

NSPc1 is a cell growth regulator that acts as a transcriptional repressor of p21Waf1/Cip1 via the RARE element

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ABSTRACT

The mammalian polycomb group proteins play an important role in cell cycle control and tumorigenesis. Nervous system polycomb 1 (NSPc1) is a newly identified transcription repressor, highly homologous with PcG protein Bmi-1. In this article, we showed that NSPc1 could promote tumor cell cycle progression and cell proliferation. Semi-quantitative RT-PCR showed that NSPc1 did not affect the expression levels of most Cyclin-dependent kinases (CDK) inhibitors except for p21Waf1/Cip1. Repression activity assays, chromatin immunoprecipitation (ChIP) and DNA pulldown assays all verified that NSPc1 represses the expression of p21Waf1/Cip1 by binding to the (–1357 to –1083) region of the p21Waf1/Cip1 promoter *in vivo*, and the repression effect is dependent on the retinoid acid response element (RARE element) within the above region of the p21Waf1/Cip1 promoter. Further analysis showed that NSPc1 could compete the RARE element site with RA receptors both *in vitro* and *in vivo*. Taken together, our results support the hypothesis that NSPc1 has a positive role in tumor cell growth by down-regulating p21Waf1/Cip1 via the RARE element, which directly connects transcriptional repression of PcGs to CDKs and RA signaling pathways.

INTRODUCTION

Polycomb group (PcG) Proteins are epigenetical chromatin modifiers that can transcriptionally silence their targets through cell division during development and therefore

provide a cellular memory mechanism (1,2). Since PcG proteins were first discovered in *Drosophila* as the regulator of homeotic (Hox) genes expression, many mammalian homologues of PcG proteins have been identified so far. The mammalian PcG proteins are classified into two major Polycomb Repressive Complexes (PRCs). Among them, Mel-18, Bmi-1, MBLR and NSPc1 are classified into the Polycomb Repressive Complex 1 (PRC1) based on their structural similarities with the *Posterior sex comb (Psc)* of *Drosophila* (3–7). Now accumulated evidence indicates that these mammalian homologues of *Psc* protein may be implicated in cell cycle control, cell proliferation and tumorigenesis (8). For instance, Bmi-1 is a proto-oncogene to induce mouse lymphoma in association with c-myc (9), and Bmi-1 can also promote cell proliferation and stem cells self-renewal by repressing the p16INK4a/p19ARF locus (10). On the contrary, Mel-18 is a transcriptional negative regulator with tumor suppressor activity (11), and could also inhibit the proliferation in differentiated B lymphocytes through a c-myc/cdc25 cascade (12).

The human NSPc1 gene encodes a protein that shares high homology with Mel-18 and Bmi-1 (4). Moreover, NSPc1 protein is mainly localized in the nucleus and has a transcription repression activity (13). Recently, NSPc1 is identified as one of the genes highly expressed in HCT-116 deprived tumors grown in C.B-17 SCID mice (14), one of the most differentially expressed genes in HeLa cells after camptothecin treatment (15) and one of the 32 marker genes in kidney cancers (16). These evidences infer that NSPc1 may play a role in the pathogenesis of tumors. However, until now little is known about whether NSPc1 has a role in tumor cell cycle control and tumorigenesis.

Cell cycle progression in eukaryotic cells is finely regulated by the sequential activation of cyclin-dependent kinases (CDKs), which are activated after interaction with their corresponding cyclins (17). The activity of these

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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CDKs is restrained by two groups of CDK inhibitors (CDKIs): the INK4 family, which includes CDKN2A/p16 ARF, CDKN2B/p15 INK4B, CDKN2C/p18 INK4C, CDKN2D/p19 INK4D; and the Cip/Kip family, consisting of CDKN1A/p21 CIP1, CDKN1B/p27 KIP1, CDKN1C/p57 KIP2 (18). The kinase inhibitor p21 Waf1/Cip1 (hereafter p21) retards S phase progression by inhibition of cyclin-dependent kinases (19) and functions as a G2 checkpoint by binding to cyclin B1–cdc2 complexes, which are integral in the G2/M transition (20,21). Deregulation in the expression of p21 would be expected to result in abnormal cell proliferation and to predispose the cells to transformation (22,23).

PcGs have been found to repress some negative cell cycle regulators to promote cell cycle transition and cell proliferation (8,24,25). For example, PcG proteins Bmi-1 and Cbx-7 have been identified to regulate cell proliferation by repressing the p16ARF/p19INK4a locus in different tissues and cell lines (9,26). In the present study, we explored the role of NSPc1 in tumor cell growth regulation and demonstrated that p21 is the direct target gene regulated by NSPc1. The underlying molecular mechanism has also been investigated.

MATERIALS AND METHODS

Cell culture and transfection

HeLa or COS-7 cells were maintained in DMEM, SH-SY5Y in RMPI 1640 with 10% heat-inactivated FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin under a humidified atmosphere of 5% CO₂ and 95% air at 37°C. Lipofectamine 2000 (Invitrogen, USA) was used to transfect cells according to the manufacturer's instructions.

