HSF4 is required for normal cell growth and differentiation during mouse lens development

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The heat shock transcription factor (HSF) family consists of three members in mammals and regulates expression of heat shock genes via a heat shock element. HSF1 and HSF2 are required for some developmental processes, but it is unclear how they regulate these processes. To elucidate the mechanisms of developmental regulation by HSFs, we generated mice in which the *HSF4* gene is mutated. HSF4-null mice had cataract with abnormal lens fiber cells containing inclusion-like structures, probably due to decreased expression of γ-crystallin, which maintains protein stability. Furthermore, we found increased proliferation and premature differentiation of the mutant lens epithelial cells, which is associated with increased expression of growth factors, FGF-1, FGF-4, and FGF-7. Unexpectedly, HSF1 competed with HSF4 for the expression of FGFs not only in the lens but also in other tissues. These findings reveal the lens-specific role of HSF4, which activates *γ-crystallin* genes, and also indicate that HSF1 and HSF4 are involved in regulating expression of growth factor genes, which are essential for cell growth and differentiation.

Introduction

All organisms respond to elevated temperatures by inducing a set of heat shock proteins (Hsps). This response is regulated mainly at the level of transcription by heat shock transcription factor (HSF), which binds to a heat shock element (HSE), which is composed of at least three inverted repeats of

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consensus sequence nGAAn (Wu, 1995). The HSF family consists of four members (HSF1–4) in vertebrates, whereas a single HSF is present in yeast, nematode, and the fruit fly (Morimoto, 1998). HSF1 and HSF2 are ubiquitous among vertebrate species, whereas HSF3 and HSF4 have been characterized only in avians and in mammals, respectively (Nakai, 1999). All of the HSFs have a DNA-binding domain and a leucine zipper-like trimerization domain (HR-A/B), and HSF trimers bind to HSE with high affinity. There is another leucine zipper-like domain (HR-C) that suppresses trimer formation except in HSF4. Therefore, HSFs stay as monomers or dimers under normal growth conditions and are converted to trimers when they are activated (Morimoto, 1998). In mammals, HSF1 is necessary for induction of heat shock genes under heat shock conditions and for acquisition of thermotolerance (McMillan *et al*, 1998; Zhang *et al*, 2002).

In addition to the role in the activation of gene expression in response to various stresses, HSFs have been shown to be involved in cell growth and differentiation (Morano and Thiele, 1999; Pirkkala *et al*, 2001). In *Drosophila*, a single HSF is necessary for oogenesis and early larval development (Jedlicka *et al*, 1997). Likewise, mouse HSF1 is required for oogenesis, placental development, and normal growth (Xiao *et al*, 1999; Christians *et al*, 2000; Zhang *et al*, 2002). Furthermore, HSF1 plays a role in eliminating injured male germ cells when these cells are exposed to stress (Nakai *et al*, 2000; Izu *et al*, 2004). Remarkably, these developmental functions of HSF1 are not mediated through the induction of Hsps. HSF2 is also involved in oogenesis, spermatogenesis, and brain formation (Kallio *et al*, 2002; Wang *et al*, 2003). Furthermore, HSF1-null or HSF2-null male mice are fertile (Xiao *et al*, 1999; Kallio *et al*, 2002; McMillan *et al*, 2002; Zhang *et al*, 2002; Wang *et al*, 2003; Izu *et al*, 2004), whereas mice deficient in both HSF1 and HSF2 are sterile with severe defects in spermatogenesis (Wang *et al*, 2004). This observation indicates the complementary roles of HSF1 and HSF2 in spermatogenesis. Although comprehensive chromatin immunoprecipitation (ChIP) analysis revealed that HSF1 binds to many genes *in vivo* in human cells under normal growth conditions (Trinklein *et al*, 2004), it is yet unclear how HSF1 and HSF2 regulate the developmental processes and which target genes are responsible for the development.

In contrast to HSF1 and HSF2 proteins, which are expressed in most tissues, the expression level of HSF4 protein is too low to be detected in many tissues except in the brain and lung (Tanabe *et al*, 1999). There are at least two isoforms, HSF4a and HSF4b, which are derived by alternative RNA splicing events. HSF4b has the potential to activate transcription, whereas HSF4a does not have this potential (Nakai *et al*, 1997; Tanabe *et al*, 1999; Frejtag *et al*, 2001; Zhang *et al*, 2001). Both isoforms lack an HR-C domain that inhibits the trimer formation. Therefore, HSF4 is constitutively a trimer that binds to HSE, suggesting that HSF4 may have physiological roles during development (Nakai, 1999). It was shown recently that mutations of HSF4 are associated with dominant

inherited cataracts in human (Bu et al, 2002). In this study, we found that HSF4 has a major HSE-binding activity specifically in the lens extract. As the lens consists of only two cell populations, it provides an excellent model of cell growth and differentiation (McAvoy et al, 1999). To elucidate the roles of HSFs in these processes, we generated mice in which the HSF4 gene was mutated. We found anomalies of the lens, and revealed novel HSF4 target genes that are essential for cell growth and differentiation.

Result

HSF4 consists of a major HSE-binding activity in the lens

In the absence of stress, there are weak, but distinct, HSE-binding activities in many tissues. Antibody supershift experiments showed that most of the HSE-binding activities consist of HSF1 and HSF2 (Fiorenza et al, 1995). HSF4 mRNA is expressed ubiquitously at low levels (Nakai et al, 1997; Tanabe et al, 1999), and HSF4 protein has been detected in the brain and lung, and cell lines such as C2C12 (Tanabe et al, 1999). To detect HSE-binding activity of HSF4, we performed gel shift assay using extracts isolated from various tissues including the skin, inner ear, and nasal cavity. HSE-binding activity was composed mostly of HSF1 in the tissue extracts as in the brain and lung extracts (Figure 1A, data not shown). Exceptionally, the HSE-binding activity in the lens extract was exclusively composed of HSF4 (Figure 1A). Furthermore, the expression level of HSF4 protein was much higher in the lens compared to those in the brain and lung, whereas the expression level of HSF1 protein was lower in the lens (Figure 1B). These results suggest unique roles of HSF4 in the lens.

We next examined the profile of HSF4 expression during lens development. We detected only a b-isoform of HSF4 (HSF4b) at any developmental stages by Western blot analysis (Figures 1C and 2D) and RT–PCR analysis (data not shown). Consistent with a previous work showing HSE-binding activity in the lens (Somasundaram and Bhat, 2000), the level of HSF4 protein reached a peak early after birth, and then decreased (Figure 1C). At embryonic day 13.5 (E13.5), cells in the posterior half of the lens vesicle elongated and differentiated to form the primary fibers. HSF4 mRNA is not expressed at E11.5, but is expressed in both epithelial cells and fiber cells at E13.5 (Figure 1D). HSF4 mRNA continued to be expressed in both cell types of the lens at least until 6 weeks after birth (data not shown).

