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HSF1 is required for extra-embryonic development, postnatal growth and protection during inflammatory responses in mice

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HSF1 is the major heat shock transcriptional factor that binds heat shock element (HSE) in the promoter of heat shock proteins (Hsps) and controls rapid Hsp induction in cells subjected to various environmental stresses. Although at least four members of the vertebrate HSF family have been described, details of their individual physiological roles remain relatively obscure. To assess whether HSF1 exhibited redundant or unique *in vivo* functions, we created *Hsf1*$^{-/-}$ deficient mice. We demonstrate that homozygous *Hsf1*$^{-/-}$ mice can survive to adulthood but exhibit multiple phenotypes including: defects of the chorioallantoic placenta and prenatal lethality; growth retardation; female infertility; elimination of the 'classical' heat shock response; and exaggerated tumor necrosis factor alpha production resulting in increased mortality after endotoxin challenge. Because basal Hsp expression is not altered appreciably by the HSF1 null mutation, our findings suggest that this factor, like *Drosophila Hsf* protein, might be involved in regulating other important genes or signaling pathways. Our results establish direct causal effects for the HSF1 transactivator in regulating critical physiological events during extra-embryonic development and under pathological conditions such as sepsis to modulate pro-inflammatory responses, indicating that these pathways have clinical importance as therapeutic targets in humans.

Keywords: development/heat shock/HSF/inflammation/stress response

Introduction

The heat shock transcription factor 1 (HSF1) gene belongs to a family of vertebrate HSF transactivators, and encodes the orthologous protein found across the eukaryotic phyla (Morimoto, 1998). Originally identified in crude extracts of heat-shocked *Drosophila* as a heat shock element (HSE) binding activity in hsp promoters and chromatin (Wu, 1984), HSF was subsequently purified and characterized from yeast and flies (Sorger and Pelham, 1987; Wiederrecht *et al*., 1987; Sorger and Nelson, 1989). A single-copy HSF gene was identified in *Saccharomyces cerevisae* (Sorger and Nelson, 1989) and *Drosophila* (Clos *et al*., 1990), respectively, and three genes encoding HSFs were isolated in plants (Scharf *et al*., 1990). The hierarchy of the HSF family in vertebrates was established by cloning two HSFs from mice, mHSF1 and mHSF2 (Sarge *et al*., 1991), two HSFs, hHSF1 and hHSF2 (Schuetz *et al*., 1991) from humans, three HSFs from chicks (Nakai and Morimoto, 1993) and, most recently, a fourth novel factor, hHSF4, from human cells (Nakai *et al*., 1997). Despite the increasing number of vertebrate HSFs over the past few years, and the notion that each imparts unique physiological functions in the cells of complex organisms (reviewed in Wu, 1995), little specific knowledge is available about the *in vivo* biological roles of individual mammalian HSFs.

The remarkably conserved heat shock response is among the best characterized endogenous mechanisms in prokaryotic and eukaryotic cells alike, essential for host defense and survival (Lindquist and Craig, 1988). The best known function of HSF is transactivation of hsp gene expression in response to heat, chemical and pathophysiological stress such as ischemia in diverse eukaryotic cell types from yeast to humans (Morimoto, 1998). The detailed mechanisms by which diverse physiological stresses converge to activate the common HSF regulatory pathway have been deciphered (Morimoto *et al*., 1994; Zhong *et al*., 1998). In addition, phylogenetic and structural studies have established that eukaryotic HSFs are organized into functional domains beginning with a conserved DNA-binding region at the N-terminus, an adjacent oligomerization domain comprised of multiple hydrophobic repeats (or leucine zippers), a nuclear localization signal and a C-terminal transactivation domain (Wu, 1995).

In contrast to HSF in yeast, HSF1 of higher eukaryotes exists in the unstressed cell as an inactive monomer whose basal activity is negatively regulated, at multiple levels, by a C-terminal hydrophobic repeat domain (Rabindran *et al*., 1993), the chaperone Hsp90 (Zou *et al*., 1998), and to a lesser degree by Hsp/c70 repressor complexes (Rabindran *et al*., 1994). In response to physiological stress, HSF monomers aggregate into homotrimers that bind avidly within the major groove of DNA at conserved nGAAn repeats and activate target hsp genes (Amin *et al*., 1988). This process is also associated with phosphorylation at serine and threonine HSF residues through signal transduction involving the mitogen-activated protein kinase pathways, which are reversible upon stress withdrawal (Knauf *et al*., 1996).

Rapid synthesis of stress proteins in the cell is widely believed to play a pivotal role in defense against proteotoxic damage during adverse (patho)physiological conditions such as myocardial infarction and sepsis (Morimoto *et al*., 1994; Wu, 1995). Stress proteins (Hsps) function as molecular chaperones to facilitate proper folding of

nascent polypeptides, maintain proteins in their native folded state, and repair or promote degradation of unfolded proteins (Gething and Sambrook, 1992; Parsell *et al*., 1993). Transgenic studies have provided compelling evidence that overexpression of stress proteins (e.g. hsp70) can mitigate the effects of pathological conditions simulating heart attacks and strokes in animals (reviewed in Benjamin and McMillan, 1998). These observations have important implications for other pathological states, including febrile illness, sepsis, cancer, Alzheimer's disease and other degenerative diseases associated with oxidative stress in aging humans. However, a lack of available robust experimental models to test directly the functional roles of stress-inducible Hsp expression or regulatory networks *in vivo* has hindered progress.

In addition to playing a key role in the response to noxious stimuli, vertebrate HSFs are widely believed to play a role in spontaneous hsp gene expression during embryogenesis, organogenesis, development and postnatal growth (Tanguay *et al*., 1993). Mutagenesis studies using a heterologous Hsp70.1 promoter to regulate transgene expression have implicated HSF1 during zygotic genome activation (Christians *et al*., 1997). *In vitro* studies of the structurally homologous factor, HSF2, have demonstrated its HSE-DNA binding both after hemin treatment during differentiation of K562 erythroleukemia cells (Sistonen *et al*., 1992) and in extracts from mouse blastocysts and embryonic tissues (Loones *et al*., 1997). Together, the available evidence that HSFs play crucial roles during development is primarily indirect and correlative; furthermore, no prior study has provided direct functional evidence for mammalian HSFs *in vivo*. Accordingly, we have undertaken a systematic approach using gene targeting to elucidate the regulatory effect that mammalian HSFs exert during physiological growth and development.

Details of the gene targeting strategy used in our laboratory to create homozygous *Hsf1*$^{-/-}$ mice have been reported (McMillan *et al*., 1998). The deletion encompasses ~80% of the DNA-binding domain and the three leucine zippers (1–3) that comprise the oligomerization domain. Although a mutant mRNA transcript was produced, with a predicted in-frame open reading frame (ORF) deletion of 301 amino acids (31 kDa), we detected no corresponding immunoreactive proteins using available HSF1 antiserum in both *in vitro* (McMillan *et al*., 1998) and *in vivo* systems. In previous *in vitro* analysis of HSF1 functions, we have demonstrated that *Hsf1*$^{-/-}$ cells derived from mouse embryos exhibited markedly increased susceptibility to heat-induced apoptosis, indicating non-redundant and essential roles for HSF1 during thermotolerance (McMillan *et al*., 1998). In this report, we have focused on the unexpected findings implicating new functions for HSF1 expression during development of the chorioallantoic placenta and postnatal growth in *Hsf1*-deficient mice. In addition, we demonstrate here that *Hsf1*$^{-/-}$ mice, despite lacking a 'classical heat shock' response, can survive into adulthood but sustain increased mortality associated with exaggerated production of the pro-inflammatory cytokine, tumor necrosis factor α (TNF-α), after endotoxemic and inflammatory challenge.

