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Original Article

Knockdown of nucleophosmin induces S-phase arrest in HepG2 cells

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Abstract

Nucleophosmin/B23 (NPM) is a universally expressed nucleolar phosphoprotein that participates in proliferation, apoptosis, ribosome assembly, and centrosome duplication; however, the role of NPM in cell cycle regulation is not well characterized. We investigated the mechanism by which NPM is involved in cell cycle regulation. NPM was knocked down using siRNA in HepG2 hepatoblastoma cells. NPM translocation following actinomycin D (ActD) treatment was investigated using immunofluorescent staining. Expression of NPM and other factors involved in cell cycle regulation was examined by Western blotting. Cell cycle distribution was measured using flow cytometry to detect 5-ethynyl-2′-deoxyuridine (EdU) incorporation. Cell proliferation was quantified by the MTT assay. Knockdown of NPM increased the percentage of HepG2 cells in S phase and led to decreased expression of P53 and $P21^{Cip1/WAF1}$. S-phase arrest in HepG2 cells was significantly enhanced by ActD treatment. Furthermore, knockdown of NPM abrogated ActD-induced G_2/M phase cell cycle arrest. Taken together, these data demonstrate that inhibition of NPM has a significant effect on the cell cycle.

Key words Nucleophosmin, p53, $p21^{Cip1/WAF1}$, actinomycin D, cell cycle

Nucleophosmin/B23 (also known as NPM, B23, numatrin, and NO38) is an abundant nucleolar phosphoprotein that shuttles between the nucleus and cytoplasm[1] and participates in various cellular processes, including cell proliferation[2], cytoplasmic-nuclear shuttle transportation[1], nucleic acid binding[3], ribosome assembly[4], and centrosome duplication[5]. NPM also displays ribonuclease activity[6] and acts as molecular chaperone[7]. The diverse functions of NPM suggest that it may act as both an oncogene and a tumor suppressor, depending on environmental stimuli.

Compared to normal resting cells, tumor and growing cells overexpress NPM. Indeed, NPM has been proposed as a potential tumor marker in human colon[8], gastric[9], and hepatocellular[10,11] cancers, all of which show markedly increased expression of NPM. NPM possesses chaperone activity and acts to prevent protein aggregation and facilitate renaturation of chemically denatured proteins. NPM also interacts with other proteins, such as alternative reading frame protein (ARF)[12,13] and murine double mimute 2 (MDM2)[14], via an N-terminal oligomerization domain. P53[15] and $P21^{Cip1/WAF1}$[16] are well-characterized cell cycle regulators that are reported to be stabilized by physical interaction with NPM; however, the precise role of NPM in cell cycle regulation has not been well documented.

Actinomycin D (ActD), a natural antibiotic that can inhibit gene transcription and lead to reduced proliferation of cancer cells, has been used clinically as an anticancer drug. The anticancer mechanism of ActD is thought to be DNA intercalation by binding guanine residues and inhibiting DNA-dependent RNA polymerase activity. Normally, chemotherapeutic drugs exert their effects via

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induction of apoptosis and cell cycle arrest. ActD has been reported to induce Fas-mediated cell death in HepG2 cells[17] and to sensitize pancreatic cancer cells to Fas-mediated apoptosis through abrogation of the DNA binding activity of NF-κB[18]. ActD has also been reported to induce S/G_2 phrase arrest in HepG2 cells[19].

In the present study, we demonstrated that decreased expression of NPM in HepG2 cells induces S-phase arrest and reduced expression of P53 and P21. In HepG2 cells, ActD treatment stimulates significant S-phase arrest; however, knockdown of NPM expression abrogates ActD-induced G_2/M phase arrest. Collectively, our data indicate that knockdown of NPM has a significant effect on the cell cycle, suggesting that NPM plays an important role in cell cycle regulation.

