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Hsp70 is required for optimal cell proliferation in mouse A6 mesoangioblast stem cells

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Abbreviations: MHC, major histocompatibility complex; HSE, heat shock element; CHBF, constitutive HSE-binding factor; RNAi, RNA interference

Key words: Hsp70; mouse mesoangioblast; Ku factor; cell doubling time; shRNA.

Synopsis

Mouse Hsp70 is preferentially induced by heat or stress stimuli. We previously found that Hsp70 is constitutively expressed in A6 mouse mesoangioblast stem cells, but its possible role in these cells and the control of its basal transcription remained unexplored. Here we report that in the absence of stress, Ku factor is able to bind the heat shock element (HSE) consensus sequence *in vitro*, and *in vivo* it is bound to the proximal hsp70 promoter. In addition, we show that constitutive hsp70 transcription depends on the cooperative interaction of different factors such as Sp1 and GAGA binding protein with Ku factor, which binds the HSE consensus sequence. We used mRNA interference assays to select knockdown cell clones. These cells were able to respond to heat stress by producing a large amount of Hsp70, and produced the same amount of Hsp70 as that synthesized by stressed A6 cells. However, severe Hsp70 knockdown cells had a longer duplication time, suggesting that constitutive Hsp70 expression has an effect on the rate of proliferation.

Introduction

Hsp70 (70kDa heat shock protein; synonyms: Hsp68; Hsp72; Hsp70A1; Hspa1b/Hspa1a; heat shock 70kDa protein 1B/1A; Hsp70.1/3; Hsp70-1/3) expression is induced in response to several types of cellular stress in all living organisms. This protein and its cognate form (Hsc70) are involved in several folding processes, such as refolding of aggregated proteins, prevention of aggregation, and folding and assembly of nascent polypeptides. They are also involved in translocation of protein through the intracellular membrane and in the interactions with signal transduction proteins [1]. Hsp70 also prevents the disruption by environmental stressors of normal cellular processes involving mitosis, meiosis, or differentiation [2,3]. Under stress, hsp70 transcriptional regulation involves a highly conserved cis-acting sequence, known as the heat shock element, HSE. HSE is the binding site of the heat shock transcription factor, HSF1 [4,5], a positive regulatory factor in the stress response. However, in a number of cases HSF1 activation is not sufficient for the induction of hsp70 gene expression [6]. Furthermore, it has been observed that some human cell lines constitutively produce Hsp70 in the absence of stress, but no significant HSF1-binding activity was found, suggesting that constitutive Hsp70 expression in these cases is an HSF1-independent process [7,8]. In some mammalian cells it has been observed that a constitutive HSE-binding factor (CHBF) binds HSE in the absence of any stress [9-11]. Purification of this factor demonstrated that it corresponds to Ku autoantigen [12,13], a heterodimer consisting of 86-kDa (Ku-80) and 70-kDa (Ku-70) polypeptides [14]. Ku appears to be involved in many cellular processes such as DNA double-strand break repair, DNA replication, regulation of telomeric termini, and transcription regulation [15]. Under some types of stress, the Ku-70 subunit has been demonstrated to regulate hsp70 gene expression [13,16,17]. Hsp70 overexpression is typical of several types of tumors, and strong evidence suggests that it plays a role in the control of cell cycling and growth. Under unstressed conditions, Hsp70 is expressed in some cases in proliferating cells during the G₁/S and S phases of the cell cycle [18,19]. Its role in

proliferation has been demonstrated by overexpression and antisense administration assays [20,21]. When studying the stress response in mouse A6 mesoangioblasts, vessel-derived stem/progenitor cells that can differentiate into different cell types of solid mesoderm such as muscle and bone [22,23], we found that these stem cells constitutively synthesize inducible Hsp70 [24]. In the mouse, hsp70.1 and hsp70.3 are the major stress-inducible genes. They are located in the major histocompatibility complex (MHC) region of mouse chromosome 17 [25,26], and encode proteins differing at only two amino acid residues, though they have completely different sequences in the 3' untranslated region (UTR) [27]. We also observed that in A6 cells, there are two classes of HSE-binding factors: one is constitutive, but the other is inducible by stress. We demonstrated that HSF1 is responsible for the stress response, but neither HSF1 nor HSF2 are involved in the basal HSE complex formation, which involves CHBF [24]. In the present study we investigated whether Ku-70 is involved in hsp70 basal transcription. We found using chromatin immunoprecipitation (ChIP) analysis that Ku factor is localized on the hsp70 proximal promoter. Our evidence also suggests that Ku factor has a prominent role in constitutive hsp70 gene transcription. Furthermore, to understand the possible role of Hsp70 constitutive expression in mesoangioblasts, we carried out stable RNA interference (RNAi) of hsp70, and demonstrated that a reduction (~50%) in Hsp70 level impairs cell proliferation, causing an increase in cell doubling time.

Experimental

Cell culture, low oxygen tension, and heat shock treatment

Mouse A6 stem cells were grown on plates treated with collagen I in Dulbecco's modified Eagle medium (DMEM) (Euroclone, Milan, Italy) supplemented with 10% fetal bovine serum in a humidified 5% CO₂ atmosphere at 37°C. For experiments with lower oxygen tension, cells were grown as previously described [28]. For heat shock, plates were sealed with Parafilm and immersed for 1 h at 42°C in a water bath.