Inactivation of the HSF4 gene causes abnormal lens fiber cells containing inclusion-like structures

To examine the in vivo functions of HSF4, we generated targeted disruptions of the mouse HSF4 gene by homologous recombination in TT2 embryonic stem (ES) cells (Inouye et al, 2003). In the targeting vector, a region containing a part of exon 2 and exons 3–8 was substituted with a neomycin-resistant gene cassette (Figure 2A). This substitution removes amino acids at position 68 to position 248 in mouse HSF4, which contains part of the DNA-binding domain and an oligomerization domain (Tanabe et al, 1999). The ES cells were electroporated with the targeting vector, and placed under positive–negative selection (Inouye et al, 2003). The surviving 176 ES clones were screened for homologous
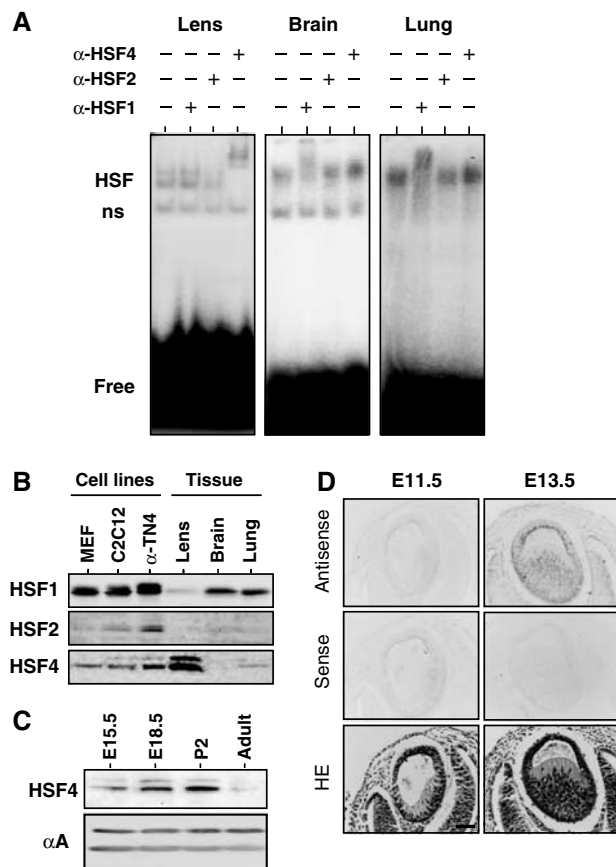

Figure 1 HSF4 consists of a major HSE-binding activity in the mouse lens. (**A**) Gel shift assay in the presence or absence of antibody for HSF1, HSF2, or HSF4. Whole tissue extracts were prepared from the lens, brain, and lung in 6-week-old mice, and were mixed with the binding reaction containing a ^{32}P-labeled HSE oligonucleotide and antibody. HSF indicates the complexes of HSFs and an HSE probe, ns indicates nonspecific binding activity, and free indicates an unbound HSE probe. (**B**) Protein levels of HSF1, HSF2, and HSF4 in cells and tissues. Cell extracts (40 μg) prepared from MEF, C2C12, and α-TN4, and tissue extracts (80 μg) prepared from the lens, brain, and lung in 2-day-old mice were subjected to Western blot analysis using each specific antibody. (**C**) Protein levels of HSF4 in the lens at E15.5, E18.5, p2, and 6-week-old (adult) were analyzed by Western blot. Level of αA-crystallin was constant during development. (**D**) In situ hybridization was performed on the eye sections of E11.5 and E13.5 embryos using sense and antisense probes specific for HSF4. The sections were also stained with hematoxylin and eosin (HE). Bar, 50 μm.

recombination by PCR and Southern blotting (Figure 2B), and correct gene targeting occurred in 22 clones. Two lines of knockout mice were generated by injecting C35 and C49 ES clones into eight-cell embryos of ICR mice, and were maintained by mating with ICR mice. Progeny of matings between heterozygotes were analyzed by PCR to identify wild-type (+/+), heterozygous (+/−), and homozygous (−/−) mutant offspring (Figure 2C), and were found to be present at the expected Mendelian ratio (49:82:43, respectively; n = 174). Body weights of HSF4-null mice were as same as those of wild-type mice at all developmental stages (data not shown). Male and female HSF4-null mice were fertile, and the histology of the testis and ovary was normal (data not shown). We detected no abnormality in the brain or lung in HSF4-null mice (data not shown).
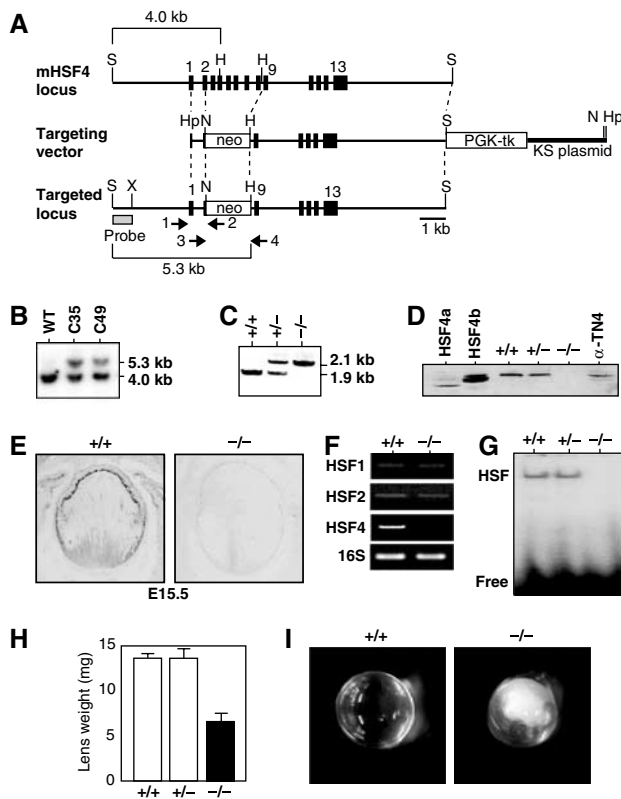
Figure 2 Cataract formation in HSF4-null mice. (**A**) Schematic representation of wild-type and mutant *HSF4* loci together with targeting vector. The targeting vector was constructed to replace a part of exon 2 and exons 3–8 with a neomycin-resistant gene cassette. Locations of an external probe used to confirm correct targeting, and locations of PCR primers used to screen targeted ES clones (primers 1 and 2) and to identify genotype (primers 3 and 4) are shown. S, SalI; H, HindIII; Hp, HpaI; N, NotI; X, XbaI. (**B**) Southern blot of SalI/HindIII-digested genomic DNAs isolated from wild-type and targeted two ES clones (C39 and C49) using ^{32}P-labeled probes described in (A). (**C**) PCR genotypic analysis for targeted locus. Mouse tail genomic DNA was isolated and was used to amplify DNA fragments by PCR using primers 3 and 4. (**D**) Western blot analysis of extracts of the lens of 6-week-old mice (+/+, +/−, −/−), 293 cells ectopically expressing HSF4a and HSF4b, and mouse lens epithelial α-TN4 cells. (**E**) *In situ* hybridization on the eye sections of E15.5 wild-type (+/+) and HSF4-null (−/−) mice using an antisense probe specific for HSF4. (**F**) mRNA levels of HSFs in wild-type(+/+) and HSF4-null (−/−) lens were examined by RT–PCR. (**G**) Whole-cell extracts were prepared from the lenses, and gel shift assay was performed using a ^{32}P-labeled HSE oligonucleotide. Positions of free probe and probe bound by HSF are shown. (**H**) Lens weights in 6-week-old wild-type and mutant mice. Means and standard deviations were estimated by analyzing each of the three mice. (**I**) Lens opacity in 6-week-old wild-type and HSF4-null mice.