Flow cytometric analysis

Flow cytometric analysis was carried out at the Flow Cytometry facility of PUMC. Briefly, cells grown on 100 mm tissue culture dishes to 70% confluency were trypsinized, pelleted and washed with pre-chilled phosphate-buffered saline (PBS). Cells were fixed at –20°C overnight with 70% ethanol, washed with PBS, and resuspended in 0.1% Triton X-100 containing RNase A (200 µg/ml, Sigma) and propidium iodide (200 µg/ml, Sigma). Cells were then incubated at room temperature for 30 min before detection. Data were collected using a Becton–Dickinson FACScan apparatus with doublet discrimination and analyzed using the CELL Quest (Becton–Dickinson) and ModFit LT 3.0.

Establishment of NSPc1 stably integrated cell pools and NSPc1 knocked down cell pools

To establish NSPc1 stably integrated HeLa cell pools, the coding sequence of NSPc1 was inserted into pcDNA3.1-V5 (Invitrogen, USA) for transfection into HeLa cells. A control set of cells was transfected with empty vector. After 24h of transfection, the growth medium was replaced with fresh DMEM containing all of the supplements listed above, as well as 600 µg/ml G418 (Gibco, USA) in order to select for geneticin-resistant cells. After 2–3 weeks of selection, pools of G418-resistant cells, denoted as 'NSPc1 + V5' and 'V5', respectively, were identified with

semi-quantitative RT-PCR and western blot. To establish NSPc1 knocked down cell pools, the IMG800-based plasmids (GeneSuppressor system, Imgenex, USA), which contains the human U6 promoter, were constructed as described by the manufacturer <http://www.imgenex.com>. Briefly, several 21-nucleotide sequences within the coding region of NSPc1 were selected as candidates and the PCR amplified fragments were inserted into the IMG800 vector. Final constructs including the region of nt 94–114 and nt 515–536 were used and named 'RNAi1' and 'RNAi2', respectively. The two RNAi constructs were then transfected into HeLa cells along with IMG800 empty vector as a control. After 24 h of transfection, the cells were switched to grow in the presence of G418 (600 µg/ml). After 2–3 weeks of selection, pools of G418-resistant cells, denoted as NSPc1-RNAi1, 2 and IMG800 (control), were also identified with the same methods. These above mentioned cell pools were then replated, expanded and maintained in complete media containing 300 µg/ml G418 as stable integrated cell pools. For experiments described below, log phase cells between 4th and 10th passages were utilized.

Semi-quantitative RT-PCR analysis

Total RNA was isolated from the stable cell pools using reagent Trizol (Invitrogen, USA) according to the manufacturer's instructions. Reverse transcription was performed using a first-strand cDNA kit (Life Technologies, USA) following the protocol recommended by the manufacturer. RT-PCR exponential phase was determined on 25–35 cycles to allow semi-quantitative comparisons among cDNAs developed from identical reactions. Each PCR regime involved a 5 min initial denaturation step at 94°C, followed by amplification cycles (e.g. 25 cycles for GAPDH and 32 cycles for NSPc1 and p21) of 94°C for 30 s, 57°C for 30 s, and final extension at 72°C for 30 s. The primers are NSPc1-up: 5'-gacacagccactgctcaa-3', NSPc1-down: 5'-tgcacatgctgagggtt ag-3'; GAPDH-up: 5'-accacagtcctatgccatcac-3', GAPDH-down: 5'-tccaccaccctgttgetgta-3'; and CDKN1a (p21Cip1/Waf1)-up: 5'-atgtggacctgtcactgtctgtga-3', CDKN1a (p21Cip1/Waf1)-down: 5'-agggtatgtacatgaggaggtgt-3'; CDKN1b (p27kip1)-up: 5'-catttgggtggaccacaaagac-3', CDKN1b (p27kip1)-down: 5'-cctccttccccaaagtta-3'; CDKN1c (p57Kip2)-up: 5'-agatcagcgcctgagaagtc-3', CDKN1c (p57Kip2)-down: 5'-gg gaccagtgtacctctctcg-3'; CDKN2a (p16Arf/Ink4a)-up: 5'-gcacca gaggcagtaaccat-3', CDKN2a (p16Arf/Ink4a)-down: 5'-cgtgag tgctcactccagaa-3'; CDKN2b (p15Ink4b)-up: 5'tagtggagaaggtg cgacag-3', CDKN2b (p15Ink4b)-down: 5'gggtgggaaattgggtaa ga-3'; CDKN2c (p18Ink4c)-up: 5'-acgtcaatgcacaaatgga-3', CDKN2c (p18Ink4c)-down: 5'-gcctgcatcaggctaacaac-3'; CDKN2d (p19Ink4d)-up: 5'-ctgcaggtcatgatgtttgg-3', CDKN2d (p19Ink4d)-down: 5'-aagccacaactgtgctct-3'; Bmi-1-up: 5'-acgcaaacatgcaaaagaa-3', Bmi-1-down: 5'-gctgtaagcaaggtcaac-3'. Equal volumes of PCR mixtures were resolved on 1.5% agarose gels and visualized by ethidium bromide staining.