Preparation of protein extracts

Protein extracts were prepared as previously described [24]. Protein concentrations of extracts were determined using the Bradford microassay method (Sigma, St. Louis, MO) with bovine serum albumin as a standard.

Western blot (immunoblot) analysis

Whole-cell extracts were subjected to 10% SDS-PAGE and transferred to an ECL-Hybond membrane (Amersham, Pittsburgh, PA) using a mini-electroblot (Biorad, Hercules, CA). After blocking for 2 h in 5% nonfat dry milk in TBST buffer (10 mM Tris-HCl, pH 7.5; 150 mM NaCl; and 0.2% Tween-20), the membranes were incubated overnight with rabbit polyclonal anti-Hsp70 (1:10,000 dilution; Calbiochem, San Diego, CA), mouse anti-actin (1:800 dilution; Neomarkers, Fremont, CA), rabbit anti-Ku-70 (1:200 dilution, Santa Cruz, Santa Cruz, CA). Alkaline phosphatase conjugated anti-rabbit and anti-mouse (1:5,000 dilution; Promega, Madison, WI) were used as secondary antibodies.

Gel mobility shift assay

Whole-cell extracts containing 45 μ g of protein were incubated with a double-stranded γ -³²P-labeled HSE oligonucleotide (5'-GGGGTCAGAATATTCTAGAATATTCTAGAATGGGGTCAGG-3'), GAGA (5'-GATCAGAGAGAGAGAGAGAAAAGAGAGAG-3') or GC (5'-TTCGATCGGGGCGGGGCGAG-3') in 30 μ l of a solution containing 10 mM HEPES, pH 7.9; 10 mM KCl; 50 mM NaCl; 1.5 mM MgCl₂; and 10% glycerol. The samples were incubated at 25°C for 20 min. The HSE-containing complexes were analyzed on 4% non-denaturing polyacrylamide gels. For antibody perturbation experiments, two concentrations (1 and 3 μ g) of Ku antibody (AbD Serotec, Raleigh, NC) were incubated with the reaction mixture for 1 h at 25°C.

beta-galactosidase and chloramphenicol acetyltransferase (CAT) assays

A6 cells were cotransfected with phsplacZ [29] and with pCAT-control vector (Promega) into six-well plates. In total, 2 μ g of each plasmid was used for the transfection. Where indicated, phsplacZ with a mutation in the HSE, GAGA or GC consensus sequence was used. Plasmids were transfected into the

cells using FUGENE (Roche, Pleasanton, CA). After 48 h, cells were washed three times with PBS and then air-dried. Cells were lysed by adding 0.65% NP-40; 10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0; and 150 mM NaCl. After incubation for 5 min, the lysis buffer containing cytoplasmic proteins was centrifuged at $12,000 \times g$ for 1 min at 4°C. The resulting 30 μ l of lysate was used for both β -galactosidase and CAT assays. To measure beta-galactosidase activity, 204 μ l of 1 mM MgCl₂, 45 mM β -mercaptoethanol, and 66 mM Na₂HPO₄, and 66 μ l of o-nitrophenyl- β -D-galactopyranoside (4 mg/ml) were added to the sample. The sample was incubated overnight at 37°C, and quantified using a spectrophotometer (420 nm) after the addition of 500 μ l of Na₂CO₃ containing 1M beta-galactosidase. For the CAT assay, samples were incubated 5 min at 68°C, then 70 μ l was added of a mix containing 20 μ l of 8 mM chloramphenicol, 20 μ l of 0.5 mM acetyl-coenzyme A, 30 μ l of 250 mM Tris-HCl, pH 7.8, and 2 μ l of ¹⁴C-acetyl-coenzyme A (54 mCi/mmol). The samples were incubated for 2 h at 37°C. Acetylated chloramphenicol was obtained by extraction with ethyl acetate. Incorporation of ¹⁴C was measured. The results from three independent experiments were quantified.

Chromatin immunoprecipitation (ChIP)

Unstressed or heat-treated (20 min at 43°C) cells were collected in flasks and treated with 0.5% formaldehyde (from a solution of 5.5% formaldehyde containing 0.1 M NaCl; 1 mM EDTA; 0.5 mM EGTA; and 50 mM Tris-HCl, pH 8) for 10 min at 25°C with gentle agitation. Reactions were terminated by the addition of a stoichiometric excess of glycine (0.125 M). Cells were collected by centrifugation for 5 min and washed once in ice-cold PBS. Following a second centrifugation, nuclei were isolated by incubation of the cells in cell lysis buffer (10 mM Tris; 10 mM NaCl; 0.2% NP-40, pH 8) containing protease inhibitors (1 μ g/ml leupeptin, 50 μ g/ml PMSF, and 1 μ g/ml aprotinin) for 10 min on ice. The volume of cell lysis buffer was 1.5-fold greater than the estimated volume of the cell pellet. Nuclei were isolated by centrifugation ($800 \times g$, 4°C, 5 min) and the pellet was lysed in enough ice-cold nuclei lysis buffer (50 mM Tris; 10 mM EDTA; 1% SDS; and protease inhibitors, pH 8.1) to

