Heat shock protein 70 inhibits hydrogen peroxide-induced nucleolar fragmentation via suppressing cleavage and down-regulation of nucleolin

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Abstract It has been reported that nucleolar fragmentation is a part of the overall apoptotic morphology, however, it is currently obscure whether and how nucleolar fragmentation can be induced by hydrogen peroxide (H_2O_2) and heat shock protein 70 (Hsp70) can prevent nucleolar fragmentation. To dissect these two questions, C_2C_{12} myogenic cells and immortalized mouse embryonic fibroblasts (MEFs) with heat shock transcriptional factor 1 (HSF1) null mutation were treated with heat shock response (HS) ($42.5 \pm 0.5^\circ C$ for 1 h and recovery at $37^\circ C$ for 24 h) and then were insulted with 0.5 mmol/L H_2O_2 . Morphological changes of nucleoli were observed under contrast microscope or electronic microscope. It was found that (1) stimulation with H_2O_2 -induced nucleolar fragmentation by mediating cleavage and down-regulation of nucleolar protein, nucleolin in C_2C_{12} myocytes and MEFs; (2) HS

suppressed nucleolar fragmentation by inducing the expression of Hsp70 in an HSF1-dependent manner as indicated by assays of transfection with Hsp70 antisense oligonucleotides (AS-ONs) or recombinant plasmids of full-length Hsp70 cDNA; (3) protection of Hsp70 against nucleolar fragmentation was related to its accumulation in nucleolus mediated by nuclear localization sequence and its inhibition against cleavage and down-regulation of nucleolin. These results suggested that H_2O_2 -induced nucleolar fragmentation and HS or Hsp70 inhibit H_2O_2 -induced nucleolar fragmentation through the translocation of Hsp70 into nucleolar and its protection against impairment of nucleolin.

Keywords Nucleolus · Nucleolar fragmentation · Hydrogen peroxide · Heat shock protein 70 · Nucleolin

Abbreviations

AS-ONs	Antisense oligonucleotides
S-ONs	Sense oligonucleotides
HS	Heat shock response
HSF1	Heat shock transcriptional factor 1
Hsps	Heat shock proteins
Hsp70	Heat shock protein 70
H_2O_2	Hydrogen peroxide
MEFs	Mouse embryonic fibroblasts
NLS	Nuclear localization sequence

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Introduction

The nucleolus is formed around ribosomal DNA (rDNA) repeats in eukaryotic cells, which cluster at chromosomal loci called nucleolar organizers. A functional nucleolus is crucial for cell survival because of involvement in ribosome

biogenesis, cell cycle progression, gene silencing, ribonucleoprotein complex formation, and sensing cell stress (Boisvert et al. 2007; Heun 2007; Boulon et al. 2010; Guerrero and Maggert 2011). In quiescent cells, or cells subjected to transcriptional arrest, a phenotype of nucleolar segregation/fragmentation is observed, in which the granular and dense fibrillar nucleolar RNA components, are separated from the central fibrillar DNA component (Halicka et al. 2000; Fraschini et al. 2005). Many evidences have suggested that the nucleolar segregation/fragmentation was induced by actinomycin D and adriamycin (Fraschini et al. 2005), roscovitine (Wojciechowski et al. 2003), cisplatin (Horky et al. 2001), heat stress (Pelham 1984; Morcillo et al. 1997) and UV irradiation (Al-Baker et al. 2004). Evidence suggests that nucleolar segregation/fragmentation is a significant and early process during apoptosis (Horky et al. 2001; Fraschini et al. 2005; Suzuki et al. 2007).

It is well known that oxidative stress is a common causative mechanism in cardiovascular disease involving ischemia–reperfusion. Reactive oxygen species produced during oxidative stress in the cardiovascular system are involved in apoptosis (Hulsmans and Holvoet 2010; Touyz and Briones 2011). Hydrogen peroxide (H_2O_2) is one of the most frequently and widely used oxidants for the study of apoptotic cell death. We have previously demonstrated that H_2O_2 treatment of cardiomyocytes and C_2C_{12} cells results in apoptosis by inducing the release of the second mitochondria-derived activator of caspases and cytochrome C from mitochondria with subsequent activation of caspase-8, -9, -3 (Jiang et al. 2005a, b; Liu et al. 2007; Sun et al. 2011). It is currently obscure whether and how H_2O_2 could induce the nucleolar fragmentation in myocytes.

