Heat shock transcription factors (HSFs) play roles not only in heat shock response but also in development of the reproductive organs, brain, and lens. Here, we analyzed sensory organs and found abnormalities of the olfactory epithelium in adult HSF1-null mice, which is developmentally related to the lens. The olfactory epithelium was normal until postnatal 3 weeks but was not maintained later than 4 weeks in HSF1-null mice. The olfactory epithelium was atrophied with increased cell death of olfactory sensory neurons. Analysis of the epithelium revealed that induction of HSP expression and reduction of LIF expression are lacking in adult HSF1-null mice. We found that DNA binding activity of HSF1 is induced in the olfactory epithelium later than 4 weeks and that HSF1 binds directly to LIF gene and inhibits its expression. HSF4 has opposing effects on LIF expression and olfactory neurogenesis. These data indicate that HSF1 is required for the precise expression of Hsp and cytokine genes that is obligatory for maintenance of olfactory neurogenesis in adult mice and suggest that stress-related processes are involved in its maintenance.

Heat shock response is characterized by induction of a set of heat shock proteins (HSPs) and is an evolutionary conserved response inherent in all organisms from bacteria to human. In eukaryotes, this response is regulated mainly by heat shock transcription factor (HSF), which can bind to a heat shock element (HSE) composed of at least three inverted repeats of consensus sequence nGAAn (1). In vertebrates, three mammalian Hsf genes (Hsf1, Hsf2, and Hsf4) and three avian HSF genes (HSF1, HSF2, and HSF3) have been identified (2, 3), and their expression profiles are temporally and spatially different during development (3, 4). In unstressed cells HSF1 is present mostly as a monomer and is converted to a trimer that binds to HSE in response to heat shock, whereas HSF4 is constitutively a trimer. A single mutation of Hsf1, Hsf2, or Hsf4 genes or combined mutations of Hsf genes in mice showed that HSFs are involved in development of the reproductive organs (5–10), brain (6, 10, 11), and lens (12–14) and in immune response (15, 16). In addition to regulating HSP expression, HSFs are involved in development partly by regulating expression of growth factor genes such as Fgf and Il-6 genes directly (13, 15). Although HSF1 binds to many genes in vivo in unstressed cells (17), we have little information about when and how HSF activity is regulated during development and maintenance of tissues.

We previously identified that HSF4 is required for development of lens in the eye partly by regulating Hsp and Fgf genes (13). HSF1 is also involved in lens development and competes with HSF4 for expression of the same Fgf genes. These observations highlighted sensory organs that receive stimuli from the external environment and are susceptible to stress. Here, we examined development of other sensory organs in HSF1- and HSF4-null mice. Surprisingly, we found abnormalities in the nasal cavity in adult HSF1-null mice that are derived from placodes expressing Pax6 gene as well as the lens (18–20). This study demonstrates that HSFs are required for maintenance of the olfactory epithelium in adult mice.

MATERIALS AND METHODS

Mice—HSF1-null (21) and HSF4-null (13) mice were maintained by crossing with ICR mice. Mice deficient for both HSF1 and HSF4 were generated as described previously (13).

Histopathology and Immunohistochemistry—Mice were systematically anesthetized with ketamine (16 mg/kg, intraperitoneal) and xylazine (16 mg/kg, intraperitoneal) and transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline (PBS). Blocks of bone containing paranasal sinuses were removed, soaked in 4% paraformaldehyde for 12 h at 4 °C, and then incubated for 10 days in a decalcification solution (5% EDTA, 0.2 m sucrose (pH 7.2)), changing the solution every day. After washing with PBS, the blocks were dehydrated, embedded in paraffin, and cut into sections 5-μm thick. The sections were stained with hematoxylin and eosin. To detect calcified bone in the nasal cavity, the Tripp-Mackay method for calcium (22) was used. Bone mass was estimated using NIH images.

