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SVH-B interacts directly with p53 and suppresses the transcriptional activity of p53

Xinyuan Zhou, Guohua Yang, Ruimin Huang[1], Xiaotao Chen, Gengxi Hu*

State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

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Abstract **We previously reported that inhibition of SVH-B, a specific splicing variant of SVH, results in apoptotic cell death. In this study, we reveal that this apoptosis may be dependent on the presence of p53. Co-immunoprecipitation and GST pull-down assays have demonstrated that SVH-B directly interacts with p53. In both BEL-7404 cells and p53-null Saos-2 cells transfected with a temperature-sensitive mutant of p53, V143A, ectopically expressed SVH-B suppresses the transcriptional activity of p53, and suppression of SVH by RNA interference increases the transcriptional activity of p53. Our results suggested the function of SVH-B in accelerating growth and inhibition of apoptosis is related to its inhibitory binding to p53.**

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Keywords: p53; SVH-B; Interaction; Transcriptional regulation

1. Introduction

Specific splicing variant involved in hepatocarcinogenesis (SVH) is closely related to the Armadillo proteins lost in epithelial cancers on chromosome X (ALEX) family, which contained at least three members: ALEX1, ALEX2, and ALEX3. These genes composed of only one single exon, are closely localized on chromosome Xq21.33–q22.2 [1]. However, *SVH* gene is localized on chromosome 7q11.22 and composed of seven exons. SVH is expressed in most human adult tissues. There are four variants of SVH, namely SVH-A, B, C and D, resulted from alternative splicing in the coding region of the SVH transcript. These four variants share the same N- and C-terminus, and all consist of a transmembrane domain and a single armadillo repeat. However, only the B variant of SVH (SVH-B) causes an accelerated-rate growth and confers tumorigenicity to non-malignant hepatocyte in nude mice, and suppression of SVH-B in hepatoma cells line BEL-7404 results in apoptotic cell death [2].

p53, a tumor suppressor protein, plays a central role in cellular processes such as growth arrest [3–5], DNA repair [5–8], and apoptosis [9–12]. The human p53 gene encodes a 393-amino acid protein and contains an N-terminus transactivation domain (TAD, amino acids 1–73), a sequence-specific DNA-binding domain (DBD, amino acids 100–293), and a C-terminus domain (CTD, amino acids 293–393) [13–15]. A number of regulators binding to p53 have been observed, and play various roles in p53 transactivation. For example, TAFII31 binds to the N-terminus of p53 and inhibits its activated transcription [16]; 53BP1 and 53BP2 bind to the central domain of p53 and enhance its mediated transcriptional activation [17]; p300 interacts with the C-terminus of p53 and stimulates its sequence-specific DNA binding [18].

In this study, we have demonstrated that SVH-B interacts directly with the DNA-binding domain of p53 and suppresses the transcriptional activity of p53. The results shed light on the molecular mechanism of growth control related to SVH-B gene.

2. Materials and methods

2.1. Cell culture and transfection

BEL-7404 was purchased from Type Culture Collection of Chinese Academy of Sciences, Shanghai. HEK-293T and Saos-2 were purchased from ATCC. These cells and their derivatives, *7RS*, *7RP-1*, and *7RP-2* cells [19], were maintained in Dulbecoo's modified Eagle's medium (Gibco) containing 10% fetal bovine serum, 100 μg/ml streptomycin sulfate, and 100 μg/ml penicillin at 37 °C in 5% CO_2. Transient transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

2.2. Plasmid construction

Full-length human SVH-A, B, C, D, and p53 were cloned into pcDNA3.1A (Invitrogen) and N-Flag-pcDNA3 (kindly provided by Dr. Gang Pei) vectors, respectively. The mutant V143A of p53 was constructed by point-mutation of Flag-p53 using Quick-Change II XL site-directed mutagenesis kit (Stratagene). Full-length human p53 and its deletion mutants were cloned into pGEX-2 T (Amersham Biosciences).

2.3. Western blot

According to our previous protocol [2], the samples were loaded on SDS–PAGE, transferred, probed with antibodies, and visualized with enhanced chemiluminescence. Antibodies against SVH, myc-tag, p53, GST (HanMan), α-tubulin (Zymed Laboratories), Flag, Flag-HRP (Sigma–Aldrich), and p53-HRP (R&D Systems) were used.

