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Phenotypic characterization of mouse embryonic fibroblasts lacking heat shock factor 2

Liliana Paslaru [a] *, M. Morange [b], Valérie Mezger [b]

[a] *"Carol Davila" University of Medicine and Pharmacy Fundeni Hospital, Bucharest, Romania*
[b] *UMR8541, École Normale Supérieure, Paris, France*

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Abstract

In murine cells, the heat shock response is regulated by a transcription factor, HSF1, which triggers the transcription of heat shock genes. HSF2 has been shown to be involved in meiosis and mouse brain development. We characterized the effects of the absence of HSF2 in mouse embryonic fibroblasts (MEFs). The temperature threshold of the heat shock response appeared lowered in *Hsf2*$^{-/-}$ MEFS as monitored by the synthesis of heat shock protein HSP70. In contrast to unstressed wild type MEFS, HSP70 and HSF1 are localized in the nucleus of unstressed *Hsf2*$^{-/-}$ MEFS, a characteristic of stressed cells. HSF1 is not activated for DNA-binding at unstressed temperature in *Hsf2*$^{-/-}$ MEFS. Therefore, the absence of HSF2 induces some but not all of the characteristics of the stress response. In addition, *Hsf2*$^{-/-}$ MEFS exhibited proliferation defects, altered morphology, remodeling of the fibronectin network.

Keywords: Heat shock response - heat shock transcription factors - HSF1 - HSF2 - mouse embryonic fibroblasts - proliferation

Introduction

Organisms have developed very conserved pathways to resist to proteotoxic stresses like heat shock by the expression of heat shock proteins (Hsps). Hsps have an important role in proteic homeostasis and cellular protection through their functions of molecular chaperones. The expression of heat shock genes is primarly regulated at the transcriptional level by transcription factors called "heat shock factors" (HSF) which bind to the heat shock promoter element (HSE)[1]. The discovery of four distinct members of this family in vertebrates has raised the question about specialized or overlapping functions of this HSFs. HSF1 is the paradigm member of the family. It is present in the cell in a latent monomeric inactive state. In response to heat shock and various proteotoxic stresses, HSF1 is rapidly converted to a trimer, is translocated to the nucleus, acquires DNA-binding activity, is phosphorylated and becomes transcriptionally active. In addition HSF1 is also involved in placental development and female fertility [2, 3]. In human and mice, HSF2

* Correspondence to: Dr. Liliana PASLARU
"Carol Davila" University of Medicine and Pharmacy,
Department of Biochemistry,
Fundeni Hospital, Bucharest , Romania.
Tel.: 00 40 21 777 31 27.

has remained an enigmatic factor until it was recently shown that it is involved in meiosis and brain development [4, 5]. Its function as a heat shock factor has nevertheless remained unclear. HSF2 is not activated by heat shock, but in contrast inactivated [6]. However, the subcellular localization of HSF2 was early reported to be stress-regulated and human HSF2 might participate in HSF1 mediated Hsp70 gene transcription upon a stress [7, 8]. In murine fibroblasts HSF2 localizes in the perinuclear region [8]. However, recent report provided evidence of human HSF2 being an integral member of the cellular stress response pathway [9, 10]. A characteristic feature of cellular stress in human cells, but not murine cells is the organization of HSF1 into specific subnuclear structures,termed stress granules [11]. According to this study, HSF2 interacts physically with HSF1 and is a novel stress-responsive component of the stress granules. Based on the analysis of deletion mutants, HSF2 influences the localization of HSF1 in stress granules, regulatory mechanism of HSF1-mediated heat shock response [10].

In an attempt to better understand the role of HSF2, we characterized the phenotype of mouse embryonic fibroblasts (MEFs) derived from our *Hsf2* knocked-out mouse strain. Since human and murine cells display different characteristics of HSF1 regulation, in particular for stress granules formation, we investigated whether the absence of HSF2 might influence the heat shock response in *Hsf2*$^{-/-}$ MEFs.

We show that the proliferation of *Hsf2*$^{-/-}$ MEFs is markedely impaired in correlation with altered cell morphology and remodeling of the fibronectin network. The constitutive expression of most Hsps is not modified. However, the expression of HSP25 is slightly increased but its phosphorylation is not significantly modified in *Hsf2*$^{-/-}$ MEFs. Interestingly, *Hsf2*$^{-/-}$ MEFs display a heat shock response at lower temperatures than wild-type MEFs. In correlation with this increased sensitivity, HSF1 and HSP70 are nuclear in unstressed *Hsf2*$^{-/-}$ MEFs, while they are mainly cytoplasmic in wild-type MEFs. Therefore, the absence of HSF2 might influence the localization of HSF1 and HSP70 or alternatively, since the nuclear localization of HSP70 and HSF1 is characteristic of stressed cells, the absence of HSF2 might be interpreted as a stress by the cell.

Materials and methods

Hsf2-null mice, genotyping

Hsf2 null mice were obtained by Valérie Mezger and heterozygous males and females were breeded. Mice and embryos were genotyped by PCR as described in Kallio *et al.*, 2002 [4].

Cell culture and heat shock

Wild type and *Hsf2*$^{-/-}$ embryonic fibroblasts null were obtained from fetuses of the litters from heterozygous crosses. After dissection, the tissues of individual embryos dissociated into individual cells by trypsine and cultured in Dulbecco's Modified Eagle Medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal calf serum, 2 mM glutamine, 50 μg/ml streptomycin and 50 units/ml penicillin. Cells were cultured *ex vivo* for at least ten passages.