Immunostaining of the paraffin sections was performed as described previously (6). Antibodies used were antiserum to mouse HSP110 (aHSP110a) (13), for human HSP90 (aHSP90b) (23), for human HSP70 (aHSP70–1) (13), for HSP60 (epHSP60–1) (13), for human HSP40 (aHSP40, which was generated by immunizing rabbit with recombinant human HSP40), and for rat HSP27 (a gift from Drs. K. Kato and H. Itoh, Aichi Human Service Center). Peroxidase-conjugated goat anti-rabbit IgG was used as a secondary antibody. Signals were detected using a DAB substrate kit (Vector Laboratories, Inc.). Sections were counterstained with methyl green.

BradUrD Incorporation and TUNEL Staining—To examine the patterns of DNA replication, incorporation of 5-bromo-2’-deoxyuridine...
(BrdUrd) was analyzed essentially as described previously (13). 3–6 week-old mice were injected intraperitoneally with 50 μg/g of BrdUrd (Sigma) diluted with PBS. 16 h after the injection, mice were anesthetized and perfused with 4% paraformaldehyde/PBS. Blocks of bone containing paranasal sinuses were dissected, fixed, and embedded in paraffin as above. Immunostaining of BrdUrd was performed essentially as described (13). Numbers of BrdUrd-positive cells were counted in all areas of the olfactory epithelium, and means and S.D. of the numbers in each microscopic field were estimated.

To detect apoptotic cells, paraffin sections prepared above were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining using an in situ cell death detection kit (Roche Diagnostics). The sections were mounted in Vectashield mounting medium with 4’6-diamidino-2-phenylindole (DAPI; Vector Laboratories) and visualized using fluorescence microscopy (Axioskop; Zeiss). Numbers of TUNEL-positive cells were counted in all areas of olfactory epithelium, and means and S.D. of percentages of TUNEL-positive cells were estimated.

Western Blot Analysis and Gel Shift Assay—Mice were killed by cervical dislocation, and skulls were immediately dissected using a razor. The olfactory epithelium including turbinates was collected by scraping in buffer C (20 mM Hepes, pH 7.9, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml of pepstatin A, 1 μg/ml of leupeptin, and 0.5 mM dithiothreitol) as previously described (24). Whole cell extracts from the brain, trachea, and oviduct were prepared by disintegrating organs from the same mice. Aliquots containing equal amounts of proteins were subjected to Western blot analysis using antiserum for mouse HSF1 (13) and the antibodies described above. To detect HSE binding activity in the tissues, 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-HSF2β, or α-HSF4b (25, 26) as described previously (13).

RT-PCR Analysis—Olfactory epithelium including turbinates was collected as described above. Total RNA was immediately isolated by using TRIzol reagent (Invitrogen). cDNAs were synthesized from 2 μg of total RNA using a cloned AMV first-strand cDNA synthesis kit that contains avian myeloblastosis virus-reverse transcriptase, oligo(dT)20, and a random hexamer (Invitrogen). 25–35 cycles of PCR reactions were performed using 1 μl from each reverse transcription product. Primers used were indicated previously for amplifying Fgf-1, Fgf-7, TNF-α, IL-1β, IL-6, and S16 ribosomal protein genes (13, 15) and other Fgf (27), Bmp (28), and Lif, Tgf, Bstdf, and Omp genes (29). The amplified DNA was stained with ethidium bromide and photographed using an Epi-Light UV FA1100 (Aisin Cosmos R&D Co.).

Chromatin Immunoprecipitation—Mice were systematically anesthetized as described above and then transcardially perfused with 1% formaldehyde in PBS. Blocks of bone containing paranasal sinuses were removed and soaked in 1% formaldehyde in PBS at 37 °C for 10 min. Olfactory epithelium including turbinates were collected by scraping the nasal epithelium. Chromatin immunoprecipitation (ChIP) was performed using a ChIP assay kit (Upstate Group, Inc.) essentially according to the manufacturer’s instructions (13). Thirty-five cycles of PCR reactions were performed to amplify a DNA fragment of Lif gene (−641 to −270 from a transcription start site). Primers used to amplify ChIP-enriched DNA were: LIF-5′, 5′-TCC CCTTT CTGTTG TCTTTC TCCA AAAT GTGTT GGTGC TCTG GGTCCG-3′. To amplify an HSE-R3 region (−474 to −270), we used primers HSE-R3−5′, 5′-AGT CCTG ACTC TTTT GCT-3′ and HSE-R3−3′, 5′-TCC TAA GTG GTT GGTG TCTG GGTTCG-3′.