Materials and Methods

Cell culture and drug treatment

The hepatoblastoma cell line (HepG2) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 mg/mL penicillin, and 100 mg/mL streptomycin in a humidified atmosphere containing 5% CO_2 at 37℃. ActD was purchased from Sigma (St. Louis, MO, USA) and used at a final concentration of 0.05 μmol/L.

RNA interference

Small interfering RNAs designed against NPM (NPM siRNA, 5′-UGAUGAAAAUGAGCACCAGTT-3′) and nonspecific control siRNA (control siRNA; 5′-GUUCUCC GAACGUGUCACGTT-3′) were synthesized by Shanghai Genepharma Co. (Shanghai, China). For RNA interference experiments, 2×10^5 HepG2 cells were seeded into 6-well plates 24 h prior to transfection, and 50 nmol/L siRNA was transfected into the cells in each well using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24–72 h, the cells were harvested for gene knockdown analysis.

Immunofluorescence

HepG2 cells were grown on cover slips, fixed for 20 min in PBS containing 4% parafomaldehyde, permeabilized in 0.1% Triton X-100 for 2× 5 min, and incubated in blocking buffer (3% donkey serum in TBS) for 1 h. The cells were incubated in primary antibody in dilution buffer (3% bovine serum albumin in TBS) for 2 h at room temperature and then washed extensively in PBS before incubation with the appropriate fluorochrome-conjugated secondary antibody for 1 h. DNA was stained using 4',6-diamidino-2-phenylindole (DAPI).

Western blot analysis

Whole cell lysates of HepG2 cells treated with siRNA or ActD were prepared in RIPA buffer, and protein concentration was quantified using the BCA assay. The lysates were boiled in 6× SDS loading buffer, separated using SDS-PAGE, and transferred to polyvinylindene fluoride (PVDF) membranes. The membranes were blocked in 5% milk solution for 1 h and incubated with primary antibodies followed by secondary anti-rabbit or anti-mouse IgG peroxidase-conjugated antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and ECL detection reagent (Amersham Life Sciences, Piscataway, NJ, USA). Anti-GAPDH (Santa Cruz Biotechnology) was used as a loading control.

Antibodies

The antibodies used in this study were as follows: mouse monoclonal anti-NPM[f] (1:2000); mouse monoclonal anti-GAPDH (1:4000), rabbit polyclonal anti-p53 (1:1000), rabbit polyclonal anti-CDK2 (1:500), rabbit polyclonal anti-cyclin A (1:500), and rabbit polyclonal anti-cyclin E (1:200) from Santa Cruz Biotechnology; and rabbit polyclonal anti-p21 (1:1000) from Cell Signaling Technology, Beverly, MA, USA.

Cell cycle analysis

HepG2 cells treated with ActD for the indicated times were collected, fixed, and permeabilized with 75% ice-cold ethanol and stored at –20℃. The cells were centrifuged, resuspended in 1 mL of lysis buffer (0.1% Triton X-100, 0.05 mg/mL propidium iodide, and 1 mg/mL RNase A), incubated for 30 min at 37℃, and analyzed using flow cytometry.

MTT cell proliferation assay

HepG2 cells were plated at a density of 1500 cells/well in 96-well plates and incubated for 24–120 h. Subsequently, 20 μL of MTT reagent was added to each well, and the plates were incubated for 3 h in 5% CO_2 at 37℃. The supernatant was then discarded and replaced with DMSO to dissolve the formazan product. Absorbance was measured at 490 nm in a spectrophotometric plate reader for 5 days continuously.

