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# Gelsolin negatively regulates the activity of tumor suppressor p53 through their physical interaction in hepatocarcinoma HepG2 cells

Joo-Hee An [1], Jung-Woong Kim [1], Sang-Min Jang, Chul-Hong Kim, Eun-Jin Kang, Kyung-Hee Choi *

*Department of Life Science (BK21 Program), College of Natural Sciences, Chung-Ang University, Seoul 156-756, Republic of Korea*

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ABSTRACT

As a transcription factor, p53 modulates several cellular responses including cell-cycle control, apoptosis, and differentiation. In this study, we have shown that an actin regulatory protein, gelsolin (GSN), can physically interact with p53. The nuclear localization of p53 is inhibited by GSN overexpression in hepatocarcinoma HepG2 cells. Additionally, we demonstrate that GSN negatively regulates p53-dependent transcriptional activity of a reporter construct, driven by the p21-promoter. Furthermore, p53-mediated apoptosis was repressed in GSN-transfected HepG2 cells. Taken together, these results suggest that GSN binds to p53 and this interaction leads to the inhibition of p53-induced apoptosis by anchoring of p53 in the cytoplasm in HepG2 cells.



## 1. Introduction

The tumor suppressor protein p53 is involved in various cellular responses such as cell-cycle control, apoptosis, differentiation, and DNA repair [1,2]. In over 50% of human cancers, the p53 gene is mutated, which reflects its importance in carcinogenesis [1,3]. In response to DNA damage, p53 accumulates in the nucleus where it transcriptionally activates the genes that encode p21, Bax, PUMA, and MDM2, as well as other genes that are involved in growth arrest and apoptosis [4–7]. Although p53 is a wild-type, abnormal cytoplasmic sequestration of p53 was revealed in a subset of human tumors such as breast, colon cancer, and neuroblastoma [8,9]. Several proteins have been shown to be involved in the regulation of p53 cellular localization, by tethering p53 in the cytoplasm. For example, an hsp70 family member, Mot2, can interact with p53 and inhibit its entrance into the nucleus. Overexpressed Mot2 in NIH3T3 cells resulted in an abrogation of nuclear translocation of wild-type p53 and the repression of p53-mediated transactivation [10,11]. Also, Bcl2 has been implicated in p53 nucleocytoplasmic translocation in murine erythroleukemia cells [12]. Furthermore, in neuroblastoma cells, MAP1B (Microtubule-Associated Protein 1B) light chain interacts with p53 and negatively regulates the activity of p53 when treated with doxorubicin by altering its localization from cytoplasm to the nucleus [13]. However, the precise molecular mechanisms underlying the regulation of p53 translocation have not yet been elucidated.

Gelsolin (GSN) controls actin organization and participates in the regulation of cell motility and morphogenesis by severing capping filament ends and nucleating actin assembly [14]. In addition to actin regulation activity, GSN has been reported to participate in the regulation of apoptotic process [15–17]. GSN can be cleaved by caspase-3 at Asp 376, producing N-half and C-half fragments that have controversial functions in apoptosis [18]. The N-half fragment can independently sever actin filaments without $Ca^{2+}$ and dismantle the actin-based cytoskeleton to cause apoptosis [18]. In contrast, full-length GSN and its C-terminal half are mostly antiapoptotic [19,20]. While in some cancers, such as colon, ovary, and prostate, GSN is decreased, high expression levels of GSN occur in a subset of non-small cell lung cancers and in the transition from noninvasive to invasive tumors [21,22]. High levels of GSN expression are thought to be an independent marker for tumor recurrence and progression in urothelial tumors, particularly for high-grade variants [21]. Recently, it has been shown that several members of the GSN protein family, including GSN, flightless, and supervillin exhibit unexpected, potential functions in regulating transcription [23–25]. For example, GSN can interact with hormone-bound androgen receptor to facilitate nuclear translocation [26]. Moreover, thyroid hormone receptor-b1 interacts with GSN and the transcriptional activity of thyroid hormone receptors is affected by GSN [27]. Therefore, it is possible to consider that GSN may participate in transcriptional regulation as a co-regulator through protein interactions.

