Heat shock protein 27 promotes cell cycle progression by down-regulating E2F transcription factor 4 and retinoblastoma family protein p130

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Abstract

Heat shock protein 27 (HSP27) protects cells under stress. Here, we demonstrate that HSP27 also promotes cell cycle progression of MRC-5 human lung fibroblast cells. Serum starvation for 24 h induced G1 arrest in these cells, and upon serum refeeding, the cells initiated cell cycle progression accompanied by an increase in HSP27 protein levels. HSP27 levels peaked at 12 h, and transcriptional up-regulation of six G2/M-related genes (CCNA2, CCNB1, CCNB2, CDC25C, CDCA3, and CDK1) peaked at 24 to 48 h. siRNA-mediated HSP27 silencing in proliferating MRC-5 cells induced G2 arrest coinciding with down-regulation of these six genes. Of note, the promoters of all of these genes have the cell cycle–dependent element or the cell cycle gene-homology region. These promoter regions are known to be bound by the E2F family proteins (E2F-1 to E2F-8) and retinoblastoma (RB) family proteins (RB1, p107, and p130), among which E2F-4 and p130 were strongly up-regulated in HSP27-knockdown cells. E2F-4 or p130 knockdown concomitant with the HSP27 knockdown rescued MRC-5 cells from G2 arrest and up-regulated the six cell cycle genes. Moreover, we observed cellular senescence in MRC-5 cells on day 3 after the HSP27 knockdown, as evidenced by increased senescence-associated β-galactosidase activity and up-regulated inflammatory cytokines. Cellular senescence was suppressed by the combined E2F-4/HSP27 or p130/HSP27 knockdown. The cellular senescence was also suppressed by the concomitant knockdown of E2F-4/HSP27 or p130/HSP27. Our findings indicate that HSP27 promotes cell cycle progression of MRC-5 cells by suppressing
expression of the transcriptional repressors E2F-4 and p130.

Heat shock protein (HSP) 27, also known as the heat shock protein family B member 1 (HSPB1), belongs to the small HSP family that commonly has a conserved C-terminal α-crystallin domain (1). In response to heat shock, HSP27 functions as an ATP-independent molecular chaperone to facilitate the proper refolding of damaged proteins (2). HSP27 is also involved in a wide range of cellular processes such as cytoskeletal organization, redox metabolism, suppression of apoptosis, and cell proliferation (2-6). HSP27 also promotes degradation of ubiquitinated proteins by the 26S proteasome (7, 8). Furthermore, HSP27 is frequently overexpressed in cancer cells and closely associated with aggressive tumor behavior, metastasis, poor prognosis, and resistance to chemotherapy (9, 10).

While the involvement of HSP27 in stressed cells and cancer cells is now well documented, there are only a few reports on the role of HSP27 in cell cycle progression and cellular senescence. For example, HSP27 reverses the G2 arrest caused by HIV-1 viral protein R (11). HSP27 promotes G1/S transition of cells arrested with serum depletion or with the nitric oxide donor glyceryl trinitrate by promoting ubiquitination and proteasomal degradation of the cyclin-dependent kinase (CDK) inhibitor p27Kip1 (8). Embryonic fibroblasts derived from Hspb1-deficient mice display reduced entry into S phase with increased expression of the CDK inhibitors p21Cip1 and p27Kip1 (12). Taken together, HSP27 may have a promoting role in cell cycle progression.

As for cellular senescence, HSP27-knockdown of MCF-7 mammary carcinoma cells led to reduced proliferation and acquisition of a spontaneous secretory phenotype, a feature of cellular senescence (13). While overexpression of HSP27 protects MCF10A human mammary epithelial cells from doxorubicin-induced cellular senescence by inhibiting p53-mediated induction of p21Cip1, the major regulator of the senescence program, depletion of HSP27 in HCT116 human colon carcinoma cells caused cellular senescence through activation of p53 and induction of p21Cip1 (14). Thus, HSP27 may also have a suppressive role in cellular senescence by interfering with p53 activation and/or function.

Previously, we have demonstrated that HSP27 plays an essential role in transforming growth factor (TGF)-β1-induced myofibroblast differentiation of normal lung fibroblasts and accordingly in the development of bleomycin-induced pulmonary fibrosis (15). During the same study, we also observed that siRNA knockdown of HSP27 significantly suppressed cell proliferation of normal lung fibroblasts. Thus, in the present study, we further explored the role of HSP27 in cell cycle progression by using MRC-5, a normal human lung fibroblast cell line (16). MRC-5 was serum-starved and -refed to synchronize the cell cycle. Upon refeeding, we found a rapid increase in HSP27 protein along with transcriptional upregulation of
six G2/M-related genes: cyclin A2, cyclin B1, cyclin B2, cdc25c, cdcA3, and CDK1 (17, 18). We further found that HSP27-knockdown of proliferating MRC-5 induced G2 arrest together with downregulation of the same six genes and upregulation of E2F-4/p130. We demonstrated that concomitant knockdown of E2F-4 or p130 with HSP27-knockdown rescued MRC-5 from G2 arrest and also prevented the downregulation of the six genes. MRC-5 also underwent cellular senescence 3 days after HSP27-knockdown as evidenced by increases in senescence-associated β-galactosidase positivity and upregulation of proinflammatory cytokines. The cellular senescence was also prevented by the concomitant knockdown of E2F-4 or p130 with HSP27-knockdown. Collectively, HSP27 plays a pivotal role in cell cycle progression of MRC-5 by downregulating the expression of E2F-4/p130, whose upregulation leads to G2 arrest through downregulation of the six G2/M-related genes, which eventually results in cellular senescence in MRC-5.

Results
HSP27 increases during cell cycle progression of serum-refed MRC-5
MRC-5 is a human diploid lung fibroblast cell line that is widely used as a model of normal human fibroblasts (15, 16). In our preliminary experiments, HSP27-knockdown by siRNA transfection significantly suppressed cell proliferation of MRC-5 (data not shown, but see Fig. 2). To test whether HSP27 was involved in cell cycle progression, we used the technique of serum-starvation and refeeding to synchronize the cell cycle of MRC-5. After 24 h of fetal bovine serum (FBS)-starvation, we re-fed MRC-5 with 5% FBS to initiate the cell cycle progression. We confirmed that while FBS-starvation increased cells at G0/G1 phase (G0/G1 = 73 ± 0.6%, S = 6 ± 0.2%, G2/M = 20 ± 0.5%), FBS-refeeding increased cells at S and G2/M phases (G0/G1 = 52 ± 0.7%, S = 20 ± 0.2%, G2/M = 28 ± 0.5%) (Fig. 1A). We found that FBS-refeeding also increased the protein level of HSP27 with a peak at 12 hours (Fig. 1B). No significant increase was seen in the phosphorylation of HSP27 (Fig. 1B). Since cell cycle progression is regulated by cyclins and CDKs (Fig. 1C), we quantified mRNA levels of these molecules together with that of HSP27 (Fig. 1D). We found that HSP27 mRNA was not upregulated by FBS-refeeding. Thus, the increase of HSP27 protein was mostly translationally and/or post-translationally controlled in FBS-refed MRC-5. On the other hand, among the 12 cell cycle-related genes examined, the six genes (cyclin A2, cyclin B1, cyclin B2, cdc25c, cdcA3, and CDK1) were strongly upregulated after 24 to 48 hours of FBS-refeeding (Fig. 1D). No such changes were seen in the mRNA levels of cyclin D1, cyclin D2, cyclin E1, CDK2, and the two CDK inhibitors p21^{Cip1} and p27^{Kip1}. Of note, all the six upregulated genes are those involved in the G2/M phases and contain the cell cycle-dependent element (CDE) and/or the cell cycle genes homology region (CHR).
in their promoters (19): cyclin A2, cdc25c, cdcA3, and CDK1 have both CDE and CHR, while cyclin B1 and cyclin B2 have CHR. These elements are known to be regulated by the binding of the E2F and retinoblastoma (RB) family proteins (19, 20).