DNA synthesis analysis

HepG2 cells were plated at 4 × 10^4 cells/well in 24-well plates, allowed to adhere, washed with PBS, and incubated in serum-free DMEM containing 10 μmol/L 5-ethynyl-2'-deoxyuridine (EdU, a BrDU substitute; Guangzhou RiboBio Co., Ltd, Guangzhou, China) for 2 h. The cells were washed with PBS, fixed, and permeabilized in PBS containing 2% formaldehyde, 0.5% Triton X-100, and 300 mmol/L sucrose for 15 min. After washing with PBS, the cells were blocked using 10% FBS in PBS for 30 min, and incorporated EdU was detected by incubation with the fluorescent-azide coupling solution (Apollo®, Guangzhou RiboBio Co., Ltd) for 30 min. The cells were washed three times with PBS containing 0.05% Tween-20 (PBST), incubated with the DNA-staining dye Hoechst 33342 for 30 min, and washed in PBS. Images were captured using a fluorescent microscope, and the average nuclear fluorescent intensity was calculated from at least 50 non–S-phase cells randomly selected in 5 different fields of view.

Statistical analyses

Each experiment was repeated at least three times. Data are presented as mean ± standard deviation (SD). Student's *t*-tests were used for statistical analysis and *P* values <0.05 were considered significant.

Results

Knockdown of NPM in HepG2 cells increases the number of S-phase cells

To determine the effect of NPM on cell cycle regulation, we knocked down NPM expression using siRNA in HepG2 cells. Expression of NPM protein was noticeably reduced 24 h after transfection of NPM siRNA (Figure 1A). Reduced expression of NPM without the occurrence of nucleo-cytoplasmic translocation was confirmed using immunofluorescence 24 h after transfection of NPM siRNA (Figure 1B). Knockdown of NPM protein expression increased the accumulation of S-phase cells to 30.7%, versus 17.8% in control cells (Figure 1C). An increase in HepG2 cell volume was also observed after NPM knockdown (data not shown).

The expression of cell cycle regulators was examined using Western blotting after transfection of NPM siRNA. In parallel to down-regulation of NPM, expressions of P53, P21, and Cyclin E were dramatically decreased compared to that of control cells. Cyclin A and CDK2 levels were not altered by NPM siRNA (Figure 1D).

NPM knockdown-induced S-phase arrest is intensified by ActD treatment in HepG2 cells

Because ActD is a classical drug used to induce cell cycle arrest and was found to be associated with the distribution of NPM in our early study, we investigated if it could affect NPM knockdown-induced cell cycle arrest. In response to ActD treatment alone, NPM was redistributed to the nucleoplasm from the nucleolus in HepG2 cells (Figure 2A). In contrast, co-treatment of HepG2 cells with NPM siRNA and ActD led to a significant reduction in NPM expression in both the nucleoplasm and nucleolus (Figure 3A). In response to ActD treatment alone, NPM expression was not altered, but P53 and P21 were up-regulated and Cyclin A was down-regulated (Figure 2B). In contrast, co-treatment of HepG2 cells with NPM siRNA and ActD significantly reduced expression of P53 and P21 and led to undetectable levels of NPM expression (Figure 3B).

Cell cycle analysis was performed in HepG2 cells treated with ActD and NPM siRNA. After 24 h of treatment with ActD, the percentage of G_2/M phase cells increased to 45.5% compared to 10.1% in control cells (Figure 2C); however, in cells treated with both NPM siRNA and ActD, only 10.7% of cells were in G_2/M phase compared to 48.3% in cells treated with control siRNA and ActD. Co-treatment with NPM siRNA and ActD had a synergistic effect on S-phase arrest in HepG2 cells, increasing the percentage of S-phase cells to 73.9% compared to 30.7% in NPM siRNA–transfected cells and 37.4% in control siRNA and ActD co-treated cells (Figure 3C).

To confirm the increased number of S-phase HepG2 cells after treatment with ActD and NPM siRNA, the cells were labeled with EdU[20] to measure active DNA synthesis. Consistent with the results of the cell cycle flow cytometry analysis, the percentage of cells that incorporated EdU was 20.1% in the control siRNA group, 32.3% in NPM siRNA-transfected cells, and 37.7% in control siRNA and ActD co-treated cells; however, in the ActD and NPM siRNA co-treated group, 72.3% of the cells were EdU positive (Figure 3D). The remarkable increase in EdU incorporation in ActD and NPM siRNA-treated cells indicated that DNA synthesis activity was enhanced because cells were trapped in S phase.