In this study, we identified that GSN physically interacts with p53 in hepatocarcinoma cell line, HepG2. We report that GSN

* Corresponding author. Address: Department of Life Science (BK21 Program), College of Natural Sciences, Chung-Ang University, 221 Heuksuk Dong, Dongjak Ku, Seoul 156-756, South Korea. Fax: +82 2 824 7302.
*E-mail address:* khchoi@cau.ac.kr (K.-H. Choi).
[1] These authors contributed equally to this work.

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binds to the p53 transactivation- and DNA binding domains and inhibits transcriptional activity of p53. Also, ectopic expression of GSN in HepG2 cells reduced the translocation of p53 to the nucleus. Our findings suggest that GSN acts as a negative regulator of p53-mediated apoptosis via GSN/p53 interaction in hepatocarcinoma cells.

## 2. Materials and methods

### 2.1. Cell culture and transfection

HepG2 and HEK 293 cells were obtained from the ATCC (American type culture collection; Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen; Carlsbad, CA) and penicillin–streptomycin (50 units/ml). Transient transfection was performed by Lipofectamine 2000 (Invitrogen) with different plasmid DNA according to the manufacturer's instructions.

### 2.2. In vivo binding assay and Western blotting

HepG2 cells were lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris–HCl, pH 7.5, 0.1% SDS, 1% Nonidet P-40, and 1 mM PMSF. For immunoprecipitation assays, the supernatants were pre-cleaned with 20 μl of protein A/G agarose bead (50% slurry) and then incubated at 4 °C overnight with 30 μl of fresh protein A/G bead in the presence of appropriate antibodies. Samples were analyzed by Western blotting using the appropriate antibodies to detect protein expression. The GSN (sc-6405) and p53 (DO-1, sc-126) protein were detected by each primary antibody (purchased from Santa Cruz, 1:500 dilution) and secondary antibody (HRP-anti-goat and -anti-rabbit, 1:5000 dilution). The polyclonal GFP (GFP-1814460), p21 (sc-397), Bax (sc-493), Histone H3 (sc-8654), and β-tubulin (sc-9104) antibodies were from Roche Diagnostics (Indianapolis, IN) and Santa Cruz Biotechnology. Western blot was detected by chemoluminescence (ECL, Santa Cruz Biotechnology).

### 2.3. Immunofluorescence staining and confocal microscopic detection

HepG2 cells were washed with phosphate-buffered saline (PBS) and fixed for 1 h in 4% paraformaldehyde. The cells were permeabilized with 0.3% Triton X-100 for 20 min, incubated with a primary antibody in blocking solution for 3 h at room temperature, washed in blocking solution, and then incubated with the appropriate secondary antibody for 30 min. Primary antibodies were used at 1:100 for p53 (DO-1, sc-126). Cy3-conjugated goat anti-mouse IgG (1:200, Amersham Biosciences) was used as secondary antibodies. DAPI (Sigma) was used for nuclear staining. Cells were incubated in 300 nM DAPI in PBS for 2 min at room temperature. Cells were visualized using a Carl Zeiss LSM-510 META laser scanning microscope (Oberkochen, Germany).

### 2.4. Luciferase assay

HepG2 cells were cultured in 60 mm dishes and transfected with the firefly luciferase p21 reporter gene (0.1 μg) and pCMV-β-galactosidase (0.1 μg) together with pEGFP-GSN, pCMV-FLAG-p53, or pCMV-FLAG control vector using Lipofectamine 2000. After 24 h of transfection, cells were lysed in reporter lysis buffer (Promega). Cell extracts were analyzed with the luciferase reporter assay system using a glomax luminometer (Promega). Luciferase activities of the p21-luciferase vector were normalized based on β-galactosidase activity of the co-transfected vector. All transfection experiments were repeated independently at least three times.