Cell extracts and western blotting

To detect the endogenous NSPc1 protein level in the stable cell pools, whole cell extracts were prepared and assayed using western blot as described before (13). Additionally, mouse monoclonal anti-FLAG (1:5000, Sigma, USA),

mouse monoclonal anti-p21WAF1/CIP1 (1:1000, Upstate, USA) and anti- β -actin (1:5000, Sigma, USA) antibodies were used for detection of their corresponding proteins. The horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:4000, Santa Cruz Biotechnology, USA) was used as the secondary antibody and signals were visualized using the enhanced chemiluminescence system (ECL, Amersham Biosciences, USA).

Cell proliferation by MTT assay

Cell proliferation was quantified using MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide assay). The HeLa stable cell pools seeded on 96-well microplates at 6000 cells/well were incubated in DMEM medium containing 300 μ g/ml of G418 for 1–7 day prior to be assayed. The medium was changed once with fresh culture medium on the third day. MTT assay was performed after 1, 3 or 5, 7 days as described before (27). Briefly, cells were incubated with 0.5 mg/ml of MTT (Sigma) for 4 h in a CO₂ incubator at 37°C. After the removal of the solution, the purple precipitates were dissolved in DMSO for 20 min at room temperature and the resultant solution was transferred to new 96-well plates. The absorbance was measured at 570 nm using a Labsystems Multiscan RC enzyme-linked immunosorbent assay (ELISA) reader. Each experiment was performed in triplicate, and the means were determined for each time point.

Colony formation assay

For colony formation assays, cells derived from NSPc1-V5 and NSPc1-RNAi1 stable cell pools were plated under sparse conditions (1000 cells per well of 6-well plate in triplicate) with 4% methyl cellulose semi-solid DMEM medium and fed with fresh DMEM growth medium containing 300 μ g/ml of G418 every 2–3 days. After 2 weeks, the plates were stained with crystal violet in 20% ethanol and photographed. The total number and the number of colonies greater than 2 mm in diameter were counted and the means and SDs were determined for each pool. The clonogenic assay was performed three times, which generated almost identical results.

Repression activity assays

Full-length of NSPc1, Bmi-1, c-Myc and RXR α was subcloned into the pcDNA6-FLAG (Invitrogen, USA) vector. The human p21Waf1/Cip1 promoter constructs (2.3 kb, 1.8 kb, mut-RARE and mut-E-box fragments of the p21 promoter construct ligated to pGL3-basic vector) were the generous gifts from Dr Gallego, C., as described previously (28). Synthetic Renilla Luciferase Report Vector (pRL-TK) was purchased from Promega. Repression activity assays were performed as described before (13). In short, exponentially growing COS-7 or HeLa cells were seeded onto 24-well plates at a density of 2×10^4 cells/well, 1 day prior to transfection. Cells were transfected with pcDNA6-FLAG-NSPc1, pcDNA6-FLAG-Bmi-1, pcDNA6-FLAG-RXR α and/or pcDNA6-FLAG-c-Myc, together with reporter plasmid pGL3-p21 promoter series (200 ng/well) and pRL-TK (100 ng/well) as an internal control. Total amounts of transfected DNA were equalized by the addition of empty vector

(pcDNA6-FLAG). Each transfection reaction was performed in triplicate. After 24 h post transfection, the medium was aspirated, and cells were harvested and assayed for luciferase activity by using the Dual-luciferase reporter assay system (Promega, USA). Results were obtained from three different transfection experiments after normalization for the internal control of TK activity. Experimental variations are indicated as means \pm SDs.

Chromatin immunoprecipitation (ChIP) assay

Approximately 2×10^8 (common PCR) or 2×10^7 (for Absolute Quantitative PCR) subconfluent HeLa cells grown in DMEM medium were collected, and native protein–DNA complexes were cross-linked by treatment with 1% formaldehyde for 15 min. The ChIP assay was generally carried out as reported earlier (29). Briefly, equal aliquots of isolated chromatin were subjected to immunoprecipitation with rabbit anti-NSPc1 antibody (13), RXRa antibody (Santa Cruz), pre-immunized sera or mouse IgG. The DNA fragments associated with specific immunoprecipitates or with negative control preimmune serum were isolated and used as templates for subsequent PCR reactions to amplify the p21 promoter sequences. The primers for the upstream region (–2264 to –1971) were: 5' primer, 5'-ttgagctctggcatagaaga-3'; 3' primer, 5'-taccagacacacttaagg-3'. The primers for the RA response element region (–1357 to –1063) were: 5' primer, 5'-agactctgagcagcctgag-3'; 3' primer, 5'-aacctcattgcagatggt-3'. The primers for the downstream core promoter region (–194 to +81) were: 5' primer, 5'-accggctggcctggaact-3'; 3' primer, 5'-tctgccgctctctcacct-3'. The primers for the GAPDH 2nd intron: 5' primer, 5'-aatgaatggcagccgtag-3'; 3' primer, 5'-agctagcctcctccacctgac-3'.