We quantified cell proliferation using MTT to determine the effect of NPM siRNA and ActD treatment on cell growth. In agreement with the results of the cell cycle analysis, knockdown of NPM using NPM siRNA significantly reduced cell proliferation compared to control siRNA–treated cells, whereas ActD treatment almost completely inhibited cell growth in both NPM siRNA– and control siRNA–transfected cells (Figure 3E).

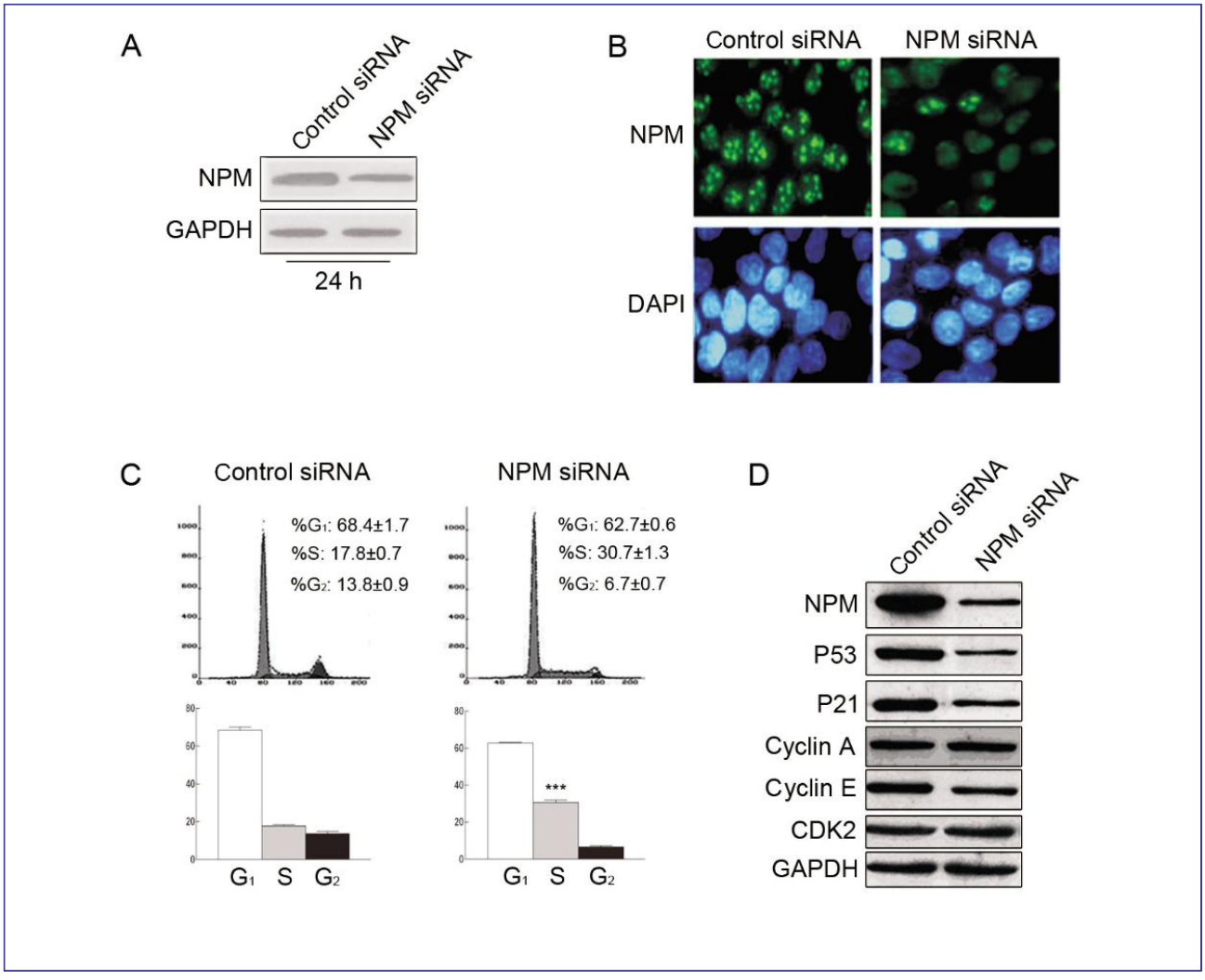

Figure 1. **Knockdown of nucleophosmin (NPM) induces S-phase arrest in HepG2 cells, accompanied by reduced P21 and P53 expression.** A, Western blot of NPM protein expression in HepG2 cells 24 h after transfection of control or NPM siRNA. B, immunofluorescent staining of NPM protein (green) and nuclear counterstain (DAPI, blue) in HepG2 cells 24 h after transfection of control or NPM siRNA. C, cell cycle analysis of HepG2 cells 24 h after transfection of control or NPM siRNA. Knockdown of NPM increased the percentage of S-phase cells. Values are presented as mean ± standard deviation (SD) of at least three independent experiments; $***P < 0.001$, compared with control siRNA-treated cells. D, Western blot of cell cycle proteins in HepG2 cells 24 h after transfection of control or NPM siRNA. The expression of P53, P21, and Cyclin E was reduced when NPM was knocked down.

Discussion

NPM is a multifunctional shuttle protein. In the present study, we found that knockdown of NPM using siRNA led to an accumulation of cells in the S phase and to decreased proliferation. NPM-induced S-phase arrest and decreased proliferation were both synergistically increased in the presence of ActD, and ActD-mediated G_2/M phase arrest was blocked by NPM siRNA. These data suggest that NPM is involved in cell cycle regulation.

Previous studies of the role of NPM in cell cycle regulation have demonstrated different results in various cells. Reduced NPM expression in osteosarcoma cell line U2OS significantly increased the number of S-phase cells, due to decreased expression of P53[15]. Down-regulation of NPM in colorectal carcinoma cell line HCT116 resulted in increased numbers of S-phase cells and G_2/M arrest, mediated by GADD45α[21]. Knockdown of NPM led to G_1 phase arrest in pulmonary adenocarcinoma cell line H157[22] and embryonic kidney epithelial cell line HEK293[23]. However, in hematopoietic