### 2.5. Preparation of sub-cellular fractions

HepG2 cells were washed with ice-cold PBS, harvested by centrifugation, and lysed in Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) for 15 min. For cell lysis, 10% (v/v) of Nonidet P-40 was added, and then cells were vortexed for 10 s. The pellet was added to ice-cold Buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride), incubated for 15 min at 4 °C, and centrifuged at 14,000 rpm for 5 min. The supernatant (nuclear extracts) was transferred to new tubes and kept frozen at −70 °C until use.

### 2.6. Flow cytometry

After cells were trypsinized approximately $1 \times 10^6$ cells were collected by centrifugation at 1000*g* for 5 min. Cells were then washed in PBS, followed by resuspension and fixation in 70% ethanol for approximately 2 h. Cellular DNA was then stained by the addition of 10 μg of propidium iodide (PI) or Annexin-V, and cells were analyzed by FACScan flow cytometer using Cellquest software (Becton–Dickinson, Franklin Lakes, NJ).

## 3. Results

### 3.1. p53 physically interacts with the actin binding protein, GSN

Using a yeast two-hybrid assay system, we have identified a variety interacting proteins of p73β, a homolog of p53, such as Amphiphysin IIb-1, p19ras and MAP1B-LC1 that are involved in p73β-mediated transcriptional regulation and apoptosis [13,28,29]. We also previously confirmed that GSN binds with p73β in yeast two-hybrid assay (Supplementary data 1A) and in overexpressed conditions of HEK 293 cells (Supplementary data 1B). Moreover, we found that the binding affinity of p53 with GSN was stronger than with p73β (Supplementary data 1C). To further determine the interaction of p53 with GSN in human cultured cells, we examined the coprecipitation of HA-p53 and full-length GFP-GSN from transiently transfected HEK 293 cells (Fig. 1A). Whole cell lysates were incubated with anti-HA antibody for immunoprecipitation and followed by Western blotting with anti-GFP antibody. The full-length GFP-GSN protein was coimmunoprecipitated with HA-p53 (lane 2) and without HA-empty control vector (lane 1). We also confirmed the interaction of p53 and GSN by Western blotting with anti-HA antibody and subsequent coimmunoprecipitation using anti-GFP antibody (Fig. 1B). Because GSN is highly expressed in HEK 293 and HepG2 cell lines, we then examined the endogenous interactions between GSN and p53. Total cell lysates were immunoprecipitated with anti-p53 antibodies (lane 2) together with a control rabbit serum (lane 1), and then Western blot analysis was performed using anti-GSN or anti-p53 antibodies. As shown in Fig. 1C, we clearly observed that endogenous GSN was specifically detected by immunoprecipitation with the anti-p53 antibody (lane 2), but not with the preimmune rabbit serum (lane 1) for both HEK 293 and HepG2 cells.

### 3.2. GSN interacts with p53 transactivation- and DNA binding domains

To define the region of p53 that is required for interactions with GSN, HepG2 cells were transfected with p53 encoding FLAG-tagged to full-length p53 (FL), the p53 transactivation domain (TAD), the