Results

Homozygous Hsf1$^{-/-}$ *mice exhibited prenatal lethality*

Table I shows the results of *Hsf1*$^{+/-}$ heterozygous (F_1) intercrosses from separate mating between germline chimeric males (ES cells, 129SvEv) to 129SvJ, inbred BALB/c and outbred ICR wild-type females, respectively. Accordingly, these targeted mutant strains are referred to herein as follows: 129SvJ×129SvEv (129-*Hsf1*), BALB/c ×129SvEv (C,129-*Hsf1*) and ICR×129SvEv (R,129-*Hsf1*), respectively. Breeding to homozygosity produced live *Hsf1*$^{-/-}$ progeny that were recovered at less than the predicted Mendelian distribution in all three genetic backgrounds (129-*Hsf1* or C,129-*Hsf1* or R,129-*Hsf1* strains; $p < 0.01$). Only two of 165 (1.2%) 129-*Hsf1* live births were homozygotes from 43 litters. In contrast, survival of F_2 *Hsf1*$^{-/-}$ mice in either the C,129-*Hsf1* (23 of 195 or 11.8%) or R,129-*Hsf1* (20 of 133 or 15%) background was an order of magnitude higher than that of the 129-*Hsf1* mutant strain ($p < 0.01$), indicating variable penetrance in different genetic backgrounds.

Based on widespread HSF1 expression *in vivo*, the lesion causing prenatal lethality in *Hsf1*$^{-/-}$ mice could manifest at various stages of embryogenesis. Table II shows the results of 129-*Hsf1* heterozygous intercrosses in the most severely affected strain resulting in embryonic lethality. Between 10.5 and 11.5 days post-coitus (d.p.c.), we found the expected Mendelian distribution of viable embryos examined ($n = 149$), indicating that haploinsufficiency of HSF1 expression affected neither fertilization nor pre-implantation in mice. Thereafter, the number of 129-*Hsf1* homozygous embryos found alive after 12.5 d.p.c. decreased dramatically such that at 13.5 d.p.c. only 35% (7/20) of these embryos were observed with beating hearts (Table II). Between 14.5 and 17.5 d.p.c., we analyzed an additional seven litters containing 43 of the 129-*Hsf1* embryos and found that <3% of *Hsf1*$^{-/-}$ embryos (1/43) of the predicted Mendelian distribution were alive (Table II; expected *Hsf1*$^{-/-}$ versus observed; $p < 0.05$). Given the paucity of 129-*Hsf1* live births (1.2%; Table I), we conclude that prenatal lethality in the most severely affected 129-*Hsf1*-deficient animals mainly occurred around 14.0 d.p.c. Interestingly, the timing of embryonic lethality was less sharply demarcated in both C,129- and R,129-*Hsf1* mutant strains, and occurred between the window from late mid-gestation to birth (Tables I and II).

HSF1 is required for development of chorioallantoic placenta

Based on the proposed crucial roles for HSF1 expression in basal Hsp expression (Fiorenza *et al*., 1995), coincident with the metabolic requirements during rapid embryonic growth, we first analyzed viable embryos in the targeted mutation strains at 11.5, 12.5 and 13.5 d.p.c., in anticipation of finding embryonic defects. No distinguishable morphological or microscopic abnormalities were detected in multiple solid or soft tissues (i.e. brain, heart, liver, kidney, striated muscle, smooth muscle, bone, cartilage and bone marrow) in different mutant strains examined extensively at varying developmental stages (Figure 1A; data not shown). Besides the absence of primary abnormalities in

Table I. Effect of genetic background on live–term births from F_1 heterozygous parents

Targeted mouse strain	Number of live births	Number of litters	Genotype of offspring (%)		
			+/+	+/–	–/–
129SvJ×129SvEv (129-*Hsf1*)	165	43	59 (35.8)	104 (63.0)	2 (1.2)**
BALB/c×129SvEv (C,129-*Hsf1*)	195	24	50 (25.6)	122 (62.6)	23 (11.8)[a]**
ICR×129SvEv (R,129-*Hsf1*)	133	16	36 (27.1)	77 (57.9)	20 (15.0)[a]*

Intercrosses of heterozygous (F_1) parents were used to determine live–term births by Southern blotting as described in Materials and methods. None of the targeted mutant strains yielded the expected Mendelian distribution.
[a]Compared with 129-*Hsf1*, $p < 0.01$.
Observed Mendelian distribution was compared with expected: *$p < 0.05$; **$p < 0.01$.

Table II. Genotype and survival of embryos from 129,*Hsf1*$^{+/-}$ parents

Age (d.p.c.)	*n*	Litter number	Average litter size	*Hsf1* genotype			*p*
				+/+	+/–	–/–	
10.5–11.5	149	20	7.5	32 (2)	73 (2)	44 (1)	NS
12.5	41	6	6.8	10 (1)	22 (2)	9 (2)	NS
13.5	74	11	6.7	20 (2)	34 (2)	20 (13)	**
14.5–15.5	27	4	6.7	7	16 (1)	4 (3)	**
16.5–17.5	16	3	5.3	8	8 (2)	0	*

The timing of prenatal lethality was determined for the most severely affected 129-*Hsf1* mutant strain. Before 12.5 d.p.c., the Mendelian distribution and number of null animals alive were similar to the wild-type animals. The numbers of dead 129-*Hsf1* embryos, indicated in parentheses, were significantly higher at 13.5 d.p.c., indicating that prenatal lethality occurred during late mid-gestation. In addition, fewer numbers of *Hsf1* null embryos were retrieved, suggesting that reabsorption had occurred with advancing aging.
NS, not significant.
*χ^2 test for actual genotype segregation versus predicted, $p < 0.05$.
**χ^2 test for embryonic lethality, compared with E10.5–11.5 group, $p < 0.01$.

organ structures, we found that no histopathological features consistent with secondary complications associated with lethality at these developmental periods, such as congestive heart failure, pulmonary congestion or other organ failure, were present in the embryo proper and adult animals.

