRI in heat-stressed mouse testis. A previous study proved HRI-dependent eIF2α phosphorylation in heat-shocked erythroid cell; however, eIF2α phosphorylation was confirmed in *HRI* -/- cell (Lu *et al*., 2001). This means that confirming heat-sensing pathway is very complicated event. Additionally, further studies with *PERK* knockout mouse

model can be conducted to establish the eIF2α kinase that responds specifically to heat stress.

A critical observation of developmental stage-specific eIF2α phosphorylation pattern in heat-stressed mouse male germ cells led me to begin this study (Figure 1C, 2). qRT-PCR, immunoblot, and immunochistochemical analyses (Figure 3, 4, 5) showed the consistent observations that each four eIF2α kinase has cell type- and developmental stage-specificity. Unfortunately, comparing the pattern of eIF2α phosphorylation and each kinase expression, I could not infer the exact kinase that is responsible for eIF2α phosphorylation by heat stress in mouse testis. These observations still indirectly provided the possibility of existence of the heat-response kinase in mouse testis among the four eIF2α kinase.

This study is crucial in that it focuses on upstream events of the formation of stress granules, which allow for the cells to undergo translational block and improve cellular survival (Arimoto *et al*., 2008). There have been many *in vivo* studies that implied causal relationships between heat stress and male germ cells (Lue *et al*., 1999; Rockett *et al*., 2001). Those studies have given some significant information about the potential mechanism involved in heat stress response. However, there had not been a study that specifically investigates the precise downstream pathways that are activated upon heat

stress. The recent study that showed phosphorylation of eIF2α in heat-stressed testis provided the additional evidence to those relationships (Kim *et al*., 2012). Heat shock is a well-known factor that phosphorylates eIF2α, but heat-sensing mechanism is not extensively studied yet. Thus, finding this mechanism that is related to eIF2α phosphorylation in heat-stressed testis is important in understanding the metabolism of male germ cells under the heat stress.

Recent studies showed that heat shock can activate ER stress pathway (Heldens *et al*., 2011; Liu *et al*., 2012; Zhu *et al*., 2012), and the results from this study also indicate that heat stress activates ER stress pathway in the mouse testes (Figure 7). While previous studies reported that ER stress pathway can be activated by PERK phosphorylation upon heat stress (Pallepati and Averill-Bates., 2011; Zhu *et al*., 2012), this study showed that phosphorylation of PERK and eIF2α is occurred rapidly in heat-treated (42°C for 20min) mouse testis. This result is interesting because, different from *in vivo* germ cells, the higher temperature (43~44°C) is needed to phosphorylate eIF2α and the extended time (over 3h) is required to phosphorylate PERK in somatic cells.

Mammalian testes contain not only germ cells, but also somatic cells; i.e., Sertoli and Leydig cells. Their roles are to support the male germ cells and to control steroidogenesis, respectively. Since these somatic cells offer suitable environment for germ cell development and maintenance, any misregulation of

their functions can negatively affect germ cells. Since there are some evidences of heat stress and its effect on the Sertoli cells and Leydig cells (Hagenas and Ritzen, 1975; Karpe *et al* 1981; Zhang *et al* 2004; Aktas and Kanter, 2009; Kanter and Aktas, 2009), it is possible that heat stress might indirectly affect germ cells via somatic cells. The present work could not confirm whether the phosphorylation of eIF2α occurred in somatic cells of the heat-stressed mouse testes. Hence, further careful observation of somatic cells and associated subcellular response in germ cells in heat-stressed testes should be conducted.

REFERENCES

Aktas C, Kanter M (2009). A morphological study on Leydig cells of scrotal hyperthermia applied rats in short-term. *J Mol Histol* **40**: 31–39.

Arimoto K, Fukuda H, Imajoh-Ohmi S, Saito H, Takekawa M. (2008). Formation of stress granules inhibits apoptosis by suppressing stress-responsive MAPK pathways. *Nat Cell Biol* **10**: 1324-32.