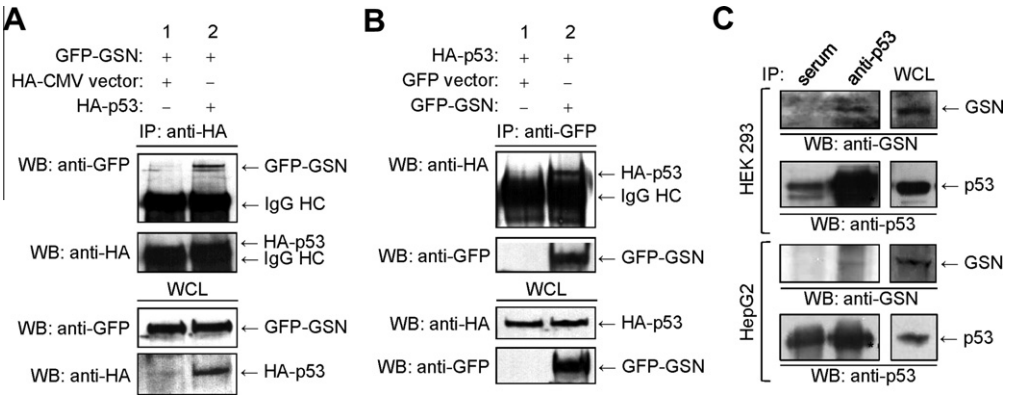


**Fig. 1.** p53 physically interacts with gelsolin (GSN). (A) HEK 293 cells were transfected with plasmids expressing GFP-tagged GSN (1 μg) together with either HA (1 μg) or HA-p53 (1 μg). Twenty-four hours after transfection, cells were harvested and lysates were immunoprecipitated with anti-HA antibody. By Western blotting, proteins were detected by using appropriate antibodies as indicated. (B) HEK 293 cells co-transfected by HA-tagged p53 (1 μg) and either GFP-tagged GSN (1 μg) or GFP (1 μg) were harvested and lysed. (C) *In vivo* interaction of endogenous p53 with GSN proteins was determined in HEK 293 cells and in HepG2 cells. Cell lysates were immunoprecipitated with anti-p53 antibodies and co-precipitated proteins were detected by Western blotting using indicated antibodies.

p53 DNA binding domain (DBD) or the p53 oligomerization domain (OD) together with the GFP-GSN expression plasmid (Fig. 2A). After whole cell lysates were immunoprecipitated with anti-FLAG antibodies, Western blotting was performed with anti-GFP and anti-FLAG antibodies. Although OD (amino acids 323–393) containing regions of p53 did not appear to interact with GSN, the TAD (amino acids 1–102) and DBD (amino acids 103–322) containing regions strongly interacted with GFP-GSN (Fig. 2B). Taken together, these results demonstrated that p53 physically interacts with GSN through its transactivation- and DNA binding domains *in vivo*.

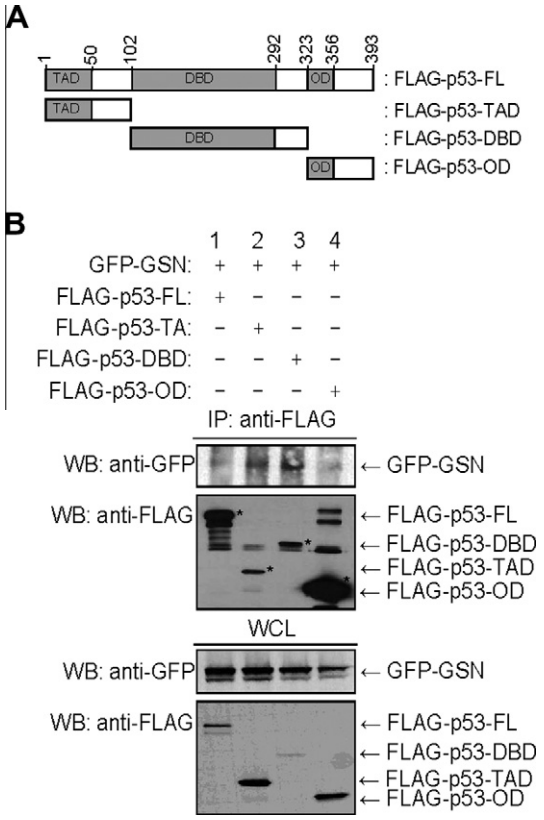


**Fig. 2.** GSN physically interacts with transactivation- and DNA binding domains of p53. (A, B) The truncated forms of FLAG-fused p53 was transfected into the HepG2 cells together with HA-fused GSN. Total cell lysates were immunoprecipitated with anti-FLAG antibody and co-precipitated GSN was detected by Western blot analysis using an anti-HA antibody.