Heat shock proteins (Hsps) are a family of molecular chaperones, which are indispensable in physiological states and exhibit a protective role in pathological processes. Many conditions of environmental stress including heat shock, oxidative stress, etc. induce the expression of Hsps (Kregel 2002; Toko et al. 2008; Khalil et al. 2011). Hsps have been classified in four families according to their molecular size: Hsp90, Hsp70, Hsp60, and small Hsps (Benjamin and McMillan 1998; Kregel 2002; Toko et al. 2008). As molecular chaperones, they function mainly to modulate the assembly, transport, and folding of other proteins. The inducible expression of Hsps is regulated by heat shock transcription factor 1 (HSF1) (Benjamin and McMillan 1998; Akerfelt et al. 2007). Hsp70 is the best characterized endogenous factor involved in protecting cells, tissues, and organs from injury under various pathological conditions in *in vivo* and *in vitro* experimental models (Yenari et al. 2005; Evdonin et al. 2006; Peng et al. 2010; Yao et al. 2011). Several groups have demonstrated that Hsp70 inhibits heat shock-induced nucleolar fragmen-

tation (Pelham 1984; Morcillo et al. 1997). However, it remains unclear whether and how Hsp70 inhibits the H_2O_2 -induced nucleolar fragmentation in myocytes.

In the present study, we hypothesized that H_2O_2 could induce nucleolar fragmentation both in C_2C_{12} myogenic cells and mouse embryonic fibroblasts (MEFs) and that Hsp70 could inhibit H_2O_2 -induced nucleolar fragmentation in myocytes by redistribution from the cytoplasm to the nucleolus.

Material and methods

Cell culture and treatment C_2C_{12} myogenic cell lines (ATCC) and embryonic fibroblasts (made by ourselves, see below) were grown in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Cells were maintained at 37°C in 95% air, 5% CO_2 in a fully humidified incubator. The cells were grown to 80% confluence and then treated with H_2O_2 .

Heat shock response (HS) was performed according to the methods reported previously (Jiang et al. 2005a, b). Briefly, subconfluent culture cells in 50-mm dishes or six-well plates were subjected to hyperthermia of $42\pm0.5^\circ C$ for 1 h with a water bath. As a control, cells were cultured under normal conditions without hyperthermia. Cells were then routinely allowed to recover for 24 h at 37°C in a humidified atmosphere containing 5% CO_2 , and then cells were used for the following experiments.

Construction of immortalized MEFs from HSF1 knock-out mice HSF1 heterozygous ($HSF1^{+/-}$) female mice with the mixed genetic background C-129XI (129XI/SvJ $\times$ BALB/C) were mated with HSF1 heterozygous ($HSF1^{+/-}$) male mice (kind gifts from Dr. Ivor Benjamin, Center for Cardiovascular Translational Biomedicine, Division of Cardiology, University of Utah School of Medicine, Salt Lake City, UT, USA). MEFs were isolated according to the method described previously (McMillan et al. 1998). Briefly, embryos were collected at E14, dissociated by incubation in 0.25% trypsin (Life Technologies, Inc.), and cultured. Genomic DNA was extracted for genotype identification from the remains of embryos. Cells were immortalized by stable transfection of SV40 large T antigen, using Lipofectamine 2000™ (Invitrogen) according to the manufacturer's instructions and screened with G418 (500 μ g/ml) for 4 weeks. Genotype identifications were performed by detecting the HSF1 gene and the replacement gene neomycin with polymerase chain reaction (PCR). The responsible primers was indicated as follows, HSF1 forward primer, 5'-TCTCCTGTCCTGTGTGCCTACG-3'; HSF1 reverse primer, 5'-CAGGTCAACTGCCTACACA

GACG-3'; neomycin forward primer, 5'-AGGACATAGCGTTGGCTACCCGTC-3'; neomycin reverse primer, 5'-GCCTGCTATTGTCTTCCCAATCG-3'.

Lipofectamine-mediated Hsp70 gene loss- and gain-of-function C₂C₁₂ cells were transfected with Hsp70 antisense oligonucleotids (AS-ONs) for gene loss-of-function assay or transfected with eukaryotic expression plasmids for gene gain-of-function assay by using Lipofectamin 2000™ according to a previously described procedure (Ito et al. 1999; Tang et al. 2007). Sequence of sense oligonucleotides (S-ONs) and AS-ONs (phosphorothionate conjugated oligo.) of Hsp70 are as follows: 5'-ATGGCCAAGAAAACAGC GATCGG-3' and 5'-CCGATCGCTGTTTTCTTGCCAT-3'. Sequence of S-ONs and AS-ONs of nucleolin are as follows: 5'-GCCAGCCTTTGCGAGCTTCACCAT-3' and 5'-ATGGTGAAGCTCGCAAAGGCTGGC-3'. Hsp70 eukaryotic expression plasmids pcDNA3.1 with full-length cDNA of Hsp70 (pcDNA3.1-Hsp70FL) or with deletion in nuclear localization sequence (pcDNA3.1-ΔNLS) was constructed according to the method described previously (Tang et al. 2007). After transfection with AS-ONs or plasmid for 24 h, cells were treated with HS and H₂O₂ for further study.