Barakat B, O'Connor AE, Gold E, de Kretser DM, Loveland KL. (2008). Inhibin, activin, follistatin and FSH serum levels and testicular production are highly modulated during the first spermatogenic wave in mice. *Reproduction* **136**: 345-59.

Barber GN (2005). The dsRNA-dependent protein kinase, PKR and cell death. *Cell Death Differ* **12**: 563–570.

Bellvé AR, Cavicchia JC, Millette CF, O'Brien DA, Bhatnagar YM, Dym M. (1977). Spermatogenic cells of the prepuberal mouse. Isolation and morphological characterization. *J Cell Biol* **74**: 68-85.

Cataldo L, Mastrangelo MA, Kleene KC (1997). Differential effects of heat shock on translation of normal mRNAs in primary spermatocytes, elongated spermatids, and Sertoli cells in seminiferous tubule culture. *Exp Cell Res* **231**: 206–213.

Chen JJ, London IM. (1995). Regulation of protein synthesis by heme-regulated eIF-2 alpha kinase. *Trends Biochem Sci* **20**: 105-8.

Deng J, Harding HP, Raught B, Gingras AC, Berlanga JJ, Scheuner D, Kaufman RJ, Ron D, Sonenberg N (2002). Activation of GCN2 in UV-irradiated cells inhibits translation. *Curr Biol* **12**: 1279–1286.

Dever TE (2002). Gene-specific regulation by general translation factors. *Cell* **108**: 545–556.

Hagenas L, Ritzen EM (1975). Impaired Sertoli cell function in experimental cryptorchidism in the rat. *Mol Cell Endocrinol* **4**: 25–34.

Han AP, Yu C, Lu L, Fujiwara Y, Browne C, Chin G, Fleming M, Leboulch P, Orkin SH, Chen JJ (2001). Heme-regulated eIF2alpha kinase (HRI) is required for translational regulation and survival of erythroid precursors in iron deficiency. *EMBO J*

20: 6909–6918.

Harding HP, Zhang Y, Ron D. (1999). Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. *Nature* 397: 271-4.

Heldens L, Hensen SM, Onnekink C, van Genesen ST, Dirks RP, Lubsen NH. (2011). An atypical unfolded protein response in heat shocked cells. *PLoS One* **6**: e23512.

Holcik M, Sonenberg N (2005). Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol* **6**: 318–327.

Hutson, J. M., Hasthorpe, S. and Heyns, C. F. (1997). Anatomical and functional aspects of testicular descent and cryptorchidism. *Endocr Rev* **18**: 259-280.

Kanter M, Aktas C (2009). Effects of scrotal hyperthermia on Leydig cells in long-term: a histological, immunohistochemical and ultrastructural study in rats. *J Mol Histol* **40**: 123–130.

Karpe B, Plöen L, Hagenäs L, Ritzén EM (1981). Recovery of testicular functions after surgical treatment of experimental cryptorchidism in the rat. *Int J Androl* **4**: 145–160.

Kedersha N, Chen S, Gilks N, Li W, Miller IJ, Stahl J, Anderson P. (2002). Evidence that ternary complex (eIF2-GTP-$tRNA_i^{Met}$)-deficient preinitiation complexes are core constituents of mammalian stress granules. *Mol Biol Cell* **13**: 195-210.

Kim B, Cooke HJ, Rhee K (2012). DAZL is essential for stress granule formation implicated in germ cell survival upon heat stress. *Development* **139**: 568–578.

Kimball SR (2001). Regulation of translation initiation by amino acids in eukaryotic cells. *Prog Mol Subcell Biol* **26**: 155–184.

Krishnamoorthy T, Pavitt GD, Zhang F, Dever TE, Hinnebusch AG (2001). Tight binding of the phosphorylated alpha subunit of initiation factor 2 (eIF2alpha) to the regulatory subunits of guanine nucleotide exchange factor eIF2B is required for inhibition of translation initiation. *Mol Cell Biol* **21**: 5018–5030.

Ladiges W, Morton J, Blakely C, Gale M. (2000). Tissue specific expression of PKR protein kinase in aging B6D2F1 mice. *Mech Ageing Dev* **114**: 123-32.