### 3.3. GSN inhibits the translocation of p53 to the nucleus

In unstressed cells, as a mediator of $G_1$ checkpoints, p53 is predominantly in nuclei at $G_1$ phase and is largely cytoplasmic during S and $G_2$ phases [30]. However, in response to stress, stabilized p53 is retained in the nucleus where it induces the expression of target genes involved in cell-cycle arrest or apoptosis. Thus, it is certain that the ability to retain p53 in the nucleus is critical for p53-mediated stress response. To investigate the localization of endogenous p53 in GSN transfectecd HepG2 cells, we transfected GFP-GSN into HepG2 cells and protein localization was visualized by anti-p53 antibody conjugated red fluorescence and self-fluorescence of GFP for GFP-GSN. As shown in Fig. 3A, lower merged panel, endogenous p53 with overexpressed GFP-GSN are co-localized in the cytosol (arrow) whereas p53 alone without GFP-GSN expression is located in the nucleus during the same phase. To quantify the localization of p53, $1 \times 10^3$ of GFP-GSN-transfected cells were counted. GFP-GSN significantly increased the proportion of cytoplasmic p53 to 73.7% compared with 28.4% of the GFP-mock transfected group (Fig. 3B). To further confirm the sub-cellular localization of p53 and GSN, HepG2 cells were transfected with GFP empty vector or GFP-GSN together with FLAG-p53 and nuclear and cytoplasmic fractions were separated (Fig. 3C, total protein expressions were visualized by Western blotting in Supplementary data 2A). Through Western blotting analysis, sub-cellular amounts of both proteins were determined. Consistent with previous data, GFP-GSN was almost exclusively located in the cytoplasmic fraction; however, cytoplasmic p53 was increased by GFP-GSN co-transfection in contrast to the mock transfected group. To ensure the complete separation of sub-cellular fractions, marker proteins (cytoplasmic marker; β-tubulin and nuclear marker; Histone H3) were immunoblotted. Taken together, these results demonstrate that p53 is sequestrated in the cytoplasm by GSN through their protein interaction.

### 3.4. GSN represses the p53-mediated apoptosis through the inhibition of its transcriptional activity

To examine whether GSN could affect p53-dependent transcriptional activation through their physical interaction, HepG2 cells were transiently co-transfected with a p53 expression plasmid and a luciferase reporter plasmid containing the p53-responsive element from the p21 promoter, together with or without the GSN expression plasmid. As shown in Fig. 4A, enhanced luciferase activity by p53 transactivation (lane 2) was repressed by GSN over-expression in a dose-dependent manner (lanes 3 and 4). Because