Transmission Electron Microscopy—Mice were systematically anesthetized and transcardially perfused with a fixative (2% paraformalde-
hyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.4). Blocks of bone containing paranasal sinuses and trachea were removed and soaked in the fixative for 16 h. Samples were then incubated for 10 days in a decalcification solution, changing the solution every day. Transmission electron microscopy was performed as described previously (13).

Olfactory Behavior—Olfactory behavior was estimated in a dark soundproof chamber with a ventilation system. Cotton balls were placed at four corners in a cage, and acetic acid solution (40%) was instilled into one of the cotton balls. Mice harboring a light reflector were placed at the center of the cage. Behaviors of the mice were recorded with an infrared digital video camera (Sony, Tokyo, Japan) for 3 min. The recorded motion pictures were analyzed with an NIH image on a personal computer (Apple), and position coordinates of mice at each time point were calculated. The cage area was divided into four (acetic acid area, cross area, and two side areas) (see Fig. 2), and the percentage of time spent at each area was estimated.

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.

RESULTS

HSF1 Is Required for Normal Development of the Nasal Cavity—Previouly, we showed that HSF4 is required for maturation of the eye lens, while HSF1 competes or cooperates with HSF4 in regulation of heat shock genes and growth factor genes (13). Therefore, we next examined the morphology of other sensory organs in HSF1- and HSF4-null adult ICR strain mice. We found normal morphology in the ear (30), skin, and taste buds (data not shown) in both types of mice. Surprisingly, we found that the structure of the nasal cavity was abnormal in 6-week-old HSF1-null mice (Fig. 1A). Mice developed chronic sinusitis with accumulation of mucus, containing mostly dead cells (data not shown). Infiltration of leukocytes was hardly detected, excluding the possibility of bacterial infection due to impaired immunoglobulin production (15). The nasal cavity area increased, which was associated with marked decreases in turbinate areas and calcified bone (Fig. 1A). Furthermore, atrophy of the olfactory epithelium was observed (Fig. 1B). The thickness of the olfactory epithelium at the septum in HSF1-null mice decreased by ~50% compared with that in wild-type mice. We found an increase in TUNEL-positive apoptotic cells, but electron microscopic analysis showed that necrotic olfactory nerve cells were dominant in the
HSF1-null olfactory epithelium. Similar phenotypes were also observed in HSF1-null mice of the C57Bl/6 strain (data not shown). These data indicated that HSF1 is required for normal development of the nasal cavity.

A major role of the nasal cavity is to sense smell. To estimate olfaction, we monitored olfactory behavior of mice. Cotton balls were placed at four corners in a cage, and acetic acid solution was instilled in one corner. We found that wild-type mice evaded the cotton ball containing acetic acid, whereas HSF1-null mice did not (Fig. 2). This result suggests that olfaction is disturbed in HSF1-null mice.

**HSF1 Is Indispensable for Maintenance of the Olfactory Epithelium in Mice over 4-Weeks-old**—We next examined developmental stages at which abnormalities appear in the nasal cavity. We detected no abnormality in the 3-week-old HSF1-null nasal cavity by morphological examination (Fig. 3A). Cell growth determined by BrdUrd incorporation and spontaneous cell death assayed by TUNEL staining were at normal levels in the 3-week-old HSF1-null olfactory epithelium (Fig. 3, D and E). In 4-week-old HSF1-null mice, however, we detected accumulation of mucus in the nasal cavity and atrophy of the olfactory epithelium (Fig. 3A). In wild-type mice, the number of BrdUrd-incorporated cells in the olfactory epithelium significantly decreased at 4 weeks compared with that at 3 weeks (Fig. 3D) (31). Notably, the number of BrdUrd-incorporated cells in the olfactory epithelium of 4-week-old HSF1-null mice (4.2 ± 2.8 cells/field) was much lower than that in the epithelium of the same age wild-type mice (0.2 ± 0.4 cells/field). Furthermore, the percentage of TUNEL-positive cells in the olfactory epithelium of 4-week-old HSF1-null mice was markedly higher than that in the epithelium of the same age wild-type mice (Fig. 3E). These results indicated that HSF1 is not required for development of the olfactory epithelium until 3 weeks of age but is indispensable for maintenance of the olfactory epithelium in mice over 4 weeks old.