Liu Y, Sakamoto H, Adachi M, Zhao S, Ukai W, Hashimoto E, Hareyama M, Ishida T,

Imai K, Shinomura Y. (2012). Heat stress activates ER stress signals which suppress the heat shock response, an effect occurring preferentially in the cortex in rats. *Mol Biol Rep* **39**: 3987-93.

Lu L, Han AP, Chen JJ (2001). Translation initiation control by heme-regulated eukaryotic initiation factor 2alpha kinase in erythroid cells under cytoplasmic stresses. *Mol Cell Biol* **21**: 7971–7980.

Lue, Y. H., Hikim, A. P., Swerdloff, R. S., Im, P., Taing, K. S., Bui, T., Leung, A. and Wang, C. (1999). Single exposure to heat induces stage-specific germ cell apoptosis in rats: role of intratesticular testosterone on stage specificity. *Endocrinology* **140**: 1709-1717.

Manche L, Green SR, Schmedt C, Mathews MB. (1992). Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol Cell Biol* **12**: 5238-48.

Munro S, Pelham HR. (1986). An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell* **46**: 291-300.

Nakamura M, Hall PF (1978). The influence of temperature upon polysomes of spermatids of rat testes. *Biochem Biophys Res Commun* **85**: 756–761.

Nakamura M, Romrell LJ, Hall PF (1978). The effects of temperature and glucose on protein biosynthesis by immature (round) spermatids from rat testes. *J Cell Biol* **79**: 1–9.

Nakamura M, Hall PF (1980). The mechanism by which body temperature inhibits protein biosynthesis in spermatids of rat testes. *J Biol Chem* **255**: 2907–2913.

Oakberg EF. (1956). A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. *Am J Anat* **99**: 391-413.

Pallepati P, Averill-Bates DA. (2011). Activation of ER stress and apoptosis by hydrogen peroxide in HeLa cells: protective role of mild heat preconditioning at 40°C. *Biochim Biophys Acta* **1813**: 1987-99.

Patil C, Walter P (2001). Intracellular signaling from the endoplasmic reticulum to the nucleus: the unfolded protein response in yeast and mammals. *Curr Opin Cell Biol* **13**:

349–355.

Russell, L. D., Ettlin, R. A., Sinha-Hikim, A. P. and Clegg, E. D. (1990). Histological and Histopathological Evaluation of the Testis. *Clearwater, FL: Cache River Press.*

Rockett, J. C., Mapp, F. L., Garges, J. B., Luft, J. C., Mori, C. and Dix, D. J. (2001). Effects of hyperthermia on spermatogenesis, apoptosis, gene expression, and fertility in adult male mice. *Biol Reprod* **65**: 229-239.

Rowlands AG, Panniers R, Henshaw EC (1988). The catalytic mechanism of guanine nucleotide exchange factor action and competitive inhibition by phosphorylated eukaryotic initiation factor 2. *J Biol Chem* **263**: 5526–5533.

Sarge KD (1995). Male germ cell-specific alteration in temperature set point of the cellular stress response. *J Biol Chem* **270**: 18745–18748.

Sarge KD, Bray AE, Goodson ML (1995). Altered stress response in testis. *Nature* **374**: 126.

Shi Y, Vattem KM, Sood R, An J, Liang J, Stramm L, Wek RC. (1998). Identification

and characterization of pancreatic eukaryotic initiation factor 2 alpha-subunit kinase, PEK, involved in translational control. *Mol Cell Biol* **18**: 7499-509.

Shikone, T., Billig, H. and Hsueh, A. J. (1994). Experimentally induced cryptorchidism increases apoptosis in rat testis. *Biol Reprod* **51**: 865-872.

Scheuner D, Song B, McEwen E, Liu C, Laybutt R, Gillespie P, Saunders T, Bonner-Weir S, Kaufman RJ. (2001). Translational control is required for the unfolded protein response and in vivo glucose homeostasis. *Mol Cell* **7**: 1165-76.