Figure 3 The lens fiber cells contain inclusion-like structures in HSF4-null mice. (**A**) Histological examination of the lens sections of the 6-week-old wild-type (a, c) and HSF4-null (b, d) mice stained with HE. Immunostaining of αB-crystallin was performed using the lens sections of wild-type (e) and HSF4-null (f) mice. The open arrow in (b) indicates the lesion where the structure of the fiber cells was not recognized. The legion indicated by a square in (b) was enlarged into (d). Arrows in (d, f) indicate cytoplasmic inclusion-like structures and stars indicate the nucleus. Magnification: (a, b) ×25; (c–f) ×1000. Bars, 10 μm. (**B**) Expression of Hsps and αA-, αB-, and γ-crystallins. Proteins isolated from the lens in 6-week-old wild-type (+/+) and mutant (+/− and −/−) mice were analyzed by Western blot analysis using each specific antibody. In the Hsp70 column, an upper band represents Hsc70 and a lower band represents an inducible Hsp70. (**C**) Immunohistochemistry of 6-week-old mice using antiserum for each specific antiserum. The bow regions (upper columns) and the epithelial layers (lower columns) are shown.

We next examined the lens, and confirmed that HSF4 expression was absent in HSF4-null mice (Figure 2D and E). Expression levels of HSF1 and HSF2 mRNAs were normal in HSF4-null lens (Figure 2F). We detected no HSE-binding activity in an extract isolated from HSF4-null lens (Figure 2G). Weight of the HSF4-null lens was lower than that of the wild-type lens (Figure 2H), and HSF4-null mice had cataract (Figure 2I). We then examined histology of the lens. In 6-week-old mice, wild-type lens fiber cells were flattened by dehydration, whereas HSF4-null fiber cells swelled (Figure 3A). Furthermore, HSF4-null fiber cells in the center
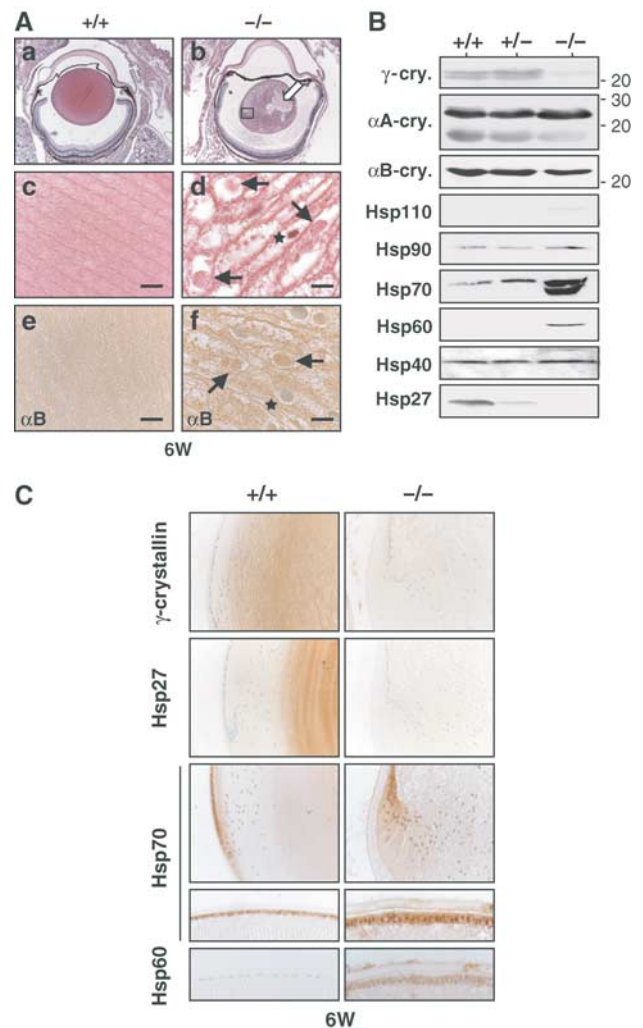
of the lens contained inclusion-like structures, which were stained heavily with eosin and were possibly protein aggregates (Figure 3A, arrows in d). These abnormalities can be observed even at 2 days after birth (data not shown). However, HSF4-null lens cells normally elongated and differentiated into fiber cells until E15.5 (data not shown). The inclusion-like structures were stained with antibodies for abundant lens proteins, αB-crystallin (Figure 3Af) and αA-crystallin (data not shown). Hsp70 was not accumulated in the inclusion (data not shown). These results suggest that

loss of HSF4 function may cause protein aggregates in the center of the lens.

Expression of γ-crystallins decreases in HSF4-null mice

As heat shock genes are candidate targets of HSF4, we examined the expression of major Hsps in the lens. We found that Hsp27 expression in the inner layer of the lens reduced in HSF4-null mice (Figure 3B and C). However, it is unclear whether reduced expression of Hsp27 leads to protein aggregates. Unexpectedly, we found that expression of Hsp60, Hsp70, Hsp90, and Hsp110 increased (Figure 3B). Immunohistochemical analysis also showed increased expression of Hsp70 and Hsp60 in the epithelial cells of HSF4-null lens (Figure 3C). These results indicate that HSF4 suppresses expression of major Hsps in epithelial cells.

As crystallins are major lens structural proteins in the lens, we examined expression of crystallins in the HSF4-null lens. The expressions of αA-crystallins, αB-crystallins, and β-crystallins were constant in the HSF4-null lens (Figure 3B, data not shown). Unexpectedly, we found that γ-crystallin expression was markedly reduced (Figure 3B and C). All six γ-crystallin gene expressions were markedly reduced in the lens of adult 6-week-old HSF4-null mice when these were estimated by semiquantitative RT–PCR analysis (Figure 4A). The expression levels were reduced even in the lens of 2-day-old HSF4-null mice, but were normal in the lens of E15.5 HSF4-null embryos. The stage at which abnormal morphology of fiber cells becomes apparent correlates with the initiation of decreased expression of the γ-crystallin genes. These results demonstrate that HSF4 is required to maintain γ-crystallin gene expression at later developmental stages. As the γ-crystallin is essential for protein stability in the lens fiber cells as described below, the abnormalities of the fiber cells are partly due to the reduced expression of γ-crystallin.

As we found HSE-like sequences in all six γ-crystallin genes near transcription start sites (Figure 4B), we also analyzed γ-crystallin expression in the lens of HSF1-null mice (Inouye *et al*, 2003). Cataract did not develop in adult HSF1-null mice (see Figure 7A), but the γ-crystallin level reduced a little (∼70% of control level) (Figure 4C). Especially, expression of the γA-crystallin gene reduced significantly (Figure 4D). The reduction of γ-crystallins was not due to the shift of γ-crystallin proteins into an insoluble fraction (data not shown) or the leakage of proteins from damaged fiber cells (Figure 4E) (Piatigorsky *et al*, 1978). We further analyzed the binding of HSF4 to the HSE-like sequence of the γF-crystallin gene. Chromatin immunoprecipitation analysis revealed that HSF4 binds to the upstream region (−349 to +6) of the γF-crystallin gene *in vivo* (Figure 4F). These results suggest that HSF4 directly binds and regulates expression of the γ-crystallin gene.