Absolute quantitative PCR

Absolute quantitative PCR was performed using the standard curve method and SYBR Green Chemistry on Applied Biosystems 7500 SDS instruments. Briefly, generate a standard curve for the target amplicon by plotting the Ct value versus amount of HeLa genomic DNA template (100, 50, 12.5, 3.12, 0.78 and 0.39 ng by serial dilution). The amount of target DNA in the given ChIP sample from normal, NSPc1 over-expressed or knocked-down HeLa cell was quantified by using the above mentioned standard curve. Real-time PCR amplification was performed in 20 μ l reaction mixture containing 1 μ l of ChIP DNA sample, 10 μ l of QuantiTect SYBR Green PCR Master Mix (Applied Biosystems) and 5 pmol of specific primers that are listed as the follows: upstream, 5'-gcaaatgttccaggcacaga-3'; downstream, 5'-tctaccacaccctgccc-3'. The data were used to calculate the ratio of total amount of precipitated DNA in different samples to the initial amount of input DNA used for immunoprecipitation. Results were expressed as means \pm SDs. An analysis of variance with ANOVA was used to examine differences between groups.

DNA pulldown assays

DNA pulldown assays were carried out following a previous description (30). COS-7 Cells were lysed by sonication in HKMG buffer (10 mM HEPES, pH 7.9, 100 mM KCl, 5 mM MgCl₂, 10% glycerol, 1 mM DTT and 0.5%

NP-40) containing protease and phosphate inhibitors. Cellular debris was removed by centrifugation. 1 mg of cell extracts were precleared with 40 μ l of streptavidin-agarose beads (Amersham Bioscience) for 1 h at 4°C, then incubated with 2 μ g of biotinylated double-stranded oligonucleotides and 40 μ g of poly(dI-dC)-(dI-dC) for 16 h at 4°C. DNA-bound proteins were collected with 60 μ l of streptavidin-sepharose beads for 1h at 4°C, washed twice with HKMG buffer, separated on a SDS-PAGE, and identified by western blot. The biotinylated double-stranded oligonucleotides were amplified using the following 5'-terminal biotinylated primers with the same sequences in ChIP detection: the primers for the RA response element region (-1357 to -1063) were: 5' primer, 5'-biotin-agactctgagcagcctgag-3'; 3' primer, 5'-aacctcattgcagatggt-3'; the primers for the downstream core promoter region (-194 to +81) were: 5' primer, 5'-biotin-accggctggcctgctggaact-3'; 3' primer, 5'-tctgcgccgtctctcact-3'. The mutant-RARE DNA amplicons were amplified using the pGL3-mutantRARE-p21-promoter constructs as the template.

RESULTS

Transiently over-expressed NSPc1 affects cell cycle distributions of HeLa and SH-SY5Y cells

Many members of the polycomb complexes have been implicated in cell cycle control. To test whether NSPc1 has a similar role in tumor cell growth as Mel-18 and Bmi-1, we transiently over-expressed NSPc1 in HeLa (PcG members enriched cell line) and SH-SY5Y (endogenous NSPc1 expressed cell line) cells. Cell cycle distributions were determined by analysis of their total DNA content. The cells with 2n DNA were classified as G₀/G₁ populations, those with 4n DNA were classified as G₂/M populations, and those with DNA content 2n-4n were assigned to be in the S phase of the cell cycle. Flow cytometric analysis showed an S phase increase and a G₂/M phase decrease in

both cell lines after transient NSPc1 over-expression (Figures 1 a-f).

NSPc1 promotes tumor cell cycle progression and enhances cell proliferation

NSPc1 stably over-expressed and knocked-down HeLa cell pools were established to verify its effect on cell proliferation. The IMG800 plasmid, which contains the human U6 promoter and a neomycin resistance gene, was used to establish the NSPc1 stably knockdown HeLa cell pool. Several constructs that direct synthesis of NSPc1 siRNAs *in vivo* were designed as mentioned in materials and methods. The expression level of NSPc1 in the above HeLa cell pools were analyzed both at the mRNA and protein level (Figure 2a).

Flow cytometric analysis demonstrated that the cell cycle distributions of the above mentioned stable HeLa cell pools showed the similar pattern as in Figure 1 (Figures 2b and c). Stable over-expression of NSPc1 in HeLa cells caused S phase increase and G₂ phase decrease or G₂/M transition (Figure 2b). While RNAi NSPc1 in HeLa cells led to the reverse effects, and it seemed that the 'RNAi1' construct was more effective (Figure 2c). Furthermore, the results from MTT assays demonstrated that the expression level of NSPc1 could also influence the proliferation rate of HeLa cells (Figure 2d and e). Since HeLa cell is a tumor cell line with colony formation ability in semi-solid culture medium, we addressed the question whether NSPc1 interferes with the colony formation ability of this tumor cell line. Considering that the siRNA synthesized from the 'RNAi1' constructs seemed to be more effective in HeLa cells (Figure 2a), we only chose the 'RNAi1' and 'NSPc1' stable cell pools to compare cell colony formation activity. As shown in Figure 2f and g, there was about an average of 25% decrease in colony number and a decrease in average colony size in the 'RNAi1' pool compared to the 'NSPc1' pool. All these results proved that NSPc1 not only promotes HeLa cell cycle progression, but enhances cell proliferation as well.