2.4. In vivo co-immunoprecipitation

Co-transfected or non-transfected HEK-293T cells were lysed for 1 h at 4 °C in immunoprecipitation (IP) buffer (10 mM Tris–HCl, pH 7.4,

*Corresponding author. Fax: +86 21 54921342.
E-mail address: hgxgene@sunm.shcnc.ac.cn (G. Hu).

[1]Present address: Department of Neurology, Molecular Pharmacology and Chemistry Program, Memorial Sloan Kettering Cancer Center, 1275 York Avenue, New York, USA.

Abbreviations: RNAi, RNA interference; TAD, transactivation domain; DBD, DNA-binding domain; CTD, C-terminus domain

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150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.5% NP-40, 0.2 mM Na_3VO_3, and 0.2 mM PMSF). Cell extracts were incubated with 2 μg/ml of mouse anti-myc, anti-SVH or IgG (HanMan) overnight at 4 °C. Immunocomplexes were isolated by incubation 2 h at 4 °C with protein A agarose (Invitrogen). Then, the immunoprecipitated samples were washed extensively with IP buffer. Western blot was performed with the corresponding mouse anti-Flag-HRP or anti-p53-HRP antibody.

2.5. Apoptosis assay

For measuring the cell apoptosis, cells were transfected with SVH-RNAi and GFP-RNAi plasmids for 48 h, Annexin-V-FLUOS (Roche) staining were performed according to the manufacturer's protocol and analyzed by flow cytometry (Becton Dickinson).

2.6. In vitro GST pull-down

HEK-293T cells transfected with Flag-tagged SVH-B were lysed for 1 h at 4 °C in IP buffer, and cell extracts were incubated with anti-Flag (M2)-agarose beads (Sigma–Aldrich). Beads were washed with IP buffer, and then eluted with the same buffer containing 0.2 mg/ml FLAG peptide (Sigma–Aldrich). Bacteria expressed proteins of p53 and its mutants were induced by 0.1 mM IPTG at 30 °C for 2 h and purified using glutathione agarose beads (BD Biosciences Pharmingen). SVH-B-Flag, and p53, its mutants, or GST alone were each mixed and incubated for 2 h at 4 °C. The beads were separated by a brief centrifugation, washed with buffer 1 (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% NP-40, and 0.2 mM PMSF). Western blot was performed with the corresponding mouse anti-Flag and anti-GST antibody.

2.7. Quantitative real-time RT-PCR assay

Total RNA extraction and reverse transcription were performed as described previously [2]. To measure mRNA level of p21, a real-time PCR assay was performed on a DNA Engine Opticon 2 (MJ Research) using the DyNAmo SYBR Green qPCR kit (FINNZYMES). Human p21 RT-PCR forward primer was CTGCCCAAGCTCTACCTTCC, and its RT-PCR reverse primer was TGGAGAAGATCAGCCGGCG. Human GAPDH RT-PCR forward primer was GGGGAGCCAAAAGGGTCATCATCT, and its RT-PCR reverse primer was GAGGGGCCATCCACAGTCTTCT. A threshold cycle value for each mRNA was determined using the Opticon Monitor software (version 2.02). All data were normalized to the GAPDH mRNA level and expressed as mRNA relative change.

2.8. RNA interference

Plasmids encoding shRNAs directed against human SVH was constructed by cloning oligonucleotides targeting nucleotides 1056–1074 (GACCTGTTCCAGGTGTTAC) of SVH sequence (NM_031905), in pSuper.retro.puro (OligoEngine), as previously described [20]. Oligonucleotides targeting green fluorescent protein (GFP) (GAACGGCATCAAGGTGAAC) was used as a control.

2.9. p53-mediated reporter assay

WWP-Luc, a p53-responsive luciferase reporter plasmid containing CDKN1A (p21) promoter [3], was kindly provided by Dr. Bert Vogelstein. To measure p53 transcription activity, BEL-7404 cells were co-transfected with WWP-Luc, pRL-TK (Promega), Flag-p53 and SVH-B, pcDNA3.1A, SVH-RNAi or GFP-RNAi individually and maintained for 48 h. The luciferase activity in cell lysates was measured by a Dual-Luciferase Reporter Assay System (Promega) following the manufacturer's protocol.