bring the final volume to 500 μ l. To increase the volume, IP dilution buffer (20 mM Tris, 150 mM NaCl, 2 mM Na-EDTA, 0.01% SDS, and protease inhibitors, pH 8.1) was added, bringing the volume to 1 ml. Sonication was performed on ice with three 10-s pulses at 100% of maximum power with a VC 600 series Vibra Cell (Sonics Materials, Newtown, CT). Following sonication, samples were centrifuged (10,000 \times g; 4°C, 10 min). Samples were precleared by overnight incubation at 4°C by addition of 100 μ l of protein G-Sepharose to bring the final volume to 1 ml. To immunoprecipitate protein-DNA complexes, samples were incubated overnight at 4°C with 2 μ g of antibody per 500 μ g of sample. Antibodies used were: anti-Ku-70 (Santa Cruz), anti-HSF1 (AbCam, Cambridge, MA), and anti-Hsp27 (Neomarkers). Immune complexes were collected by incubation with 30 μ l of protein G-Sepharose. Protein G-Sepharose was pre-treated for 2 days at 4°C with 1 mg/ml of BSA and 130 μ g/ μ l of salmon sperm DNA. Following antibody binding and protein G-Sepharose absorption, protein G-Sepharose pellets were washed twice with buffer 1 (20 mM Tris; 50 mM NaCl; 2 mM EDTA; 0.1% SDS; and 1% Triton X-100, pH 8.1), once with buffer 2 (10 mM Tris; 0.25 M LiCl; 1 mM EDTA; 1% NP-40; and 1% deoxycholate, pH 8.1), and twice with TE (0.01 M Tris; and 1 mM EDTA, pH 8.0). Immune complexes were eluted twice at room temperature with 75 μ l of elution buffer (0.1 M NaHCO₃, and 1% SDS). RNase (3 μ g/ml) and 0.3 M NaCl were added, and crosslinking was reversed by incubation for 4 h at 65°C. Samples were digested with 0.2 mg/ml proteinase K for 12 h at 45°C. Glycogen (5 μ g) was added, and immunoprecipitated DNA was purified by extraction once each with phenol:chloroform and chloroform, followed by ethanol precipitation. Purified DNA was resuspended in 20 μ l of water. Samples were analyzed by PCR. Oligonucleotides used for the amplification were sense 5'-TACTCTCTAGCGAACCCAG-3', and antisense 5'-GACAAGCGAAGACAAGAGAA-3'. PCR reactions contained 20 μ l of immunoprecipitated DNA, 0.5 μ M of hsp70.1 and hsp70.3 primers, 0.5 U Taq DNA polymerase (Eppendorf, Westbury, NY), 0.2 mM dNTP mixture, and 1X Taq buffer (Eppendorf), in a total volume of 50 μ l. Twenty-eight cycles of amplification were used.

Metabolic labeling with ³⁵S-methionine

Cells were incubated in methionine-free medium (Sigma) containing 10% fetal bovine serum and exposed to stress treatment. After the treatment, 20 $\mu\text{Ci/ml}$ of ^{35}S -L-methionine (1,000 Ci/mmol, Amersham) was added and cells were labeled for 3 h and then chased with unlabeled L-methionine. Samples were collected 0 h, 24 h, and 48 h after labeling. The incorporation of ^{35}S -L-methionine into proteins was determined by trichloroacetic acid precipitation. Whole-cell extracts containing 30 μg of protein were analyzed using 10% SDS-PAGE and fluorography.

hsp70.1/3 RNA interference (RNAi)

MISSION shRNA lentiviral vectors (Sigma), consisting of short hairpin sequences cloned into a lentiviral vector, were used. One shRNA specific for both hsp70.1 and hsp70.3 (phsp70.1/3) was chosen from an RNAi lentiviral library. The target sequence chosen is called 599, and the sequence of shRNA cloned in the plasmid is as follows: CCGGCGACGGCATCTTCGAGGTGAACTCGAGTTCACCTCGAAGATGCCGTCGTTTTTGGGC CGCTGCCGTCGAAGCTCCACTTGAGCTCAAGTGGAGCTTCTACGGCAGCAAAAAC. A6 cells were transfected with vector 599 and selected for resistance to puromycin, and 20 clones with different levels of knockdown (between 15 and 55%) were obtained. For all subsequent experiments, we used clone A6-NM3, which exhibited 55% knockdown of the Hsp70 protein.

Flow cytometry

Cells were collected and washed with PBS. The pellets were treated with 0.1% $\text{C}_6\text{H}_5\text{Na}_3\text{O}_7$, 0.1% NP-40, 50 $\mu\text{g/ml}$ propidium iodide, and 0.06 mg/ml RNase A for 4 h at room temperature in the dark. The cells were analyzed on a BD FACSCanto (Franklin Lakes, NJ) using FACSDiva Software. Analysis was performed using Modfit 3.2 software.

Proliferation assay

Cells were plated at a concentration of 4,000 cells/100 μl in 96-well multiwell plates. At 24, 48, and 72 h after plating, cells were incubated for 4 h at 37°C with 10 μM BrdU, and the assay was performed

using a cell proliferation ELISA kit (Roche), according to the manufacturer's instructions. The reaction products were quantified by measuring absorbance at 450 nm using a scanning multiwell spectrophotometer (ELISA reader; Celbio, Milan, Italy).