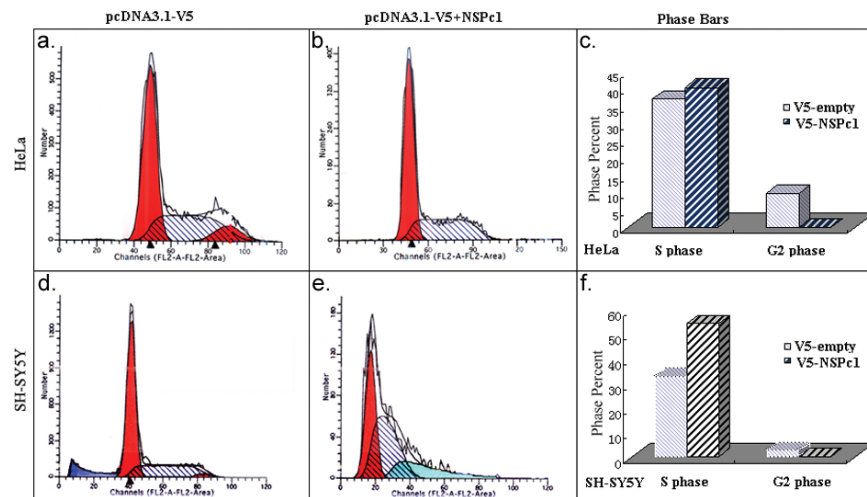


Figure 1. Flow cytometric analysis of HeLa and SH-SY5Y cells transiently transfected with pcDNA3.1-v5+NSPc1 or control vector. A representative experiment out of at least three performed is shown both in cell phase distribution and bar diagrams. (a-c) HeLa cell, (d-f) SH-SY5Y cell.