Nucleoli staining After H₂O₂ treatment, cells cultured on the coverslips were washed twice with cold phosphate-buffered saline (PBS). Nucleoli staining was performed according to the procedure described previously (Horky et al. 2001). Briefly, after rinse with PBS, Cells were fixed with 4% paraformaldehyde/1% Triton X-100/PBS for 10 min at room temperature. Then cells were allowed to dry on the slides, briefly immersed in 1% toluidine blue in 1% sodium tetraborate solution, rinsed in water, mounted in 80% glycerol, and immediately photographed under a contrast microscope. The numbers of stained nucleoli per cell were counted from five visual fields of each sample from at least three samples.

Western blot analysis For Western blot analysis, cells were scraped and collected in 10-ml tube, washed twice with PBS and resuspended with 5 vol. of lysis buffer (50 mM Tris-HCl (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% Nonidet P-40) supplemented with protease inhibitor mixture (Roche Applied Science). The cell lysate was incubated on ice for 30 min and then centrifuged at 10,000×g for 10 min at 4°C. The protein content of the supernatant was determined by the Bradford assay (Bio-Rad) and diluted to 1 mg/ml. After added appropriate 6× SDS loading buffer, equal amounts of protein (20–30 µg) were loaded and separated on 10% SDS-PAGE and then transferred electrophoretically onto nitrocellulose membranes. Blots were blocked with 2% albumin in TBST (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween)

overnight at 4°C and then probed with primary antibody. The immune complexes were visualized with an HRP-conjugated secondary antibody and DAB staining kit (Boster Biological Technology, China). Bands of target protein were scanned and quantified with the Band Leader software (Shanghai, China).

Electron microscopy Cells were harvested and rinsed twice with cold PBS at the appropriate time after H₂O₂ treatment. The specimens were fixed in a mixture of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 1 h at 4°C, postfixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in a graded series of ethanol, and embedded in epoxy resin. Ultrathin section were made on a LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a JEOL 1200 EX electron microscope.

Isolation of nucleoli Nucleoli was isolated from C₂C₁₂ myogenic cells as previously described (Lam et al. 2007). Briefly, the cytoplasm extracts were prepared from about 5×10⁷ cells firstly. The pellets containing about 5×10⁷ nuclei were washed three times with PBS, resuspended in 5 ml buffer A (10 mM HEPES-KOH [pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT), and dounce homogenized ten times using a tight pestle. Dounced nuclei were centrifuged at 228×g for 5 min at 4°C. The nuclear pellet was resuspended in 3 ml 0.25 M sucrose/10 mM MgCl₂, and layered over 3 ml 0.35 M sucrose/0.5 mM MgCl₂, and centrifuged at 1,430×g for 5 min at 4°C. The clean, pelleted nuclei were resuspended in 3 ml 0.35 M sucrose/0.5 mM MgCl₂, and sonicated for 6×10 s using a microtip probe and a Misonix XL 2020 sonicator at power setting 5. The sonicate was checked using phase contrast microscopy, ensuring that there were no intact cells and that the nucleoli were readily observed as dense, refractile bodies. The sonicated sample was then layered over 3 ml 0.88 M sucrose/0.5 mM MgCl₂ and centrifuged at 2,800×g for 10 min at 4°C. The pellet contained the nucleoli. The nucleoli were then washed twice by resuspension in 500 µl of 0.35 M sucrose/0.5 mM MgCl₂, followed by centrifugation at 2000×g for 2 min at 4°C.

Immunocytochemistry C₂C₁₂ cells were grown on 18×18-mm glass coverslips. After 24 h, cells were transfected with full-length Hsp70 plasmid for 24 h, and then cells were exposed to 0.5 mmol/L H₂O₂. At the appropriate time, cells were washed twice with PBS and fixed with 2% paraformaldehyde for 15 min at 4°C. Samples were then treated with 3% H₂O₂ for 10 min at 4°C and were permeabilized using antibody buffer (0.5% Triton X and 0.2% NP-40 in PBS) for 30 min at 4°C and blocked for 30 min at 4°C using antibody buffer containing 5% BSA. Cells were then

incubated with mouse anti-Hsp70 monoclonal antibody (final concentration of 2 $\mu\text{g/mL}$) (Stressgen) overnight at 4°C. After thorough washing in antibody buffer, appropriate anti-mouse secondary antibodies coupled with biotin (Boster Biological Technology, China) were applied to the samples for 1 h at 4°C. After thorough washing in antibody buffer, appropriate anti-biotin third antibody coupled with HRP was applied to the samples for 1 h at 4°C. Coverslips were then washed three times for 15 min with PBS, mounted on glass slides using glycerol:PBS solution and sealed. Cells were photographed under the contrast microscope.

Expression of results and statistical analysis Data are represented as means $\pm$ S.E.M. of the number of independent experiment indicated (*n*) or as examples of representative experiments performed on at least three separate occasions. Significance of differences between groups was determined by two-tailed Student's *t* test or Kruskal–Wallis test. $P < 0.05$ was considered significant.