Cell duplication time

To calculate cell duplication time we used Equation 1 below:

$$\ln 2 \cdot \frac{t' - t}{\ln C' - \ln C}$$

With C' = concentration at time t' (final); and C = concentration at time t (beginning). Cell concentration was calculated in the exponential growth phase.

Statistical analysis

Quantitative data are represented as the mean \pm S.E.M., or the mean \pm S.D. of three experiments. The statistical analyses were performed with GraphPad (InStat, Inc., San Mateo, CA) software (version 3.00) using Student's t-test for two-group comparisons. Densitometry analysis was performed using ImageJ open-source software. Results were normalized to actin expression levels.

Results and discussion

A6 stem cells synthesize Hsp70 in low oxygen tension culture conditions

We previously found that A6 cells, a clone of mouse mesoangioblasts [22], constitutively synthesize the inducible form of Hsp70 [24]. Immunofluorescence studies have also shown that Hsp70 and Hsc70 localize differently inside the cell; Hsp70 primarily associates with microfilaments and the cell cortex [30].

Before investigating constitutive hsp70 gene regulation and a possible role for this protein, we tested whether constitutive synthesis of Hsp70 was due to hyperoxic stress. A6 cells were cultured in low oxygen tension, following the procedure described by Wright and Shay [28]. Cells cultured under unstressed laboratory conditions are exposed to a hyperoxic environment, because oxygen tension in

most mammalian tissues ranges from 1% to 6%. Immunoblot assays of cell lysates demonstrated that constitutive Hsp70 synthesis in A6 cells occurs in low oxygen tension in the same amount as well as under 20% oxygen (Fig. 1, lane 2 and 1 respectively). This result indicates that high oxygen tension is not involved in Hsp70 basal expression and confirms our previous findings.

CHBF/Ku factor binds the HSE consensus sequence *in vitro*

Relatively little is known about the regulation of hsp70 genes at the transcriptional level in basal growth conditions. In A6 cells, we previously detected two distinct HSE-protein binding complexes using biochemical analysis: one, HSF1, was inducible by stress, but the other was constitutive, and supershift analysis showed that neither HSF1 nor HSF2 were involved in this HSE-complex formation [24]. We therefore hypothesized that in A6 stem cells, constitutive expression of Hsp70 might be due to CHBF, as previously described both in HeLa cells and in rat cells [9,17]. To test whether Ku might be the constitutive HSE binding factor, both *in vitro* and *in vivo* experiments were performed. First, the presence of Ku autoantigen in A6 cells was demonstrated by immunoblot assays with antibody against Ku-70 (Fig. 2A). Furthermore, DNA-binding perturbation assays with ³²P-labeled HSE-oligonucleotide and increasing amounts of anti-Ku-70 antibody were performed to test Ku DNA-binding ability. As the antibody interferes with the complex formation in a dose dependent manner (Fig. 2B lanes 3, 4), it is possible to conclude that Ku was the factor responsible for the constitutive DNA binding activity. The specificity of the complex was previously demonstrated by competition assays with an excess of the unlabeled HSE oligonucleotide [24]. These results indicated that Ku factor is present in A6 cells and is also able to bind the HSE consensus *in vitro* to form a constitutive complex, confirming our previous findings.

CHBF/Ku factor binds the hsp70 promoter *in vivo*

The *in vivo* relevance of the observed Ku binding activity was analyzed in the context of chromatin in A6 cells by chromatin immunoprecipitation (ChIP) of endogenous Ku. ChIP can directly detect the association of any protein with its *in vivo* binding site. Anti-Ku antibodies efficiently and specifically

immunoprecipitated the proximal hsp70 promoter (Fig. 2C lane 1), indicating that there is a stable association between the endogenous Ku factor and the hsp70 promoter *in vivo*. To confirm the specificity of the binding of Ku to the hsp70 promoter under unstressed growth conditions, we performed ChIP assays after heat shock. No DNA amplification was observed in samples treated with anti-Ku antibody (Fig. 2C lane 2). On the contrary, as expected, DNA amplification was observed using anti-HSF1 antibodies under stress conditions, but no amplification occurred under unstressed growth conditions (Fig. 2C lanes 4 and 3, respectively). Control sample treated with anti-Hsp27 or without anti-Ku antibody did not show DNA amplification (Fig. 2C lanes 5 and 7, respectively). This result indicates that in A6 cells under physiological cell growth conditions, Ku is localized on the proximal promoter of the hsp70 gene, suggesting its involvement in regulation of constitutive hsp70 gene transcription.

CHBF/Ku is involved in hsp70 promoter regulation under basal conditions

We investigated whether transcription factors other than Ku are bound to the hsp70 gene promoter under non-stress conditions. The hsp70 proximal promoter contains, near the HSEs, two consensus sequences, for GAGA and the CG box. These two sequences are known to be involved in the basal transcription of many genes. DNA-binding assays showed that both elements form *in vitro* DNA-protein complexes and their specificity was demonstrated by competition assays (Fig. 3A and 3B, respectively). To gain insight into the regulation of hsp70 expression, we analyzed the basal activity of the proximal promoter of the hsp70 gene using gene reporter assays. To this aim, A6 cells were transfected with a plasmid containing the beta-galactosidase reporter gene under the control of the hsp70 gene promoter with point mutations in the GAGA or GC consensus sequence. Beta-galactosidase activity was measured and transfection efficiency was monitored by co-transfection with a pCAT-control vector. The beta-galactosidase values are reported in Fig. 4B. MutGCphsplacZ and mutGAGaphsplacZ showed lower basal-level activity of 65.7% and 75.0%, respectively, compared to the control vector phsplacZ. This result indicates partial involvement of these two factors in the