For Western experiments, cells were plated in 24-well plates and incubated at 37°C in 7,5% CO_2 water-saturated atmosphere during the exponential growth phase. The heath shock treatment was performed by immersing the culture dishes in a waterbath at 42°C, 43°C and 44°C, during the indicated times, and cells were brought to recovery at 37°C for 4 h; during the last hour of incubation at 37°C, (^{35}S)-methionine was added (300 μCi/ml; 120 μCi/well)

Cell morphology

Embryonic fibroblasts were cultured in cell culture dishes (ϕ = 10 cm) and stained with Coomassie blue.

Cell growth analysis

4 x 10^4 cells were plated in a 6 cm diameter culture plate at day 0. At the indicated times, cells from triplicate plates were dissociated to individual cells and counted.

Gel electrophoresis, Western blots and EMSA

(^{35}S)-methionin-labeled cells were rapidly washed in PBS. Protein extracts were prepared by resuspending the

cells in 50µl of Laemmli sample buffer with 5% β-mercaptoethanol and the samples heated for 10 min at 90°C [12].

Samples were analysed by SDS-10% polyacrylamide gel electrophoresis; a volume corresponding to the same amount of cells, was deposited on each lane. The gels were fixed, dried and submitted to autoradiography. 2-D gel electrophoresis were performed as described in Western blot analysis and EMSA performed as described in [13]. Anti-mouse HSF2 rabbit polyclonal antibody was used at dilution 1:10,000 (Sarge *et al.*, 1993). Monoclonal antibody against HSF1 (Ab-4, Neomarkers) was used at 1:100. Anti-mouse HSP27 (Neomarkers) was used at 1:1000 dilution.

Immunocytochemistry

Cells were grown on gelatine-coated sterile glass coverslips, then rinsed with PBS at 4°C and fixed in 4% paraformaldehyde for 15 min at room temperature. Cells were rinsed in PBS, blocked in 3% BSA/PBS, incubated with primary antibodies in 3% BSA/PBS for 1h at room temperature or overnight at 4°C and rinsed 3 times in PBS. Cells were incubated with secondary antibodies, Hoechst 33342 (Sigma) and FITC-phalloidin when necessary, finally rinsed 3 times in PBS before being mounted in Mowiol 40-88 (Aldrich-Chemie). Anti-HSP70 mouse monoclonal antibody was obtained from Stressgen (SPA 810) and used at 1:100 dilution. CY3 coupled-anti mouse secondary antibody was used at 1:400 dilution. Anti-HSF1 rat monoclonal antibody (Ab-4, Neomarkers) was used at 1:200 dilution. Secondary antibodies: FITC-conjugated goat anti-mouse IgG (Jackson), Anti- mouse fibronectin monoclonal cellular antibody (Sigma) was used at 1:400 dilution. The fluorescence images were analysed with Zeiss microscope DMRB coupled to a digital camera Leica DC300F and Leica FW4000 program.

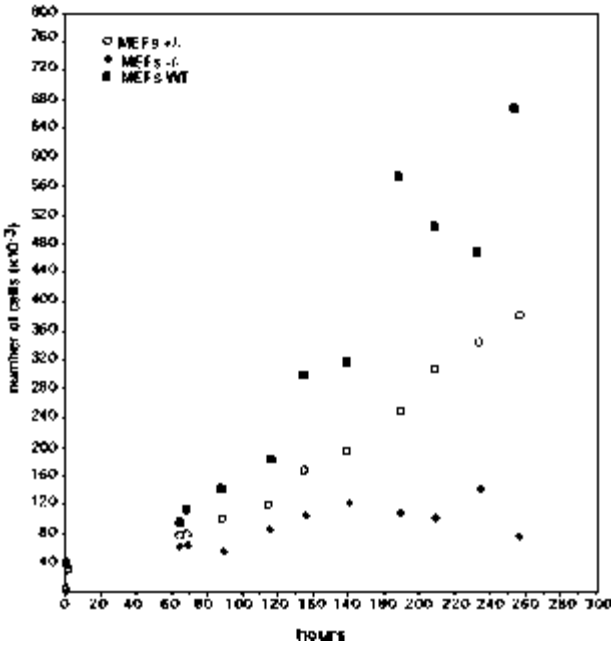

Fig. 1 The absence of HSF2 compromises MEFs proliferation. Example of the proliferation rate of WT (dark squares), homozygous (black diamonds) or heterozygous (empty squares) MEFS. 40 x10^{-3} cells were seeded at time zero. Cells were counted at the indicated time (hours). The number of cells plotted (x10-3) corresponds to the average value of triplicates. Errors bars were smaller thant the size of each symbol and therefore not drawn. Such type of experiments was performed on four distinct litters of embryons and gave reporducible results. Compared to wild type or homozygous MEFs which display a very reproducible phenotype, heterozygous MEFs populations vary in their proliferation rates, being intermediate between WT and homozygous. A population of heterozygous MEFs with an intermediate proliferation rate is illustrated in this figure.