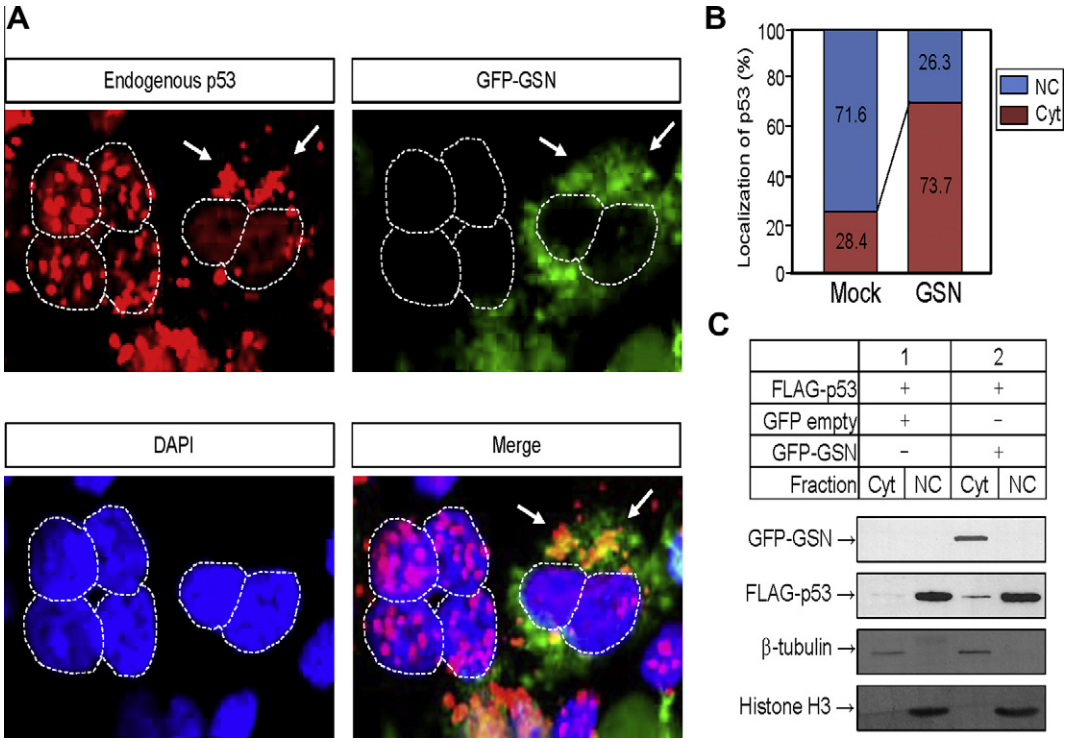


**Fig. 3.** GSN inhibits the nuclear translocation of p53. (A) Endogenous p53 levels in GFP-GSN-transfected HepG2 cells were analyzed by immunofluorescence. Cells were fixed and immunostained with anti-p53 and then visualized with red dye or self-fluorescence of GFP, respectively. (B) The localization of p53 was counted in GFP-GSN-transfected $1 \times 10^3$ HepG2 cells. (C) After HepG2 cells were transfected with FLAG-p53 and GFP-GSN, their nuclear and cytoplasmic fractions were separately prepared as described in Section 2. Western blot analysis was performed using anti-FLAG and GFP antibodies. Whole cell lysates served as controls (Supplementary data 2A).

GSN inhibited the transcriptional activity of p53, these results raised the possibility that GSN may inhibit p53-mediated apoptosis. To investigate whether GSN could reduce p53-mediated apoptosis, HepG2 cells were transfected with FLAG-p53 together with GFP-GSN encoding plasmids. We then examined the expression levels of endogenous p21 and Bax, which are transcriptional target genes of p53, by immunoblot analysis. As shown in Fig. 4B, the expression levels of p21 and Bax were increased by the overexpression of p53. However, the increased p21 and Bax levels were reduced by GSN expression (lanes 3 and 4). These results suggested that GSN repressed p53-mediated transcriptional activities through the interaction of GSN and p53.

To determine whether GSN inhibits p53-mediated apoptosis, HepG2 cells were co-transfected with the FLAG-p53 and HA-GSN expression plasmids. When we examined the cellular DNA content profile by flow cytometric analysis, co-expression of cells with p53 and GSN resulted in a significant decrease, by 1.85%, in cell-cycle arrested DNA patterns (Fig. 3C). Moreover, apoptotic cells were monitored by double staining with PI and Annexin-V (Fig. 3D). The p53-mediated apoptosis, Annexin-V positive and PI negative stained portion, which represents an early-apoptotic pattern, was significantly decreased by GSN overexpression to 12.8% compared with 29.9% in cells transfected solely with FLAG-p53. Taken together, these results indicate that GSN represses p53-induced apoptosis in hepatocarcinoma HepG2 cells.