regulation of the hsp70 gene promoter, which drives transcription of the reporter gene. We had previously found that transcription of the reporter gene under control of the hsp70 promoter with the HSE deleted (Δ HSEphsPlacZ) dropped to 52% with respect to that of the phsPlacZ control [24]. Together, these experiments show that the deletion of the HSE consensus sequence decreases the expression of the reporter gene much more than mutation of the GAGA and GC consensus sequences. Previous studies of Hsp70 transcription under stress (i.e., IPTG, arsenite, sodium, salicylate) indicated that high HSF1 DNA-binding activity is sometimes insufficient for Hsp70 mRNA synthesis, and that dissociation of the HSE-Ku complex is necessary [10,16,17]. Based on the results presented here and our previous data [24], we suggest that CHBF/Ku factor is involved in basal transcription of hsp70 in cooperation with GC and GAGA box binding factors in the absence of stress.

hsp70 RNA interference assay (RNAi) and isolation of knockdown clones

To identify a possible role for basally expressed Hsp70, we knocked down both hsp70 mRNAs and studied the effects of silencing on A6 stem cell physiology. To choose between transient or stable RNA interference (RNAi) methods, we first studied the turnover of Hsp70 protein. After 1 h of shock at 42°C, A6 cells were labeled with 35 S-methionine, then chased with unlabeled methionine. Total cell lysates were prepared 3 h, 24 h, and 48 h after heat treatment. The twin Hsp70 proteins differ for only two amino acid residues, and they co-migrate as a single band on an electrophoresis gel [27]. The results of autoradiography indicated that the Hsp70 band was still evident 48 h after chase (Fig. 5 lane 3), showing that turnover of the protein is slow. For this reason, we used a vector containing a specific RNAi sequence targeting both hsp70.1/3 mRNAs for stable RNA silencing. A6 cells were transfected with a hsp70 shRNA vector or with a mock vector as negative control. Stable integrants were selected using puromycin 72 hours after the transfection, and after 15 days in the selective medium, resistant pools of mock-transfected cells and hsp70 shRNA (NM) cells were obtained. The level of Hsp70 protein of each cell clone was analyzed by western blot analysis and quantified using densitometric analysis (Fig. 6). The Hsp70 protein level in hsp70 knockdown cell clones was decreased to 16-50%

that of A6 control cells. Negligible Hsp70 reduction was observed in mock-transfected cell clones (SH) (Fig. 6). Levels of hsp70.1 and hsp70.3 mRNAs of knockdown, mock-transfected, and A6 control cell clones were investigated using semi-quantitative RT-PCR. The results indicated a reduction of both hsp70.1 and hsp70.3 mRNAs in all knockdown cell clones although the reduction was variable. No reduction in hsp70 mRNA was observed in mock-transfected cell clones (data not shown).

Heat shock response in hsp70 knockdown A6 cells

We investigated whether a decreased level of Hsp70 in NM3 cells affects normal cell physiology. Hsp70 knockdown clones did not show alterations in cell morphology under unstressed growth conditions (data not shown). Moreover, to examine the influence of hsp70 knockdown on the ability of NM clones to respond to heat stress, we compared the Hsp70 level in NM3 and A6 cells before and after heat stress using western blot analysis. A6 and NM3 cells were subjected to hyperthermic treatment (42°C for 1 h) and then allowed to recover at 37°C. During recovery, NM3 cells synthesized a large amount of the protein, 8.6-fold greater than the basal level using densitometric analysis (Fig. 7). A 4.6-fold increase in protein level in stressed compared to unstressed A6 cells was observed (Fig. 7). The result showed that the total amount of Hsp70 after heat stress was almost the same in A6 and NM3 cells. A similar result was obtained for all other NM cell clones subjected to heat stress (data not shown). Thus, NM cell clones, when subjected to stress, produce an amount of Hsp70 necessary to protect themselves from injury, suggesting tight regulation of Hsp70 expression under unstressed and stress conditions.

Decreased cell proliferation and longer doubling time in hsp70 knockdown A6 cells

Hsp70 function has been mainly ascribed to the protection of the cells, but there is growing evidence for its involvement in cell proliferation [31]. For this reason we tested whether the suppression of Hsp70 elicits cell proliferation. The effect of hsp70 silencing on the cell cycle was determined using cell growth curves. Fig. 8 shows results for A6 cells, along with NM3, and SH2 cells representing knockdown and mock-transfected clones, respectively. A6 and SH2 cells expressing the same level of