To determine whether prenatal lethality associated with *Hsf1* deficiency involved extra-embryonic sites, we next characterized extensively the chorioallantoic placenta. By mid-gestation, the chorioallantoic placenta consists of three morphologically distinct layers: the decidua layer of mixed origin, and the spongiotrophoblast and labyrinthine trophoblast giant cell layers, both of embryonic origin. Beginning at 11.5 d.p.c., all three layers were found to be indistinguishable in both wild-type (Figure 1B, a, c and e) and heterozygous *Hsf1*$^{+/-}$ (data not shown) concepti of the 129-*Hsf1* or C,129-*Hsf1* hybrid background. In contrast, a marked thinning of the spongiotrophoblast layer at 11.5 d.p.c. disrupted this normal architecture in *Hsf1*$^{-/-}$ embryos of both 129-*Hsf1* and C,129-*Hsf1* strains (data not shown), which was more pronounced at 13.5 d.p.c. (Figure 1B, b, d and f). In agreement with our histological studies, *in situ* hybridization studies using a lineage-specific marker for spongiotrophoblast—the tyrosine kinase receptor for vascular endothelial growth factor, *Flt-1* (Breier *et al.*, 1995)—confirmed that the *Flt-1* transcript was dramatically reduced in the *Hsf1*$^{-/-}$ placentas compared with wild-type placentas in both 129-*Hsf1* and C,129-*Hsf1* strains at 13.5 d.p.c. (Figure 1C, arrowheads in b and d, respectively). In contrast, expression of the proliferin transcript, *PLF-1A*, a specific marker for trophoblast giant cells of the labyrinth (Carney *et al.*, 1993), was unaltered in *Hsf1*-deficient mice, indicating that the spongiotrophoblast defects occurred without other compensatory changes or proliferative responses in these cells at 13.5 d.p.c. (data not shown).

To determine whether surviving *Hsf1*$^{-/-}$ animals at later developmental stages exhibited the wild-type or mutant morphology, we intercrossed homozygous male and heterozygous *Hsf1* female mice and analyzed the concepti of C,129-*Hsf1* strain at 18.5 d.p.c. Whereas the placentas of C,129-*Hsf1* heterozygous animals exhibited a normal appearance with hypercellularity and vascularization, prominent abnormalities such as reduced organ size, fibrin deposits, vacuolization, degeneration and significant hemorrhages were detected in the labyrinth, even in mutant embryos alive at 18.5 d.p.c. (Figure 1B, i and j). Therefore, the consistent spongiotrophoblast defects in both strains at late mid-gestation, 13.5 d.p.c. (Figure 1B, f and j, arrow), along with histopathological abnormalities at late gestation, indicated that deficiencies of HSF1 functions were confined and restricted to extra-embryonic tissues of the chorioallantoic placenta throughout *in utero* development.

Spontaneous Hsp expression is unaffected in Hsf1-deficient mice

To determine first whether embryonic viability was associated with alterations in Hsp gene expression in the embryo proper, we examined representative members of the Hsp multigene family, beginning at mid-gestation. By Western blot analysis, we found equivalent concentrations of Hsp90α, Hsc70, Hsp70 and Hsp25 proteins between 11.5 and 13.5 d.p.c. in both wild-type and 129-*Hsf1* homozygous embryos (Figure 2A). In particular, indirect immunohistochemical labeling of sagittal sections of wild-type and *Hsf1*$^{-/-}$ embryos at 13.5 d.p.c. correlated with quantitative findings in that Hsp90β and Hsc70 proteins, for example, were expressed ubiquitously with a remarkably high level of Hsp90β labeling in the central nervous