Results

H₂O₂-induced nucleolar fragmentation in C₂C₁₂ myocytes

Stress-induced nucleolar fragmentation was previously reported in cisplatin- or adriamycin-treated HeLa cells and heat shock-treated COS cells (Morcillo et al. 1997; Horky et al. 2001; Fraschini et al. 2005). Here, C₂C₁₂ myocytes were exposed to 0.5 mmol/L of H₂O₂ for 6, 12, and 24 h and then cells were stained with 1% toluidine blue. Results showed that untreated C₂C₁₂ cell has generally one or two compact nucleoli localized in the central portion of the nucleus, while H₂O₂-treated cell showed many small nucleolar fragments (5–12 dots) scattered in the nucleus (Fig. 1a and supplementary Figure 1). The numbers reached the maximum (average 9.5 stained dots) at 12 h after exposure to H₂O₂.

Dose–effect analysis showed that 0.5 mmol/L of H₂O₂ is the best concentration to induce nucleolar fragmentation (Fig. 1a). A low concentration of H₂O₂ (0.

Fig. 1 Heat shock response (HS) inhibited H₂O₂-induced nucleolar fragmentation in C₂C₁₂ myogenic cells. **a** Time and dose course of H₂O₂-induced nucleolar fragmentation. Cells were cultured on slides and treated with or without 0.5 mmol/L H₂O₂ for appropriated time (*left panel*) or treated with different concentration of H₂O₂ for 12 h (*right panel*). Then cells were stained with toluidine blue (TB) for 10 min at room temperature as described under **Material and methods** and then were imaged with phase contrast microscopy. The numbers of stained nucleolus were counted from five visual fields of each sample from at least three samples. * $P < 0.05$ and ** $P < 0.01$ compared to H₂O₂-untreated control (Con) cells. **b** Inducible expression of Hsps by HS. Cells were cultured in 100-ml flasks and bathed for 1 h at 42.5 $\pm$

0.5°C and recovered for 24 h at 37°C. Then the whole cell lysates were extracted and Western blotting was performed as mentioned under **Material and methods**. Several Hsps were detected and β -actin also was tested as loading control. Data was from one of three independent experiments and the density of protein band was analyzed by using Bandleader 3.0 software. ** $P < 0.01$ compared to unheated control (Con) cells. **c** Inhibitory effect of HS on H₂O₂-induced nucleolar fragmentation. HS or without HS treatment cells were exposed to 0.5 mmol/L H₂O₂ for 12 h and then were stained by using TB and numbers of stained nucleolus was measured as mentioned in **a**. ** $P < 0.01$ compared to H₂O₂-untreated control (Con) cells or HS plus H₂O₂-treated (HS + H₂O₂) cells

25 and 0.125 mmol/L) almost can not induce fragmentation of nucleoli. Although high concentration (1 and 2 mmol/L) led to nucleoli fragments, it was less than that induced by 0.5 mmol/L of H_2O_2 . Cell survival analysis showed that H_2O_2 promoted proliferation of C_2C_{12} cells instead of apoptosis or necrosis at low concentration (0.25 and 0.125 mmol/L), while treatment with high concentration of H_2O_2 (1 and 2 mmol/L) induced necrosis rather than apoptosis as indicated by analysis of lactate dehydrogenase release and apoptotic percentage (supplementary Figure 2).

HS inhibited nucleolar fragmentation by inducing the expression of Hsps in an HSF1-dependent manner

C_2C_{12} myogenic cells were subjected to heat shock ($42 \pm 0.5^\circ\text{C}$ for 1 h and recovery for 24 h at 37°C). Trypan blue exclusion was done to assess the percentage of cell death and less than 5% of cells exhibited an altered morphology or uptake of trypan blue, indicating that membrane integrity was not disrupted (data not shown). PI staining and flow cytometry assay showed that percentage of apoptotic cell death is less than 5% (supplementary Figure 3). Nucleoli staining displayed that after recovery for 24 h at 37°C , heat-shocked cells showed also one or two compact and central localized nucleoli (supplementary Figure 4). A high level expression of Hsp25, Hsp70, and Hsp90 was shown by immunoblotting in heat-shocked cells as compared with control cells (Fig. 1b). Heat-shocked cells were then stimulated with 0.5 mmol/L H_2O_2 for 12 h. Toluidine blue staining showed less nucleolar fragments in

nucleus (Fig. 1c and supplementary Figure 4) than cells treated only with H_2O_2 . These results suggested that HS inhibit H_2O_2 -induced nucleolar fragmentation in C_2C_{12} myogenic cells and it might be related to HS-inducible expression of Hsps.