Hsp70 exhibited similar growth curves, whereas NM3 cells displayed a reduced growth rate. The effect of hsp70 silencing on the cell cycle was determined by fluorescence-activated cell sorting (FACS) analysis of cell cycle phase distribution after propidium iodide staining of exponentially growing cells. A6 cells and SH2 and NM3 clones showed similar cell-cycle phase distribution profiles as reported in supplementary data (Fig. S1 and table S1). The same result was obtained for all other NM cell clones (data not shown). This finding indicates that knockdown of hsp70 did not alter cell-cycle phase distribution profiles. We also measured cell duplication time and found that stable suppression of Hsp70 results in a marked decrease in the cell growth rate. A6 cells have a doubling time of 16 hours, as previously described for mouse mesoangioblasts [22], but NM3, NM15 and NM17 cells exhibited a longer doubling time of about 25 hours, and mock-transfected shRNA cells did not exhibit a detectable change in growth rate. NM clones 3, 15, and 17 showed ~50% reduction in Hsp70 level. In contrast, NM clones 1, 5, and 8, which had levels of Hsp70 reduced by only 16% and 25%, showed a doubling time ranging between 16 and 17.7 hours, similar to that of A6 cells (Table I). The differences in population doubling time cannot be attributed to cell-cycle differences, as demonstrated by FACS analysis (data not shown). Flow cytometry can be used to estimate the length of G₁, S and G₂ phases when certain conditions are met: the mean generation time for a cell population is known, the fraction of proliferating cells is high, the frequency of cell loss is low, and cells are reasonably homogeneous with respect to their cell cycle kinetics [32]. The calculated cell-doubling times and cell-cycle analysis were used to provide an estimate of the duration of G₀/G₁, S, and G₂/M cell-cycle phases in A6, NM3, and SH2 cells. NM3 and SH2 are representative of ~50% knockdown clones and mock-transfected clones, respectively. Data presented in Table II indicates that cells expressing low levels of Hsp70 have a longer duration of the G₀/G₁ phase compared to cells expressing a basal level of Hsp70 (14.9 vs. 7.5 hours).

Together, these data indicate that a relevant reduction in Hsp70 level negatively influences cell proliferation, while a lower reduction has no effect, demonstrating that there is a correlation between the amount of basally expressed Hsp70 and cell proliferation.

Other previous work supports our data, confirming that Hsp70 is involved in both mitosis and meiosis. Consistent with such a function, Hunt and co-workers [33] demonstrated that the doubling time of MEF Hsp70.1/3^{-/-} cells obtained from knockout mice is longer than that of Hsp70.1/3^{+/+} MEFs. MCF-7 breast cancer cells overexpressing Hsp70 [31] have a shorter doubling time, providing further evidence of involvement of Hsp70 in the cell cycle. A role in meiosis was also demonstrated for Hsp70.2, a member of the Hsp70 family expressed specifically in mouse spermatogenic cells. Hsp70.2, expressed during the meiotic phase of spermatogenesis, is required for desynapsis of the synaptonemal complex and the completion of meiosis I in spermatocytes [34,35]. Constitutive Hsp70 involvement in mitosis and embryo cleavage was also demonstrated in *P. lividus* embryos, a model of embryonic development. Soon after fertilization and during early cleavage, basally expressed Hsp70 is located on the mitotic apparatus, and blockage of its function impairs mitosis and embryo development [36,37].

In conclusion, we suggest that a basal level of Hsp70 expression may be required for optimal cell proliferation of mesoangioblast cells, which have a short doubling time.

Conclusions

In A6 stem cells, basally expressed Ku factor binds the HSE consensus sequence in the hsp70 proximal promoter and is positively involved in its basal transcription in the absence of stress. Hsp70 expressed under basal growth conditions influences the growth rate of A6 stem cells. A 50% reduction in the level of Hsp70 causes an increase in cell doubling time and prolongs the G₀/G₁ phase of the cell cycle.

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Figure Legends

Figure 1 Hsp70 synthesis under low oxygen tension

Western blot analysis for Hsp70 was carried out using total cell extracts obtained from A6 cells grown in 8% O₂ and control cells. Molecular size is indicated in kilodaltons. After immunoblotting, the relative intensity of Hsp70 was quantitated by normalization to the level of actin (the internal control), using ImageJ software. The data represent a typical result from three independent experiments.

Figure 2 Presence of Ku factor in A6 cells and *in vivo* and *in vitro* DNA binding

(A) Immunoblot analysis for Ku in A6 control cells. Nuclear lysate was run on SDS-PAGE gels and analyzed with Ku-specific antibody to evaluate the presence of Ku-70. (B) Effect of anti-Ku-70 antibody on the migration of DNA-protein complexes with extracts of untreated cells. Double-stranded oligonucleotides representing the HSE sequence were synthesized and end-labeled with [γ -³²P]dATP. Whole-cell extracts of A6 cells were incubated with double-stranded oligonucleotides end-labeled with [γ -³²P]dATP in the presence or absence of antibodies as indicated. Lane 3: 1 μ g, and lane 4: 3 μ g of anti-Ku-70 antibody were incubated with 45 μ g of A6 cell extract. Lane 1: free probe. The position of the Ku-specific retarded band is indicated by an arrow. The data shown represent typical results of a series of three experiments. (C) Ku binds the proximal hsp70 promoter. Chromatin

immunoprecipitation (ChIP) assays were performed as described in the Experimental section. The immunoprecipitates were subjected to PCR analysis using a primer pair spanning the hsp70 proximal promoter (depicted schematically). HSEs and primer pairs (arrows) are indicated; GAGA and the GC box are not shown. For heat shock, A6 cells were exposed for 20 min at 43°C. M, 100 bp DNA ladder (Fermentas). Lane 1, lane 3, and lanes 5-7: DNA obtained from unstressed A6 cells. Lane 2 and lane 4: DNA obtained from heat shocked A6 cells. Lanes 1-2: immunoprecipitates with anti-Ku-70 antibody; lanes 3-4: immunoprecipitates with anti-HSF1 antibody; lane 5: immunoprecipitates with anti-Hsp27 antibody as a control; lane 6: input chromatin as a control; lane 7: immunoprecipitates treated without anti-Ku-70 antibody. The data represent a typical result from three independent experiments.