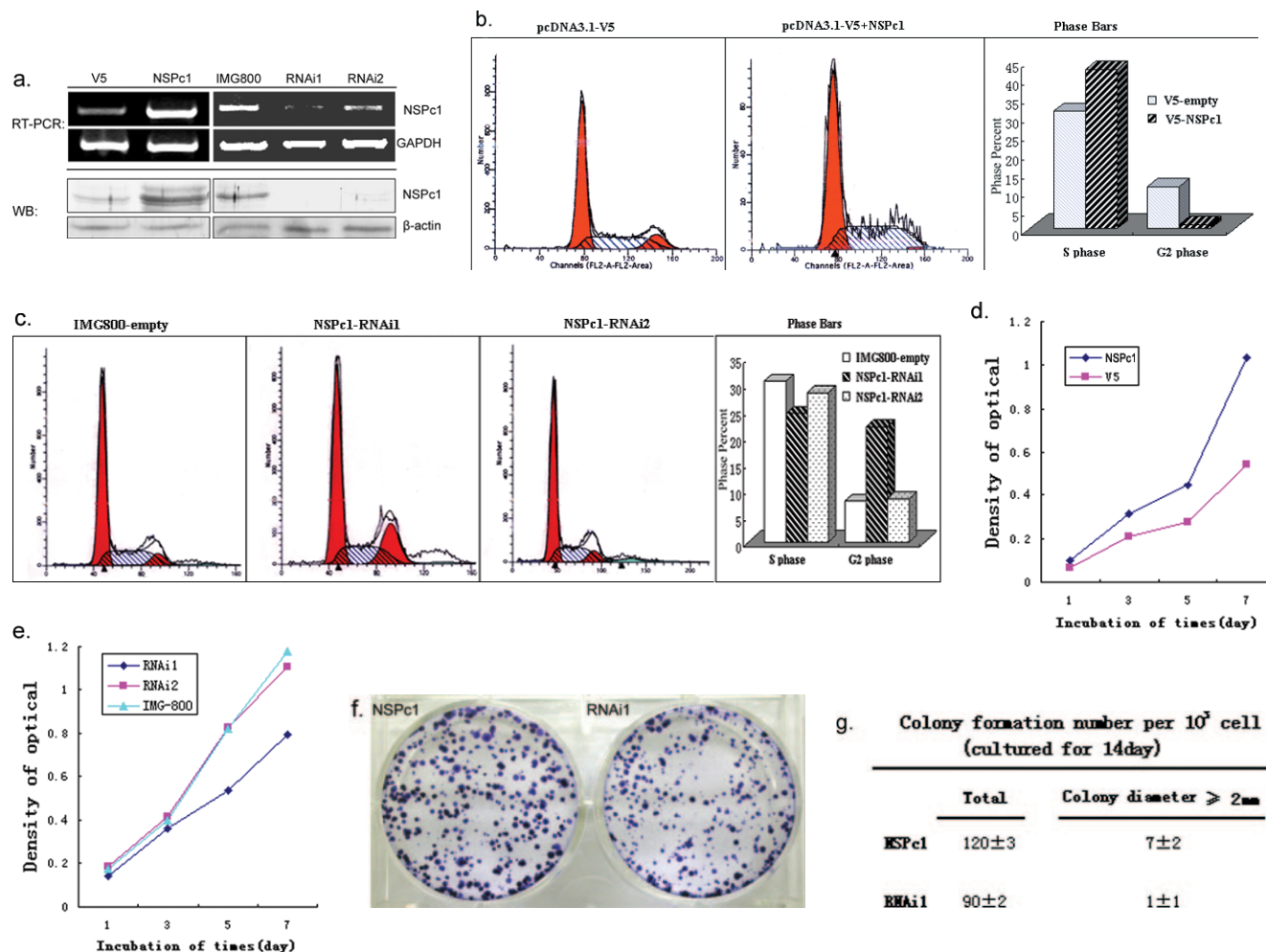


Figure 2. Effects of NSPc1 on HeLa cell cycle progression, cell proliferation and cell colony formation. (a) Establishment of the NSPc1 stably over-expressed and NSPc1 knocked-down HeLa cell pools. Up: changes in mRNA level of HeLa stably transfected cells pool confirmed by semi-quantitative RT-PCR. PCR products generated by primer sets for NSPc1 were normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH); Down: changes in protein expression confirmed by Western blots (normalized by β -actin protein level). (b and c) Flow cytometric analysis of HeLa stable cell pools. (d and e) MTT assay of HeLa stable cell pools. (f and g) Colony formation assay. Culture dishes fixed and stained with 0.1% crystal violet at the end of the experiment. The numbers of total colonies and colonies greater than 2 mm in diameter were counted and the means and SDs of three independent experiments were shown. Abbreviations: V5, pcDNA3.1-V5 cell pool; NSPc1, pcDNA3.1-V5+NSPc1 cell pool; IMG800, IMG800 cell pool; RNAi1, NSPc1-RNAi1 cell pool; RNAi2, NSPc1-RNAi2 cell pool. Note: a representative experiment out of three performed is shown in (b-f).

NSPc1 specifically represses the expression of CDK inhibitor p21

PcGs have been found to repress some negative cell cycle regulators to promote cell cycle transition and cell proliferation (8,24,25). For example, the p16ARF/p19INK4a locus has been verified as the key target of PcG proteins Bmi-1 and Cbx-7, and the two CDKIs from this locus are partially responsible for the effect of Bmi-1 and Cbx-7 on cell growth regulation (9,26). Therefore, we speculated that NSPc1, as a typical PcG member, could also promote cell growth through repressing some negative cell cycle regulators, specially the CDK inhibitor family members. To find out the possible CDK inhibitors involved, the expression levels of the CDK inhibitors were assessed in our stable HeLa cell pools with semi-quantitative RT-PCR. GAPDH and Bmi-1 were amplified as controls. As shown in Figure 3a, only the expression of p21 was repressed. Western blot results showed the similar expression pattern of p21 (Figure 3b).

NSPc1 represses p21 promoter activity in a dose dependent manner but independent of p53

To elucidate the molecular mechanisms of the repression of p21 by NSPc1, we examined the effect of over-expressed NSPc1 on the transcription activity of the p21 promoter by using dual luciferase report assay. Considering that the proximal 2.3 kb region of the p21 promoter has been well studied (31), we mainly focused on this 2.3 kb region in the following repression activity assays. The plasmid pGL3Basic-2.3kb p21 promoter and its derivative plasmids, which were described by Liu *et al.* (28), were kindly represented by Dr Gallego C. and used in the current work. Meanwhile, c-Myc was included as a positive control since it represses the expression of the luciferase reporter gene driven by a p21 promoter (32). As shown in Figure 4a, over-expression of NSPc1 in COS-7 cells led to a \sim 45% decrease of relative luciferase activity compared with control, although over-expression of c-Myc led to a stronger repression (\sim 60% decrease) on the p21

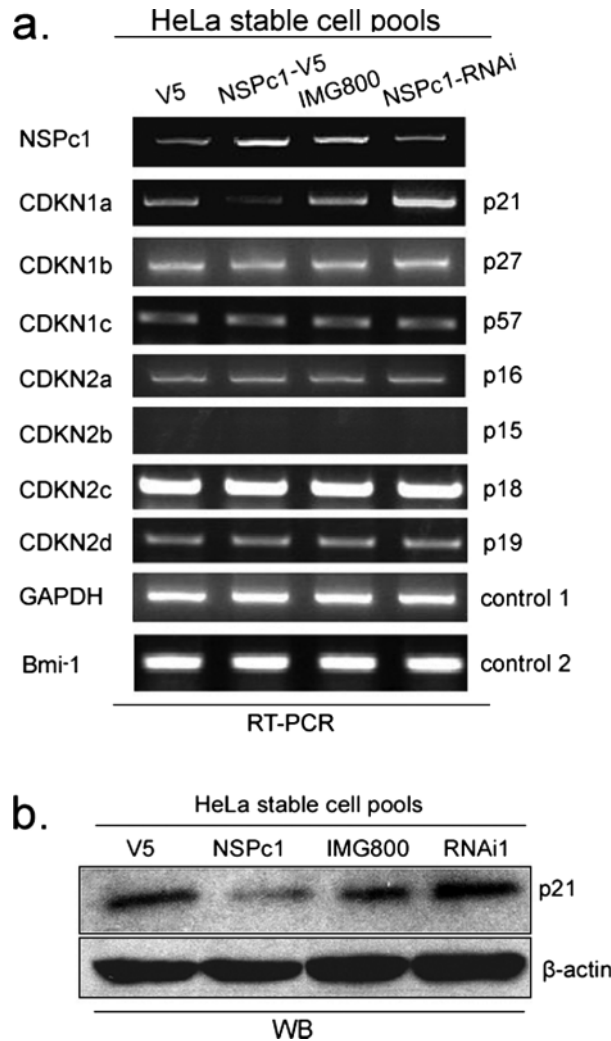


Figure 3. NSPc1 specifically represses the expression of the CDK inhibitor p21. (a) Semi-quantitative RT-PCR results (normalized by GAPDH level) show the expression level of CDKs in various HeLa stable cell pools. Bmi-1 was detected as a control of NSPc1-RNAi specificity. Similar results were observed in three independent experiments. (b) Western blot results confirmed that the expression levels of p21 protein in various stable cell pools were reversely correlated with NSPc1 (normalized by β -actin protein level).