3. Results and discussion

3.1. SVH-B is the predominant SVH protein in BEL-7404 cells

Using an anti-SVH monoclonal antibody, overexpressed Flag-tagged SVH-A, B, C, and D proteins were successfully detected both in immunoblotting and immunoprecipitation assay (Figs. 1A and B). However, endogenous SVH proteins from BEL-7404 cells could not be detected by Western blot probably due to their low expression level (data not shown). Then the endogenous SVH proteins in BEL-7404 (Fig. 1C) and HeLa cells (data not shown) were immunoprecipitated and enriched using this antibody. The precipitated proteins were validated by mass spectrometry. Together with the molecular weight information, it showed that SVH-B protein was the predominant format in both cell lines tested (Fig. 1C and data not shown).

3.2. RNAi suppression of SVH induces apoptosis in a p53-dependent manner

We previously reported that specifically suppression of SVH-B by antisense oligonucleotides treatment could induce apoptosis in BEL-7404 cells [2]. It was well known that tumor suppressor p53 plays a pivotal role in apoptosis [9–12]. So, we investigate whether SVH-B inhibition induced apoptosis is p53-dependent. Because BEL-7404 cells have wild type p53 expression, we generated the p53-RNAi stably transfected BEL-7404 cells to completely ablate p53 [19]. RNAi ablation of SVH was also confirmed in BEL-7404 and Saos-2 cells transfected with Flag-tagged SVH-B gene plasmid (Fig. 2A). Then, SVH-RNAi or GFP-RNAi plasmid as control was transfected into BEL-7404 cells with wild type p53 [parental (*Par*) or *7RS* cells] and p53-null (*7RP-1* or *7RP-2* cells), respectively. It showed that SVH-RNAi treatment increased apoptosis incidence in the parental BEL-7404 cells (from 12.01% to 17.74%) and control cell line *7RS* (from 14.2% to 19.88%). However, in the p53-null BEL-7404 cells, there was no significant change (Fig. 2B). The above results indicated that p53 is involved in apoptosis induced by RNAi ablation of SVH.

3.3. SVH-B interacts with p53 both in vitro and in vivo

Since SVH-B induced apoptosis is p53-dependent, we performed both co-immunoprecipitation (Co-IP) and GST pull-down assays to study whether SVH-B could interact with p53 directly. In HEK-293T cells, Flag-tagged p53 plasmid was co-transfected with myc-tagged SVH-B plasmid. Using an anti-myc antibody, exogenous Flag-tagged p53 protein was co-immunoprecipitated with exogenous myc-tagged SVH-B protein (Fig. 3A). We also applied endogenous Co-IP assay in HEK-293T cells. As Fig. 3B shown, after incubation of SVH antibody, endogenous p53 protein was co-immunoprecipitated with endogenous SVH protein. It suggested that SVH protein could interact with p53 protein in vivo.

The interaction domain of p53 protein with SVH protein was detected by in vitro GST pull-down assay. Series deletion of p53 gene were constructed to map the binding domain for SVH protein, including Full length (1–393), Deletion 1 (1–101, containing N-terminus transactivation domain), Deletion 2 (98–322, containing DNA-binding domain), and Deletion 3 (293–393, the C-terminus domain). It was shown in Fig. 3C that only the formats of Full length and Deletion 2 of p53 protein could pull down the SVH-B protein and the Deletion 2 was more efficient than the whole protein. It indicated that the DNA-binding domain of p53 is indispensable for the interaction between SVH-B and p53 proteins and some domains out of the DNA-binding domain might inhibit the interaction.