The γ-crystallin gene expression is directly regulated by many transcription factors, including Pax-6 (Kondoh, 2002), Sox-1 (Nishiguchi *et al*, 1998), c-Maf (Kim *et al*, 1999), and Prox-1 (Lengler *et al*, 2001), and their regulations are essential for differentiation into fiber cells. In the HSF4-null lens, however, expression levels of these transcription factors were normal (data not shown). It is unclear why HSF4 is not required for expression of γ-crystallin genes during embryonic development, although Sox-1, c-Maf, and Prox-1 transcription factors are essential for the expression.
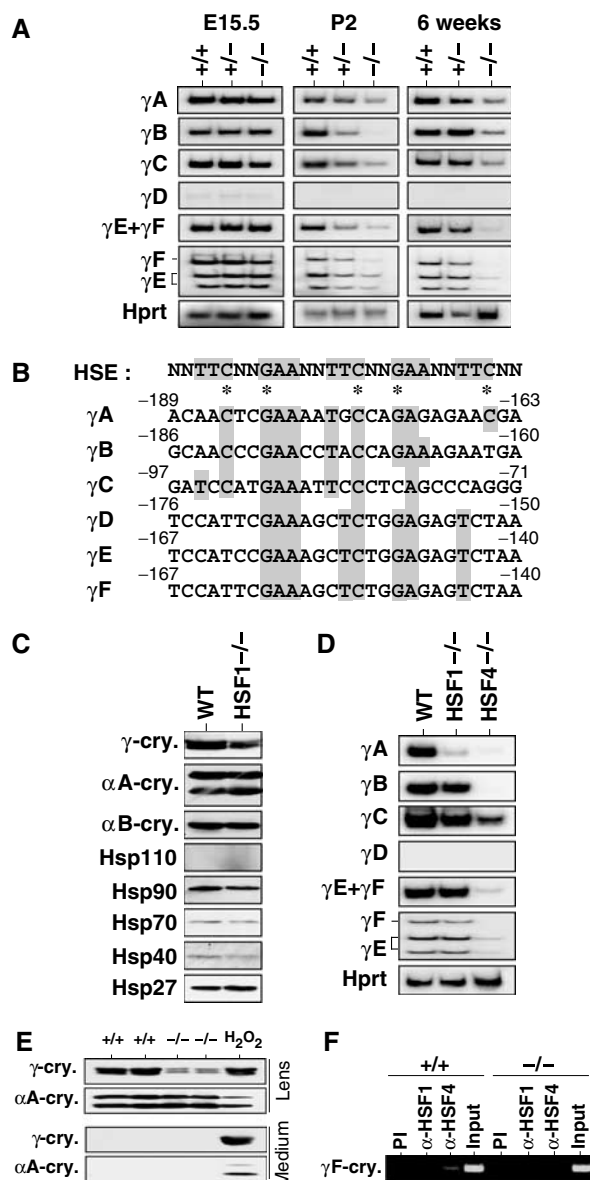

Figure 4 Expression of the *γ-crystallin* genes is markedly reduced in HSF4-null lens. (**A**) RT–PCR analysis of mRNAs of the *γ-crystallin* genes using specific primers. Total RNAs were isolated from lenses in 6-week-old mice, 2-day-old mice (P2), and 15.5 dpc embryos (E15.5). Images of autoradiography are shown. To identify γE and γF, the PCR products amplified with the same set of primers were digested with *Bgl*II. (**B**) Promoter sequence alignment of the six mouse *γ-crystallin* genes. Sequences identical to the consensus HSE sequences are shown in gray boxes. The asterisks indicate key nucleotides essential for HSF1 binding. (**C**) Expression of crystallins and Hsps in 6-week-old wild-type (WT) and HSF1-null (HSF1$^{-/-}$) mice examined by Western blot analysis. (**D**) RT–PCR analysis of mRNAs of the *γ-crystallin* genes using total RNAs isolated from lenses in 6-week-old mice. Images of autoradiography are shown. (**E**) Lenses were removed from 2-week-old wild-type (+/+) and HSF4-null (−/−) mice, and were incubated in medium at 37°C for 24 h. To damage lens cells, the lenses from wild-type mice were incubated in the presence of 1 mM H_2O_2. Proteins in the lens and medium were analyzed by Western blot analysis using antiserum specific for αA- or γ-crystallin. (**F**) ChIP-enriched DNAs from 2-week-old wild-type (+/+) and HSF4-null (−/−) lenses using preimmune serum (PI), anti-HSF1 serum (α-HSF1), and anti-HSF4 serum (α-HSF4) as well as an input DNA were amplified using primers specific for the *γF-crystallin* gene by PCR analysis. The DNA fragment (−349 to +6) was amplified.

HSF4 regulates proliferation and differentiation of lens epithelial cells by suppressing FGF expression

In addition to the abnormalities of the fiber cells, we observed abnormal epithelial cell morphology. Epithelial cells were found in the anterior of the lens and had cuboidal morphology in the wild-type lens. However, the epithelial cells in adult HSF4-null mice were columnar (Figure 5A). Furthermore, the epithelial cells in the bow region, where epithelial cells differentiated into fiber cells, were highly elongated in 2- and 6-week-old mice (Figure 5A). Electron micrographs showed the columnar morphology of the epithelial cells, and also showed that fiber cells near the epithelial layer were rich in organelles such as mitochondria (Figure 5B). Denucleation in fiber cells was also inhibited (Figure 5B). Furthermore, epithelial cells detached from the capsule that was stained with PAS (see Figure 7A). Numbers of epithelial cells per section in HSF4-null mice were the same as those in wild-type mice during embryonic development, but increased even at 2 days postpartum (Figure 5C). 5-bromo-2′-deoxyuridine (BrdU)-incorporated epithelial cells were observed throughout the epithelial layer in E18.5 mice and the number of the BrdU-positive cells increased in HSF4-null mice (Figure 5D). These results indicate increased proliferation of epithelial cells and premature differentiation of fiber cells in HSF4-null mice.

Fibroblast growth factors (FGFs) regulate growth and differentiation of lens epithelial cells (McAvoy *et al*, 1999). Transgenic mice expressing a low dose of FGF-1 exhibited epithelial cell hyperplasia, which is associated with premature elongation of epithelial cells (Robinson *et al*, 1995). These phenotypes are also observed in transgenic mice expressing FGF-4, FGF-7, FGF-8, and FGF-9 (Lovicu and Overbeek, 1998), and are similar to those observed in HSF4-null mice. Therefore, we examined expression of the FGFs by RT–PCR analysis. It clearly revealed that FGF-1, FGF-4, FGF-7, but not FGF-2, were expressed at higher levels in HSF4-null lens than those in wild-type lens (Figure 6A). *In situ* hybridization analysis showed high expression of FGF1 mRNA in HSF4-null epithelial cells and fiber cells (Figure 6B). To examine the roles of HSF4 in the FGF expression in other cell types, we overexpressed HSF4 into mouse embryo fibroblast (MEF) cells using an adenovirus vector, and found that HSF4 suppressed FGF-7 expression (Figure 6C). Furthermore, ChIP analysis revealed that HSF4 bound to the upstream region (-615 to $+10$) of the *FGF-7* gene *in vivo* in the lens (Figure 6D). This region contains five HSE consensus sequences (Finch *et al*, 1995; Fasciana *et al*, 1996). These data strongly suggest that HSF4 represses FGF expression by directly binding to *FGF* genes.