The inducible expression of Hsps is regulated mainly by transcription factor HSF1 (Akerfelt et al. 2007; Toko et al. 2008). In the present study, MEFs were immortalized from $\text{HSF1}^{-/-}$ and $\text{HSF1}^{+/+}$ mice which genotype was confirmed by PCR respectively (Fig. 2a). To make sure that deficiency of HSF1 abrogate inducible expression of Hsps and block the protection of HS against nucleolar fragmentation, MEFs were heated for 1 h at $42 \pm 0.5^\circ\text{C}$ and recovered for 24 h at 37°C , and then whole cell lysate was prepared to detect the expression of inducible Hsp70. Immunoblotting assay showed that HS-induced high expression of Hsp70 in $\text{HSF1}^{+/+}$ MEFs but not $\text{HSF1}^{-/-}$ MEFs (Fig. 2b). Twelve hours after heat-treated or untreated MEFs exposure to H_2O_2 , nucleoli staining showed that HS inhibited significantly H_2O_2 -induced nucleolar fragmentation in $\text{HSF1}^{+/+}$ MEFs but not in $\text{HSF1}^{-/-}$ MEFs (Fig. 2c). These results suggested that HS protected H_2O_2 -mediated nucleolar fragmentation via HSF1-induced Hsps expression.

Down-regulation of Hsp70 abolished the protection of HS against nucleolar fragmentation

It is well documented that Hsp70 is the most important and abundant member in Hsps superfamily (Benjamin and McMillan 1998; Evdonin et al. 2006; Akerfelt et al. 2007; Toko et al. 2008). To clarify whether Hsp70 played

Fig. 2 HSF1 deficiency abolished the protection of HS against nucleolar fragmentation. **a** Mouse embryonic fibroblasts (MEFs) genotypes identified by PCR and 1.2% agarose gel electrophoresis. MEFs were transfected with pSV3-neo recombinant plasmid containing SV40 large T antigen and CMV promoter. After selection with G418 for a month, the genotypes were identified by PCR. "Control" is negative control without DNA template. **b** Expression of Hsp70 shown by Western blotting. The HSF1 wide type (+/+) and HSF1 knock-out homozygous (-/-) MEFs were treated with HS as in

Fig. 1b and the expression of Hsp70 was detected by Western blotting. Result from three parallel experiments. $**P < 0.01$ compared to unheated control (Con) cells or heated $\text{HSF1}^{-/-}$ cells. **c** nucleolar fragmentation shown by TB staining. $\text{HSF1}^{+/+}$ and $(-/-)$ MEFs with or without HS treatment were exposed to H_2O_2 for 12 h and nucleolar fragmentation was detected by TB staining and analyzed as described under Material and methods. $**P < 0.01$ compared to H_2O_2 -untreated control (Con) cells; $##P < 0.01$ compared to H_2O_2 -untreated HS cells or HS plus H_2O_2 -treated $\text{HSF1}^{+/+}$ cells

Fig. 3 Hsp70 antisense (AS) oligonucleotides (ONs) abrogated the protection of HS against nucleolar fragmentation. **a** Expression of Hsp70 in AS-ONs-transfected C₂C₁₂ cells shown by Western blotting. Cells were transfected with AS-ONs or sense (S)-ONs for 12 or 24 h as described under [Material and methods](#) before HS, and then cells lysates were prepared and Western blotting was performed to test the expression of Hsp70 and β -actin. The bars indicated standard errors of the mean calculated from results derived from three independent experiments. ** P <0.01 versus control (Con) cells; ## P <0.01 com-

pared to HS-treated (HS) or S-ONs-treated cells. **b** Effect of AS-ONs on the protection of Hsp70 against nucleolar fragmentation. Cells were transfected with AS-ONs or S-ONs for 12 h before HS, and then cells were exposed to 0.5 mmol/L H₂O₂ for 12 h. Nucleolus was stained by TB and the numbers of stained nucleolus were counted as mentioned above. ** P <0.01 compared to H₂O₂-untreated cells. ## P <0.01 versus H₂O₂ alone treated control (Con) cells or AS plus HS plus H₂O₂-treated cells (AS). **c**, nucleolar fragmentation shown by electron microscopy. Scale bar represents 0.83 μ m (magnification $\times$ 12,000)

important functions in the protection of HS against nucleolar impairment, we synthesized artificially the AS-ONs of Hsp70 according to the sequence described previously (Ito et al. 1999). Results showed that HS-induced expression of Hsp70 protein were down-regulated completely at 12 or 24 h after transfection with AS-ONs. However, the corresponding S-ONs did not affect HS-enhanced expression of Hsp70 in C₂C₁₂ myogenic cells (Fig. 3a). Down-regulation of Hsp70 was not due to the decrease of cell numbers because only 10% apoptosis mediated by AS-ONs transfection is not enough to eliminate the expression of Hsp70 (supplementary Figure 3).