Results

MEFs lacking HSF2 display reduced proliferation rate and altered morphology

Three heterozygous females were mated with three heterozygous males. A total of 34 embryos recovered at day E14.5 postcoitum were genotyped by PCR analysis (see Materials and Methods). Nine wild type embryos, 9 homozygous embryos and 16 heterozygous embryos. MEFs were prepared from individual embryos. Since *Hsf2*$^{-/-}$ embryos showed a delay in replating requirements compared to wild type, we decided to investigate the proliferation the *Hsf2*$^{-/-}$ MEFs. Forty thousands wild-type MEFS or *Hsf2*$^{-/-}$ MEFs were plated and cells were counted at the indicated times. Compared to wild-type MEFs, *Hsf2*$^{-/-}$ MEFs reproducebly showed proliferation

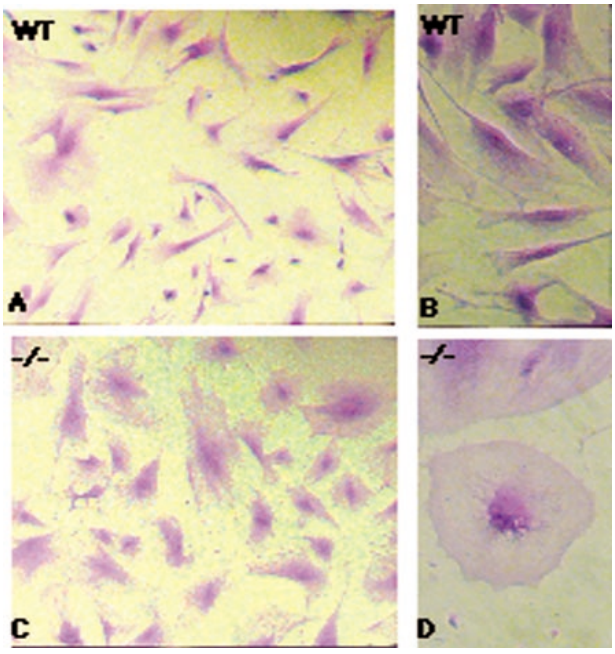

Fig. 2 Altered morphology of *Hsf2*$^{-/-}$ MEFs. Wild type (A and C) or *Hsf2*$^{-/-}$ MEFS were stained with Coomassie blue. A and C: 5 fold magnification. B and D: 20 fold magnification. Note that *Hsf2*$^{-/-}$ MEFs display enlarged size.

defects. One typical example of the growing properties of a wild type and a *Hsf2*$^{-/-}$ MEF population was plotted in Fig. 1. Wild-type MEF number of cells increased by approximately 8x fold in 6 days and were still proliferating 10 days after plating, In contrast, *Hsf2*$^{-/-}$ MEF number of cells rapidly reached a plateau and increased in number only three fold approximately in 6 days. Heterozygous MEFs exhibited variability in their proliferating properties. Some heterozygous populations proliferated almost equally well as wild-type MEFs and some had a profile an intermediate proliferation profile as illustrated in Fig. 1.

In correlation with proliferation defects, *Hsf2*$^{-/-}$ MEFs displayed an altered morphology (Fig. 2) reminiscent of senescence features. The cell surface of *Hsf2*$^{-/-}$ MEFs was globally larger, cells spread out and cell shape lost their fibroblastic characteristics and rounded out (Fig. 2, D compared with B). Cells with more than one nuclei were apparent (Fig. 5, A).

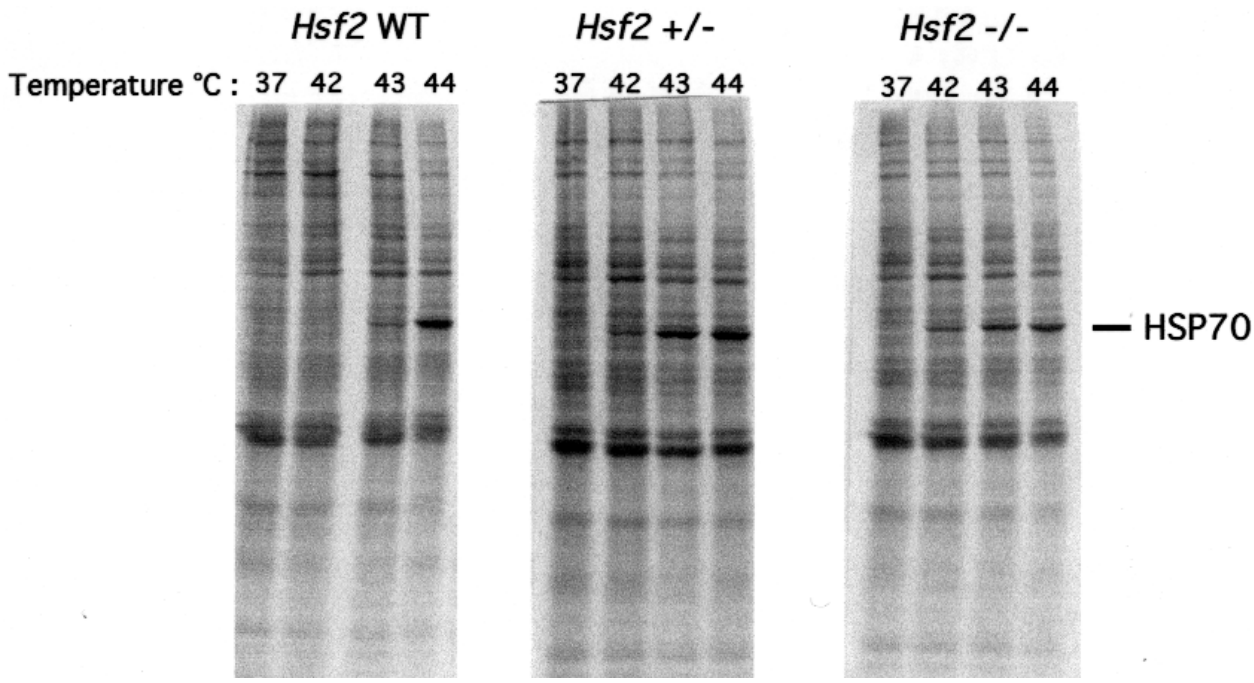

Fig. 3 The temperature threshold of the induction of synthesis of Heat Shock Protein 70 is lowered in the absence of HSF2. A. MEFs were exposed for 2 hours at 42°C or 1 hour at 43°C or 30 minutes at 44°C and labelled by (35S) methionine. The induction of synthesis of HSP70 is detected as low at 41°C in *Hsf2*$^{-/-}$ MEFs. B. Western blot analysis of the same samples with anti-HSP70 (upper panel) or anti-HSC70 monoclonal antibodies.