HSF1 and HSF4 have opposing effects on FGF expression

We next examined involvement of HSF1 in the regulation of FGF expression. We found that the epithelial cell morphology was normal in the HSF1-null lens (Figure 7A), but the expression of FGF-1 and FGF-7 was decreased (Figure 7D). As HSF1 also bound directly *FGF-7* gene (Figure 6D), HSF1 and HSF4 may regulate expression of *FGF* genes via HSE. To elucidate the interplay between HSF1 and HSF4 in FGF expression, we analyzed mice deficient for both HSF1 and HSF4 (double-null). We found that the high levels of FGF-1, FGF-4, and FGF-7 expression in the HSF4-null lens returned
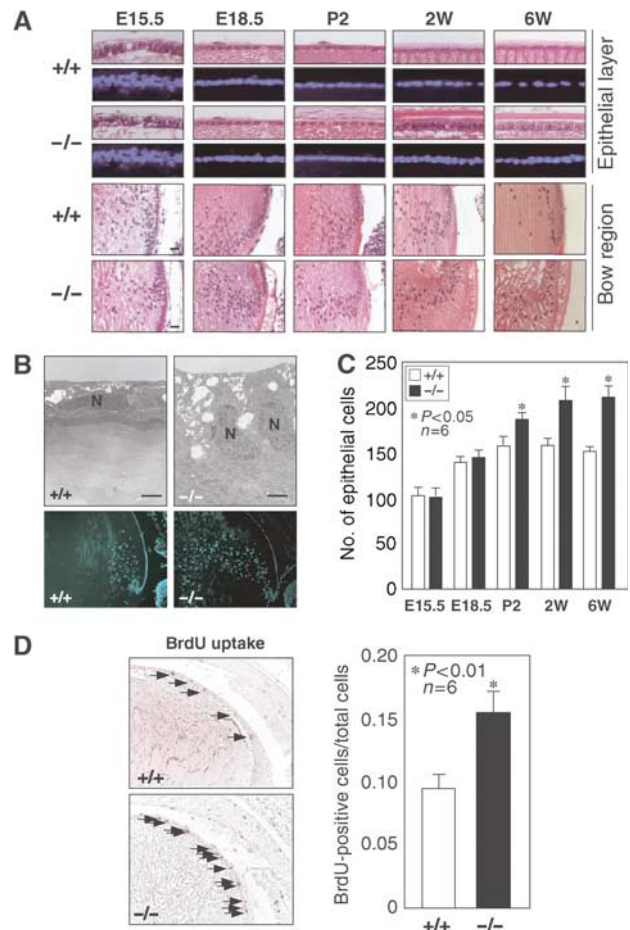

Figure 5 Increased proliferation and premature differentiation of the lens epithelial cells in HSF4-null mice. (**A**) Histological examination of the lens sections of 6-week-old, 2-week-old, 2-day-old, E18.5, and E15.5 wild-type ($+/+$) and HSF4-null ($-/-$) mice. Sections were stained with HE and DAPI. The epithelial layer (upper columns) is only found in the anterior of the lens. The bow regions, where epithelial cells differentiate into fiber cells, are also shown in the lower columns. (**B**) Transmission electron microscopic analysis (upper columns) and DAPI staining (lower columns) of 2-week-old wild-type and HSF4-null lens. The nuclei of the epithelial cells are indicated as N. Bar, 2 μm. (**C**) Numbers of total epithelial cells per section in six lenses were counted. The stars indicate $P<0.05$. (**D**) BrdU incorporation in the lens epithelial cells of E18.5 mice. The arrows indicate cells incorporated with BrdU. Percentages of BrdU-positive cells are shown. The stars indicate $P<0.01$.

to normal levels in the double-null lens (Figure 7D). Consistent with the expression levels of FGFs, the increased number of epithelial cells in the HSF4-null lens reverted to almost the normal number and elongated epithelial cells returned to a fairly normal morphology in the double-null lens (Figure 7A and B). Furthermore, high levels of Hsp70 and Hsp60 expressions in HSF4-null epithelial cells returned to normal levels in the double-null cells (Figure 7A and C). These results suggest that HSF1 may compete with HSF4 for the expression of FGFs and Hsps.

To reveal whether HSFs regulate FGF expression in other tissues, we examined FGF expression in the lung, where HSF4 and HSF1 are expressed (Tanabe *et al*, 1999), as well as in the testis. We did not detect any distinct abnormality in the two tissues (data not shown). Although expression of both FGFs and Hsps was not altered in HSF4-null testis, FGF-1
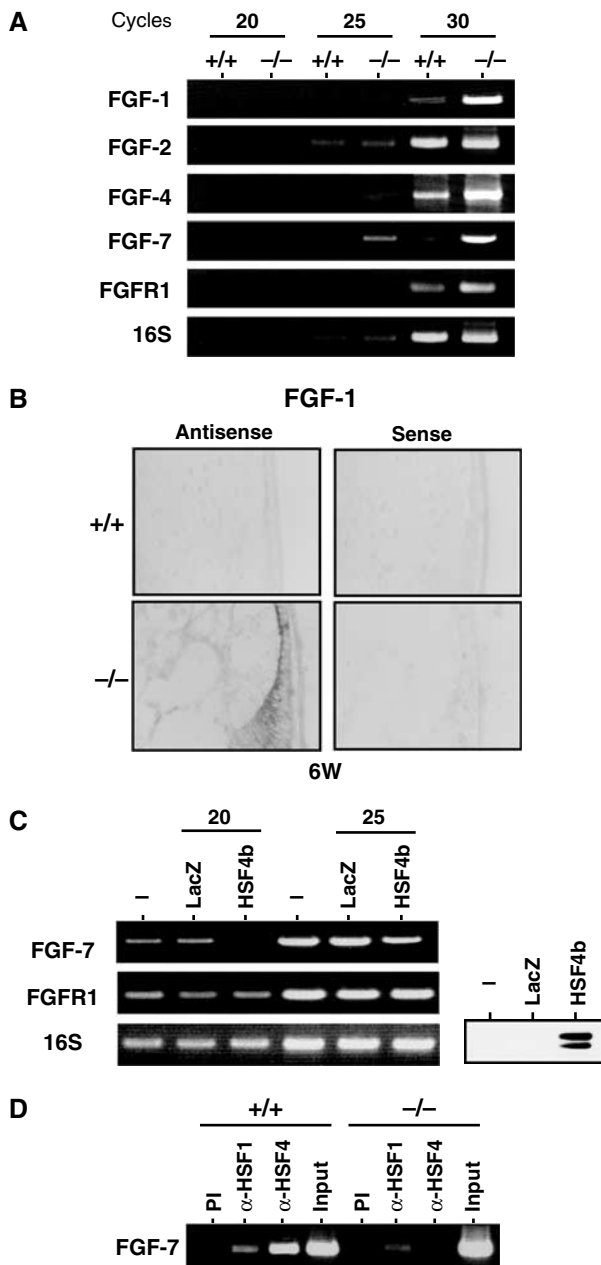
A