Nucleolus staining revealed that Hsp70 AS-ONs abolished remarkably the protection of HS against nucleolar fragmentation while S-ONs did not disturb HS-mediated protection (Fig. 3b). Further electron microscopic analysis showed a very dense and central nucleolus within the nucleus of normal control C₂C₁₂ myogenic cell, and scattered nucleolar segments in H₂O₂-treated cell, and showed that HS maintained an almost normal morphology

of nucleolus after H₂O₂ exposure, and AS-ONs of Hsp70 abrogated the protection of HS (Fig. 3c). These results suggested apparently that Hsp70 played important roles in the protection of HS against nucleolar impairment.

Over-expression of Hsp70 inhibited nucleolar fragmentation

To further understand the protection of Hsp70 against H₂O₂-induced nucleolar fragmentation, a eukaryotic expression plasmid containing the full-length cDNA of Hsp70 (pcDNA3.1-Hsp70) was constructed and delivered into C₂C₁₂ cells as described previously (Tang et al. 2007). The over-expression of Hsp70 was confirmed by using immunoblotting assay (Fig. 4a). Cells transfected with Hsp70 plasmids were exposed to H₂O₂ for 12 h. Nucleolus staining showed that numbers of nucleolar segments induced by H₂O₂ in Hsp70-expressed cells was less than that of control plasmid-transfected cells (Fig. 4b). It provided important evidence for Hsp70-attenuated H₂O₂-induced nucleolar fragmentation in myocytes.

Fig. 4 NLS is essential motif for accumulation of Hsp70 in the nucleus and nucleolus and its protection against nucleolar fragmentation. **a** Expression of Hsp70 in cells transiently transfected with FL Hsp70 or its deletion mutant at nuclear localization sequence (NLS). Cells were transfected transiently with FL Hsp70 (FL) or its NLS-truncated mutant (Δ NLS) or control plasmid (neo) for 24 h, and the whole cell lysates were prepared for Western blotting assay. At the same time, HS-treated (HS) or untreated (Con) cells were used as the positive or negative control respectively. Relative density was analyzed as mentioned above. $**P < 0.01$ compared to negative control cells (Con) or control plasmid-transfected cells (neo). **b** Nucleolar fragmentation shown by TB staining. After 24-h transfection with FL Hsp70 (FL) or its NLS-truncated mutant (Δ NLS) or control plasmid (neo), cells were treated with or without 0.5 mmol/L H₂O₂ for 12 h, and then cells were stained by TB and the numbers of stained nucleolus were counted as mentioned above. $**P < 0.01$ compared to H₂O₂-untreated cells, and $##P < 0.01$ compared to NLS-truncated

mutant (Δ NLS) or control plasmid (neo) cells. **c** Subcellular distribution of Hsp70 shown by immunocytochemistry. The cells transfected transiently with full-length (FL) Hsp70 were grown on coverslips. After 30 min or 1 h of treatment with H₂O₂, cells were fixed with 4% paraformaldehyde and then were incubated with mouse anti-Hsp70 monoclonal antibody and biotin-coupled goat anti-mouse IgG and HRP-conjugated anti-biotin antibody respectively and then stained with DAB staining kits (Boster Biotech, China). The arrow indicated the nucleolus and scale bar is 25 μ m (amplification $\times 400$). **d** Subcellular distribution of Hsp70 shown by Western blotting. Cells were treated with or without 0.5 mmol/L H₂O₂ for 1 h after transfection for 24 h with FL Hsp70 (FL) or its NLS-truncated mutant. Subcellular fractions were prepared as described under [Materials and methods](#), HS-treated cells were used as positive control, and Bax or fibrillarin were choose as the marker for cytoplasmic (Cyto) and nucleolar (Nucl) fractions respectively. Result is representative of three independent experiments

NLS is the essential motif for Hsp70 attenuating nucleolar fragmentation

We further found that Hsp70 localized in cytosol under normal condition and redistributed into nucleus and nucleolus under oxidative stress (exposure to H₂O₂) as indicated by immunocytochemistry assay (Fig. 4c). It is reported that redistribution from cytoplasm to nucleus and nucleolus was driven by NLS motif of Hsp70 (Akerfelt et al. 2007; Tang et al. 2007). In the present study, we made a deletion mutant in NLS domain of Hsp70 and generated a eukaryotic expression plasmid (pcDNA3.1-Hsp70- Δ NLS; Tang et al. 2007). After confirmation of the expression of Hsp70- Δ NLS (Fig. 4a), cells were treated with H₂O₂ for 12 h. Nucleolus staining revealed that Hsp70- Δ NLS abolished the protection of Hsp70 against nucleolar fragmentation (Fig. 4b). Role of Δ NLS is not due to apoptosis induced by Δ NLS transfection (supplementary Figure 3).