## 4. Discussion

In this study, we found that GSN regulates the transcriptional activity of p53 through their physical interaction. We also demonstrated that the transactivation- and DNA binding domains of p53 were required for interaction with GSN (Fig. 2). Ectopic expression of GSN repressed the p53-mediated transcriptional activity on the p21-promoter (Fig. 4A). Furthermore, p53-mediated apoptosis is significantly inhibited by GSN expression in hepatocarcinoma cells (Fig. 4D).

Previous studies have demonstrated that GSN not only acts as an actin regulatory protein, which is important for cytoskeleton reorganization, cell morphology, and motility, but can also play an important role in apoptosis [14]. These controversial functions in apoptosis cause different expression in cancer. In many cancers including breast, stomach, colon, lung, bladder, prostate, and kidney, expression of GSN is reduced, while it is up-regulated in non-small lung cancer and in tumor transition from noninvasive to invasive state [21,22]. Furthermore, the role of GSN as a cofactor for transcription factor has been reported [26,27]. In response to dihydrotestosterone (DHT), androgen receptor (AR) translocate from cytosol to nucleus for transactivating target gene as a transcription factor, whereas GSN which interact to androgen receptor surrounded the nucleus during AR accumulated inside the nucleus. In addition, functional analyses demonstrate that GSN enhances AR activity in the presence of either androgen or hydroxyflutamide (HF). Thus, GSN plays a role as a cofactor for transcription factor.

Using a yeast two-hybrid assay, we have identified a variety of p73β (p53 homolog) interacting proteins that are involved in regulation of p73β-mediated transcriptional activation and apoptosis. We also found GSN acts as a binding partner of p73β and confirmed this interaction *in vivo* (Supplementary data 1A and B). However, we found that the binding affinity of GSN with p53 is stronger than p73β (Supplementary data 1C) and it specifically binds to the TAD and DBD region of p53 (Fig. 2). Several reports have demonstrated that p53 has the potential to interact with the cytoskeleton proteins. p53 associates with the microtubules and is transported to the nucleus by the dynein motor complex [31,32]. The association of p53 with microtubules allows for its transportation to the peri-nuclear region and subsequent entrance into the nucleus.