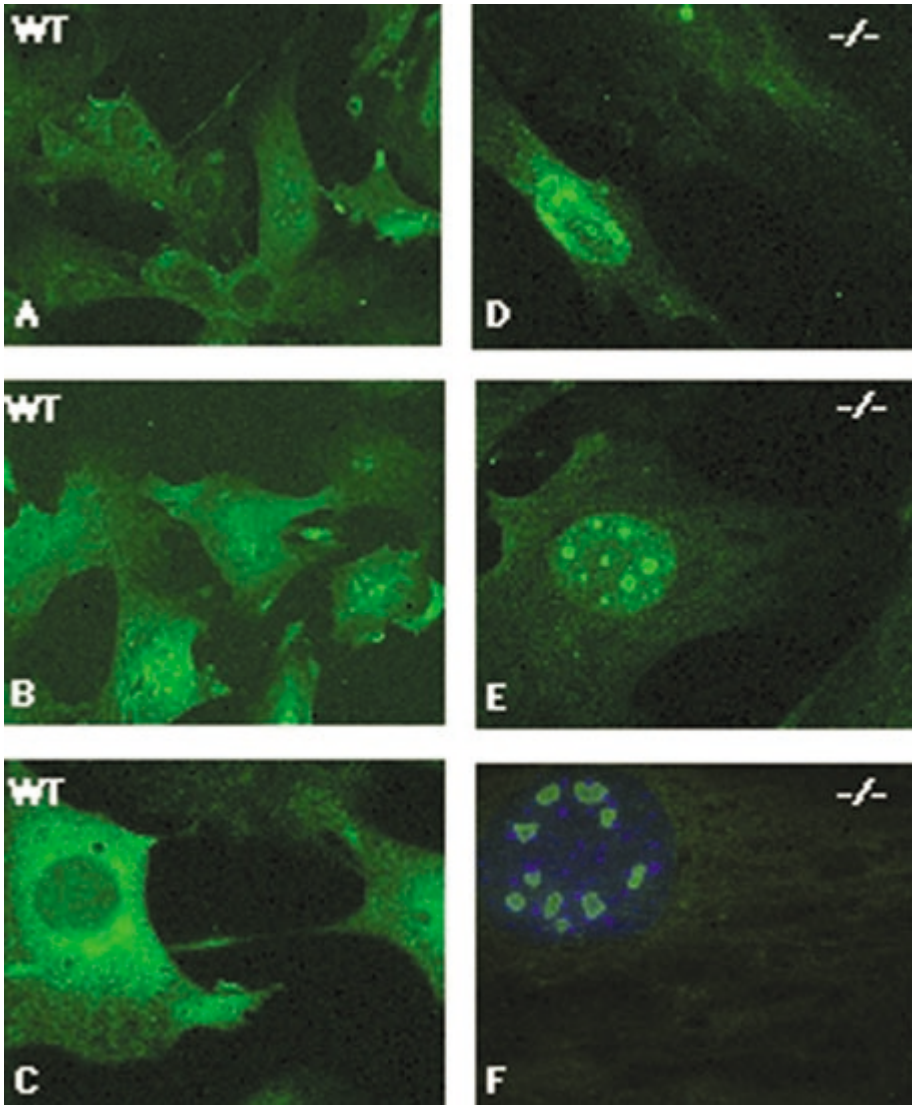

Fig. 4 HSP70 is mainly nuclear in unstressed *Hsf2*$^{-/-}$ MEFs. The subcellular localization of HSP70 in untreated MEFS was challenged by immunocytochemistry with an anti-HSP70 antibody. A, B and C : wild type MEFs. D, E and F : *Hsf2*$^{-/-}$ MEFs. Note that wild-type MEFS display a classical pattern of HSP70 distribution in which most of the HSP70 content is present in the cytoplasm, whereas a significant portion of the HSP70 content is nuclear in *Hsf2*$^{-/-}$ MEFs. In some cells, this nuclear localisation appears as granular structures that do not colocalize with Hoechst (F). Magnification: 40 x.

Hsf2$^{-/-}$ MEFs show altered sensitivity to heat shock

Although HSF2 is not activated by heat shock,, a recent reports suggests that HSF1 and HSF2 might interact [9, 10]. We therefore reasonned that the absence of HSF2 might influence the ability of cells to respond to heat shock. To check this hypothesis, wild type, heterozygous and *Hsf2*$^{-/-}$ MEFs were submitted to heat shock of various severities. MEFs of any genotype were able to respond to heat shock at 43°C (1 hour) and 44°C (30 min.), as indicated by the increase in HSP70 synthesis after metabolic labelling with (^{35}S)-methionine (Fig. 3, compare lanes 3 and 4 to lane 1; lanes 7 and 8 to lane 5; lanes 11 and 12 to lane 9). Milder heat shock conditions (2 hours at 42°C) did not induce HSP70 synthesis in wild type MEFS, but surprinsigly reproducibly induced HSP70 synthesis in *Hsf2*$^{-/-}$ MEFs (Fig. 3, lane 10 compared to lane 9). We investigated by Western blot whether HSF1 levels were altered in *Hsf2*$^{-/-}$ MEFs. However, no differences in the levels of HSF1 were detected in *Hsf2*$^{-/-}$ MEFs compared with with wild-type MEFS (data not shown) which was also observed in any other checked tissues of the knocked-out mice [4]. The absence of HSF2 might not act on HSF1 levels but possibly on the activated state of HSF1, in particular on its DNA-binding activity. HSF1 HSE-binding activity was analysed by electrophoretic mobility shift assay (EMSA) with a double stranded oligonucleotide containing a HSE sequence. However, HSF1 did not show any significant HSE-