In non-stressed cells, p53 is continuously shuttling in and out of the nucleus, and its subcellular distribution varies throughout the cell cycle. Under stress conditions, p53 is accumulated in the nucleus where it induces or inhibits the expression of stress response genes [21]. We examined the subcellular locali-

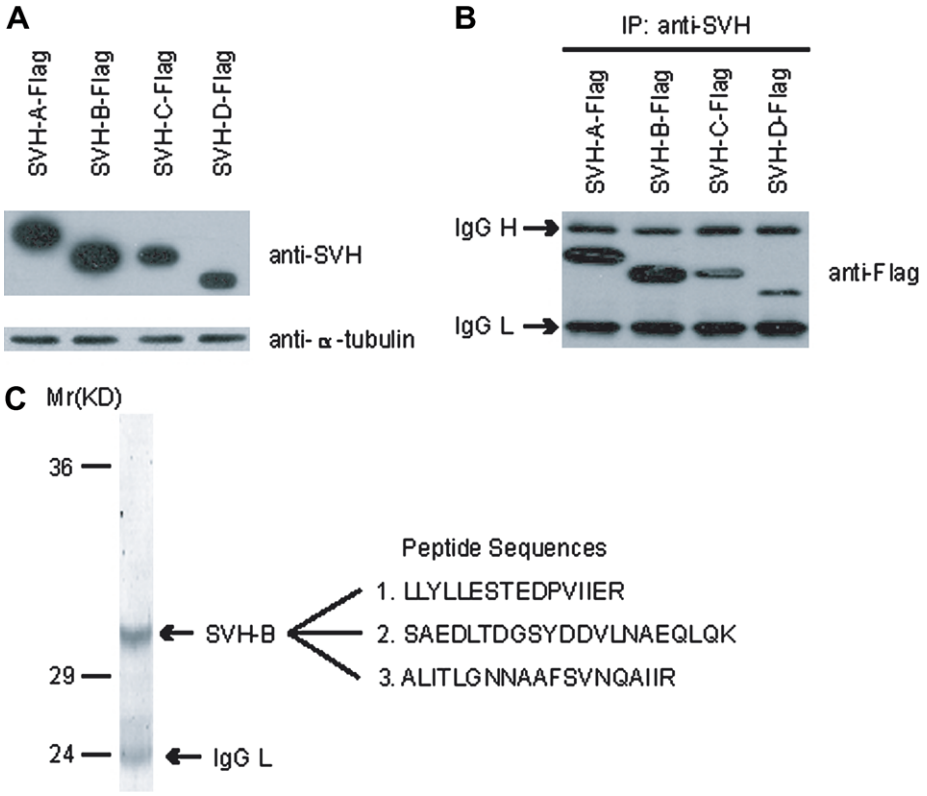

Fig. 1. SVH-B is the predominant SVH protein in BEL-7404 cells. (A) Four exogenous SVH proteins were detected by Western blot using a SVH monoclonal antibody. HEK-293T cells were transfected with Flag-tagged SVH-A, B, C, and D. Cells lysates were blotted with a SVH antibody. Alpha-tubulin was shown as a loading control. (B) Confirmation of SVH antibody's specificity by immunoprecipitation. Exogenous Flag-tagged SVH proteins were immunoprecipitated with the SVH antibody and were detected with anti-Flag antibody. IgG was shown as a loading control. (C) SVH-B is the predominant endogenous format in BEL-7404 cells. Lysates from BEL-7404 cells were immunoprecipitated with the SVH antibody, separated by SDS–PAGE and stained by Coomassie-blue solution. The major band was cut and purified for peptide sequencing by mass spectrometry. The peptide sequences indicating SVH-B were presented.