B

FGF-1

6W

C

D

Figure 6 Expression of FGFs is high in HSF4-null lenses. (**A**) RT–PCR analysis of mRNAs of the FGF-related genes using specific primers. RT–PCR was performed using total RNAs isolated from lenses of 6-week-old wild-type ($+/+$) and HSF4-null ($-/-$) mice, and DNA bands were stained with ethidium bromide. Representative data are shown. (**B**) *In situ* hybridization was performed on 6-week-old wild-type ($+/+$) and HSF4-null ($-/-$) lenses using sense and antisense probes specific for FGF-1. (**C**) RT–PCR analysis of mRNAs of FGF-related genes in MEF cells overexpressing HSF4b or LacZ as a control. MEF cells were transfected with adenovirus expressing HSF4b or LacZ. At 48 h after transfection, mRNA levels of FGF-7 and FGFR1 were estimated by RT–PCR. Western blot analysis of HSF4 protein is shown. (**D**) ChIP-enriched DNAs from 2-week-old wild-type ($+/+$) and HSF4-null ($-/-$) lenses using preimmune serum (PI), anti-HSF1 serum (α-HSF1), and anti-HSF4 serum (α-HSF4) as well as an input DNA were amplified using primers specific for the *FGF7* gene by PCR analysis. The DNA fragment (-615 to $+10$) was amplified.

expression was markedly reduced in HSF1-null lung (Figure 7E). Furthermore, the FGF-1 expression was reverted in double-null lung. Expression of major Hsps was also

reduced in HSF1-null lung, and expression of some Hsps was partially reverted in double-null lung (Figure 7E). These results suggest that HSF1 competes with HSF4 for the expression of FGFs and Hsps in general. As only an HSF4b isoform is expressed in the lung (Tanabe *et al*, 1999), HSF4b may act as a repressor of constitutive gene expression in many cell types.

It is noteworthy that lens morphology was more affected in double-null mice than in HSF4-null mice. Double-null mice developed severe cataract (data not shown). Weight of the double-null lens (4.57 ± 0.17 mg) in 6-week-old mice was similar to that of the HSF4-null lens (4.24 ± 0.69 mg). As the posterior lens capsule is thin, capsular rupture was observed in about half of the HSF4-null lens (data not shown). In contrast, the capsule ruptured in all double-null lens, and lens extrusion was heavily accumulated in double-null mice (Figure 7A, arrows). This observation may be explained by the fact that both HSF1 and HSF4 act as activators at least for *γ-crystallin* genes.

Discussion

In this study, we showed that expression of HSF4 is uniquely high in the mouse lens among major tissues. Analysis of HSF4-null lens revealed a specific role of HSF4, that is, it is required to maintain lens-specific *γ-crystallin* gene expression in fiber cells. Furthermore, analysis of abnormal growth and differentiation of epithelial cells revealed the general role of HSF1 and HSF4, which is to competitively regulate expression of FGFs.

Specific role of HSF4 in lens development
The lens is characterized by its high degree of transparency, which is essential for refracting light and focusing it onto the retina. Development of the lens occurs by differentiation of peripheral epithelial cells into elongated fiber cells, which accumulate in concentric layers and lose their nuclei and other organelles (McAvoy *et al*, 1999; Kondoh, 2002). The concentration of proteins in the lens fiber cells is extremely high, as much as 450 mg/ml of proteins in the center of the lens (Fagerholm *et al*, 1981). As proteins in the center of the lens cannot turnover and must remain stable and soluble throughout the life of the organism (Zigler, 1994; Bhat, 2003), it is important to stabilize proteins.

To stabilize proteins in that extreme condition, crystallins constitute nearly 90% of the soluble protein in the lens fiber cells (Zigler, 1994). The ubiquitous vertebrate crystallins consist of two unrelated families of proteins, the α-crystallins and the β, γ-crystallins (Wistow and Piatigorsky, 1988). The former belongs to a small Hsp/α-crystallin superfamily. The latter belongs to a β, γ-crystallin superfamily that has a repeat of a characteristic Greek key βγ motif (Wistow and Piatigorsky, 1988). Members of this family in soil bacteria and in slime mold are both induced in response to stresses such as dehydrated environments, and have roles in protein stabilization under dehydrated conditions (Wistow, 1990). We showed that HSF4 expression is extremely high in the lens among major tissues and is required for maintaining γ-crystallin expression in postnatal fiber cells. HSF might have originally played a role in the regulation of protein folding by upregulating Hsps. In the lens, however, the *HSF4*
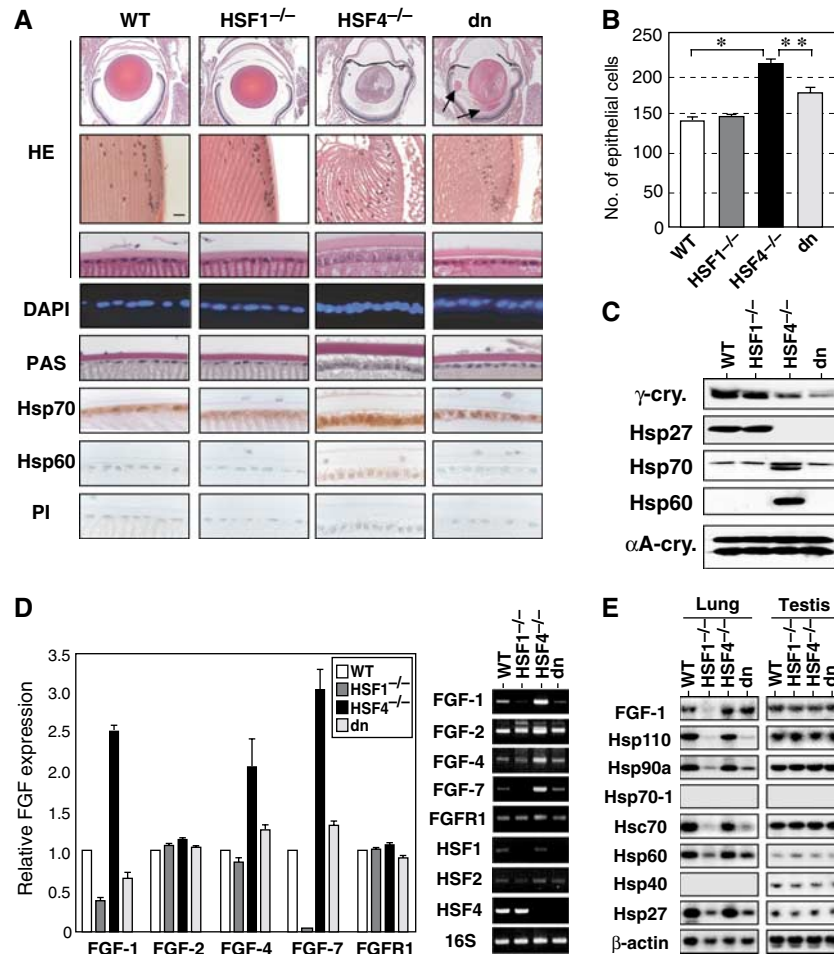
Figure 7 HSF1 competes with HSF4 for the expression of FGFs. (**A**) Histological examination of the lens sections of 6-week-old wild-type, HSF1-null, HSF4-null, and double-null (dn) mice. Sections were stained with HE and DAPI, PAS, or immunostained using a preimmune serum (PI) or an antiserum specific for Hsp70 or Hsp60. Lens extrusion was heavily accumulated in double-null mice (arrows). (**B**) Numbers of total epithelial cells per section in six lenses were shown on the right. The stars indicate $P < 0.01$. (**B**) *In situ* hybridization was performed on 6-week-old wild-type ($+/+$), and HSF4-null ($-/-$) lenses using sense and antisense probes specific for FGF-1. (**C**) Proteins isolated from the lenses in 6-week-old mice were analyzed by Western blot analysis using each specific antibody. (**D**) RT–PCR analysis of mRNAs of the FGF-related genes in wild-type, HSF1-null, HSF4-null, and double-null (dn) mice. RT–PCR analysis was performed using total RNAs isolated from lenses in 6-week-old mice. Relative FGF expression levels are estimated from three experiments. Representative data are shown on the right. (**E**) Northern blot analysis of FGF-1 and Hsp mRNAs using total RNAs isolated from the lung and testis.

gene may have evolved to adapt to the unique dehydrated environment of the lens.