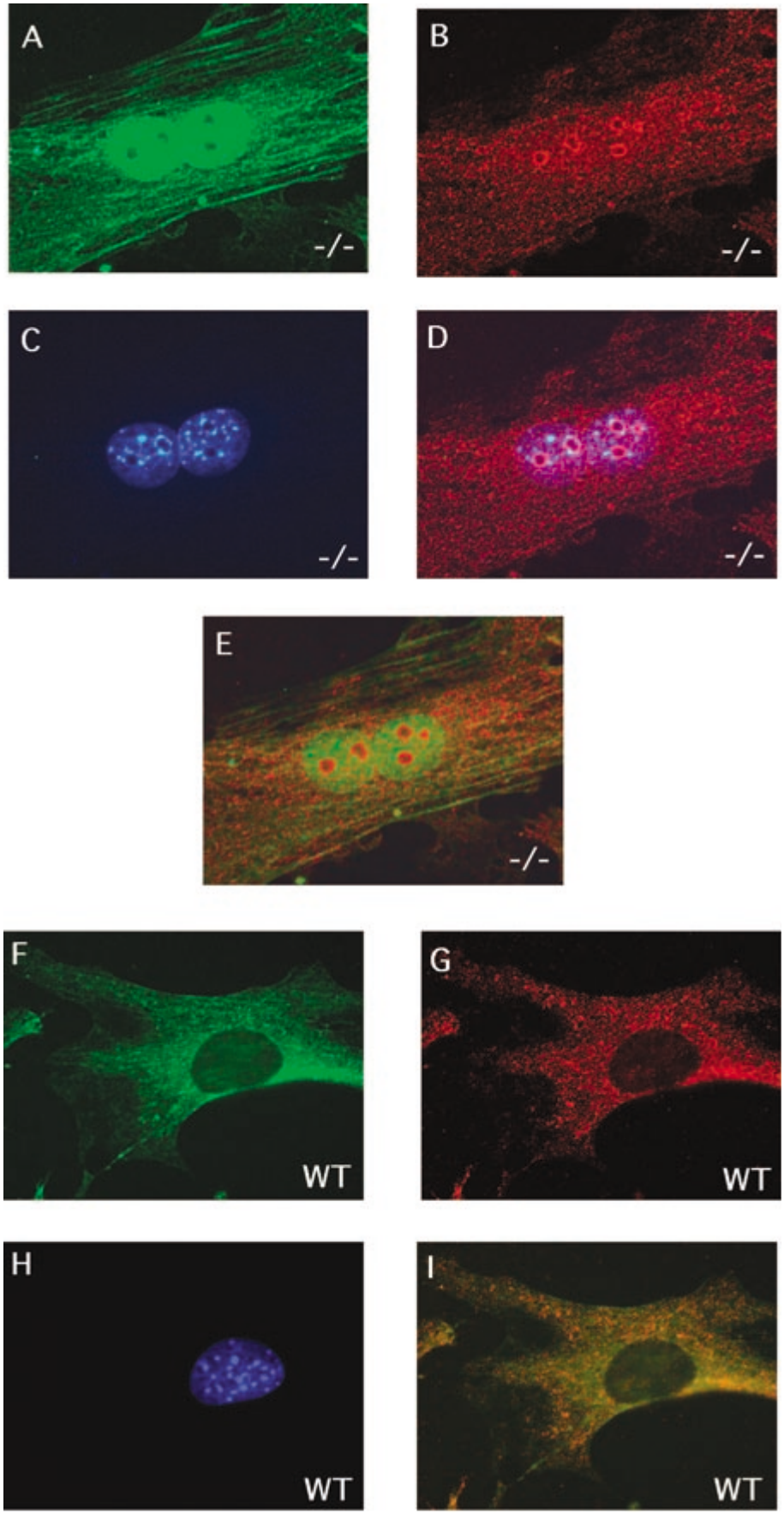

Fig. 5 HSF1 is mainly nuclear in unstressed *Hsf2*$^{-/-}$ MEFs. HSF1 (A and F) and HSP70 (B and G) intracellular localization was investigated in wild type (F, G, H, I) or *Hsf2*$^{-/-}$ (A, B, C, D) MEFs. C and H: Hoechst staining coressponding to A and F, respectively. E and I: merge of A and B and F and G, respectively.