Wang XZ, Lawson B, Brewer JW, Zinszner H, Sanjay A, Mi LJ, Boorstein R, Kreibich G, Hendershot LM, Ron D. (1996). Signals from the stressed endoplasmic reticulum induce C/EBP-homologous protein (CHOP/GADD153). *Mol Cell Biol* **16**: 4273-80.

Wek SA, Zhu S, Wek RC. (1995). The histidyl-tRNA synthetase-related sequence in the eIF-2 alpha protein kinase GCN2 interacts with tRNA and is required for activation in response to starvation for different amino acids. *Mol Cell Biol* **15**: 4497-506.

Wykes SM, Nelson JE, Visscher DW, Djakiew D, Krawetz SA. (1995). Coordinate expression of the PRM1, PRM2, and TNP2 multigene locus in human testis. *DNA Cell*

Biol **14**: 155-61.

Yin, Y., Hawkins, K. L., DeWolf, W. C. and Morgentaler, A. (1997). Heat stress causes testicular germ cell apoptosis in adult mice. *J Androl* **18**: 159-165.

Zhan K, Vattem KM, Bauer BN, Dever TE, Chen JJ, Wek RC (2002). Phosphorylation of eukaryotic initiation factor 2 by heme-regulated inhibitor kinase-related protein kinases in Schizosaccharomyces pombe is important for resistance to environmental stresses. *Mol Cell Biol* **22**: 7134–7146.

Zhang P, McGrath BC, Reinert J, Olsen DS, Lei L, Gill S, Wek SA, Vattem KM, Wek RC, Kimball SR, Jefferson LS, Cavener DR. (2002). The GCN2 eIF2alpha kinase is required for adaptation to amino acid deprivation in mice. *Mol Cell Biol* **22**: 6681-8.

Zhang ZH, Hu ZY, Song XX, Xiao LJ, Zou RJ, Han CS, Liu YX (2004). Disrupted expression of intermediate filaments in the testis of rhesus monkey after experimental cryptorchidism. *Int J Androl* **27**: 234–239.

Zhu H, Guo FJ, Zhao W, Zhou J, Liu Y, Song F, Wang Y. (2012). ATF4 and IRE1α inhibit DNA repair protein DNA-dependent protein kinase 1 induced by heat shock.

Mol Cell Biochem **371**: 225-32.

국문초록

포유동물의 웅성생식세포는 그들의 성공적인 발달을 위하여 체온 이하의 온도로 유지되어야 한다. 본 연구실에서의 선행 연구에 의하면, 열 스트레스에 대한 방어 기전으로서 생쥐 웅성생식세포 내 eIF2α (진핵 세포 번역 개시 인자 eIF2의 α 소단위체) 의 즉각적 인산화가 보고되었다. 이번 연구에서는 eIF2α 단백질이 모든 단계의 생쥐 웅성생식세포에서 발현되고 있음을 확인하였다. 그러나 흥미롭게도, 열 스트레스에 의한 eIF2α 인산화는 생쥐 웅성생식세포의 이른 태사기 정모세포 단계까지의 세포질에서만 관찰되었다. 따라서 열 스트레스에 의해 웅성생식세포의 eIF2α 를 인산화 시키는 상위의 인산화 효소를 찾고자, 현재까지 알려진 네 가지의 eIF2α 인산화 효소 (HRI, PKR, PERK, 그리고 GCN2) 의 발현 정도를 생쥐 정소에서 확인하는 실험을 수행하였고, 그 결과 각각의 eIF2α 인산화 효소들은 발달단계에 따라 특징적인 발현이 있음을 보였다. 특히 생쥐 정소 내 PERK 단백질이 열 스트레스에 의해 즉각적으로 인산화된다는 것이 확인되었다. 이러한 결과들을 바탕으로, 본 연구는 PERK 단백질이 생쥐 웅성생식세포에서 열 스트레스를 감지하여 eIF2α 를 인산화하는 상위의 효소로서 작용할 수 있음을 제안한다.

주 요 어 : 열 스트레스, 생쥐 정소, eIF2α 인산화, eIF2α 인산화 효소

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