**Induction of HSP Expression and Activation of HSF1 in Puberal Olfactory Epithelium Lack in HSF1-null Mice—**As expression of Hsp genes and some growth factor genes was directly regulated by HSF1 and HSF4 (13, 15), we first analyzed expression of HSPs in the developing olfactory epithelium. We found that in wild-type mice expression levels of HSP27, HSP70, HSP90, and HSP110 were high in the sustentacular cells at 3 weeks of age and that the HSP levels increased in both sustentacular cells and olfactory sensory neurons at 4 and 6 weeks of age (Fig. 3A). In 3-week-old HSF1-null mice, expression levels of HSPs in the olfactory epithelium were similar to those in the same age wild-type mice. Notably, expression levels of HSPs in both sustentacular cells and oolfactory sensory neurons in HSF1-null mice decreased at 4 and 6 weeks of age. As a result, marked differences in expression levels of HSP27, HSP70, HSP90, and HSP110 were detected in the olfactory epithelium of 6-week-old wild-type and HSF1-null mice (Fig. 3, A–C). These results show that the appearance of abnormal cell growth and death of olfactory sensory neurons in HSF1-null mice is temporally correlated with decreased expression of HSPs.

HSF2 and HSF4 expressions are regulated during development (7, 13, 32); however, we do not know the developmental regulation of HSF1 expression or its activity. Therefore, we next examined whether HSF1 could be activated in the puberal olfactory epithelium. We found that levels of HSF1 protein were constant in the olfactory epithelium of 3-, 4-, and 6-week-old mice (Fig. 4A). HSF1 bands were composed of three major bands on SDS-polyacrylamide gel, but the profiles of these bands did not change during the observation period. Notably, we found that the DNA binding activity of HSF1 significantly increased in olfactory epithelium extracts of 4- and 6-week-old mice compared with that in an extract of 3-week-old mice (Fig. 4B). Thus, induction of HSPs is accom-
panied by activation of HSF1 in the olfactory epithelium of puberal mice.

LIF Expression Increases in the HSF1-null Olfactory Epithelium—As growth, differentiation, and death of olfactory sensory neurons are regulated by many cytokines such as FGFs, BMPs, TGFs, and LIF (33–35), we analyzed expression of these cytokines by RT-PCR. Although some Fgf genes are direct target genes of HSFs (13), only a modest decrease in expression of FGF-18 and TGF-β/H9252 (28 and 32%, respectively) was observed in the HSF1-null olfactory epithelium (Fig. 5 A). In marked contrast, we found that LIF expression was notably higher in the HSF1-null olfactory epithelium as well as in the lung (Fig. 5, A and B). In wild-type mice, LIF expression was high at 3 weeks but markedly decreased at 6 weeks (Fig. 5 C). In contrast, LIF expression continued to be high in the HSF1-null olfactory epithelium. As LIF inhibits maturation of olfactory sensory neurons (36) and overexpression of LIF leads to a reduction in thickness of the olfactory epithelium by inducing cell death (29), overexpression of LIF may partly cause abnormal development of HSF1-null olfactory sensory neurons.

In addition to olfactory sensory neuron abnormalities, formation of calcified bone was strongly suppressed (Fig. 1 A), probably due to abnormal gene regulation in the olfactory epithelium (37). Inflammatory cytokines tumor necrosis factor α (TNF-α) and IL-1β (38–40) as well as LIF (41) act for bone resorption, and HSF1 suppresses expression of TNF-α and IL-1β (42–44). We also found increased expression of TNF-α and IL-1β in the HSF1-null epithelium (data not shown).