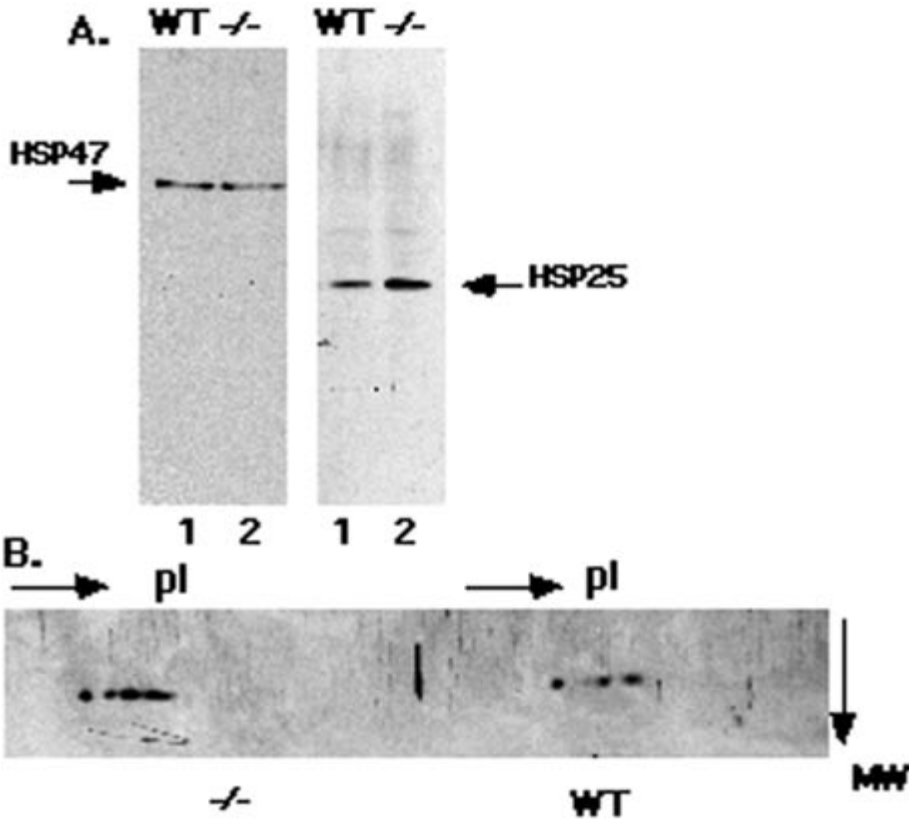

Fig. 6 A. HSP25 levels, but not HSP47, are increased in *Hsf2*$^{-/-}$ MEFs. HSP47 (left panel) and HSP25 (right panel).
B. Immunoblot detection on 2-D gels of HSP25 phosphorylation.

binding activity in the absence of heat shock in *Hsf2*$^{-/-}$ MEFs. So, the fact that heat shock response was triggered at lower temperature in *Hsf2*$^{-/-}$ MEFs was not due to the fact that HSF1 was present at higher levels or in a pre-activated state. Moreover, since *Hsf2*$^{-/-}$ MEFs displayed proliferation defects and since HSF1 was shown to be activated for DNA-binding at the G1 phase [14], we checked whether the differences in temperature sensitivity between wild-type and *Hsf2*$^{-/-}$ MEFs were due to differences in their proliferation properties. Indeed, when wild type and *Hsf2*$^{-/-}$ MEFs were arrested in G_0 by serum deprivation for 3 days and then stimulated to reenter the cell cycle by serum addition, wild-type MEFs, like *Hsf2*$^{-/-}$ MEFs displayed a heat shock response 2 hours after serum stimulation, namely they induced HSP70 synthesis at 42°C for 2 hours (data not shown). Therefore, the differences in temperature threshold of *Hsf2*$^{-/-}$ MEFs compared to wild-type MEFS might be a secondary effect due to differences in proliferation properties rather than to an intrinsic decrease in the temperature threshold of the heat shock response.

HSP70 localization in unstressed MEFs is altered in the absence of HSF2

To further analyse the effect of *Hsf2* gene inactivation on the stress response, HSP70 localization was investigated by immunocytochemistry in unstressed MEFs. In wild-type unstressed MEFs, HSP70 was found in a classical localization, predominantly cytoplasmic (Fig. 4, A, B and C) Surprisingly, however, HSP70 appeared mainly nuclear in *Hsf2*$^{-/-}$ unstressed MEFs (Fig. 4, D, E and F). Interestingly, in some nuclei HSP70 appeared as large granules which do not colocalize with Hoechst staining (Fig. 4, E and F). Nuclear relocalization of a previous cytoplasmic HSP70 has been reported upon heat shock, [15 ; 16] suggesting that *Hsf2*$^{-/-}$ MEFs may behave as stressed cells, even at normal temperature, at least for HSP70 localization. Therefore, though HSF1 is not activated at normal temperature or at 42°C in *Hsf2*$^{-/-}$ MEFs, the lack of HSF2 might induce a kind of stress conditions, not sufficient to trigger HSF1 activation, but efficient enough to relocalize HSP70 and lower the temperature threshold of the heat shock response.