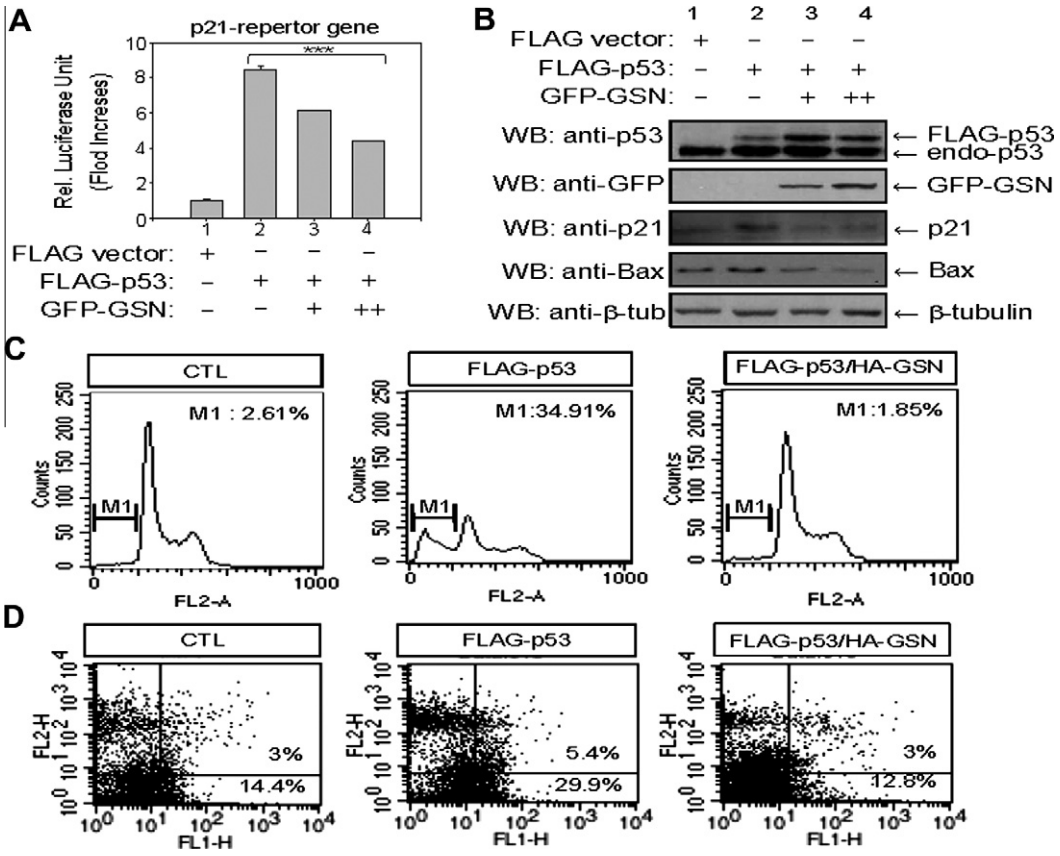


**Fig. 4.** GSN represses the p53-mediated apoptosis in HepG2 cells. (A) HepG2 cells were co-transfected with combined expression plasmids of FLAG-p53 and GFP-GSN, together with a luciferase reporter plasmid containing the p21 promoter. The data were normalized to β-galactosidase activity and are expressed in relative fold increase of luciferase units (RLU). Statistical significance is represented by Tukey's *post hoc* test (***, $p < 0.001$). (B) HepG2 cells were co-transfected with expression plasmids of FLAG-p53 and GFP-GSN and then protein expressions of FLAG-p53, GFP-GSN, endogenous p21, and Bax were detected through immunoblotting using the respective antibody. Expression of β-tubulin was included as a loading control. (C, D) Twenty-four hours after indicated plasmid transfection, cells were subjected to Propidium iodide (PI) or PI/Annexin-V double staining and then FACS analysis was performed as described in Section 2. Propidium iodide negative and Annexin-V positive cells were analyzed as apoptotic phase. Cell lysates were immunoblotted with anti-FLAG and anti-HA antibodies and β-tubulin expression was included as a loading control.

Moreover, we examined the physiological significance of the binding between GSN and p53 by assessing modulations in the transcriptional activity of p53 (Fig. 4A). It is possible that GSN might repress transcriptional activity of p53 through specific interactions with the transactivation domain and DNA binding domain of p53. GSN binding to the transactivation domain might prevent the association of co-activators of p53, such as p300/CBP, PCAF, and TIP60. Also, binding of GSN to the DNA binding domain may reduce the DNA binding affinity to its target gene promoters. However, because GSN was mostly located in the cytoplasm, it raised the possibility that GSN mainly inhibits the activity of p53 through protein interaction in the cytoplasm rather than in the nucleus. Cytoplasmic sequestration of p53 by its binding proteins—such as small Hsp alphaB-crystallin, adenoivirus E1B protein, Parc, and mortalin—inactivate p53 function in some cancers [11,33–36]. These proteins colocalize with p53 in cytosol and inhibit the translocation of p53 to the nucleus. Cytoplasmic sequestration of p53 represses the transcriptional function of p53 and reduces the p53-mediated apoptosis.

The effects of GSN on cellular physiology have mostly focused on cytoskeleton structure and its related mechanisms. Other possible functions remain to be fully elucidated. In this context, it is important to note that our findings indicate a functional linkage between GSN and p53 *in vivo*, showing that GSN associates with and negatively regulates transcriptional activity of p53 in HepG2 hepatocarcinoma cells. Our findings may aid in a molecular understanding of possible defects in the regulation of p53 in chemoresistant hepatocarcinoma cells. Furthermore, these examinations should be considered when knockdown of GSN is used as a cancer therapy to enhance p53-mediated apoptotic activity during chemotherapy.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.07.034.

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