Because NLS is a motif which mediates Hsp70 translocation from cytoplasm to nucleus and nucleolus, deletion of NLS would keep exogenous-Hsp70 to localize

in cytosol even under stress. Here, nucleoli were isolated and nucleolar protein fraction and cytoplasmic fraction were measured by immunoblotting. Results showed that Hsp70 (induced by HS or plasmids transfection) localized mainly in cytoplasmic fraction of H₂O₂-untreated cells, and redistributed into nucleolus after treatment with H₂O₂. However, Hsp70- Δ NLS did not enter into the nucleolus after exposure to H₂O₂ (Fig. 4d). These results suggested that NLS is the essential motif for Hsp70 attenuating H₂O₂-induced nucleolar fragmentation.

Down-regulation and cleavage of nucleolin resulted in nucleolar fragmentation

Nucleolin is an abundant nucleolar functional protein which plays important role in the regulation of nucleolar stability (Kito et al. 2003; Ma et al. 2007; Ugrinova et al. 2007). In present study, we further found that nucleolin was cleaved after 3-h treatment of H₂O₂ as indicated by decrease of density of intact 110-kDa nucleolin protein band and increase of density of 80-kDa fragment band, and that

intact 110-kDa nucleolin protein was almost disappeared at 12 and 24 h after exposure to H_2O_2 . However, H_2O_2 did not alter the expression of fibrillarin, an important nucleolar structure protein (Fig. 5a). Further results illustrated that AS-ONs-mediated down-regulation of nucleolin led to nucleolar fragmentation and apoptosis (Fig. 5b, c and supplementary Figure 3). These results revealed that nucleolar impairment was associated with H_2O_2 -induced cleavage and down-regulation of nucleolin.

Hsp70 attenuated nucleolar fragmentation via inhibiting down-regulation and cleavage of nucleolin

In present study, we have demonstrated that Hsp70 inhibited H_2O_2 -induced nucleolar fragmentation via NLS-mediated accumulation of Hsp70 in nucleus and nucleolus. It is unclear whether Hsp70 suppress H_2O_2 -induced down-regulation and cleavage of nucleolin. At 6 and 12 h after exposure to H_2O_2 , cleavage and down-regulation of nucleolin was inhibited significantly by over-expression of Hsp70 as indicated by immunoblotting assay (Fig. 5d, e). Moreover, results showed that over-expression of deletion mutant in NLS abrogated the protection of Hsp70 against H_2O_2 -induced cleavage and down-regulation of nucleolin (Fig. 5e). These results convinced us that inhibition of cleavage and down-regulation of nucleolin contributed to Hsp70-mediated protection against nucleolar impairment.

Discussion

The nucleolus represents a highly dynamic nuclear compartment (Boisvert et al. 2007; Heun 2007; Boulon et al. 2010), easily visible by light microscopy. Nucleolar morphology can be evaluated histologically using the toluidine blue staining. Numerous studies have reported that nucleolar fragmentation is part of the overall apoptotic morphology (Horky et al. 2001; Fraschini et al. 2005; Suzuki et al. 2007). Here, we reported that oxidative stress (exposure to H_2O_2) resulted into nucleolar segments (nucleolar fragmentation) in C_2C_{12} myogenic cells and MEFs.

Nucleolin is a ubiquitous, nonhistone nucleolar phosphoprotein exists in exponentially growing eukaryotic cells and is present in abundance at the dense fibrillar and granular regions of nucleolus. As a multifunctional nucleolar protein, the spectrum of nucleolin research has expanded to include chromatin decondensation, cytoplasmic nucleolar transport of ribosomal components, preribosomal particles and the regulation of nucleolar stability (Srivastava and Pollard 1999; Ma et al. 2007; Storck et al. 2007; Ugrinova et al. 2007). In present study, we reported that nucleolar fragmentation was in part resulted from H_2O_2 -mediated cleavage and down-regulation of nucleolin. This result was consistent with the previous reports from two groups (Ma et al. 2007; Ugrinova et al. 2007). In

Fig. 5 Hsp70 inhibited H_2O_2 -induced cleavage and down-regulation of nucleolin in the nucleus and nucleolus. **a** H_2O_2 -induced cleavage and down-regulation of nucleolin. **b** Down-regulation of nucleolin mediated by AS-ONs. Cells were transfected with AS-ONs specific against nucleolin for 24 h, then cell lysates were prepared and immunoblotting were performed. **c** Nucleolar fragmentation shown by TB staining. After 24-h transfection with AS-ONs, cells were stained by TB and the numbers of stained nucleolus were counted as mentioned above. * $P < 0.05$ compared to untransfected or S-ONs