**HSP27-knockdown induces G2 arrest**

To examine the role of HSP27 in the cell cycle progression of MRC-5, we next performed HSP27-knockdown experiments using siRNA transfection. As shown in Fig. 2A, HSP27-knockdown significantly decreased cell proliferation. This was not due to increased cell death (the percentage of dead cells: control-knockdown, 3.0 ± 0.4; HSP27-knockdown, 2.4 ± 0.4). Furthermore, as shown in Fig. 2B, HSP27-knockdown significantly increased cells at G2/M phase (G0/G1=66 ± 0.1%, S=4 ± 0.5%, G2/M=30 ± 0.4%) compared with control-knockdown (G0/G1=70 ± 0.7%, S=9 ± 0.3%, G2/M=21 ± 0.2%) (Fig. 2B). Although cells at G2 and M are diploid and indistinguishable by propidium iodide staining, M-phase cells can be distinguished by phospho-histone H3 positivity (21). Since HSP27-knockdown did not increase cells positive for phospho-histone H3 (data not shown), the cell cycle arrest was mainly at G2. We also examined the viability of cells by using Muse Count & Viability Kit. No significant difference in dead cell count was found between cells transfected with HSP27 siRNA (2.4 ± 0.4%) and control siRNA (3.0 ± 0.4%) (P=0.29). Thus, we concluded that HSP27-knockdown induced G2 arrest in MRC-5.

**HSP27-knockdown induces downregulation of the six cell cycle regulatory genes**

HSP27-knockdown efficiently decreased not only HSP27 mRNA but also the mRNAs of the six cell cycle regulatory genes that were upregulated in FBS-refed MRC-5: cyclin A2, cyclin B1, cyclin B2, cdc25c, cdcA3, and CDK1 (Fig. 2C). On the other hand, although cyclin D1 mRNA was significantly upregulated by HSP27-knockdown, the mRNA levels of cyclin D2, cyclin E1 and CDK2 as well as those of the two CDK inhibitors p21Cip1 and p27Kip1 were not affected by HSP27-knockdown. These results suggested that HSP27 was involved in the upregulation of the six G2/M cell cycle progression molecules in MRC-5.

**E2F-4 and p130 increase by HSP27-knockdown**

As mentioned earlier, the six genes (cyclin A2, cyclin B1, cyclin B2, cdc25c, cdcA3, CDK1) that were downregulated by HSP27-knockdown commonly carry the CDE/CHR or CHR element in their promoters (19). Thus, their expression can be regulated by the E2F family proteins and RB family proteins (RB1, p107 and p130) that bind to these elements (19, 20). Among the E2F family proteins, E2F-1 to E2F-3 positively and E2F-4 to E2F-8 negatively regulate the cell cycle (19). Since the HSP27-knockdown strongly decreased the expression of the six genes, we quantified the
mRNA levels of the repressor E2F family proteins in MRC-5 upon HSP27-knockdown. By using qPCR, we found that the expression of E2F-4 was significantly increased upon HSP27-knockdown (Fig. 3A), while the expression of E2F-5 to E2F-8 was negligible in MRC-5 (data not shown). Among the RB family proteins, HSP27-knockdown increased the expression of p130 (Fig. 3A) but not RB1 (supporting Fig. 1), while the mRNA level of p107 was quite low in this cell line regardless of HSP27-knockdown (supporting Fig. 1). We also confirmed strong increases of E2F-4 and p130 proteins by HSP27-knockdown using immunoblot analysis (Fig. 3B) and immunocytochemistry (Fig. 3C).

To examine whether HSP27 could directly interact with E2F-4 and/or p130, we conducted co-immunoprecipitation experiments and found no evidence for the direct binding of HSP27 to E2F-4 or p130 (data not shown). Since HSP27 was reported to enhance ubiquitination and degradation of intracellular proteins such as p27kip1 and IκB (7, 8), we also conducted the protein chase experiment using cycloheximide to determine the effect of HSP27-knockdown on the half-life of E2F-4 and p130. Although we expected slower degradation of E2F-4 and/or p130 by HSP27-knockdown, we actually found enhanced degradation of E2F-4 and p130 by HSP27-knockdown compared with control-knockdown (Fig. 3D).

**E2F-4-knockdown rescues G2 arrest by HSP27-knockdown**

To determine the role of E2F-4 in the G2 arrest of MRC-5 by HSP27-knockdown, we performed single and double knockdown experiments. Immunoblot analysis confirmed the efficient knockdown of HSP27 and E2F-4 by HSP27 siRNA and E2F-4 siRNA, respectively (Fig. 4A). While the single knockdown of HSP27 decreased cell number (Fig. 4B) and induced G2 arrest (Fig. 4C), the single knockdown of E2F-4 had no such effect on cell number (Fig. 4B) or cell cycle progression (Fig. 4C). However, the double knockdown of HSP27 and E2F-4 reversed the effect of HSP27-knockdown on cell number (Fig 4B) and G2 arrest (Fig. 4C). Furthermore, among the six G2/M regulatory genes that were downregulated by HSP27-knockdown, the downregulations of at least cyclin B2, cdc25c, cdcA3, and CDK1 were significantly prevented by the concomitant knockdown of E2F-4 (Fig. 4D). These results suggested that upregulation of E2F-4 was mostly responsible for the G2 arrest induced by HSP27-knockdown.

**p130-knockdown also rescues G2 arrest by HSP27-knockdown**

We next performed similar experiments for p130. Immunoblot confirmed efficient knockdown of HSP27 and p130 by HSP27 siRNA and p130 siRNA, respectively (Fig. 4E). We also observed that the concomitant knockdown of p130 rescued
the effect of HSP27-knockdown on cell number (Fig. 4F) and cell cycle (Fig. 4G). However, since p130 is a general cell cycle repressor, the single knockdown of p130 significantly increased cell numbers (Fig. 4F). Furthermore, by qPCR, we found that single p130-knockdown significantly upregulated mRNAs for HSP27, cyclin A2, cyclin B1, cyclin B2, cdc25c, and cdcA3 (Fig. 4H). Since the HSP27/p130-double knockdown led to a similar level of HSP27 as control cells, but a lower level of p130 expression, this might allow us to assess the effect of low p130 without a significant change in the level of HSP27. Thus, the reduction of p130 alone led to the upregulation of the six cell cycle-associated genes.