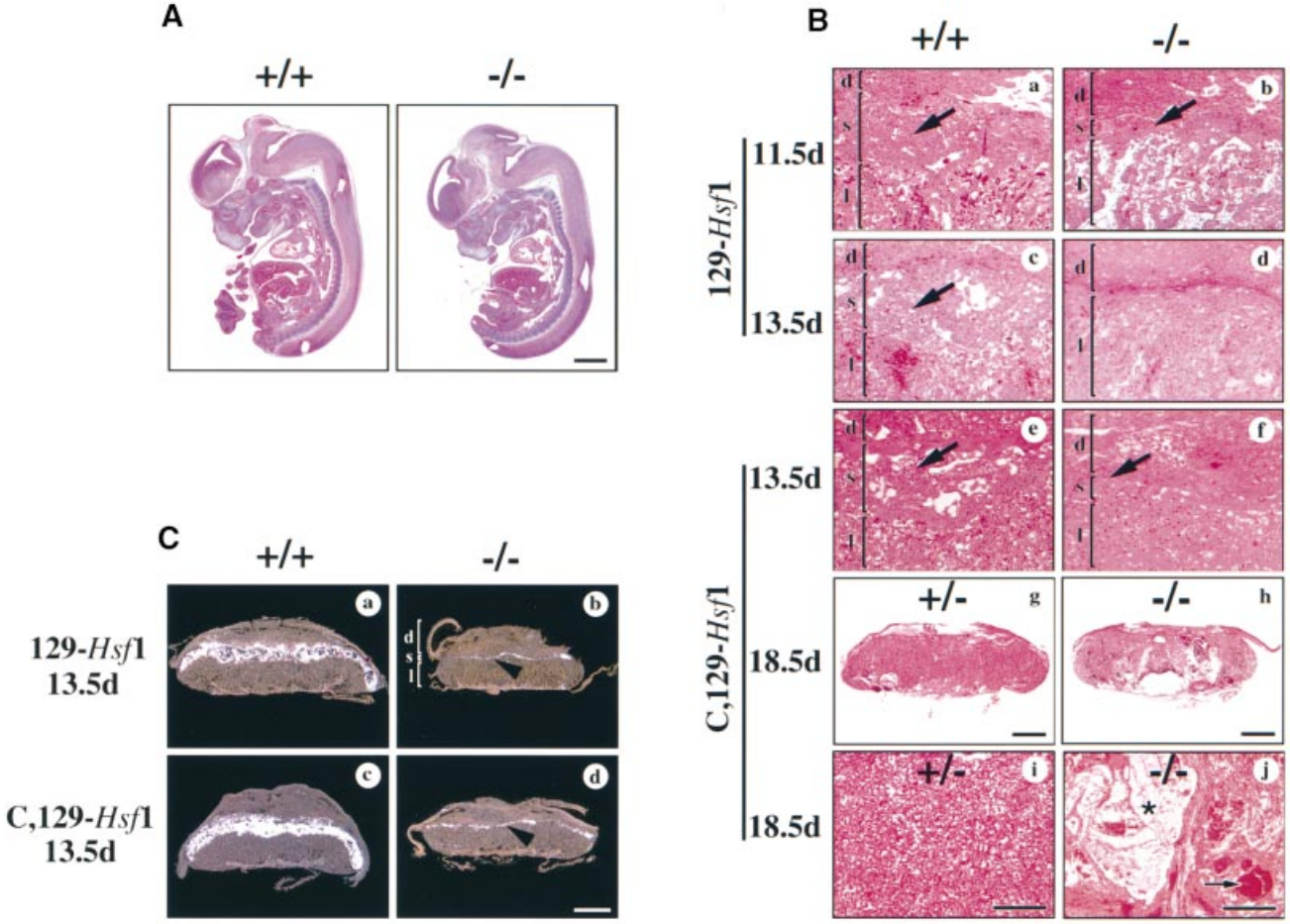

Fig. 1. Gene disruption of *Hsf1*$^{-/-}$ causes placental insufficiency and prenatal lethality in mice. (**A**) Histological staining of sagittal sections of viable wild-type and *Hsf1*$^{-/-}$ embryos, shown here at 13.5 days in the C,129-*Hsf1* hybrid strain, indicated no gross morphological abnormalities in the embryo proper (see the text for details). Scale bars = 3 mm. (**B**) Histopathological abnormalities are localized in the extra-embryonic tissues of mutant *Hsf1*$^{-/-}$ mice. Three morphologically distinct layers of embryonic origin constitute the normal chorioallantoic placentas in wild-type control animals (a, c, e), whereas striking reductions in the spongiotrophoblast layer were found in *Hsf1*$^{-/-}$ concepti of different genetic strains beginning at mid-gestation (b, d, f). Note that the greatly reduced spongiotrophoblast layer in *Hsf1*$^{-/-}$ placentas of 129-*Hsf1* strain at 11.5 d.p.c. was virtually absent at 13.5 d.p.c. (a–d). This phenotype correlated with substantially reduced survival and prenatal lethality at ~E14.0 in 129-*Hsf1* mice (Table II). In the C,129-*Hsf1* strain, discontinuous tissues of the spongiotrophoblast could still be seen at 13.5 d.p.c. between the decidua and labyrinthine trophoblast layers (f, arrow). d, decidua; l, labyrinth; s, spongiotrophoblast. Scale bars = 250 μM, except g and h = 1 mm. (**C**) Expression of vascular endothelial receptor *Flt1* identifies the spongiotrophoblast defect. *In situ* hybridization indicated that the lineage-specific marker *Flt-1* transcript was expressed abundantly in the spongiotrophoblast layer of wild-type embryo placentas at 13.5 d.p.c. (a, c) but was dramatically reduced in *Hsf1*$^{-/-}$ placentas (b, d). The figure also shows a subtle but consistent difference in the amount of spongiotrophoblast layer between C,129-*Hsf1* and 129-*Hsf1* targeted mutant strains (b, d, arrowheads). At 18.5 d.p.c., littermates were obtained from *Hsf1*$^{+/-}$ females and fertile *Hsf1*$^{-/-}$ males intercrossed in C,129-*Hsf1* hybrid strain, and examined [(B), g–j]. Note that the well-organized and highly vascular regions are prominent in the labyrinth at 18.5 d.p.c. in a normal heterozygous control, whereas the placenta of *Hsf1*$^{-/-}$ [(B), h and j] mutant is replaced by marked vacuolization [(B), h and j, arrow], hemorrhages, edematous regions and fibrin deposits [(B), j, asterisk]. Scale bar, 1 mm.

system, dorsal root ganglia, liver and lung, whereas Hsp25 was concentrated in cardiac and skeletal muscles (Figure 2B). Similar patterns of basal expression were obtained from immunohistological analyses in wild-type, *Hsf1*$^{+/-}$ and *Hsf1*$^{-/-}$ adult animals in the C,129-*Hsf1* background, indicating that both spatial and temporal Hsp expression patterns were unaltered by inactivation of the *Hsf1* gene (discussed below).

We next tested the hypothesis that extra-embryonic tissue was an important target for the heat shock response. Immunohistochemical staining of 13.5 d.p.c. 129-*Hsf1* embryos indicated that the HSF1 protein was localized in the nucleus of trophoblastic cells in all layers of wild-type mice, and was expectedly absent in *Hsf1*$^{-/-}$ placentas (Figure 2C). However, we observed neither spatial nor temporal differences in the expression patterns of Hsp90α, Hsp70 and Hsp25 proteins between wild-type and homozygous animals in all layers of the placenta (data not shown), indicating the placental defects could be dissociated from the expression of these Hsp proteins.

Hsf1-deficient mice exhibited postnatal growth retardation

In assessing the potential effects of placental defects on postnatal growth and development, we observed that both male and female *Hsf1*$^{-/-}$ mice appeared smaller than either wild-type or heterozygous littermates. Beginning the first postnatal week, the average weights of C,129-*Hsf1* homozygotes (male = 26; female = 29) were significantly lower than those of wild-type (F_2) littermates (male = 16; female = 7), a result that is consistent with but does not conclusively establish that *Hsf1* deficiency *in utero*

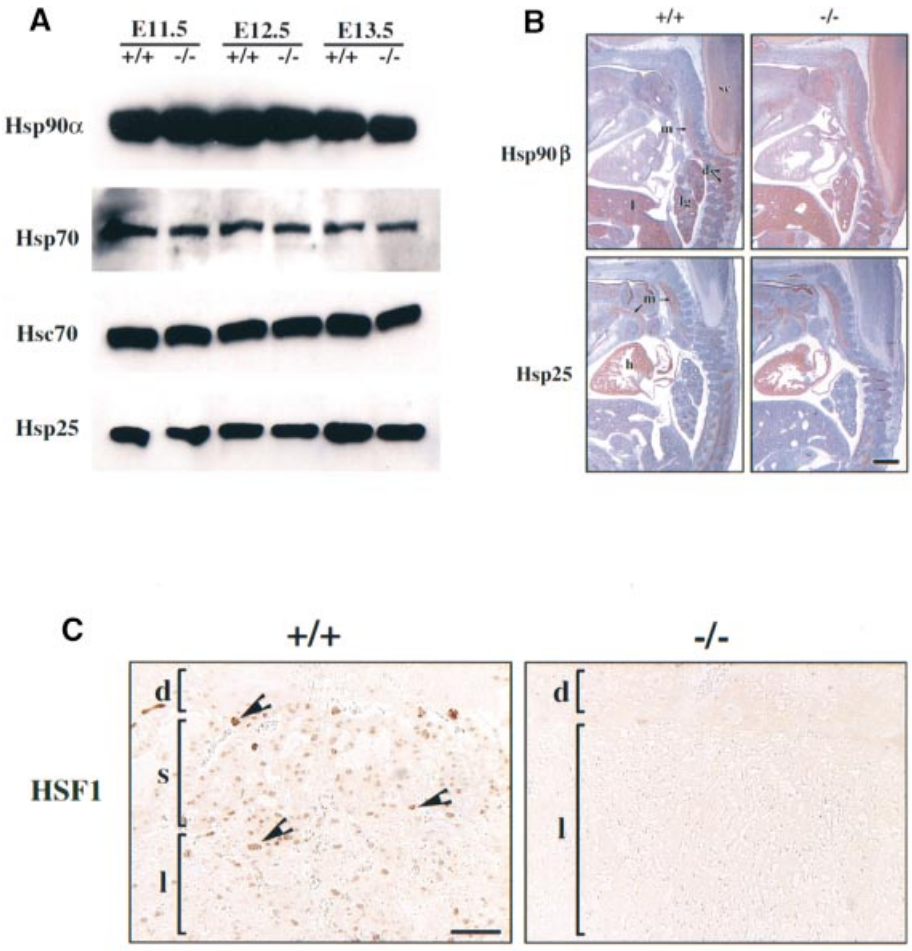

Fig. 2. Spontaneous Hsp expression was unaffected by *Hsf1* deficiency. (**A**) Developmental expression patterns of heat shock proteins from 11.5–13.5 day embryos were examined using Western blot analysis. No differences were found in constitutive expression patterns of major Hsp classes in wild-type and C,129-*Hsf1*$^{-/-}$ animals. Because of weak staining, twice the amount of the highly inducible Hsp70 protein was loaded and the exposure time was doubled. (**B**) Representative immunohistochemistry of sagittal sections of embryos at 13.5 d.p.c. in wild-type and C,129-*Hsf1* mutant embryos. In agreement with Western blot analysis, there were no differences between the wild-type and *Hsf1* homozygous animals by immunohistochemical analysis. Hsp90β was expressed ubiquitously but high levels could be seen in the central nervous system, dorsal root ganglions, liver, lungs and skeletal muscles; in contrast, Hsp25 was localized mainly in the heart and skeletal muscles. d, dorsal root ganglion; h, heart; l, liver; lg, lung; m, skeletal muscle; sc, spinal cord. (**C**) Immunohistochemical staining showed that HSF1 protein was as expected absent in the placentas of *Hsf1*$^{-/-}$ embryos but was localized intensely in the nucleus of trophoblast cells, especially in the spongiotrophoblast of wild-type placentas (Figure 1C, arrowheads). Weak staining of HSF1 protein in decidua of the mutant was derived from maternal *Hsf1*$^{+/-}$ tissues (d, decidua; l, labyrinth; s, spongiotrophoblast).