Figure 3 GAGA and SP1 DNA-binding activity *in vitro*

EMSA assays performed using total extract from untreated cells, with GAGA (A) or GC (B) consensus sequences, are shown. Double-stranded oligonucleotides representing GAGA or Sp1 sequences were synthesized and end-labeled with [γ - 32 P]dATP. Whole-cell extracts of A6 cells were incubated with double-stranded oligonucleotides end-labeled with [γ - 32 P]dATP in the presence or absence of unlabeled (cold) oligonucleotides. Lane 1: free probe; lane 2: protein extracts with labeled consensus sequence; lane 3: same as lane 2, plus unlabeled consensus sequence as specific competitor, lane 4: same as lane 2 plus unlabeled specific competitor, corresponding to a DNA fragment of *P. lividus*. A representative gel from three experiments is shown. Position of the specific retarded bands is indicated by arrows.

Figure 4 Basal activities of complete and mutated hsp70 proximal promoter

(A) Scheme of the different hsp70 promoters employed in beta-galactosidase assays showing the consensus sites for transcription factors relative to the transcription starting point (+1). (B) Promoter activities of the phsplacZ, mutGCphsplacZ, mutGAGaphsplacZ and Δ HSEphsplacZ constructs. Transfection efficiency was monitored by co-transfection with the pCAT-control vector. Each bar is the

mean \pm S.E.M. of three independent experiments. Significance was calculated using Student's t-test, and p-values *vs.* control of 0.0112 (*), 0.0013 (**), and 0.0062 (***) are indicated.

Figure 5 Hsp70 half-life in A6 cells

To determine the half-life of Hsp70 protein, we exposed A6 stem cells to heat shock for 1 h at 42°C, pulse-labeled with [³⁵S] methionine for 3 h and then chased with unlabeled methionine. Protein synthesis was observed 0 h, 24 h, and 48 h after labeling. The filled triangle indicates Hsp70. Molecular sizes are indicated in kilodaltons. A representative gel from three experiments is shown.

Figure 6 Use of shRNA to knock down Hsp70 protein expression

Analysis of Hsp70 level in A6 control cells and in A6 cells with knockdown of Hsp70 by specific shRNA (NM) or mock shRNA (SH) as control. After immunoblotting, the relative intensity of Hsp70 was quantitated by normalization to the level of actin (the internal control), using ImageJ software. Each bar is the mean \pm S.D. of three independent experiments. Significance was calculated using Student's t-test, and p-values *vs.* control of < 0.01 (*).

Figure 7 Hsp70 level in A6 and NM3 cells after heat shock

After immunoblotting, the relative intensity of Hsp70 was obtained by normalization to the level of actin (the internal control), using ImageJ software. Each bar is the mean \pm S.E.M. of three independent experiments. Significance was calculated using Student's t-test, and p-values *vs.* control of < 0.01 (*) are indicated.

Figure 8 Effect of hsp70 knockdown on cell growth

A6, NM3, and SH2 cells were seeded in plates, and cell counts were determined at various intervals. Proliferation was assessed by measuring incorporated BrdU using a colorimetric ELISA kit. Absorbance is plotted against days of cell growth. The data represent the mean \pm S.D. of three independent experiments. Significance was calculated using Student's t-test, and p-values *vs.* control of < 0.01 (*) and < 0.001 (**) are indicated.

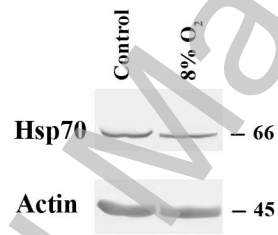
Table I. Hsp70 level and doubling time of A6, NM and mock cells.

Cell lines	% Hsp70 protein	Duplication time (h)
A6	100	16
siRNA NM3	50.5 (+/- 5.0)	25.0 (+/- 2.0)
siRNA NM1	75.0 (+/- 7.9)	17.7 (+/- 2.7)
si RNA NM5	81.4 (+/- 8.1)	17.0 (+/- 0.8)
siRNA NM8	86.0 (+/- 10.9)	16.2 (+/- 1.0)
siRNA NM15	52.2 (+/- 7.0)	25.9 (+/- 1.2)
siRNA NM17	54.8 (+/- 5.0)	27.0 (+/- 0.6)
mock SH2	95.0 (+/-3.1)	17.0 (+/- 0.2)
mock SH3	102.0 (+/- 4.0)	17.0 (+/- 0.2)
mock SH5	101.0 (+/- 3.0)	17.8 (+/- 1.0)
mock SH6	95.0 (+/- 2.8)	15.4 (+/- 1.4)

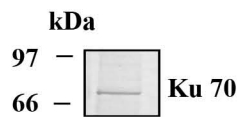
Table II Estimated duration (in hours) of G₀/G₁, S, and G₂/M, phases^a