**HSP27-knockdown leads to cellular senescence**

We also examined a long-term effect of HSP27-knockdown on MRC-5. After 3 days of HSP27-knockdown, we observed significant increases in cells positive for senescence-associated β-galactosidase (SA-β-gal) (Fig. 5A) and significant upregulation of pro-inflammatory cytokines IL-1α and IL-8 (Fig. 5B). These results demonstrated that HSP27-knockdown of MRC-5 induced not only cell cycle arrest but also cellular senescence after day 3. Again the concomitant knockdown of E2F-4 or p130 with HSP27-knockdown effectively prevented the increase of β-galactosidase-positive cells. Of note, although HSP27-knockdown significantly decreased the mRNA level of p16, a senescence inducer molecule (22), it did not affect the protein level of p16 (Fig. 5C).

**HSP27-knockdown induces cellular senescence in G2 arrested cells**

Since HSP27-knockdown induced G2 arrest, we also examined whether increased cellular senescence by HSP27-knockdown could also be associated with the G2 phase, whose cells can be identified by intense Ki-67 positive nuclear staining and/or cytoplasmic cyclin B1 staining (23, 24). In control-knockdown cells, SPI-DER-βGal® positive senescent cells were negative for Ki-67 (Fig. 5B) or cytoplasmic cyclin B1 (data not shown). On the other hand, in HSP27-knockdown cells, we found that some SPI-DER-βGal® positive senescent cells had Ki-67 positive nucleoli or cyclin B1 positive cytoplasm, suggesting that increased senescent cells in HSP27-knockdown were mostly at the G2 phase (Fig. 5D).

**Cell cycle arrest by HSP27-knockdown is not associated with oxidative cellular damages**

Cell cycle arrest occurs under unfavorable conditions, such as nutrient deficiency or DNA damages. Since we used MRC-5 maintained in 5% FBS-Opti-MEM in HSP27-knockdown experiments, the nutrient deficiency might not be the likely cause of the cell cycle arrest. On the other hand, since HSP27 has an antioxidant property (2), oxidative damages might be increased by HSP27-knockdown, thereby leading to DNA damages and
cell cycle arrest. To test this possibility, we analyzed protein oxidation, protein ubiquitination, and DNA damages in HSP27-knockdown MRC-5. We found no apparent increases in protein oxidation or ubiquitination by HSP27-knockdown (Fig. 6A). The phospho-histone 2X, a marker of cellular DNA damages, was also not increased by HSP27-knockdown either (Fig. 6B). Thus, oxidative damages were not the likely cause of the cell cycle arrest induced by HSP27-knockdown.

**Cell cycle arrest by HSP27-knockdown is independent of p53**

We further examined the possible involvement of p53, a key molecule of cell cycle arrest (25). Although p53 was increased by HSP27-knockdown, p21<sup>Cip1</sup>, the CDK inhibitor and one of the major downstream mediators of p53 function, was not affected (Fig. 6C). In addition, pifithrin-α, a p53 inhibitor (26), did not prevent the decrease in cell number or cell cycle arrest by HSP27-knockdown (Fig. 6D). Pifithrin-α also did not prevent the downregulation of mRNA levels of the cell cycle-associated genes by HSP27-knockdown either (data not shown). We concluded that the cell cycle arrest induced by HSP27-knockdown was mostly independent of p53.

**Discussion**

The cell cycle is a process composed of four phases: G1 (pre-DNA synthesis), S (DNA synthesis), G2 (pre-division) and M (mitosis) (27, 28). Cyclins, CDKs, and CDK inhibitors (p21<sup>Cip1</sup> and p27<sup>Kip1</sup>) are the key molecules that directly regulate the cell cycle progression (Fig. 1C) (17, 27). The E2F family of transcription factors also control the cell cycle at the transcriptional level as activators (E2F-1 to E2F-3) or repressors (E2F-4 to E2F-8) (29). The repressor proteins, especially E2F-4 and E2F-5, are known to function in association with the RB proteins (30, 31).

In the present study, we used MRC-5, a normal human lung fibroblast cell line (16), and demonstrated a rapid increase in HSP27 protein with a peak at 12 h in serum-starved and -refed MRC-5 (Fig. 1). Furthermore, the six genes associated with G2/M cell cycle progression (cyclin A2, cyclin B1, cyclin B2, cdc25c, cdcA3, and CDK1) were strongly upregulated in serum-refed MRC-5 (Fig. 1). Of note, all these six genes carry CDE and/or CHR in their promoters, where the complex of a repressive E2F and RB family proteins is known to bind (19, 20). By using the siRNA gene silencing technique, we showed that HSP27-knockdown of proliferating MRC-5 led to G2 arrest with concurrent downregulation of the same six genes (Fig. 2) and upregulation of the transcriptional repressor complex E2F-4/p130 (Fig. 3). We further showed that the G2 arrest as well as the downregulation of the six cell cycle-related genes induced by HSP27-knockdown was prevented by concomitant knockdown of E2F-4 or p130 (Fig. 4). Since HSP27 is known to enhance the intracellular protein degradation through
ubiquitination and proteosomal degradation (7, 8), we considered that HSP27 might promote the degradation of E2F-4 and/or p130. However, by a protein chase assay using cycloheximide, we observed no slowing down of E2F-4 or p130 degradation by HSP27-knockdown (Fig. 3). Collectively, HSP27 plays a pivotal role in G2 progression of MRC-5 by upregulating the G2/M-associated genes via downregulation of E2F-4/p130 (Fig. 7).

Although the mechanism(s) of downregulation of E2F-4/p130 by HSP27 remains unclear at present, HSP27 may have an effect on the promoter activity of these molecules, since HSP27 has been reported to bind some transcription factors and enhance their activities (32). Indeed, we detected a significant increase of the p130 promoter activity in HSP27-knockdown MRC-5 (supporting Fig. 2). We also found the presence of HSP27 in the nucleus of phosphorylated-histone H3 positive M phase cells (supporting Fig. 2). These results are consistent with a notion that HSP27 negatively regulates the transcription of p130.