contributed to growth retardation at birth (Figure 3B and C) ($p < 0.01$). In addition, the average body weights of C,129-*Hsf1*$^{-/-}$ mice lagged behind their wild-type and heterozygous littermates, and this difference increased further after weaning, strongly suggesting that HSF1 expression was required for normal postnatal growth (Figure 3B and C). Growth retardation was independent of sex; male homozygotes were 77% of normal (Figure 3B), whereas female homozygotes were 78% of normal after 8 weeks (Figure 3C; +/+ versus –/–; $p < 0.01$). We also observed that average weights of *Hsf1*$^{-/-}$ mice in another hybrid strain (e.g. B6,129-Hsf1) were similar to those reported presently in C,129- and R,129-*Hsf1* strains (Figure 3C; data not shown). Because the plasma levels of sex steroid (estradiol, estrone and progesterone) and glucocorticoid (corticosterone) were similar in wild-type ($n = 4$) and C,129-*Hsf1*$^{-/-}$ ($n = 4$) adult female mice (data not shown), growth retardation in *Hsf1* inactivation appeared to be unrelated to disruption of the adrenal–pituitary axis.

We routinely obtained litters from intercrosses between wild-type or heterozygous females and multiple strains of *Hsf1*$^{-/-}$ males, indicating that this mutation has no effect on fertility in males. However, no *Hsf1*$^{-/-}$ females ($n = 8$) produced any litters when mated for extended periods (~3 months) with fertile males of all three genotypes. More thorough and detailed analyses of HSF1's potential roles in female infertility in pre-implantation (e.g. early cleavage) and post-implantation development (e.g. decidua response) are in progress and will be reported elsewhere (manuscript in preparation).

Disruption of HSF1 eliminates the heat shock response in adult Hsf1 null mice

Our previous *in vitro* studies using mouse embryonic fibroblasts (MEFS) have demonstrated that the null

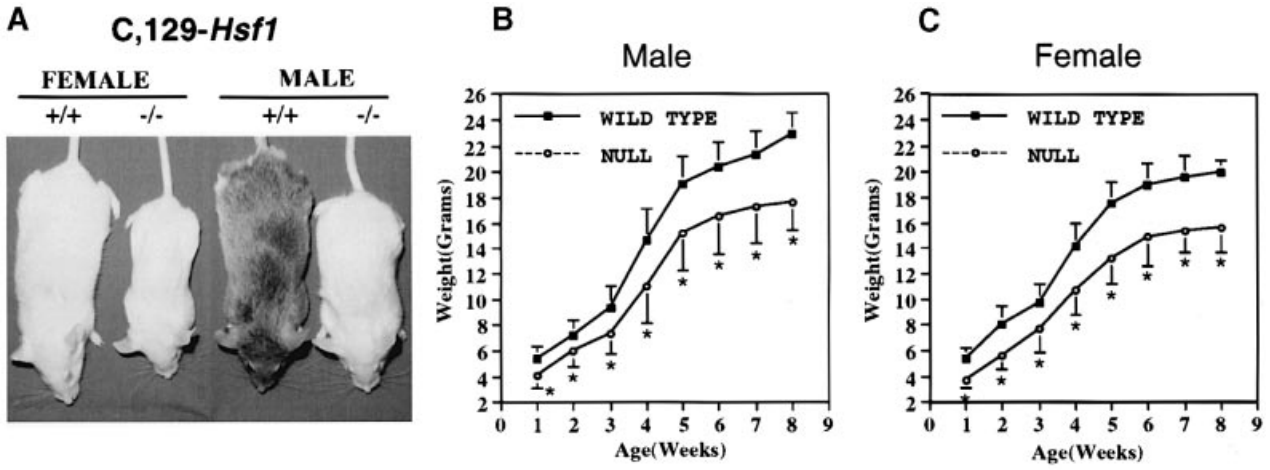

Fig. 3. (**A**–**C**) *Hsf1*-deficient mice exhibit growth retardation at birth. In both sexes of C,129-*Hsf1* strains, *Hsf1*$^{-/-}$ adult mice were visibly and significantly smaller than their wild-type littermates, beginning after the first postnatal week. Mice were weighed every week after birth such that by 8 weeks, *Hsf1*$^{-/-}$ males (n = 16) weighed 77.3% of wild-types males [n = 26, (B)] and *Hsf1*$^{-/-}$ females (n = 7) weighed only 78.2% of wild-type females [n = 29, (C)] from week 1 through week 8 (p <0.01).

mutation completely abrogated the capacity of *Hsf1*$^{-/-}$ cells to acquire thermotolerance, and thereby significantly increased their susceptibility to and reduced their protection against heat-induced apoptosis (McMillan *et al*., 1998). No differences in thermal protection were found between wild-type and heterozygous *Hsf1* cells. Whereas no HSF1 protein was detected in any tissues of *Hsf1*$^{-/-}$ mice, we found that expression of HSF1 protein was reduced to ~50% of wild-type levels in C,129-*Hsf1* heterozygous animals (Figure 4A). To assess whether other HSFs, besides HSF1, conferred heat shock responsiveness *in vivo*, adult mice of all three genotypes in the hybrid C,129-*Hsf1* background were subjected to heat shock (42°C for 15 min) and the heat shock response was assessed after 24 h. In agreement with our *in vitro* analysis (McMillan *et al*., 1998), concentrations of the major classes of Hsps (Hsp70/c70, Hsp90α,β, Hsp60 and Hsp25) were increased dramatically in multiple tissues (e.g. heart, brain, liver and kidney), but similar levels were observed in wild-type and *Hsf1*$^{+/-}$ animals after heat shock (Figure 4B and C). However, Hsp induction was predictably abolished in all tissues of *Hsf1*$^{-/-}$ mice, indicating that other members of the mammalian HSF family do not compensate during inducible hsp gene expression *in vivo* (Figure 4B and C).

Disruption of Hsf1 increases mortality and exaggerates TNF-α production during endotoxemia

Although we observed no adverse outcomes among all three genetic groups under confinement and normal handling in their cages, we next tested the hypothesis that *Hsf1* inactivation would reduce stress tolerance of the organism in response to systemic challenges. Several previous studies have demonstrated that pre-treatments with either heat stress or chemicals (e.g. sodium arsenite), which induced multiple Hsps, improved the survival in several rodent models challenged by endotoxin, a component of lipopolysaccharide (LPS) of Gram-negative bacteria (Hotchkiss *et al*., 1993; Ribeiro *et al*., 1994). To assess the functions of constitutive Hsps, and physiological consequences of a muted heat shock response *in vivo*, wild-type (n = 19), heterozygous (n = 18) and homozygous C,129-*Hsf1* mice (n = 20) of both sexes were administered LPS intraperitoneally (i.p.), and survival rates were monitored for 96 h. The LD_{50} of *Escherichia coli* LPS (25 mg/kg body weight) was determined in preliminary studies using this mixed genetic background (data not shown). Figure 5A shows that the survival rate of C,129-*Hsf1*$^{-/-}$ mice was significantly lower (35% after 4 days) than that of wild-type controls and heterozygotes (63.2 and 60% after 4 days, respectively) after LPS exposure, with death in 40% of mutant mice occurring after 48 h (+/+ versus –/–; χ^2 test for survival, p <0.05). Thus, laboratory mice bearing inactivation of HSF1 expression exhibited increased mortality during pathological challenges mimicking lethal inflammatory stimuli in humans.