promoter. But over-expression of Bmi-1 could not repress the transcriptional activity of the p21 promoter at all, which eliminated the possibility that the inhibition by NSPc1 is an artifact. Co-transfection of NSPc1 with c-Myc has a slight synergistic transcriptional repression effect on the p21 promoter (~70% decrease, data not shown). Furthermore, we found that NSPc1 inhibited p21 promoter-mediated luciferase expression in a dose-dependent manner in COS-7 cells (Figure 4c). Since COS-7 cells express abundant p53 proteins while HeLa cells lack functional p53 (33), the same dose-dependent assay was also performed in HeLa cells to see whether the p53 protein is involved in the repression by NSPc1 (Figure 4d). Results obtained from HeLa cells were consistent with those from COS-7 cells, which indicated the independence of p53 protein in NSPc1-mediated repression *in vivo*.

p21 promoter repression by NSPc1 is dependent on the RA response element (RARE element)

In order to find out whether the NSPc1 protein is able to bind the p21 promoter *in vivo*, we performed ChIP assay in HeLa cells. A schematic map of the p21 promoter was drawn in Figure 5a (31). As shown in Figure 5a and b, PCR product representing the region (-1357 to -1063) of p21 promoter was immunoprecipitated with both RXR α antibody (Santa Cruz) and NSPc1 antisera (13), while no band was visible using pre-immunized sera or IgG. In contrast, no PCR products were visible when using the primers for the downstream (-194 to +84) or the upstream (-2264 to -1971) regions of p21 promoter. Real time ChIP done in various HeLa cell pools confirmed the specific binding of NSPc1 on the p21 promoter region (-1357 to -1063) (Figure 5c).

To find out whether the effect of NSPc1 on p21 relies on some important *cis*-elements in the proximal p21 promoter (Figure 5a), such as the RARE element within the (-1357 to -1063) region, several mutated constructs of the p21 promoter were used to detect the repression activity of NSPc1. As shown in Figure 5d, in COS-7 cells, NSPc1 can inhibit the activity of the 2.3 kb (wt, wild type), the 1.8 kb (with the pivotal p53 binding site deleted) and the 2.3 kb (with the proximal 1, 2 E-box mutated) fragments of the p21 promoter, but not the RARE-mutated fragment. A similar loss of function of NSPc1 on p21 RARE-mutated promoter was also observed in HeLa cells (data not shown). All of these results indicated that the RARE element within the p21 promoter region (-1357 to -1063) is crucial for NSPc1-mediated repression of p21 promoter activity, which also suggested that the RARE element is a potential functional binding site of the NSPc1 protein.

RXR α deprives the repression effect of NSPc1 on the p21 promoter through competing RARE element

RARE element is known as the binding site for RA receptors, such as RXR α , β , γ and RAR α , β , γ (34). To explore whether any type of RA receptors can interfere with NSPc1 repression on p21, we over-expressed RXR α with the reporter genes in COS-7 cells and observed an induction of the basal level of p21 promoter activity. When equal amounts of RXR α were co-expressed with NSPc1, RXR α markedly overcame the repression of NSPc1 (Figure 6a). Moreover, RXR α relieved the repression of NSPc1 on the p21 promoter in a dose dependent manner (Figure 6b, left). Increasing amounts of NSPc1 (maximum to 2-fold of RXR α) could not resume the repressive effect on p21 once a certain amount of exogenous RXR α existed (Figure 6b, right).

As RXR α is a native binding factor of RARE element, we speculated that the over-expressed RXR α occupies the RARE region and results in excluding NSPc1 from the p21 promoter. The following DNA pulldown assays sequentially verified not only the binding ability of NSPc1 dependent on the RARE element of p21 promoter, but the competitive binding relationship between NSPc1 and RXR α as well. A series of DNA pulldown assays were performed with different biotinylated DNA probes representing the regions (-1357 to -1063 and -194 to +84) of the p21 promoter (Figure 5a). It is evident from the results that the probes of region (-1357 to -1063), but not of the region (-194 to