The cell cycle arrest of proliferating cells occurs under unfavorable conditions such as starvation and DNA damage. Starvation induces G1 arrest (33), while DNA damages induce G2 arrest (34). Furthermore, there are two types of cell cycle arrest: reversible and irreversible. The former is called quiescence, while the latter senescence (35). The cellular senescence is controlled by two signaling pathways: one is the p53 to p21Cipl pathway and the other is the p16 to phosphorylated retinoblastoma protein pathway (36). Yet, another signaling pathway that does not involve p53 or p16 has been recently described in fibroblasts (37). In this case, the accumulation of GATA4, which is digested by autophagy in physiological conditions, activates the transcription factor NF-κB to initiate the expression of inflammatory cytokines and cellular senescence (37). Furthermore, while 2 days of cell cycle arrest in vitro can be reversible, cell cycle arrest may be irreversible after 3 to 4 days (38, 39). Reversible cell cycle arrest is converted to irreversible senescence through a process called geroconversion, a futile growth activity during the cell cycle arrest, which is mainly regulated by mammalian target of rapamycin (mTOR) signaling (39, 40). Senescent cells are also known to exhibit senescence-associated secretory phenotype (SASP) by producing inflammatory cytokines, metalloproteinases, and growth factors (41, 42). The SASP phenotype is initiated by NF-κB and p38 MAPK signaling (43) and maintained in an autocrine fashion by the SASP factor IL-1α (44). The production of various SASP factors is considered to be beneficial for the host by promoting immune clearance of damaged cells and preventing proliferation of potential cancer cells (45, 46). However, the SASP factors may also exacerbate age-related diseases such as atherosclerosis (47) and type 2 diabetes mellitus (42).
In the present study, we also observed cellular senescence in MRC-5 on day 3 after HSP27-knockdown as evidenced by positivity of senescence-associated β-gal and production of proinflammatory cytokines (Fig. 5). Although mTOR is considered to play a key role in the conversion from quiescence to senescence (39, 40), we detected no significant increase in the mTOR activity by HSP27-knockdown as shown by immunoblot of the mTOR substances, phospho-p70 S6 kinase and phospho-4E-BP1 (data not shown). We also observed no significant increase of p16 (Fig. 5) or p21Cip1 (Fig. 6) in HSP27-knockdown MRC-5. Thus, the cellular senescence induced by HSP27-knockdown in MRC-5 may be independent of mTOR, the p53/p21 pathway or the p16 pathway. Since the cellular senescence induced by HSP27-knockdown was also suppressed by the concurrent knockdown of E2F-4 or p130, the G2 arrest induced by upregulation of E2F-4/p130 may eventually lead to cellular senescence in MRC-5 by still unknown mechanisms.

In conclusion, we have demonstrated that HSP27 promotes cell cycle progression of MRC-5 by negatively regulating E2F-4/p130 expression. Increased expression of E2F-4/p130 in HSP27-knockdown MRC-5 leads to G2 arrest by downregulating the expression of the six G2/M-related genes (cyclin A2, cyclin B1, cyclin B2, cdc25c, cdcA3, and CDK1). The G2 arrest induced by upregulation of E2F-4/p130 eventually results in cellular senescence in MRC-5. Thus, HSP27 may indirectly prevent cellular senescence by preventing upregulation of E2F-4/p130. By datamining of NCBI's Gene Expression Omnibus microarray database, we indeed found decrease of HSP27 mRNA in some senescence cells: liver stellate cells (48) and premalignant lung cells (49). At present, we do not know how HSP27 negatively regulates the E2F-4/p130 expression but the transcriptional regulation may be partly involved (supporting Fig. 2). It also remains to be seen whether the promoting effect of HSP27 on cell cycle progression is a general phenomenon or cell-type specific phenomenon; in HSP27-knockdown in normal human lung fibroblast (NHLF) and lung adenocarcinoma cell line A549, we found that p130 mRNA levels were increased in both cell lines, while E2F4 levels of NHLF or A549 were unchanged or slightly increased, respectively (supporting figure 4). This suggests that the promoting effect of HSP27 on cell cycle progression is general.

**Experimental procedures**

**Cell culture and treatment**

MRC-5 was obtained from Riken Cell Bank (Tsukuba, Japan) and maintained in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, St Louis, MO) supplemented with 5% FBS, streptomycin (100 µg/ml) and penicillin (100 U/ml) at 37°C in 5% CO₂/95% air. To synchronize the cell cycle, cells were cultured in a 24-well plate to ≤70% confluency, washed with phosphate-buffered
saline (PBS), and placed in serum-free DMEM for 24 h. Then, FBS was added to the medium to make the final concentration of 5%. DNA damages were induced in cells by exposing to ultraviolet light ($\lambda=253.7$ nm, $120 \mu$W cm$^{-2}$) for 15 minutes (min) and culturing for 4 h.

**Immunoblot analysis**

Cells were washed with PBS and solubilized with CellLytic® (Sigma-Aldrich) containing Protease Inhibitor Cocktail Complete® (Roche Diagnostics, Mannheim, Germany) and Phosphatase Inhibitor Cocktail® (Toyobo, Osaka, Japan). After 15-min mixing at room temperature, cell debris was removed by centrifugation. In some experiments, nuclear and cytosolic fractions were prepared by using NucBaster™ Protein Extraction Kit (Merck Millipore, Billerica, MA), following the manufacturer’s instructions. Samples were electrophoresed on a sodium dodecyl sulfate (SDS)-polyacrylamide gel under reducing conditions and electrophoretically blotted onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked in 5% skim milk and probed with a primary antibody. Mouse antibodies against $\alpha$-tubulin and $\beta$-actin were obtained from Sigma-Aldrich; goat anti-HSP27 antibody, rabbit anti-E2F-4 antibody, rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), rabbit anti-p53 antibodies and mouse anti-ubiquitin antibody were from Santa Cruz (CA); mouse monoclonal antibody against p130, RB1 and p21$^{\text{Cip1}}$ were from BD Biosciences (San Diego, CA); rabbit polyclonal antibodies against phospho-HSP27 (Ser82), cyclin B1, p130, p16, and histone H3 were from Cell Signaling (Beverly, MA). After washing, membranes were reacted with a horseradish peroxide (HRP)-conjugated secondary antibody and developed using Luminata™ Forte Western HRP Substrate (Merck Millipore). Signal intensities were quantified using ImageJ software (NIH, Bethesda, MD). Protein carbonylation, a major form of protein oxidation, was determined using Oxiselect™ protein carbonyl immunoblot kit (Cell Biolabs Inc, San Diego, CA) following the manufacturer’s instructions.

**siRNA transfection**

The siRNAs listed in Table 1 were obtained from GeneDesign (Osaka, Japan). Transfection of siRNA was performed by Lipofectamine RNAiMax (Invitrogen). Briefly, cells were suspended in 5% FBS Opti-MEM (Thermo Fisher Scientific, Waltham, MA) and seeded in a 12-well plate at $2\times10^4$ cells/ml. After 24 h culture, Lipofectamine RNAiMax-siRNA complex was added to cells. The final siRNA concentration in each well was 20 nM. The HSP27 siRNA that we used in this study has been extensively used by many previous studies as the gold standard HSP27 siRNA (50-52) and was also found to be most effective in this study (data not shown). The results obtained by E2F-4a siRNA and p130a siRNA were shown in this paper, while the data obtained by
E2F-4b siRNA and p130b siRNA reproduced the results by E2F-4a siRNA and p130a siRNA, respectively (data not shown). In some experiments, cells were treated with 20 μM cycloheximide (Sigma-Aldrich) or with 10 μM pifithrin-α (Cayman chemical, Ann Arbor, MI). The pifithrin-α concentration used was pre-determined by its protective effect against doxorubicin or 5-fluorourasil-induced cell growth inhibition (53) (supporting Fig. 3). Cell lysates for immunoblot analysis were prepared as stated above.

**Cell proliferation assay**
Cells were seeded in a 96-well plate and transfected with control siRNA or HSP27 siRNA. Cell numbers were quantified by Cell Counting Kit-8 (CCK8, Dojindo Laboratory, Kumamoto, Japan). In brief, we added 10 μl of CCK8 reagent to each well and incubated for 1 h. Then, the medium from each well was transferred to a new 96-well plate. Optical density at 450 nm was measured on a microplate reader (ARVO; Perkin Elmer, Waltham, MA).