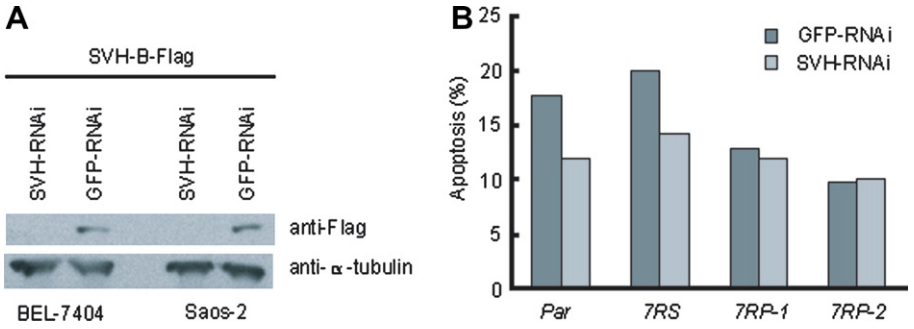

Fig. 2. Apoptosis induced by suppression of SVH is p53-dependent. (A) SVH-RNAi inhibited expression of Flag-tagged SVH-B gene in BEL-7404 and Saos-2 cells. BEL-7404 and Saos-2 cells were co-transfected with Flag-tagged SVH-B and SVH-RNAi or control GFP-RNAi plasmids. The cell lysates were blotted with anti-Flag antibody. Alpha-tubulin was shown as a loading control. (B) Effect of SVH ablation on apoptosis incidence in wildtype p53 (*Par* or *7RS* cells) and p53-null (*7RP-1* or *7RP-2* cells). Cells were transfected with SVH-RNAi or GFP-RNAi plasmids as indicated. After 48 h, Annexin-V-FLUOS staining were performed and analyzed by flow cytometry. Results shown represent three independent experiments.

zation of SVH protein in BEL-7404 cells. The ectopically expressed SVH protein was allocated mostly in the endoplasmic reticulum (ER) [2], but the endogenous SVH protein also localized in mitochondria and cell nucleus (data not shown). In addition, SVH has two predicted nuclear export signals (NES) and it would be accumulated in the cell nucleus by mutation of NES, although SVH-B could not change the subcellular localization of p53 (data not shown). It suggested that SVH-B mostly interacted with p53 in the cell nucleus and suppressed the transcriptional activity of p53 throughout its binding to the specially DNA element.

3.4. SVH-B inhibits the transactivation activity of p53

The DNA-binding domain in p53 protein is the most important functional unit for p53 to recognize and bind to the element of its specific downstream target gene [3–11]. Because SVH-B interacts with the DNA-binding domain directly, it is necessary to explore whether the binding of SVH-B could