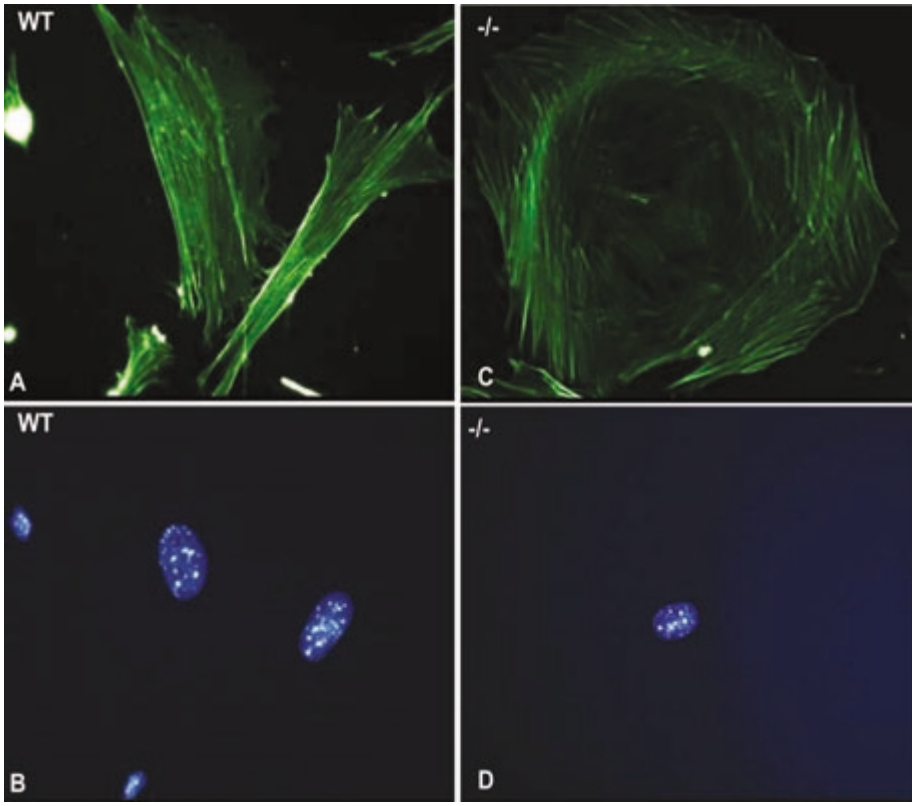

Fig. 7 Actin cytoskeleton stained by phalloidin in wild type (A) or *Hsf2*$^{-/-}$ MEFs (B).

Since HSF1 has been shown to become nuclear after heat shock in murine cells, we examined the subcellular localization of HSF1 at normal temperature in wild type and *Hsf2*$^{-/-}$ MEFs. HSF1 was found to be mainly cytoplasmic in unstressed wild type MEFs (Fig. 5, F and H) as was HSP70 (Fig. 5G). In contrast, HSF1 was mainly nuclear in unstressed *Hsf2*$^{-/-}$ MEFs (Fig. 5 A and C) as was HSP70 which was enriched at a likely perinucleolar location (Fig. 5, B and D). Therefore, although HSF1 does not show any HSE-binding activity in unstressed *Hsf2*$^{-/-}$ MEFs, it displays a nuclear localization comparable to what is found in stressed cells. In human cells, HSF1 was shown to be regulated by its interaction with HSP70 [9 ; 17] and its subcellular localization seems to be an important regulatory mechanism of the HSF1 mediated heat shock response. However, we did not detect any obvious colocalization of HSP70 and HSF1 (Fig. 5, E and I).

Therefore, *Hsf2*$^{-/-}$ MEFs display some features of heat shocked cells. The synthesis of HSP70 upon heat shock is triggered at lower temperature compared to wild type cells. Although HSF1 does not show any constitutive or increased HSE-binding activity in unstressed or stressed *Hsf2*$^{-/-}$ MEFs, respectively, *Hsf2*$^{-/-}$ MEFs might sense a kind of stress since the localization of HSF1 and HSP70 appears predominantly nuclear.

HSP25 expression and phosphorylation is altered in *Hsf2*$^{-/-}$ MEFs

The expression profile of other HSPs was compared in wild type and *Hsf2*$^{-/-}$unstressed MEFs by Western blot and/or their localization was investigated by immunolocalization. HSP47 levels and subcellular localization were not modified in *Hsf2*$^{-/-}$unstressed MEFs (Fig. 6 A left panel). In contrast, HSP25 levels were reproducibly increased in unstressed *Hsf2*$^{-/-}$ MEFs (Fig. 6, 1 right panel). The phosphorylation state of HSP25 was not markedly modified in *Hsf2*$^{-/-}$ MEFs as evidenced by 2-D denaturing gel analysis (Fig. 6, A and B). The signification of this enhanced phosphorylation is not known. Since p38 kinase was shown to be responsible for HSP25 phosphorylation in various systems [18;19] p38 levels and localization were analysed in wild type and *Hsf2*$^{-/-}$ MEFs. No differences could be observed (data not shown).