**Cell counting and cell cycle analysis by MUSE**
Cells in a 12-well plate were transfected with siRNA and cultured for 2 days. Then, cells were harvested by trypsin-EDTA and centrifuged at 100 g for 5 min. Cell pellets were re-suspended in 330 μl of PBS. A portion of cell suspension (30 μl) was mixed with Muse Count & Viability Kit (Millipore) to determine cell numbers and viability using MUSE cell analyzer (Millipore). A remaining cell suspension (300 μl) was mixed with 700 μl of ethanol and fixed at -20°C for 4 h. After washing twice with PBS, cells were suspended in Muse cell cycle kit (Millipore) and the cell cycles were determined using MUSE cell analyzer.

**Quantitative PCR**
Total RNAs were extracted from cells using RNeasy Mini Kit (Qiagen). RNA samples (500 ng each) were reverse-transcribed using High Capacity RNA-to-cDNA Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was performed using THUNDERBIRD® SYBR® qPCR Mix (Toyobo, Osaka, Japan) and ABI Step one real-time PCR system with β-actin or GAPDH as a reference control. The sequences of the sense and antisense primers used for amplification are listed in Table 1.

**Immunocytochemistry**
Cells were cultured in a multi-well glass chamber plate (BD Falcon, Franklin Lakes, NJ), washed with PBS, and fixed with ice-cold ethanol at -20°C for 4 min. After washing, cells were treated with Dakocytomation Protein Block (Dako, Glostrup, Denmark) for 1 h. Then, cells were incubated with anti-E2F-4 or anti-p130 antibody. After washing, cells were incubated with Alexa 488 or Alexa 555 fluorescein-labeled secondary antibodies (Invitrogen, Carlsbad, CA) and DNA staining reagent To-PRO3 (Invitrogen). After washing and
mounting, images were taken by a confocal laser microscope (Carl Zeiss GmBH, Jena, Germany).

**Cytochemical staining for β-galactosidase**

Cells in a 24-well plate were fixed with 4% paraformaldehyde at room temperature for 15 min. After washing with PBS, cells were incubated in a β-gal staining solution (100 mM sodium phosphate buffer pH 6.0, 2 mM MgCl₂, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 1 mg/ml X-gal). After incubation at 37°C for 18 h, cells were washed with PBS and counterstained with 1 μg/ml of 4',6-diamidino-2-phenylindole (DAPI). Four images were taken randomly, and total and β-gal-positive blue cells were counted by ImageJ software.

To detect whether senescence cells were at the G2 phase, fluorometric senescence cells detection reagent SPiDER-βGal® (Dojindo Laboratory) was used. One day after control and HSP27 siRNA transfection, cells were detached, seeded in an 8-well chamber slide (BD Falcon), and cultured 2 more days. After washing, cells were fixed with 4% paraformaldehyde and treated with SPiDER-βGal®, following the manufacture’s instruction. After incubation with this reagent, cells were fixed again and then stained with Ki-67 (Abcam, Cambridge, UK) or cyclin B1 (Cell Signaling) antibodies, which were visualized with Alexa 633 fluorescein-labeled secondary antibody (Invitrogen) and under confocal microscopy.

**Statistical analysis**

Quantitative data are presented as the mean ± SEM. Statistical analysis was performed using Student's t-test. P<0.05 was considered statistically significant.
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Conflict of interest

The authors declare that they have no conflicts of interest with the contents of this article.
References

1. Garrido, C., Paul, C., Seigneuric, R., Kampinga, H. H. (2012) The small heat shock proteins family: the long forgotten chaperones. *Int J Biochem Cell Biol.* **44**, 1588-1592

2. Arrigo, A. (2017) Mammalian HspB1 (Hsp27) is a molecular sensor linked to the physiology and environment of the cell. *Cell Stress Chaperones.* **22**, 517-529

3. Rocchi, P., Jugpal, P., So, A., Sinneman, S., Ettinger, S., Fazli, L., Nelson, C., Gleave, M. (2006) Small interference RNA targeting heat-shock protein 27 inhibits the growth of prostatic cell lines and induces apoptosis via caspase-3 activation in vitro. *BJU Int.* **98**, 1082-1089

4. Hayashi, N., Peacock, J. W., Beraldi, E., Zoubeidi, A., Gleave, M. E., Ong, C. J. (2012) Hsp27 silencing coordinately inhibits proliferation and promotes Fas-induced apoptosis by regulating the PEA-15 molecular switch. *Cell Death Differ.* **19**, 990-1002

5. Wettstein, G., Bellaye, P. S., Micheau, O., Bonniaud, P. (2012) Small heat shock proteins and the cytoskeleton: an essential interplay for cell integrity? *Int J Biochem Cell Biol.* **44**, 1680-1686

6. Christians, E. S., Ishiwata, T., Benjamin, I. J. (2012) Small heat shock proteins in redox metabolism: implications for cardiovascular diseases. *Int J Biochem Cell Biol.* **44**, 1632-1645

7. Parcellier, A., Schmitt, E., Gurbuxani, S., Seigneurin-Berny, D., Pance, A., Chantome, A., Plenchette, S., Khochbin, S., Solary, E., Garrido, C. (2003) HSP27 is a ubiquitin-binding protein involved in I-kappaBalpha proteasomal degradation. *Mol Cell Biol.* **23**, 5790-5802

8. Parcellier, A., Brunet, M., Schmitt, E., Col, E., Didelot, C., Hammann, A., Nakayama, K., Nakayama, K. I., Khochbin, S., Solary, E., Garrido, C. (2006) HSP27 favors ubiquitination and proteasomal degradation of p27\(^{kip1}\) and helps S-phase re-entry in stressed cells. *FASEB J.* **20**, 1179-1181

9. Garrido, C., Brunet, M., Didelot, C., Zermati, Y., Schmitt, E., Kroemer, G. (2006) Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. *Cell Cycle.* **5**, 2592-2601

10. Wu, J., Liu, T., Rios, Z., Mei, Q., Lin, X., Cao, S. (2016) Heat Shock Proteins and Cancer. *Trends Pharmacol Sci.* **38**, 226-256

11. Bukrinsky, M., Zhao, Y. (2004) Heat-shock proteins reverse the G2 arrest caused by HIV-1 viral protein R. *DNA Cell Biol.* **23**, 223-225

12. Acunzo, J., Andrieu, C., Baylot, V., So, A., Rocchi, P. (2014) Hsp27 as a therapeutic target in cancers. *Curr Drug Targets.* **15**, 423-431
13. Horman, S., Fokan, D., Mosselmans, R., Mairesse, N., Galand, P. (1999) Anti-sense inhibition of small-heat-shock-protein (HSP27) expression in MCF-7 mammary-carcinoma cells induces their spontaneous acquisition of a secretory phenotype. *Int J Cancer.* **82**, 574-582