Clone	G ₀ /G ₁	S	G ₂ /M
A6	7.5	6.9	1.6
SH2	7.9	7.1	1.9
NM3	14.9	7.2	2.8

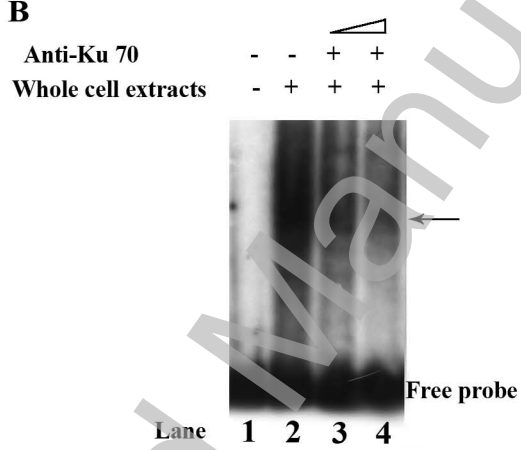
^aMean cell doubling times and cell cycle analysis data were used to determine the estimated duration of G₀/G₁, S, and G₂/M, phases.



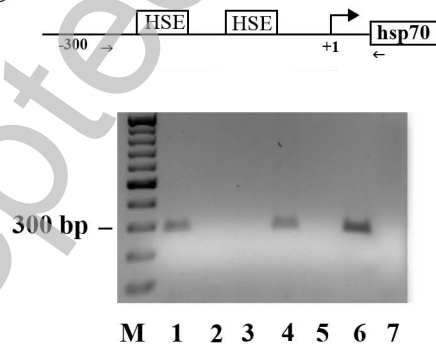
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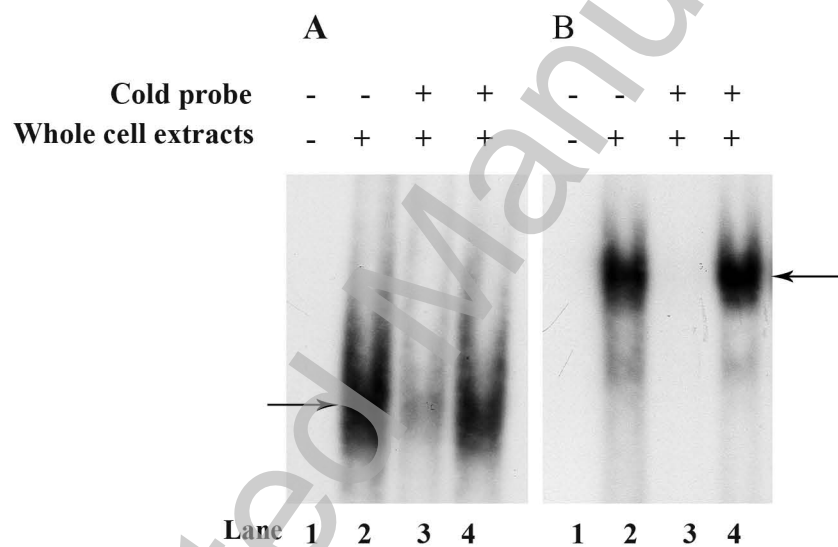


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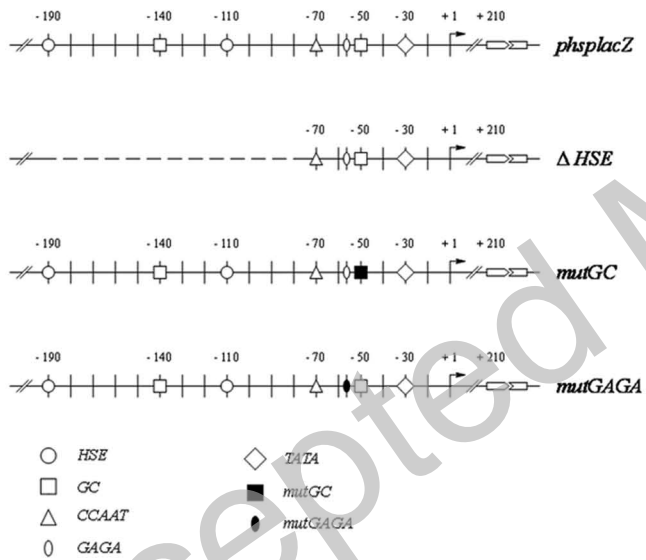


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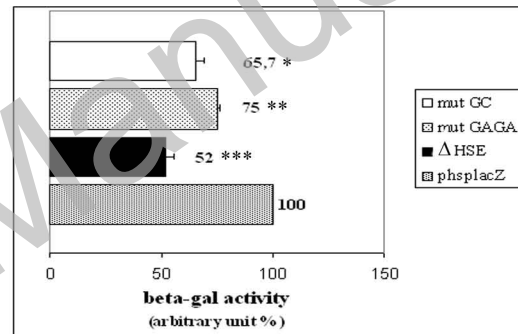


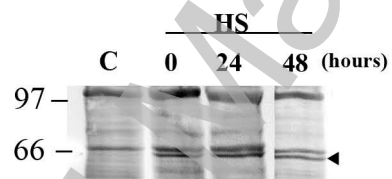


A



B





THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20082309