Multiple factors might influence differences in susceptibility to LPS treatment, including the heat shock response, co-stimulatory effects on NFκB and IκB activation and degradation, increased oxidative stress/damage, and acute-phase proteins such as pro-inflammatory cytokines. Consistent with earlier studies (Hotchkiss *et al*., 1993), we found negligible changes in the concentrations of stress-inducible Hsp70 in multiple tissues (liver, heart) and time points (12–24 h) after endotoxin treatment (data not shown). To determine whether HSF1 expression exerted direct effects on cytokine production, we examined levels of TNF-α and interleukin 10 (IL-10) using quantitative ELISA during the acute-phase response after endotoxin administration (see Materials and methods). Within 90 min after LPS, robust induction of TNF-α production in the plasma of C,129-*Hsf1*$^{-/-}$ (n = 8) animals had significantly increased by 2-fold above the levels of heterozygous (n = 11) control animals (Figure 5B; +/– versus –/–; p <0.05). In contrast, the anti-inflammatory cytokine IL-10 was not significantly different and slightly greater in *Hsf1*$^{-/-}$ (n = 13) than heterozygous (n = 11) animals (Figure 5C). Thus, reduced survival of *Hsf1*$^{-/-}$ animals

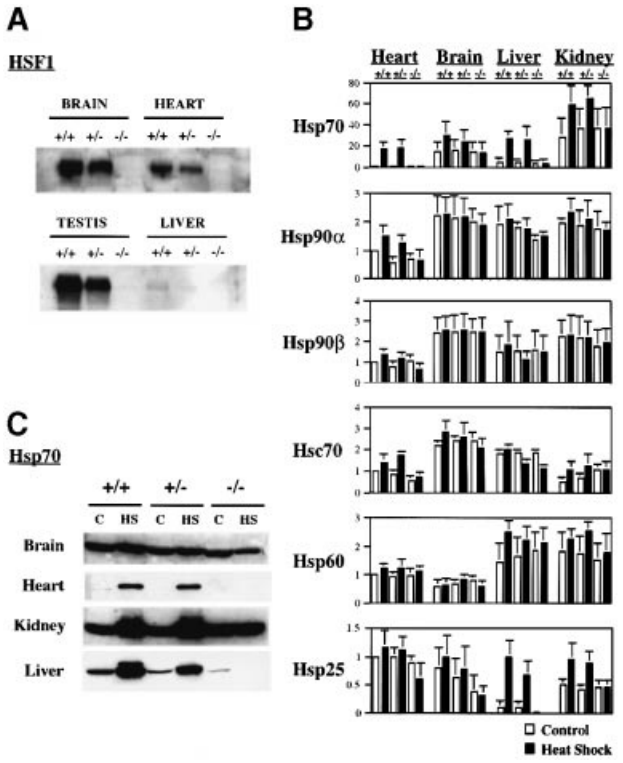

Fig. 4. (**A**) Disruption of HSF1 protein in adult wild-type and *Hsf1*$^{-/-}$ mice. Concentrations of HSF1 protein were assessed by Western blot analysis in the brain, testis, heart and liver of adult wild-type mice (C,129-*Hsf1* strain). HSF1 protein was correspondingly reduced by ~50% in heterozygous animals; however, neither full-length HSF1 nor truncated HSF1 protein was found in any tissues of *Hsf1*$^{-/-}$ mice. (**B**) *Hsf1* null mutation abolishes the heat shock response *in vivo*. Groups of wild-type (n = 3), heterozygous (n = 3) and null (n = 3) adult C,129-*Hsf1* mice were anesthetized and subjected to heat shock (42°C for 15 min) and recovered for 24 h as described in Materials and methods. Control animals of all three genotypes (n = 3 animals/group) were anesthetized but not exposed to heat shock conditions. Animals were killed after 24 h and protein extracts from different tissues were analyzed by Western blotting. Note that the average constitutive levels of the major Hsp classes under non-stressed conditions were unaltered in all genotypes (+/+, +/–, –/–) of C,129-*Hsf1*$^{-/-}$ mice. The heat shock response was completely abolished in *Hsf1*$^{-/-}$ mice, which was in agreement with previous *in vitro* analysis (McMillan *et al.*, 1998). Differences in the heat shock response were assessed by densitometry scans using representative scans for Western blotting (e.g. Hsp70 protein), as shown in (**C**). The fold changes for Hsps were plotted in arbitrary units as the ratios obtained under heat shock and non-stressed conditions.

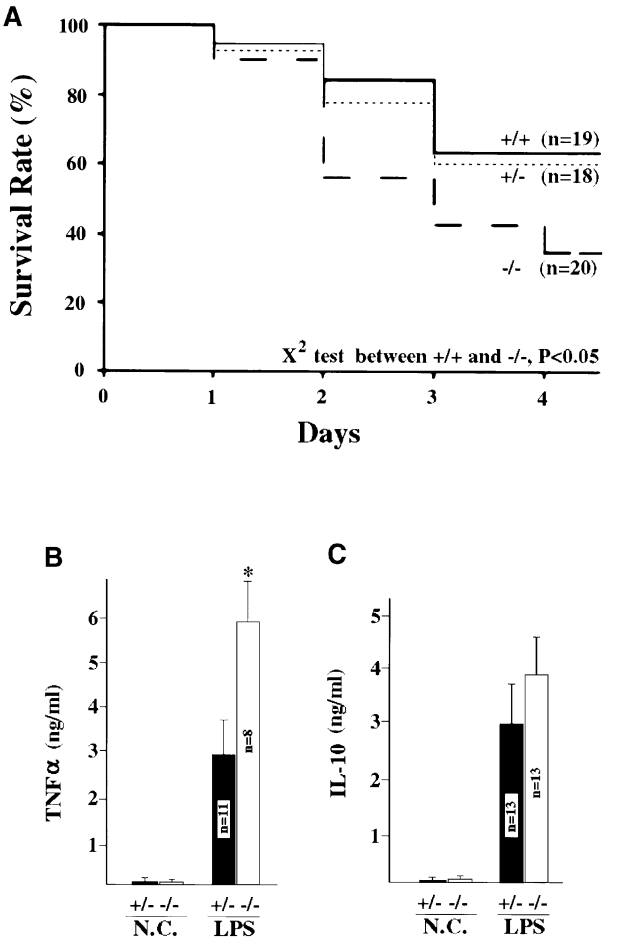

Fig. 5. (**A**) Reduced survival and increased TNF-α production of *Hsf1*-deficient mice after LPS challenge. *Escherichia coli* LPS was administered as a single dose (25 mg/kg) i.p. to groups of wild-type (n = 19), heterozygous (n = 18) and null (n = 20) adult C,129-*Hsf1* mice, and the survival rates were monitored for 4 days. A statistically significant higher survival rate was found for wild-type than *Hsf1*$^{-/-}$ adult mice after 4 days (63.2% versus 35.0%) after LPS (χ^2 test for survival analysis between wild-type and *Hsf1*$^{-/-}$ mice, $p < 0.05$). (**B** and **C**) Heterozygous mice, which exhibited similar survival rates as wild-type controls, were treated similarly to *Hsf1*$^{-/-}$ animals with LPS and the plasma levels of inflammatory cytokines (TNF-α and IL-10) were determined 90 min after LPS treatment using ELISA. In *Hsf1*$^{-/-}$ animals, the plasma levels of TNF-α were significantly increased by 100% above heterozygous controls exhibiting a lower and similar mortality to wild-type animals (A). LPS did not significantly increase levels of Hsps, in agreement with others (data not shown), indicating that alterations in the production of pro-inflammatory cytokine can be dissociated from the heat shock response.