14. O'Callaghan-Sunol, C., Gabai, V. L., Sherman, M. Y. (2007) Hsp27 modulates p53 signaling and suppresses cellular senescence. *Cancer Res.* **67**, 11779-11788

15. Park, A-M., Kanai, K., Itoh, T., Sato, T., Tsukui, T., Inagaki, Y., Selman, M., Matsushima, K., Yoshie, O. (2016) Heat Shock Protein 27 Plays a Pivotal Role in Myofibroblast Differentiation and in the Development of Bleomycin-Induced Pulmonary Fibrosis. *PLoS One.* **11**, e0148998

16. Jacobs, J. P., Jones, C. M., Baille, J. P. (1970) Characteristics of a human diploid cell designated MRC-5. *Nature.* **227**, 168-170

17. Schafer, K. A. (1998) The cell cycle: a review. *Vet Pathol.* **35**, 461-478

18. Schwartz, G. K., Shah, M. A. (2005) Targeting the cell cycle: a new approach to cancer therapy. *J Clin Oncol.* **23**, 9408-9421

19. Muller, G. A., Engeland, K. (2010) The central role of CDE/CHR promoter elements in the regulation of cell cycle-dependent gene transcription. *FEBS J.* **277**, 877-893

20. van den Heuvel, S., Dyson, N. J. (2008) Conserved functions of the pRB and E2F families. *Nat Rev Mol Cell Biol.* **9**, 713-724

21. Hans, F., Dimitrov, S. (2001) Histone H3 phosphorylation and cell division. *Oncogene.* **20**, 3021-3027

22. He, S., Sharpless, N. E. (2017) Senescence in Health and Disease. *Cell.* **169**, 1000-1011

23. Scholzen, T., Gerdes, J. (2000) The Ki-67 protein: from the known and the unknown. *J Cell Physiol.* **182**, 311-322

24. Lindqvist, A., van Zon, W., Karlsson Rosenthal, C., Wolthuis, R. F. (2007) Cyclin B1-Cdk1 activation continues after centrosome separation to control mitotic progression. *PLoS Biol.* **5**, e123

25. Georgakilas, A. G., Martin, O. A., Bonner, W. M. (2017) p21: A Two-Faced Genome Guardian. *Trends Mol Med.* **23**, 310-319

26. Gudkov, A. V., Komarova, E. A. (2005) Prospective therapeutic applications of p53 inhibitors. *Biochem Biophys Res Commun.* **331**, 726-736

27. Vermeulen, K., Van Bockstaele, D. R., Berneman, Z. N. (2003) The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell Prolif.* **36**, 131-149

28. Williams, G. H., Stoeber, K. (2012) The cell cycle and cancer. *J Pathol.* **226**, 352-364
29. Kang, C., Xu, Q., Martin, T. D., Li, M. Z., Demaria, M., Aron, L., Lu, T., Yankner, B. A., Campisi, J., Elledge, S. J. (2015) The DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4. *Science*. **349**, aaa5612

30. Macaluso, M., Montanari, M., Giordano, A. (2006) Rb family proteins as modulators of gene expression and new aspects regarding the interaction with chromatin remodeling enzymes. *Oncogene*. **25**, 5263-5267

31. Litovchick, L., Sadasivam, S., Florens, L., Zhu, X., Swanson, S. K., Velurugan, S., Chen, R., Washburn, M. P., Liu, X. S., DeCaprio, J. A. (2007) Evolutionarily conserved multisubunit RBL2/p130 and E2F4 protein complex represses human cell cycle-dependent genes in quiescence. *Mol Cell*. **26**, 539-551

32. Friedman, M. J., Li, S., Li, X. (2009) Activation of gene transcription by heat shock protein 27 may contribute to its neuronal protection. *J Biol Chem*. **284**, 27944-27951

33. Krizhanovsky, V., Yon, M., Dickins, R. A., Hearn, S., Simon, J., Miething, C., Yee, H., Zender, L., Lowe, S. W. (2008) Senescence of activated stellate cells limits liver fibrosis. *Cell*. **134**, 657-667

34. Plesca, D., Crosby, M. E., Gupta, D., Almasan, A. (2007) E2F4 function in G2: maintaining G2 arrest to prevent mitotic entry with damaged DNA. *Cell Cycle*. **6**, 1147-1152

35. Gire, V., Dulic, V. (2015) Senescence from G2 arrest, revisited. *Cell Cycle*. **14**, 297-304

36. Campisi, J., d'Adda di Fagagna, F. (2007) Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol*. **8**, 729-740

37. Thurlings, I., de Bruin, A. (2016) E2F Transcription Factors Control the Roller Coaster Ride of Cell Cycle Gene Expression. *Methods Mol Biol*. **1342**, 71-88

38. Campisi, J. (2013) Aging, cellular senescence, and cancer. *Annu Rev Physiol*. **75**, 685-705

39. Blagosklonny, M. V. (2014) Geroconversion: irreversible step to cellular senescence. *Cell Cycle*. **13**, 3628-3635

40. Terzi, M. Y., Izmirli, M., Gogebakan, B. (2016) The cell fate: senescence or quiescence. *Mol Biol Rep*. **43**, 1213-1220

41. Leontieva, O. V., Lenzo, F., Demidenko, Z. N., Blagosklonny, M. V. (2012) Hyper-mitogenic drive coexists with mitotic incompetence in senescent cells. *Cell Cycle*. **11**, 4642-4649

42. Tchkonia, T., Zhu, Y., van Deursen, J., Campisi, J., Kirkland, J. L. (2013) Cellular senescence and the senescent secretory phenotype: therapeutic opportunities. *J Clin Invest*. **123**, 966-972

43. Salminen, A., Kauppinen, A., Kaarniranta, K. (2012) Emerging role of NF-kappaB signaling in the induction of senescence-associated secretory phenotype (SASP). *Cell Signal*. **24**, 835-845
44. Orjalo, A. V., Bhaumik, D., Gengler, B. K., Scott, G. K., Campisi, J. (2009) Cell surface-bound IL-1alpha is an upstream regulator of the senescence-associated IL-6/IL-8 cytokine network. *Proc Natl Acad Sci U S A.* **106**, 17031-17036

45. Xue, W., Zender, L., Miething, C., Dickins, R. A., Hernando, E., Krizhanovsky, V., Cordon-Cardo, C., Lowe, S. W. (2007) Senescence and tumour clearance is triggered by p53 restoration in murine liver carcinomas. *Nature.* **445**, 656-660

46. Rodier, F., Campisi, J. (2011) Four faces of cellular senescence. *J Cell Biol.* **192**, 547-556

47. Zhou, X., Perez, F., Han, K., Jurivich, D. A. (2006) Clonal senescence alters endothelial ICAM-1 function. *Mech Ageing Dev.* **127**, 779-785

48. Shin, J., Hong, S., Lee, S. O., Kim, T., Park, I., An, S., Lee, W., Lim, J., Kim, K., Yang, Y., Lee, S., Jin, D., Lee, M. (2008) Serum starvation induces G1 arrest through suppression of Skp2-CDK2 and CDK4 in SK-OV-3 cells. *Int J Oncol.* **32**, 435-439