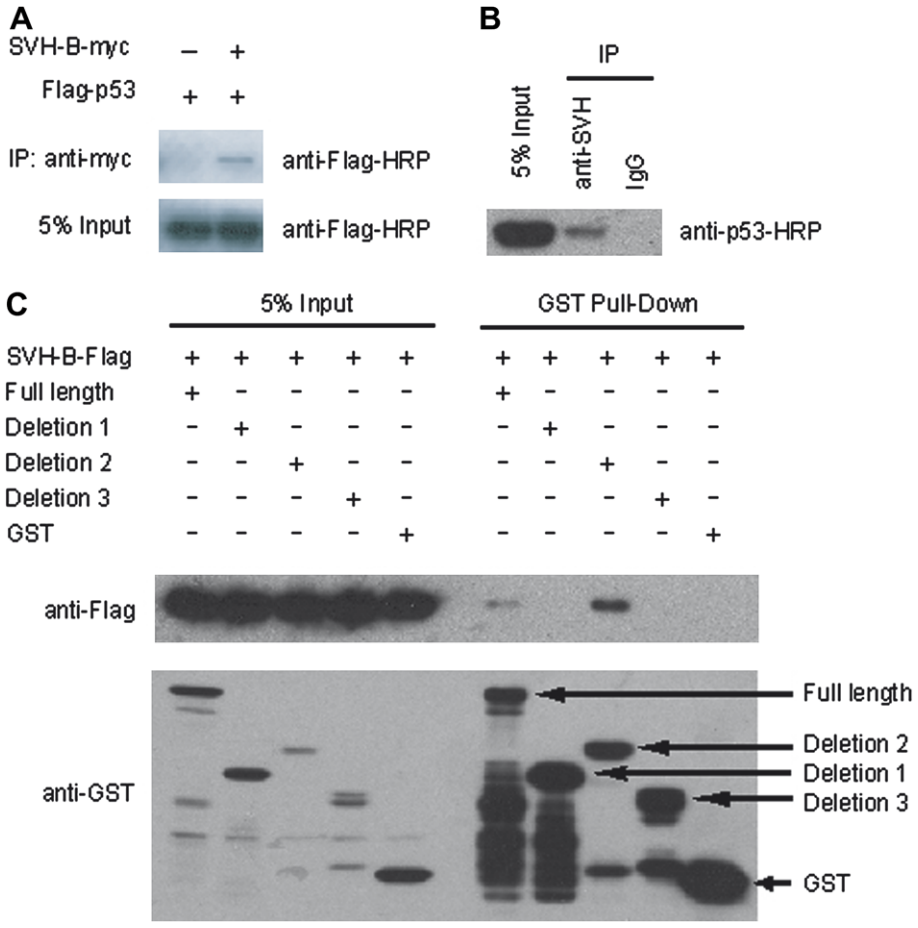

Fig. 3. Direct interaction between SVH-B and p53. (A) Exogenous co-IP assay. HEK-293T cells were co-transfected with myc-tagged SVH-B and Flag-tagged p53 plasmids and then subjected to immunoprecipitation with anti-myc antibody. Co-immunoprecipitated Flag-tagged p53 protein was detected with anti-Flag-HRP antibody. (B) Endogenous co-IP assay. Cell lysates from HEK-293T cells were incubated with anti-SVH antibody or mouse IgG, immunoprecipitated proteins were detected by anti-p53-HRP antibody. (C) Mapping the interaction region of p53 protein with SVH-B protein. The purified Flag-tagged SVH-B protein was incubated with GST-fused p53 full length, or deletion mutants, or GST alone as indicated, and then isolated using glutathione-agarose beads. Bound proteins were detected with anti-Flag antibody. Arrows indicate the expression of p53 deletion mutants.

decrease transactivation activity of p53. CDKN1A (p21) gene is a well-known downstream target gene of p53 [3]. By overexpression of SVH-B in BEL-7404 cells, mRNA expression level of p21 gene was reduced 45% (Fig. 4A). A p53-responsive luciferase reporter plasmid under the control of p21 promoter containing the p53-binding sites, WWP-Luc, was used. Overexpression of SVH-B also made a 57% decrease of luciferase activity in p21 reporter (Fig. 4B). The similar results were also obtained in QSG-7701 and HEK293 cells (data not shown). In addition, suppression of endogenous SVH-B using RNA interference in BEL-7404 cells, could enhance both p21 transcripts expression level (Fig. 4C) and activity of p21 transcriptional reporter (Fig. 4D).

To generate a temperature-inducible p53 transactiviation system, p53-null Saos-2 cells were transfected with a temperature-sensitive mutant of p53, $p53^{V143A}$, which can induce transactivation of downstream target gene at 32 °C or below, but usually loose the transactional activity at 37 °C [22]. As shown in Fig. 4E, p21 expression was induced to peak (sevenfolds comparing to the baseline) after 12 h cultured at 32 °C by ectopically expressed $p53^{V143A}$. With the presence of $p53^{V143A}$ at 32 °C, overexpression of SVH-B dramatically inhibited the expression of p21 by 90% (Fig. 4F), whereas SVH-RNAi increased the p21 expression by 2 folds comparing to the control GFP-RNAi (Fig. 4G). Thus, in two different cell lines with endogenous wildtype p53 or inducible-p53, we demonstrated that SVH-B inhibited the transcriptional activity of p53.

It is well known that the transcriptional activity plays a pivotal role in mediating the p53 response. A lot of proteins have been shown to interact with and play roles in p53 transactivation. Some directly inhibit its transcriptional activity (such as TAFII31) [16]; some indirectly enhance it (such as 53BP1 and 53BP2) [17]; some directly increase it (such as p300) [18]; others change the stability of p53 (such as MDM2 and Hsp90) [23,24]. Here, we have also demonstrated that SVH-B interacts directly with the DNA-binding domain of p53 and suppresses the transcriptional activity of p53. It could partially explain the growth accelerating function of SVH-B and its possible involvement in hepatocarcinogenesis [2].