correlated mainly with exaggerated production of the pro-inflammatory cytokine TNF-α, but not with stress-inducible Hsp expression, after single-dose endotoxemic challenge in mice (Figure 5A–C), implicating a direct role of HSF1 deficiency in pathological states in mice.

Discussion

To date, genetic studies of the *in vivo* functions of orthologous HSFs have indicated that yeast HSF is essential for viability (Sorger and Pelham, 1988), whereas *Drosophila* HSF protein is required for oogenesis but is dispensable for growth and viability under non-stress conditions (Jedlicka *et al.*, 1997). Since the discovery of multiple HSF transactivators in vertebrates, our understanding of their precise physiological roles has lagged substantially behind extensive analysis of their biochemical, structural and regulatory properties, especially during the stress response. Surprisingly, exhaustive studies of homozygous animals excluded a primary role for HSF1 expression in the development of the embryo proper in mice. HSF1 expression was unexpectedly required for extra-embryonic development, beginning at mid-gestation (Table I). Interestingly, the severity of prenatal lethality and survival of *Hsf1* mutant mice were influenced by the genetic background. Although this well-recognized effect is not infrequent in gene knockouts in mice (Threadgill

et al., 1995), variable penetrance of *Hsf1* mutation on survival suggested that the presence of genetic modifiers is involved in extra-embryonic development.

Hsf1 deficiency and chorioallantoic placenta development

Genetic studies in mice have implicated several factors in determining the cell fate and differentiation of major trophoblast cell lineages in post-implantation development. Normal histomorphological appearance of the decidua layer, containing cells of both maternal and fetal origin, and the labyrinth, containing trophoblast giant cells, in *Hsf1*$^{-/-}$ animals indicated that HSF1 expression was dispensable in the development of these cell lineages. All *Hsf1*$^{-/-}$ placentas examined in both 129-*Hsf1* and C,129-*Hsf1* strains exhibited similar histopathological abnormalities of the spongiotrophoblast lineage, indicating that the cell and tissue requirements were specific for HSF1. In the most severely affected 129Sv strain (1.2% live births), *Hsf1*$^{-/-}$ embryos perished at late mid-gestation, which limited the recovery of additional *Hsf1*$^{-/-}$ embryos due to reabsorption. The timing of prenatal death in the C,129-Hsf1 (11.8%) and R,129-Hsf1 (15%) strains is likely to have occurred between embryonic day (E) 14.5 and birth, which exhibited increased survival rates (Table II).

The mechanism for the unexpected findings of placentation defects caused by HSF1 inactivation remains to be elucidated fully but several possibilities are envisioned. Prior studies have shown that disruption of either *Mash2* (Guillemot *et al*., 1994) or the estrogen-receptor-related receptor β (ERR-β) (Luo *et al*., 1997) results in arrested differentiation of the spongiotrophoblast layer. In contrast to *Mash2*$^{-/-}$ or *ERR*-β$^{-/-}$, in which embryonic lethality occurred at early mid-gestation (~10.5 d.p.c.), disruption of *Hsf1*$^{-/-}$ produced embryonic lethality later in mid-gestation at ~14.0 d.p.c., suggesting possible requirements for downstream targets of HSF1 expression that play critical roles for maintenance of the differentiated spongiotrophoblast. Since we recovered viable C,129-*Hsf1* concepti that exclusively exhibited the characteristic abnormalities in extra-embryonic tissue, we conclude that the changes found in the labyrinth at late gestation (18.5 d.p.c.) were secondary to the primary lesions of the spongiotrophoblast at mid-gestation.

In contrast to earlier studies indicating induction of hsp70 expression in the placentas at 8.5 d.p.c. and in the yolksac at 11.5 d.p.c. in mice (Kothary *et al*., 1987), we found that extra-embryonic abnormalities were disassociated from appreciable changes in basal Hsp levels, at late mid-gestation (Figure 1C; data not shown). Interestingly, immunohistochemical studies of placentas in human subjects have indicated no direct correlation between expression patterns of multiple Hsps (e.g. Hsp72/73, Hsp60 and Hsp27) and gestational age or clinical endpoints in later pregnancy (Divers *et al*., 1995; Li *et al*., 1996). We hypothesize that HSF1 *in vivo* functions, like the *Drosophila hsf* protein (Jedlicka *et al*., 1997), might target novel non-hsp genes in placental mammals.

Accordingly, multiple experimental approaches are being used currently to determine whether the complex placental phenotype involves members of the Hsp family and other novel targets, either alone or in combination. For example, future experiments such as rescue of the trophoblast defect are needed to clarify putative HSF1 *in vivo* functions in growth, both *in utero* and in postnatal development. In addition, homozygous *Hsf1* mice of both sexes can survive to late adulthood (>2 years) with the notable exception that females, unlike *Hsf1*$^{-/-}$ males, were infertile. Characterization of female *Hsf1*$^{-/-}$ sterility, still in progress, will be reported separately (E.Christians and I.J.Benjamin, unpublished results).

HSF1 is a positive and negative regulator

We have demonstrated that *Hsf1* inactivation does not affect HSF2 expression, yet the heat shock stress response was completely abolished in *Hsf1* null cells (McMillan *et al*., 1998). Interestingly, Tanabe *et al*. (1998) have shown recently that inactivation of avian HSF3 revealed an unexpected requirement, along with chick HSF1, for full expression of stress tolerance in chick cultured cells, raising the important possibility that other vertebrate HSFs may share similar interactions. However, in multiple tissues of *Hsf1* null animals, unimpaired HSF2 expression does not compensate for HSF1, as the main stress-inducible transactivator, during mammalian heat shock response.

In agreement with earlier studies in mice (Hotchkiss *et al*., 1993), susceptible C,129-*Hsf1* wild-type or heterozygous control animals, which were capable of Hsp induction, exhibited insignificant changes in Hsp concentrations after LPS treatment (I.J.Benjamin, unpublished results). Since HSF1 activation has been shown to suppress pro-interleukin 1β gene (proIL-1β) in human monocytes after LPS stimulation *in vitro* (Cahill *et al.*, 1996), evidence for significantly greater TNF-α production in *Hsf1*$^{-/-}$ animals than in heterozygous controls is the first direct evidence supporting HSF1's *in vivo* role as a negative regulator. Thus, suppression of the pro-inflammaory cytokine TNF-α by HSF1 establishes a bona fide compensatory endogenous mechanism for protection during pathological challenges in mammals.