HSF1 Binds Directly to the Lif Gene and Inhibits Its Expression—To determine whether HSF1 binds directly to the Lif gene, we searched HSE consensus sequences on the Lif gene. We found 15 upstream sites similar to an HSE consensus sequence in region within 100 to 1000 bp from a transcription start site (Fig. 6, A and B) (45). ChIP analysis showed that HSF1 binds to the upstream region (641 to 270) of the Lif gene in the olfactory epithelium of 8-week-old mice (Fig. 6 C). Furthermore, we found that HSF1 exclusively binds to an HSE-R3 region (474 to 270) (Fig. 6 D). HSF1 did not bind to that region at 3 weeks but did at 4 weeks (Fig. 6 E). These results indicate that HSF1 binds directly to the Lif gene in the adult olfactory epithelium.

As HSF1 competes with HSF4 for expression of FGFs in lens epithelial cells (13), we next examined the olfactory epithelium in mice deficient for both HSF1 and HSF4 (double-null) (Fig. 7 A). In contrast to severely damaged turbinates and atrophied olfactory epithelia in HSF1-null mice, the morphology of the turbinates and olfactory epithelium was partially restored in double-null mice. Increased expression of LIF in the HSF1-null olfactory epithelium was also restored in part in the double-null olfactory epithelium (Fig. 7B). These results indicate that HSF1 and HSF4 have opposing effects on LIF expression in the olfactory epithelium as well as on FGF expression in the lens. Consistent with
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In this study, we showed that HSF1 is required for the maintenance of the olfactory epithelium in mice. We also showed previously that HSF4 is required for maintenance of the lens (13). Taken together, the requirement of HSF1 or HSF4 in each component of sensory organs is restricted to mice of a postnatal age, at which time these organs are exposed to environmental stimuli (35, 46). Interestingly, the developmental relationship between the two components has been well documented. The three major sensory organs of the head develop from interactions of the neural tube with a series of epidermal thicknesses called cranial placodes (47). The olfactory placode gives rise to the olfactory epithelium, the lens placode to the lens, and the otic placode forms the inner ear. Among them, lens and olfactory precursors arise from a common territory surrounding the anterior neural plate at early developmental stages (48). Furthermore, a transcription factor, PAX6, is expressed in both the olfactory and lens placodes and is required for eye and nasal development (18–20). Although different HSFs play dominant roles in the two sensory organs, our results demonstrating an HSF requirement for tissue maintenance further clarify the strong relationship between the two components.

One of major findings in this study was that HSE binding activity of an active HSF1 (HSF1ΔRD) (6) into HSF1-null mouse embryo fibroblasts reduced expression of LIF, whereas overexpression of HSF4b increased that (Fig. 7C). Taken together, these observations indicate that HSF1 directly binds to the LIF gene and inhibits its expression and that HSF4 competes with HSF1 on LIF expression.

**DISCUSSION**

Proliferation and differentiation of the olfactory sensory neurons are regulated by many cytokines (33–35), including Fgf genes, whose expressions are regulated by HSFs in the lens (13). Expressions of some of these genes decreased only modestly in the HSF4-null olfactory epithelium (Fig. 5). In marked contrast, we found that the level of LIF expression continued to be high in the adult HSF1-null olfactory epithelium. Although LIF expression is induced in injured olfactory sensory neurons (29), our results indicate that HSF1 directly suppresses LIF expression in the olfactory epithelium of adult mice as well as in mouse embryo fibroblast cells. LIF is a multifunctional cytokine and its expression is induced in many tissues; it is essential for normal development of olfactory sensory neurons as well as hippocampal development and blastocyst implantation (52). Our results suggest that LIF expression should be inhibited by HSF1 during olfactory development. Continuous overexpression of LIF may greatly affect intracellular signalings in olfactory sensory neurons, in which decreased HSP expression causes altered protein homeostasis.
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