Six genes, $\gamma A\gamma B\gamma C\gamma D\gamma E\gamma F$-*crystallin* genes, have been identified in the same chromosome in the mouse and human, which might arise by multiple gene duplication, and their expressions are independently regulated during development (Zigler, 1994; Heon *et al*, 1999; Kondoh, 2002). Surprisingly, all six γ-*crystallin* genes have conserved HSE sequences near their transcription start sites. These HSE sequences consist of at least three *n*GAA*n* units, and are recognized by HSF4 and HSF1. In this regard, the HSE sequences in the developmentally regulated γ-*crystallin* genes are similar to those in heat shock genes. As HSF4 stays as a trimer that binds to HSE with high affinity (Nakai *et al*, 1997; Tanabe *et al*, 1999), it is easy to conclude that HSF4 plays a role in the constitutive expression of γ-*crystallin* genes by binding to conventional HSE. In contrast, vertebrate HSF1 is mostly a monomer in the absence of stress (Morimoto, 1998). Nevertheless, HSF1 regulates constitutive Hsp90α expression in chicken B lymphocytes (Tanabe *et al*, 1998; Nakai and Ishikawa,

2001), and regulates Hsp27 expression in the mouse heart (Yan *et al*, 2002). As the HSE sequences in the γ-*crystallin* genes consist of more than three *n*GAA*n* units, only a low quantity of HSF1 may bind to and regulate the γ-*crystallin* genes during development.

HSF1 and HSF4 competitively regulate FGF expression, which is essential for cell growth and differentiation

A low dose of FGFs stimulates proliferation of lens epithelial cells, whereas higher doses induce cell differentiation (McAvoy and Chamberlain, 1989; Robinson *et al*, 1995; Lovicu and Overbeek, 1998). Analysis of FGF expression revealed that expression of FGF-1, FGF-4, and FGF-7 increases in HSF4-null lens. Consistently, loss of HSF4 function resulted in increased proliferation and premature differentiation of the lens epithelial cells. Furthermore, HSF1 activates the similar set of FGFs in the lens that is repressed by HSF4. Therefore, abnormal levels of FGFs in HSF4-null or HSF1-null lens reversed to normal levels in double-null lens, and proliferation and differentiation of the epithelial cells were

almost normal in double-null lens. We found that many HSE consensus sequences are located in the upstream region of the *FGF-7* gene, and that the *FGF-7* gene is a direct target of HSF4 and HSF1. These results demonstrate that HSF1 and HSF4 competitively regulate expression of FGFs that are essential for cell growth and differentiation. As FGFs regulate growth and differentiation of various cell types (Burgess and Maciag, 1989), regulation of FGFs by HSFs might be an important regulatory pathway for development in general.

We previously predicted the existence of two HSF4 isoforms, HSF4a and HSF4b, which are derived from alternatively spliced mRNA species (Tanabe *et al*, 1999). However, we could detect only HSF4b in the lens, as in other tissues. HSF4b has a weak but distinct potential to activate transcription in some cells (Tanabe *et al*, 1999). In fact, HSF4b can activate γ-*crystallin* and *Hsp27* genes in the fiber cells. In marked contrast, it represses *FGF* and *Hsp* genes in epithelial cells. These results demonstrate that HSF4b can be an activator or a repressor depending on the cell type, and suggest that there may be a cell type-specific coregulator of HSF4. Although HSF1 inhibits induction of TNF-α or IL-1β in response to endotoxin shock (Xiao *et al*, 1999; Xie *et al*, 2002), this is the first demonstration that constitutive expression of target genes is repressed by a member of HSF family. In general, HSF4 may be a repressor that competes with HSF1 as an activator.

In this study, we showed abnormality of both the lens epithelial cells and fiber cells in HSF4-null mice. These abnormalities may be partly due to reduced expression of γ-crystallins and increased expression of FGFs. In addition, we observed that Hsp27 expression reduced significantly in the fiber cells and that Hsp70 and Hsp60 increased in the epithelial cells. It is possible that the changes in expression levels of Hsps cause abnormal lens development. Although HSF1-null and HSF2-null mice exhibit only restricted phenotypes, our results suggest that HSFs may be involved in fine regulation of Hsp genes and growth factor genes, the regulation of which is not strictly necessary for development. Rather, HSFs might delicately regulate expression of these genes, which maintain homeostasis of the body under normal physiological condition.

Materials and methods

Generation of HSF4-null mice

A DNA fragment containing the mouse *HSF4* gene was isolated from a mouse 129 SvJ lambda genomic library (Stratagene) using an HSF4 cDNA probe and the gene structure was characterized by restriction mapping and sequencing. A 7.5 kb *Hin*dIII/*Sal*I fragment and a 0.8 kb PCR-amplified fragment were inserted into a plasmid pKO (Inouye *et al*, 2003) to generate a targeting vector. The linearized targeting vector was electroporated into TT2 ES cells (C57Bl/6 × CBA F1 origin). We selected targeted ES cells with G418 and gancyclovir and identified homologous recombination with PCR and Southern blot. We injected HSF4+/− two ES clones (C35 and C49) into eight-cell embryos of ICR mice and crossed the chimeras into ICR females. We crossed first-generation heterozygote mice (F1) into ICR or C57Bl/6 mice and screened the progeny by PCR and Southern blot. The progeny that carried the targeted allele were maintained by crossing into ICR or C57Bl/6 mice. We analyzed the eye phenotypes of mice crossed for more than six generations.

HSF1-null mice maintained by crossing into ICR mice were used (Inouye *et al*, 2003). To generate mice deficient for both HSF1 and HSF4, male and female mice with a genotype of HSF1+/−; HSF4+/− were crossed. Wild-type (+/+), heterozygous (HSF4+/−), and homozygous (HSF4−/−) mutant offspring were

present at the expected Mendelian ratio even in the presence of the HSF1 mutation.

Gel shift assay
Lenses and other tissues isolated from mice were frozen at −80°C until use. The lens extracts were prepared in buffer C as previously described (Nakai *et al*, 1995). Aliquots containing 80 μg of proteins were subjected to gel shift assay using an ideal HSE oligonucleotide as a probe in the presence or absence of antiserum for each HSF, α-HSF1γ, α-HSF2δ, or α-HSF4b (2.0 μl of 1:10 diluted antiserum in phosphate-buffered saline (PBS)) (Nakai *et al*, 1997).