Implications of the Hsf1 knockout for future studies of disease pathogenesis

In conclusion, new findings for crucial requirements of HSF1 *in vivo* functions in post-implantation development suggest possible new avenues to identify novel genes and signal transduction pathways likely to impact reproductive development in mammals. Because Hsp chaperones have been shown to confer survival and protection against proteotoxic damage, upregulation of stress 'heat shock' proteins (Hsps) is widely believed to play a pivotal role in defense strategies during adverse (patho)physiological conditions. Such cytoprotective properties of Hsps have been implicated in inherited human genetic disorders such as cystic fibrosis, in acquired diseases such as Alzheimer's disease, ischemic heart disease and cancer, and in degenerative diseases of aging, all of which are likely to have abnormal protein structures as common pathogenic mediators (Dobson and Ellis, 1998). That *Hsf1* mutant mice can survive to adulthood should immediately accelerate applications of this experimental model to decipher whether direct causal relationships involve members of the stress-inducible Hsps in physiological health and pathological conditions.

Materials and methods

Targeting mutation strains and characterization of the Hsf1 null allele

Hsf1 mice were created using homologous recombination with a gene-targeting vector in ES cells as described recently (McMillan *et al*., 1998). Germline male chimeras (ES cells, 129SvEv) or 129SvJ heterozygous males carrying the mutant *Hsf1* allele were mated with wild-type females of 129SvJ (129,*Hsf1*), BALB/c (C,129-*Hsf1*), C57BL/6J (B6,129-*Hsf1*) and ICR (R,129-*Hsf1*) strains to produce their offspring (F_1). Mendelian ratios of live births were determined from the offspring (F_2) of the heterozygous intercrosses. *Hsf1* mice in the 129Sv background were confirmed independently using a battery of available markers (Simpson *et al*., 1997). Sequence analysis of the mutant HSF1 transcript by PCR showed that an in-frame splicing event from nucleotide +118 to +724 has deleted 606 bp and, subsequently, 202 amino acids within the HSF1 ORF, corresponding to ~80% the DNA-binding domain plus leucine zippers 1–3 (Sarge *et al*., 1991).

Histology

Staged mouse embryos and placentas were harvested and fixed at 4°C in 4% paraformaldehyde overnight, dehydrated in ethanol and infiltrated with paraffin. Paraffin-embedded samples were then sectioned at 5 μm and subsequently stained with hematoxylin and eosin (H & E) for histological examination or sectioned at 4 μm for immunohistochemistry and *in situ* hybridization.

Immunohistochemistry

Embryos and placentas were harvested and fixed as for histology. Sections were reacted overnight at 4°C with rabbit polyclonal anti-HSF1 (1:2000), a kind gift from R.Morimoto; rabbit polyclonal anti-Hsp90α (1:16 000) and -β (1:50) (anti-Hsp86 and 84, respectively) from Affinity Bioreagents, Inc.; rabbit polyclonal anti-Hsp70 (1:100 000), a kind gift from R.Tanguay; and rat monoclonal anti-Hsc70 (1:30) and rabbit polyclonal anti-Hsp25 (1:16 000) from Stressgen Biotechnologies Corp. (Victoria, BC, Canada). After incubation with the appropriate horseradish peroxidase (HRP)-conjugated second antibody at room temperature for 1 h, slides were developed with DAB chromogen (Dako Corp., Carpinteria, CA). Some slides were counterstained with H & E.

Immunoblotting

Whole-cell extracts were prepared from various organs by homogenization. Twenty micrograms of protein were electrophoresed and transferred to Immobilon-P membrane (Millipore), and probed with the aforementioned antibodies with the following modifications: rabbit polyclonal anti-HSF1 (1:5000), rabbit polyclonal anti-Hsp90α and -β (1:5000), rabbit polyclonal anti-Hsp70 (1:100 000), goat polyclonal anti-Hsp60 (1:5000), rat monoclonal anti-Hsc70 (1:5000) and rabbit polyclonal anti-Hsp25 (1:10 000). After incubation in the appropriate HRP-conjugated second antibody (Vector Laboratories), blots were developed using the Renaissance Chemiluminescence System (NEN Life Science Products).

In situ hybridization

Dr Janet Rossant provided plasmids pFlt-1, pMASH-2 and pGPLF-IA containing cDNA sequences of Flt-1, MASH-2 and PLF-IA genes, respectively. Flt-1 and MASH-2 were used as molecular markers specific for spongiotrophoblast cells and PLF-IA for trophoblast giant cells of the placentas (Carney *et al*., 1993; Guillemot *et al*., 1994; Breier *et al*., 1995). Antisense and sense riboprobes were generated by *in vitro* transcription using the Maxiscript kit (Ambion Inc., TX) and labeled with [^{35}S]UTP (800 Ci/mmol). *In situ* hybridization was performed on paraffin-embedded placental sections as described (Wilkinson *et al*., 1987). The slides were developed, counterstained with hematoxylin, and examined under the microscope using bright and darkfield optics.

Whole-body hyperthermic and LPS challenge

Wild-type and homozygous adult mice of both sexes in the C,129-*Hsf1* background were anesthetized with 1.2% Avertin (Aldrich, WI) 250 mg/kg body weight i.p., treated with a sublethal heat shock by increasing the rectal temperature to 42°C for 15 min using a 250 A infrared heat lamp (General Electric), and then returned to their cages for 24 h recovery. Anesthetized control mice were kept under similar conditions without heat shock, and their body temperature maintained at 37 ± 0.5°C. Mice in both experimental groups were administered 0.2 ml of 0.9% sodium chloride i.p. to compensate for insensitive water loss during the procedure and recovery periods.

For the LPS study, adult C,129-*Hsf1* animals, 4–5 months old, were injected i.p. with 25 mg/kg body weight of LPS from *E.coli* serotype 0111:B4 (Sigma) diluted in pyrogen-free normal saline and mortality monitored continuously for 4 days.

Cytokine assays

TNF-α and IL-10 levels in plasma were determined using an ELISA as described by the manufacturer (R & D Systems, Minneapolis, MN). Ninety minutes after administration of LPS or non-pyrogenic saline, *Hsf1*$^{-/-}$ or heterozygous control mice were killed with sodium pentobarbital (55 mg/kg i.p.) and blood samples were extracted by intracardiac puncture. Plasma samples were collected in ice-cold heparinized (200 U/tube) Eppendorf tubes, centrifuged at 700 *g* for 10 min at 4°C. All assays were performed in duplicate using diluted (1:10) samples and a standard curve that was generated according to the manufacturer's instructions.

Densitometry

Western blots from three different animals of each genotype with or without heat shock were scanned and analyzed using IP Lab Gel 1.5C (Signal Analytics Corp., Vienna, VA). To compare the fold differences between genotypes in the respective tissues, the expression of each Hsp was normalized to the wild-type control (non-heat shocked) animals and the average fold changes from three separate experiments are shown.

Statistics

Differences in group means were compared by Student's *t*-test and differences in mortality after LPS treatment were assessed using the χ^2 test for survival. A p value of <0.05 was considered statistically significant.

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