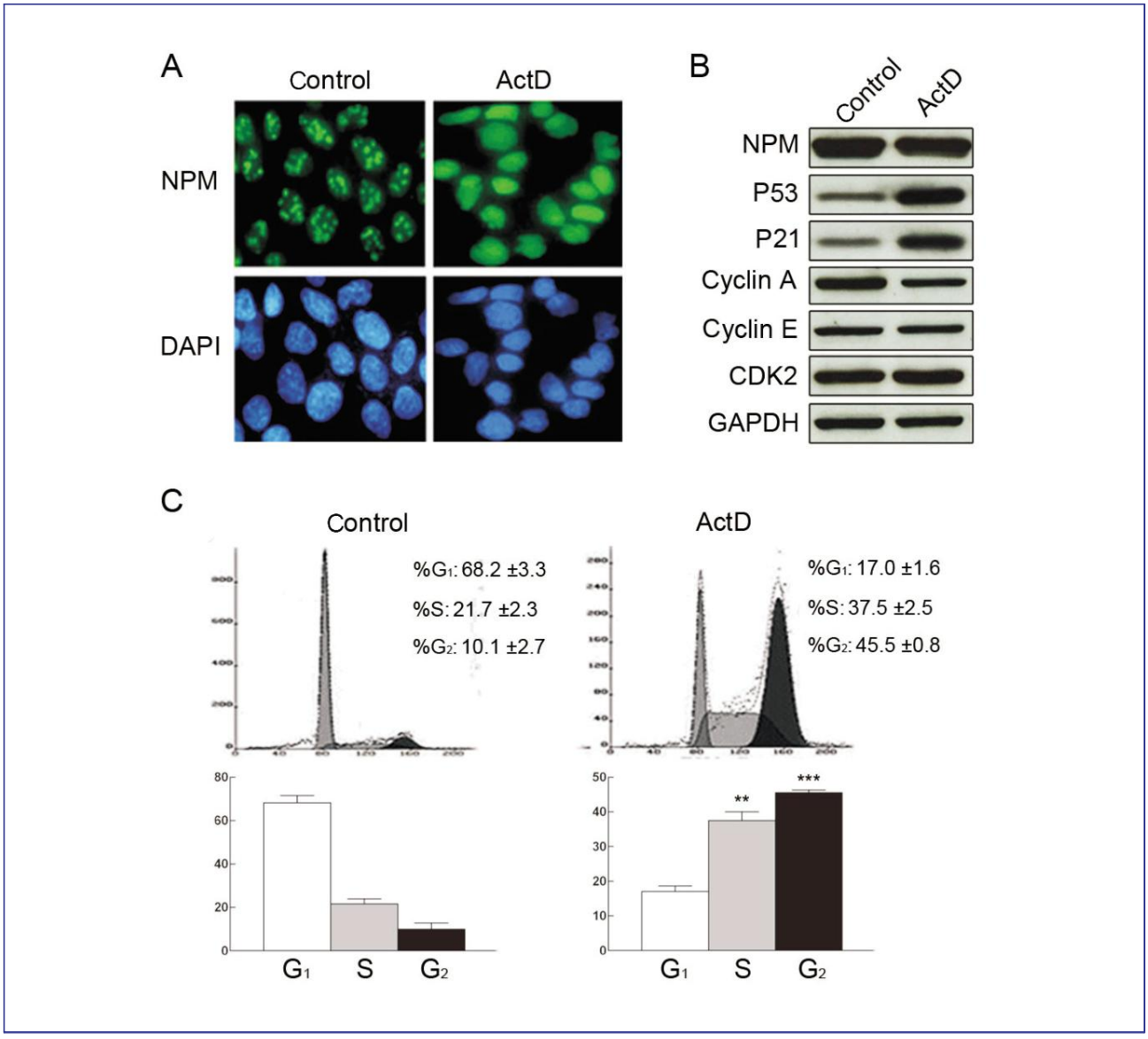

Figure 2. **Actinomycin D (ActD) treatment induces S and G_2/M phase arrest in HepG2 cells.** A, immunofluorescent staining of NPM protein (green) and nuclear counterstain (DAPI, blue) in HepG2 cells treated with 0.05 μmol/L ActD for 24 h. NPM translocated from nucleolus to nucleoplasm following ActD treatment. B, HepG2 cells were treated with ActD for 24 h and analyzed by Western blotting to detect expression of cell cycle regulation factors. ActD treatment increased the expression of P53 and P21. C, cells were treated with ActD for 24 h and flow cytometry cell cycle analysis was performed. S and G_2 phase arrest was induced following ActD treatment. All values are presented mean ± SD of at least three independent experiments; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with control cells.

stem/progenitor cells, overexpression of NPM led to increased numbers of S-phase cells[24], in contrast to the findings of previous studies that showed NPM could stabilize P53 and P21 through its molecular chaperone activity[16,25]. These inconsistencies in the effect of NPM knockdown may be related to the diverse biological characteristics of hematopoietic stem/progenitor cells. In the present study, knockdown of NPM in HepG2 cells significantly increased the percentage of S-phase cells, accompanied by decreased P53 and P21 expression. ActD treatment significantly enhanced NPM knockdown-mediated S-phase arrest, and additionally, NPM knockdown decreased ActD-induced G_2/M phase arrest. Our data suggest that NPM is important for the S/G_2 and G_2/M transition in HepG2 cells.