49. Collado, M., Gil, J., Efeyan, A., Guerra, C., Schuhmacher, A. J., Barradas, M., Benguria, A., Zaballos, A., Flores, J. M., Barbacid, M., Beach, D., Serrano, M. (2005) Tumour biology: senescence in premalignant tumours. *Nature.* **436**, 642

50. Zhu, Z., Xu, X., Yu, Y., Graham, M., Prince, M. E., Carey, T. E., Sun, D. (2010) Silencing heat shock protein 27 decreases metastatic behavior of human head and neck squamous cell cancer cells in vitro. *Mol Pharm.* **7**, 1283-1290

51. Deng, W., Zhang, Y., Gu, L., Cui, J., Duan, B., Wang, Y., Du, J. (2016) Heat shock protein 27 downstream of P38-PI3K/Akt signaling antagonizes melatonin-induced apoptosis of SGC-7901 gastric cancer cells. *Cancer Cell Int.* **16**, 5

52. Burban, A., Sharanek, A., Hue, R., Gay, M., Routier, S., Guillozou, A., Guguen-Guillouzo, C. (2017) Penicillinase-resistant antibiotics induce non-immune-mediated cholestasis through HSP27 activation associated with PKC/P38 and PI3K/AKT signaling pathways. *Sci Rep.* **7**, 1815

53. Liu, X1., Chua, CC., Gao, J., Chen Z, Landy, CL., Hamdy, R., Chua, BH. (2004) Pifithrin-alpha protects against doxorubicin-induced apoptosis and acute cardiotoxicity in mice. *Am J Physiol Heart Circ Physiol.* **286**, H933-939.
FOOTNOTE

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Table 1 Primers and siRNAs used for this study

| primer  | Forward                              | Reverse                             |
|---------|--------------------------------------|-------------------------------------|
| HSP27   | TCCCTGGATGTCAACCACCTCC             | TCTCCACCACGCGCCATCT                |
| Cyclin A2 | GGTACTGAAGTCCGGGAACC             | TGAACGCAGGCTGTCTTTACTG            |
| Cyclin B1 | TACTGGGTCCGGAAGTCACT          | AGCATCTTCTTGCGACACACA            |
| Cyclin B2 | CCAGAGAGCGACACAGTAGCT         | GAGAAGACCCCTTGGAGCACC            |
| Cyclin D1 | GGCAGAGGAGAGACAAACAGA      | CTCTCTAGGCTTGCAAGCCCTTG          |
| Cyclin D2 | TGCGAAGAGGACACATCCACCC       | GCGAAGAAACGGTGACAGGA             |
| Cyclin D3 | TGCACTGATTCTTCGGCTCT        | TCGAGGATGGCGGGTGATATGA         |
| Cyclin E1 | GGAAGAGGAGGCAAACGTGA       | TTTATTGTCCCAAGGCTGGCTCAA       |
| CDC25c  | ACTGCCACTCGAGTTACCAC       | AGCTGTGCTGGGAGCTACATT            |
| cdcA3   | TCCACTAGTGCTGGCATCC          | TAGGAGAGCAGGGGATCTGAG            |
| CDK1    | CTGGGGTGACTCCTCGTACTCT       | GGAGTGGCCAAAGCTGGTCTGGA           |
| CDK2    | TCAAGCTGGCTGGATGTCAATTCA      | CAGTGAGAGAGACAGAGCATCCAT          |
| p21    | CTGGAGACTCTCGAGCCGCTA      | CAGGACGTCAGCTTGCTCGTACT          |
| p22    | TTGGAGGAGGAGGCAAGCTGA    | TCCAGCTCCTCCGACAGTGGTGATGAG       |
| p16    | CCCACGCACGAGTGAAGATTA    | ACCAGCCTGTCCAGGAAG               |
| E2F-4   | GGAAGCCTCACGCTCACAATA       | TGGAGCTCACCAGTCTTGTCCTGG         |
| E2F-5   | GTGGCTACAGCAAGCATCA         | TGGCCAAAAGTGATCTACCA             |
| E2F-6   | CAGGCCTTCCTGCTGAGAT         | CCTGCTCCACTCCACAAACAA            |
| E2F-7   | AGGCCAAAGCAGAAACAGA         | TCCACACCAAGACTGAGAC              |
| E2F-8   | AGTGCTGCTGAGCCCTGAGAT      | TCCAGCTTGTGTTGGGATGTTG           |
| RB1     | GAACATCGAATCATGAATCCCT     | AGAGGACAAGCAGATCCACTAACTGAT      |
| p107    | CCCGCACAAGAGAAATGGTGAGAG  | ACAGACGCGTTTGGGGAAGGG            |
| p130    | ATTTGGCATGGAAGACAGA        | ATCTGCCCCTTCCAGGTCTT             |
| IL-1α   | CGCCATGACTCAGAGGAAGA       | AGGGCGTCATCCAGGATGAA            |
| IL-1β   | AATCTGTACCTGTCTGGATGTT     | TGGGTAATTTTTGATCTACACTCT         |
| IL-6    | GGTACATCCTCGACAGGCATCT   | GTGCTGCTTTTGGCTCTGGTCTC         |
| IL-8    | CTGGAGCATGCTTCCCTGATT     | TCCCTTTTGGAGTGCTGTCTGAG          |
| β-actin | GGACATCCGGCAAGACCTGTA    | TGCACTGCTGTGGGGAAGTGGT          |
| GAPDH   | GATCCACCAGTCGGCAAATT      | GATGTTGATGGGATTTCCATTG          |

| siRNA   | Forward                              | Reverse                             |
|---------|--------------------------------------|-------------------------------------|
| HSP27   | UGAGAGACUGCCGCGCCAGAUA               | UUACUUGGCACAGCCAGUCUAUU             |
| E2F-4a *| GCGGCAGGUAUAAACGACAUUdTdT          | AAGUGCUAAUAAUCCGCGCCGdTdT          |
| E2F-4b  | GGCAGAGAGACUGAGGGAGCUGdTdT       | CAGCUCCGACACUCUCUGCdTdT            |
| p130a   | GACGACAGUCAUUACGACAGUC          | AAAUCCAGUAAAGCUCAGUC              |
| p130b   | UACUUUAUAGAAACGACAGUC        | GAUGCUUAUAAUAGUAAU                 |
| control | UUUCUGAAGCAGCGAGCAGUdTdT      | ACGUGACACGCUUCGAGAAdTdT           |

* We prepared two siRNA sets for E2F-4 and p130 and confirmed similar results.
E2F-4a and p130a siRNA were chosen for following study.
Figure legends

Figure 1. Upregulation of HSP27 in serum-starved and -refed MRC-5. Cells were serum-starved for 24 hours (h), refed with 5% fetal bovine serum (FBS), and harvested at indicated time points. (A) Cell cycle analysis. Cells were serum-starved for 24 h and treated with or without FBS for 24 h. Cell cycles were determined using a MUSE cell analyzer. The representative results of four independent experiments are shown. (B) Immunoblot analysis. Protein levels of HSP27 and p-HSP27 were analyzed using an immunoblot analysis of cell lysates from control and FBS-treated MRC-5. For a loading control, β-actin was used. The representative results from four independent experiments are shown in the left. Quantitative data are shown in the right by mean ± SEM (n=4). *P<0.05. (C) A depiction of the cell cycle with cyclins, cyclin dependent kinase (CDKs), and related molecules involved in each cell cycle phase. (D) Expression of cell cycle regulatory genes. RNAs were isolated from cells treated with FBS for 0 h (open), 12 h (hatched), 24 h (gray), or 48 h (closed). The relative expression levels of cell cycle regulatory genes were determined using quantitative PCR (qPCR) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control for normalization. *P<0.05 vs without FBS (0).