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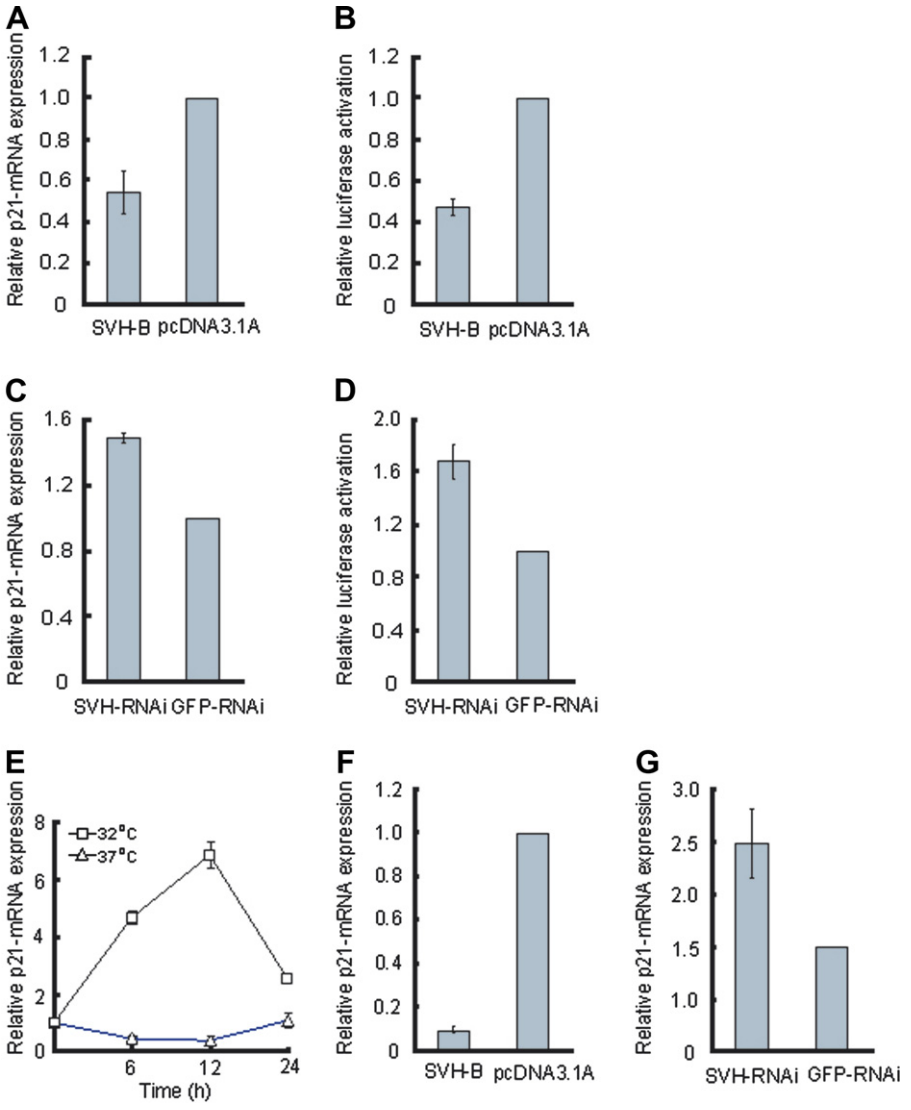

Fig. 4. SVH-B inhibits the transcriptional activity of p53 gene. (A,C) Effects of the SVH-B over-expression (A) and suppression (C) on the p21-mRNA expression in BEL-7404 cells. mRNA level of p21 gene was measured by quantitative real-time PCR, normalized mRNA level of GAPDH housekeeping gene. Cells transfected with control plasmids (pcDNA3.1A or GFP-RNAi) was defined as 1. (B,D) Effects of the SVH-B overexpression (B) and suppression (D) on the luciferase activity of p21 reporter in BEL-7404 cells. WWP-Luc and pTK-RL plasmids were co-transfected as transfection efficiency control. Luciferase activity was measured after 48 h transfection. (E) Temperature induced p21 mRNA expression in $p53^{V143A}$ transfected Saos-2 cells. Forty-eight hours after $p53^{V143A}$ transfection, Saos-2 cells were cultured at 32 °C or 37 °C for indicated time. The p21-mRNA expressions were measured by quantitative real-time PCR. (F,G) Effects of the SVH-B over-expression (F) and suppression (G) on the p21-mRNA expression in $p53^{V143A}$ transfected Saos-2 cells. Saos-2 cells were co-transfected with $p53^{V143A}$ and indicated plasmids for 48 h, and maintained at 32°C for 12 h. The mRNA levels of p21 were measured by quantitative real-time PCR. Data are shown as means ± S.D. of three independent experiments.

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