Our data also indicate that the localization of NPM correlates with cell cycle regulation. NPM translocates from the nucleolus into the nucleoplasm during

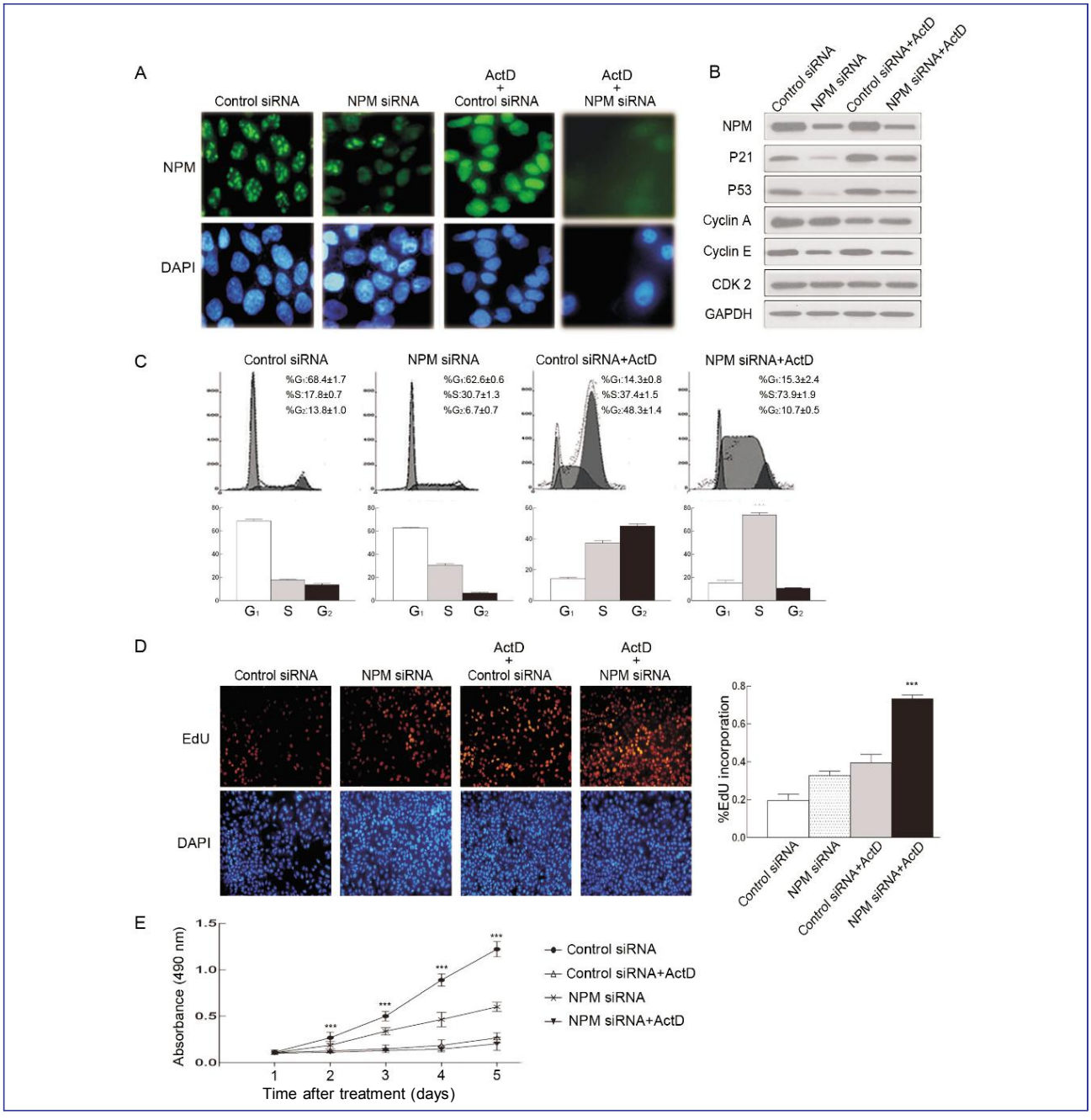

Figure 3. **NPM knockdown–induced S-phase arrest is intensified by ActD in HepG2 cells.** A, immunofluorescent staining of NPM protein (green) and nuclear counterstain (DAPI, blue) in HepG2 cells 24 h after transfection of control or NPM siRNA, with and without 0.05 μmol/L ActD, respectively. NPM translocation from the nucleolus to nucleoplasm following ActD treatment was largely inhibited after transfection of NPM siRNA. B, Western blot of cell cycle proteins in HepG2 cells 24 h after transfection of control or NPM siRNA. NPM siRNA and ActD co-treatment reversed the reduction in P21 and P53 expression observed in cells transfected with NPM siRNA. C, NPM or control siRNA–transfected HepG2 cells were treated with or without 0.05 μmol/L ActD for 24 h and cell cycle was analyzed using flow cytometry. NPM siRNA and ActD co-treatment abrogated ActD-induced G_2 phase arrest and led to enhanced S phase arrest. D, NPM siRNA and ActD synergistically induced S-phase arrest in HepG2 cells. NPM or control siRNA-transfected HepG2 cells were treated with or without 0.05 μmol/L ActD for 24 h and incubated with 5-ethynyl-2′-deoxyuridine (EdU) for 2 h. EdU incorporation was determined using fluorescence microscopy in at least 50 non–S-phase cells randomly selected from a single field. Data points are presented as mean ± SD of 5 different fields; $^{**}P < 0.01$; $^{***}P < 0.001$, compared with siRNA control. E, MTT cell viability assay of HepG2 cells treated with NPM siRNA and ActD. Values are presented as mean ± SD of at least three independent experiments; $^{***}P < 0.001$, compared with control siRNA-treated cells.