Figure 2. Cell cycle arrest by HSP27-knockdown. Cells were transfected with control siRNA (cont-KD) or HSP27 siRNA (HSP27-KD). (A) Cell proliferation. CCK8 kit was used to monitor cell proliferation. Open and closed circles indicate control siRNA and HSP27 siRNA transfected cells, respectively. Data are shown as mean ± SEM (n=6). *P<0.05. (B) Cell cycle analysis. Two days after siRNA transfection, cell cycles were determined using a MUSE cell analyzer. The representative results of four independent experiments are shown. (C) Expression of cell cycle regulatory genes. Two days after siRNA transfection, the relative expression levels of the indicated cell cycle regulatory genes were determined using qPCR with GAPDH as an internal control for normalization. *P<0.05 vs control (decrease); †P<0.05 vs control (increase).

Figure 3. HSP27-knockdown upregulates E2F-4 and p130 expression. (A) Quantitative PCR. The relative expression levels of E2F-4 and p130 genes were determined using qPCR with GAPDH as an internal control for normalization. *P<0.05. (B) Immunoblot analysis. Protein levels of E2F-4 and p130 in nuclear extracts from cells transfected with control or HSP27 siRNA were determined by immunoblot analysis. For a loading control, histone H3 was used. Quantitative data are shown as mean ± SEM (n=4). *P<0.05. (C) Immunofluorescence staining. Cells were transfected with control siRNA or HSP27 siRNA and cultured for 48 h. Immunofluorescence staining was performed for E2F-4 (red) and p130 (green).
Nuclear staining was performed with To-Pro3 (blue). Scale bar = 20 µm. (D) Protein chasing experiment. Cells were transfected with control or HSP27 siRNA. After 48 h, cells were treated with 20 µM cycloheximide. Cell lysates were prepared at indicated time points. Immunoblot analysis was performed for E2F-4 and p130. Signal intensities were measured with ImageJ software. Data are shown as mean ± SEM (n=4). *P<0.05.

Figure 4. E2F-4/p130 mediates cell cycle arrest by HSP27-knockdown. Cells were transfected with control siRNA, HSP27 siRNA, E2F-4 siRNA and/or p130 siRNA as indicated and cultured for 48 h. (A, E) Immunoblot analysis. Protein levels of HSP27, E2F-4 or p130 in cell lysates were determined by immunoblot analysis. For a loading control, β-actin was used. (B, F) Total cell numbers. *: † P<0.05 vs control siRNA; †P<0.05 vs HSP27 siRNA. (C, G) Cell cycle analysis. (D, H) Expression of cell cycle regulatory genes. qPCR was performed with GAPDH as an internal control for normalization. †P<0.05 vs HSP27 siRNA; *P<0.05 vs control siRNA.

Figure 5. Cellular senescence induced by HSP27-knockdown. (A) β-Galactosidase staining. Cells were transfected with control siRNA, HSP27 siRNA, HSP27 siRNA + p130 siRNA, or HSP27 siRNA + E2F-4 siRNA. Three days after transfection, cells were fixed with 4% paraformaldehyde, stained for β-gal (blue), and counter-stained with DAPI. Total cells and cells positive for β-gal were analyzed by ImageJ software. The representative results are shown on the left. Quantitated data are shown on the right as mean ± SEM (n=4). *P<0.05 vs control siRNA; †P<0.05 vs HSP27 siRNA. (B) Expression of pro-inflammatory cytokines. Three days after siRNA transfection, the relative expression levels of proinflammatory cytokines were determined by qPCR. *P<0.05 vs control siRNA. (C) Expression of p16. The mRNA and protein levels of p16 were determined by qPCR (left) and immunoblot assay (right), respectively, with GAPDH as the internal control for normalization. Data are shown as mean ± SEM (n=4). *P<0.05. (D) Cell cycle analysis of senescent cells. Control siRNA- or HSP27 siRNA-transfected cells were re-seeded in an 8-well chamber slide after 24 h culture. Three days after transfection, cells were fixed with 4% paraformaldehyde and stained with SPiDER-βGal (red), followed by immunofluorescence staining with antibody against Ki-67 (blue) or cyclin B1 (blue). Scale bar = 20 µm. Arrows indicate senescence cells.

Figure 6. Analysis of oxidative cell damage. Cells were transfected with control siRNA (cont) or HSP27 siRNA (HSP27). (A) Protein oxidation and ubiquitination. These were determined by Oxiselect™ protein carbonylation immunoblot kit and immunoblot analysis with anti-ubiquitin antibody, respectively. (B)
DNA damage analysis. Immunoblot analysis was performed for a DNA damage marker phospho-Histone 2X (P-H2X). Cells treated with ultraviolet light were used as a positive control (UV). (C) Immunoblot analysis. Protein levels of p53 and p21^Cip1 in cell lysates were determined by immunoblot analysis. GAPDH shown as a loading control is identical to that of figure 5C. (D) Effect of pifithrin-α, a p53 inhibitor. One day after control siRNA or HSP27 siRNA transfection, cells were incubated with or without 10 μM of pifithrin-α (pif) for 24 h. Cell number and cell cycle were determined using a MUSE cell analyzer. Cell number data are shown as mean ± SEM (n=4). *P<0.05. The representative cell cycle results of four independent experiments are shown.
Figure 1. Upregulation of HSP27 in serum-starved and -refed MRC-5. Cells were serum-starved for 24 hours (h), refed with 5% fetal bovine serum (FBS), and harvested at indicated time points. (A) Cell cycle analysis. Cells were serum-starved for 24 h and treated with or without FBS for 24 h. Cell cycles were determined using a MUSE cell analyzer. The representative results of four independent experiments are shown. (B) Immunoblot analysis. Protein levels of HSP27 and p-HSP27 were analyzed using an immunoblot analysis of cell lysates from control and FBS-treated MRC-5. For a loading control, β-actin was used. The representative results from four independent experiments are shown in the left. Quantitative data are shown in the right by mean ± SEM (n=4). *P<0.05. (C) A depiction of the cell cycle with cyclins, cyclin dependent kinase (CDKs), and related molecules involved in each cell cycle phase. (D) Expression of cell cycle regulatory genes. RNAs were isolated from cells treated with FBS for 0 h (open), 12 h (hatched), 24 h (gray), or 48 h (closed). The relative expression levels of cell cycle regulatory genes were determined using quantitative PCR (qPCR) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control for normalization. *P<0.05 vs without FBS (0).
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Heat shock protein 27 promotes cell cycle progression by down-regulating E2F transcription factor 4 and retinoblastoma family protein p130

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