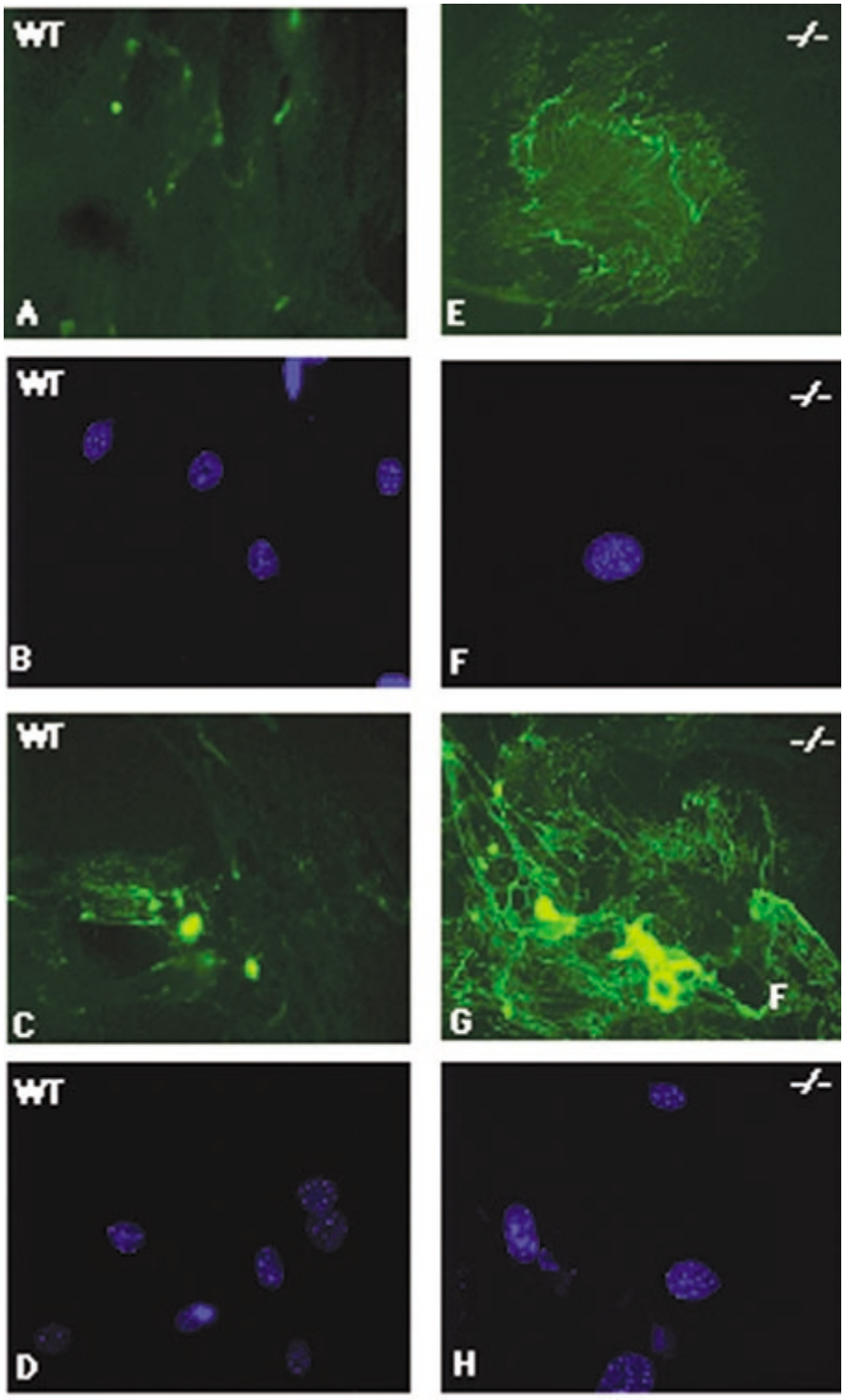

Fig. 8 Fibronectin network is disturbed in *Hsf2*$^{-/-}$ MEFs. Immunocytochemistry with anti-fibronectin in wild type (A, C) and *Hsf2*$^{-/-}$ MEFs (E and G). B : Hoechst staining of view A ; D : Hoechst staining of view C ; F : Hoechst staining of view E ; H : Hoechst staining of view G. Magnification: 400 x.

Actin cytoskeleton in *Hsf2*$^{-/-}$ MEFs

Since HSP25 is known to interact with actin and since the morphology of *Hsf2*$^{-/-}$ MEFs was altered, MEFs were incubated with phalloidin in order to stain the actin cytoskeleton. Although no major differences appeared in *Hsf2*$^{-/-}$ MEFs compared with wild type MEFs, enrichment in actin fiber

density and spreading of the actin network to the entire surface of the cell were observed in *Hsf2*$^{-/-}$ MEFs (Fig. 7, A and B). This likely reflect the rounded up morphology of the *Hsf2*$^{-/-}$ MEFs.

Fibronectin network in *Hsf2*$^{-/-}$ MEFs

The extracellular protein fibronectin is part of the extracellular matrix and disregulated in cancer cells [20]. In correlation with the marked proliferation defects observed in *Hsf2*$^{-/-}$ MEFs, these cells displayed an increase in fibronectin staining in immunocytochemistry experiments, as well as revealed profound remodeling of the fibronectin network (Fig. 8, A and C compared with E and G).

In conclusion, the absence of HSF2 in MEFs results in two major characteristics. First, *Hsf2*$^{-/-}$ MEFs display impaired proliferation in correlation with altered morphology (enlarged cells) reminiscent of senescence. The actin cytoskelton seems only mildly if not affected, but the extracellular fibronectin network is profoundly enhanced in correlation with these proliferation defects.

The levels of HSP47 are unchanged in *Hsf2*$^{-/-}$ MEFs. However, HSP25 levels and phosphorylation are modified in MEFS.

The second main characteristic of the *Hsf2*$^{-/-}$ MEFs is that they display a heat shock response at lower temperatures than wild-type MEFs. In correlation with this increased sensitivity, HSF1 and HSP70 are nuclear in unstressed *Hsf2*$^{-/-}$ MEFs while they are mainly cytoplasmic in wild-type MEFs. Therefore, the absence of HSF2 might be sensed as a stress by the cells. However, this is not sufficient to trigger HSF1 HSE-binding activity as detected in *in vitro* experiments. Moreover, this higher sensivity of *Hsf2*$^{-/-}$ MEFs to heat is not intrinsic, but is likely linked to the proliferation defects. However, this situation of a nuclear HSF1 at normal temperatures but devoid of any HSE-binding activity is reminiscent of found in the preimplantation mouse embryo. Indeed, HSF1 is a maternal factor present in the one-cell stage embryo. In an atypical manner, HSF1 is strictly nuclear at this stage, although it does not display any HSE-binding activity in EMSA [21]. Interestingly HSF2 is absent in the one-cell stage embryo. It starts to be synthesized in the two-cell stage embryo and increases in the following stages. Concommitantly, HSF1 relocalizes in the cytoplasm, as classical in unstressed murine cells. Therefore, HSF2 might be involved in the establisment of a normal heat shock response or in the maintenance of HSF1 in a cytoplasmic inactive HSF1 in non-stress conditions. The effect of the absence of HSF2 in *Hsf2*$^{-/-}$ MEFs on the status of HSF1 might not be direct. These stress-like features may derive at least partially from the proliferative defects induced in the absence of HSF2 since wild-type and *Hsf2*$^{-/-}$ MEFs synchronized by serum deprivation display a similar threshold of heat shock response.

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