transfected cells. **d** Inhibition of Hsp70 on H_2O_2 -induced cleavage and down-regulation of nucleolin. Cells transfected transiently with full-length Hsp70 or control plasmid (Neo) were treated with H_2O_2 for 6 and 12 h, then cell lysates were prepared and immunoblotting were performed. **e** NLS-mediated inhibition of Hsp70 on H_2O_2 -induced cleavage and down-regulation of nucleolin. Cells transfected transiently with full-length Hsp70 or control plasmid (Neo) or NLS truncated mutant (ΔNLS) were treated with H_2O_2 for 12 h, then cell lysates were prepared and immunoblotting were performed

reality, nucleolus is a highly dynamic subnuclear body which is a complexity of rDNA, rRNA, and proteins. It has been demonstrated that nucleolin is the most important nucleolar chaperone for maintaining nucleolar integrity and function by recruiting many essential molecules (proteins, RNA, and DNA) because of its property of interaction with these molecules (Srivastava and Pollard 1999). As nucleolin is depleted, several important nucleolar proteins (for example: B23, RNA polymerase I, and fibrillarin, etc.) will lose their affinities for other nucleolar components, therefore, the structure and function of nucleoli are disrupted (Ugrinova et al. 2007).

Nucleolin has an intrinsic protease activity for autodegradation which is activated under apoptotic stresses (Fang and Yeh 1993; Srivastava and Pollard 1999). The enzymatic domain is located within C-terminal of nucleolin protein, and this region contains four RNA binding domains (Fang and Yeh 1993; Srivastava and Pollard 1999). Here, we reported H₂O₂-induced cleavage of nucleolin which might be related to activation of its autodegradation activity. Further evidences should be provided to confirm this hypothesis. There are two important mechanisms by which nucleolin are associated with apoptosis. On the one hand, cleaved nucleolin activates autolytic endonucleases, which fragment DNA to cause apoptosis (Srivastava and Pollard 1999). On the other hand, nucleolin binds to mRNAs of several apoptotic genes to stabilize or destabilize them, for example, nucleolin stabilizes Bcl-2 (Otake et al. 2007), GADD54 mRNA (Zhang et al. 2006) and destabilizes p53 mRNA (Takagi et al. 2005).

It was well documented that HS activates effectively the endogenous protection and plays important protection against cell injury by inducing expression of Hsps (Kregel 2002; Wang and Xiao 2007; Toko et al. 2008). As reported, the inducible expression of Hsps was regulated by HSF-1 (Benjamin and McMillan 1998; McMillan et al. 1998; Akerfelt et al. 2007). In present study, we found that HS inhibited the H₂O₂-induced nucleolar fragmentation in C₂C₁₂ myogenic cells and MEFs by inducing the expression of Hsps which depended on HSF1. Among HS-mediated inducible expression of Hsps, Hsp70 is the most abundant expression in myocytes (Benjamin and McMillan 1998; Kregel 2002). However, the role of Hsp70 is not fully understood in HS-mediated protection against nucleolar fragmentation. In present study, gene loss- and gain-of-function assays revealed that HS attenuated nucleolar fragmentation via expression of Hsp70 and its redistribution into nucleus and nucleolus.

It has been reported that Hsp70 functions mainly as molecular chaperones to protect cells or organelles by modulating the assembly, transport, and folding of other proteins and by disaggregating, refolding the aggregated and unfolded proteins under stress (Mayer and Bukau 2005; Tang et al. 2007; Wang and Xiao 2007; Kampinga and

Craig 2010). Here, we found that Hsp70 translocates into nucleoli under oxidative stress. However, it is still unclear why Hsp70 accumulates in the nucleoli. We thought that it is a reasonable interpretation that accumulation of Hsp70 in nucleolus was driven by stress-mediated impairment of nucleolar proteins. What made us think so is not only that nucleolin, an important nucleolar protein, was cleaved and down-regulated after H₂O₂ exposure, but also that PARP-1 (poly(ADP ribose) polymerase-1) and XRCC1 (X-ray repair cross complementing 1) were impaired during apoptotic stimulation (Kotoglou et al. 2009). It has been demonstrated that the bipartite NLS motif is a necessary and important functional domain for Hsp70 translocating to the nucleus under stress (Tang et al. 2007). When NLS was truncated, Hsp70 cannot redistribute into nucleus under oxidative stress (H₂O₂ exposure) and lost its protective effect on the specific nucleolar molecular targets (for example: nucleolin, PARP-1, XRCC1, etc.) which contributed to the regulation of nucleolar stability.

Although we clarified that accumulation of Hsp70 in nucleolus inhibited cleavage and down-regulation of nucleolin by which Hsp70 attenuated H₂O₂-induced nucleolar fragmentation, it is still obscure that how Hsp70 suppresses cleavage and down-regulation of nucleolin. As an important molecular chaperone, investigation is needed to further reveal whether the protective effects of Hsp70 against nucleolar fragmentation can be attributed to the interactions between Hsp70 and other nucleolar proteins (for example, nucleolin), therefore Hsp70 inhibits the autodegradation activity of nucleolin and maintains stability of nucleolin.

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