Western blot analysis
To isolate soluble and insoluble lens extracts, lenses were homogenized in a buffer containing 0.1 M sodium phosphate (pH 7.4) and 0.1 M NaCl. After centrifugation at 13 000 *g* for 20 min at 4°C, the supernatants were removed and the pellets were dissolved in a sodium dodecyl sulfate (SDS) loading buffer. Aliquots containing equal amounts of proteins were subjected to Western bolt analysis using antibodies against Hsp90 (a kind gift from Dr Y Miyata), Hsp40 (StressGen, SPA-400), Hsp27, αA-crystallins, and αB-crystallins (Kato *et al*, 1998), and for γ-crystallin (a kind gift from Dr H Kondoh). To detect Hsp110, Hsp70, and Hsp60, we generated antiserum specific for each Hsp. Recombinant mouse Hsp110 (amino acids 577–877), human Hsp70 (amino acids 1–641), or mouse Hsp60 (amino acids 358–554) fused to glutathione-*S*-transferase was immunized into rabbits in a TiterMax (CrtRx Co., Georgia) water-in-oil emulsion.

To examine leakage of crystallins from lens, lenses were removed from 2-week-old mice and incubated in Dulbecco's modified Eagle's medium (DMEM) medium containing 10% fetal bovine serum at 37°C for 24 h in the presence or absence of 1 mM H_2O_2 as a control (Tumminia *et al*, 1994). Proteins leaked from the damaged lens cells into the medium were precipitated with 10% TCA, and subjected to Western blot analysis using antiserum specific for αA- or γ-crystallin.

MEFs, mouse myoblasts C2C12, and mouse lens epithelial cells α-TN4 were cultured in DMEM containing 10% fetal bovine serum. To examine protein levels of HSFs, the cells and tissues were homogenized in NP-40 lysis buffer (150 mM NaCl, 1.0% Nonidet P-40, 50 mM Tris (pH 8.0), 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin) by sonication, and centrifuged at 12 000 *g* for 10 min. Equal mounts of protein were loaded on SDS–PAGE, and transferred onto nitrocellulose membranes. The membranes were blotted with α-HSF2α (Nakai *et al*, 1995) and α-mHSF4t (Tanabe *et al*, 1999). To detect HSF1, we generated antiserum raised against recombinant mouse HSF1 (amino acids 227–503) as described above.

Histopathology and immunohistochemistry
The eyes were dissected, fixed in NEO-Fix (Merck) at room temperature for 16–24 h, embedded in paraffin and cut into sections 5 μm thick. Sections were stained with hematoxylin and eosin, or with 4′,6′-diamidino-2-phenyindole (DAPI). Immunostaining of the paraffin sections was performed as described previously (Nakai *et al*, 2000) using antibodies as described above. Signals were detected using a DAB substrate kit (Vector Laboratories, CA).

To examine the patterns of DNA replication, incorporation of BrdU was analyzed using immunohistochemistry. Pregnant mice at 18.5 days postcopulation (dpc) were injected intraperitoneally with 50 μg/g BrdU (Sigma) diluted with PBS. At 1 h after the injection, fetal heads were dissected, fixed, and embedded in paraffin as described above. Immunohistochemical staining was performed essentially as described (Nishiyama *et al*, 2002). Numbers of BrdU-positive cells in the lens epithelial cells were counted in three wild-type and three HSF4-null embryos.

In situ hybridization
In situ hybridization was performed as described previously (Izu *et al*, 2004). The plasmid pmHSF4b-N containing 5′-terminal 436 base pairs of mHSF4 cDNA was generated by removing a *Hin*dIII fragment from pmHSF4b-1 (Tanabe *et al*, 1999), and was used to generate a digoxigenin-labeled probe (Roche, Mannheim, Germany). An FGF7 cDNA fragment corresponding to the full protein coding region was amplified by RT–PCR, subcloned into a pGEM7 vector (Promega), and used to generate a digoxigenin-labeled probe.

RT–PCR analysis

Total RNA was isolated from lenses in 6-week-old mice, 2-day-old mice, 15.5 dpc embryos, and from MEF cells. cDNAs were synthesized from 1 μg of total RNA by using avian myeloblastosis virus-reverse transcriptase (Invitrogen) and random hexamer primers. The PCR reaction was performed as described previously using primers for amplifying *HSF1* and *HSF2* genes (Fiorenza *et al*, 1995), *HSF4* and *S16 ribosomal protein* genes (Tanabe *et al*, 1999), and *γ-crystallin* genes (Nishiguchi *et al*, 1998) in the presence of [α-^{32}P]dCTP. The reactions were subjected to electrophoresis on a 4% polyacrylamide gel, and the autoradiogram is shown. To examine expression of FGFs, PCR reactions were performed using specific primer sets for *FGF* genes (Hajihosseini and Heath, 2002). The amplified DNA was stained with ethidium bromide and photographed using Epi-Light UV FA1100 (Aisin Cosmos R&D Co., Japan).

Chromatin immunoprecipitation

Four lenses isolated from 6-week-old mice were treated at 37°C for 15 min with 10 ml of 1% formaldehyde/DMEM containing 10% FCS, and then incubated in 0.125 M glycine at room temperature for 5 min. After washing with PBS two times, the lenses were suspended in 1 ml of PBS, homogenized using a Dounce homogenizer, and centrifuged at 2000 r.p.m. for 2 min. The pellet fractions were suspended in 200 μl of SDS lysis buffer (1% SDS, 10 mM ethylenediamine tetraacetic acid, 50 mM Tris–HCl (pH 8.1)), incubated at 4°C for 10 min, and then sonicated. The lysates were added with 8 μl of 5 M NaCl and incubated at 65°C for 4 h. DNA was recovered by phenol/chloroform extraction. ChIP was performed using a ChIP assay kit (Upstate, New York) essentially according to the manufacturer's instructions. A total of 30 and 40 cycles of PCR reactions were performed to amplify γF-crystallin and FGF-7 DNA fragments, respectively. An anti-mHSF4t serum or an anti-cHSF1c serum (Tanabe *et al*, 1999) was used for immunoprecipitation. Primers used to amplify ChIP-enriched DNA were as follows:

gamma F1, 5′-ATC TCC TTG GGT CAG CAG ATC C-3′; gamma F2, 5′-ATG GGA TGG TGC TGT TGA G-3′; FGF7 F1, 5′-GAT TAG ACG TGG CCA TTG ATT-3′; FGF7 F2, 5′-TGT GAG TGC GTG GAG CCT TT-3′.

Transmission electron microscopy

The lenses from 2-week-old mice were fixed with a fixative (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4) for 2 h at room temperature and stored at 4°C for several weeks. After washing with 0.1 M cacodylate buffer (pH 7.4) three times (5 min each), cells were postfixed with ice-cold 1% OsO$_4$ in the same buffer for 2 h. The samples were rinsed with distilled water, stained with 0.5% aqueous uranyl acetate for 2 h at room temperature, dehydrated with ethanol, and embedded in Poly/Bed 812. Ultrathin sections were cut, doubly stained with uranyl acetate and lead citrate, and viewed with a JEM 1010 transmission electron microscope (JEOL) (Fujikura *et al*, 2002).

Statistical analysis

Significant values were determined by analyzing data with the Mann–Whitney's U test using StatView version 4.5J for Macintosh (Abacus Concepts, Berkley, CA). A level of $P < 0.05$ was considered significant.

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