prophase, localizes in the cytoplasm during prometaphase, and appears in the nucleolus at late telophase or early G_1 phase[26,27]. During liver regeneration after partial hepatectomy, NPM translocation in regenerative hepatocytes is associated with the liver cell cycle [28]. Similar redistribution of other proteins is correlated with cell cycle regulation. Cytoplasmic P21 promotes the G_0/G_1 transition in osteosarcoma cell lines U2OS and SAOS-2[29], whereas nuclear P21 arrests the G_1/S and G_2/M transition[30,31]. Inhibition of nuclear $P27^{kip1}$ reduced G_1/S arrest in fibroblast cell line COS7, whereas knockdown of cytoplasmic P27 inhibited U87 (glioblastoma cell line) and COS7 cell motility and survival without altering cell cycle progression[32]. In the present study, ActD was used to disturb NPM localization and led to significant G_2/M arrest, which could be abrogated by NPM knockdown. However, knockdown of NPM failed to impact G_2/M transition in the absence of ActD treatment; therefore, we presume that ActD-induced G_2/M arrest in HepG2 cells is mainly mediated by nucleoplasmic, but not nucleolar, NPM.

NPM interacts with other proteins to function as an effector for several cell cycle regulators, including retinoblastoma protein[33], Myc[34], P53[35], P21[16], GCN5[36], MDM2, and ARF. NPM can also monitor the kinase activities of MEK[37], Cyclin E/CDK2[38], Cdk1, and Cdc25C[39]. NPM is essential for ribosome assembly, functioning in the processing and transporting ribosomal RNA[40], which suggests that NPM plays an important role in protein synthesis during S phase and may be involved in the S/G_2 phase transition. In our study, inhibition of NPM reduced the expression of P53 and P21, which may mediate NPM-induced G_2/M regulation. On the other hand, knockdown of NPM may slow down ribosome assembly, subsequently leading to an insufficiency of proteins required to maintain the cell cycle process, and therefore induce an accumulation of S-phase cells. Altogether, NPM plays an important roll in cell cycle regulation.

Acknowledgments

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