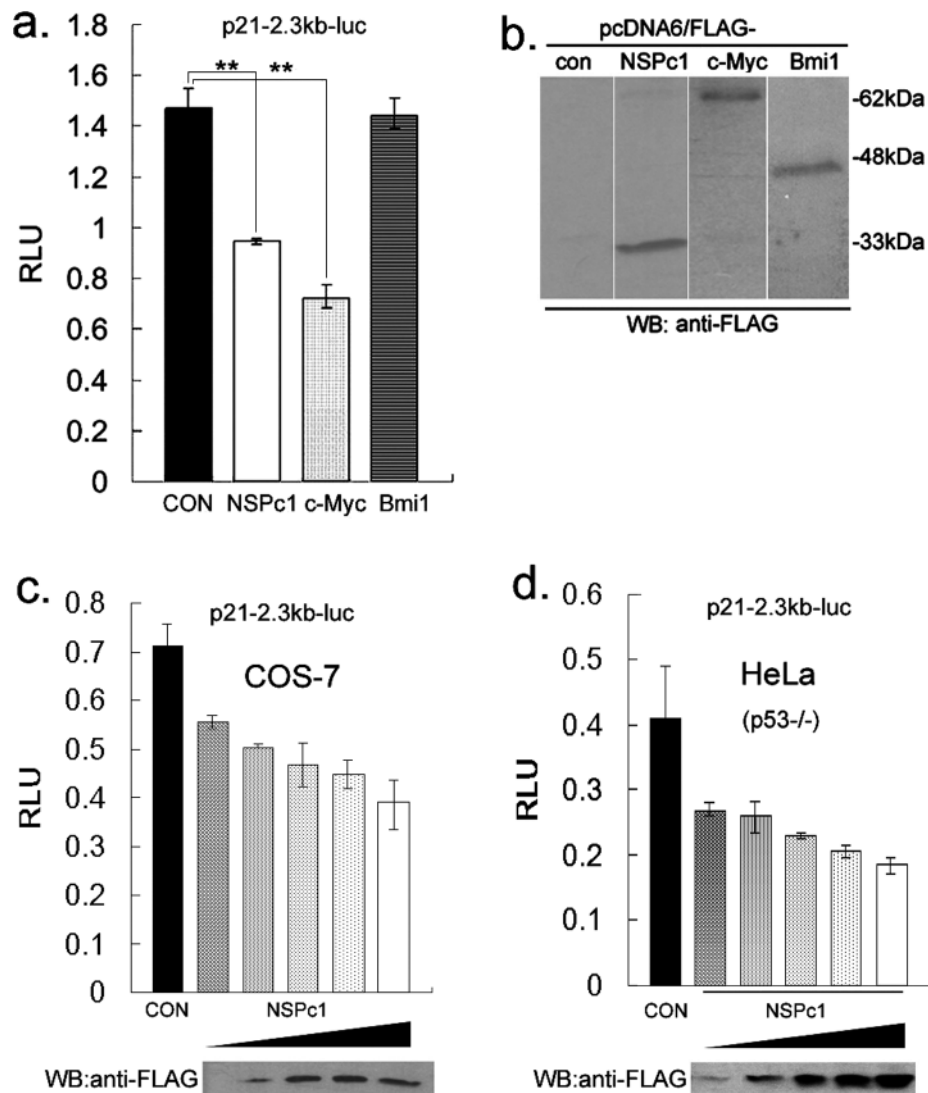


Figure 4. Transcriptional repression effect of NSPc1 on p21 promoter activity. (a) Repression activity assays of NSPc1. COS-7 cells were serially co-transfected with equal amounts of the pcDNA6-FLAG, pcDNA6-FLAG-NSPc1 or pcDNA6-FLAG-Bmi-1 plasmid (300 ng/well) together with the reporter plasmid pGL3-p21-2.3 kb-promoter construct (200 ng/well) and the internal control plasmid pRL-TK (100 ng/well). (b) Western blot results verified that each expression construct was normally expressed in COS-7 cells. (c and d) Dosage-dependent transcriptional repression of p21-promoter by NSPc1. COS-7 or HeLa cells were co-transfected with increased amounts of pcDNA6-FLAG-NSPc1 plasmid (100, 200, 300, 400, 500 ng/well) together with the reporter plasmid and pRL-TK plasmid as above. Abbreviations: RLU, relative luciferase unit; CON, control (pcDNA6-FLAG-empty vector). Error bars, SDs. $**P < 0.001$ versus control.

+84), could recruit both NSPc1-V5 and RXR α -V5 *in vitro* (Figure 6c). Here SP1-V5 and Bmi-1-V5 were included as the positive and negative controls. However, NSPc1-V5 did not bind the probes of region (-1357 to -1063) any more after RARE element was mutated, which suggested that NSPc1 binding the region (-1357 to -1063) of the p21 promoter is dependent on RARE element. Furthermore, their binding amounts on the RARE-contained region of the p21 promoter were positively correlated with the input amount of COS-7 extracts, which contain over-expressed NSPc1-V5 or RXR α -V5, respectively (Figure 6d). This let us to the hypothesis that RXR α can compete with NSPc1 for the RARE element *in vivo*. So further *in vivo* DNA pull-down assay used NSPc1-V5 over-expressed COS-7 extracts before or after retinoic acid induction as the input samples (Figure 6e). Up-regulated endogenous RA receptors including

RXR α competed for the RARE binding sites of the p21 promoter probe (-1357 to -1063) with over-expressed NSPc1-V5. This result further confirmed the competition between NSPc1 and RXR α for the RARE element *in vivo*.

DISCUSSION

In the current study, we showed that NSPc1 increased the fraction of S phase cells and promoted the G₂/M phase transition in HeLa and SH-SY5Y cells (Figure 1). These effects were then confirmed in stable HeLa cell pools (Figure 2b and c). Detection on the expression of most of CDKIs showed that the cell growth characteristics of the HeLa stable cell pools were related to the alteration of p21 expression both at the RNA and the protein levels

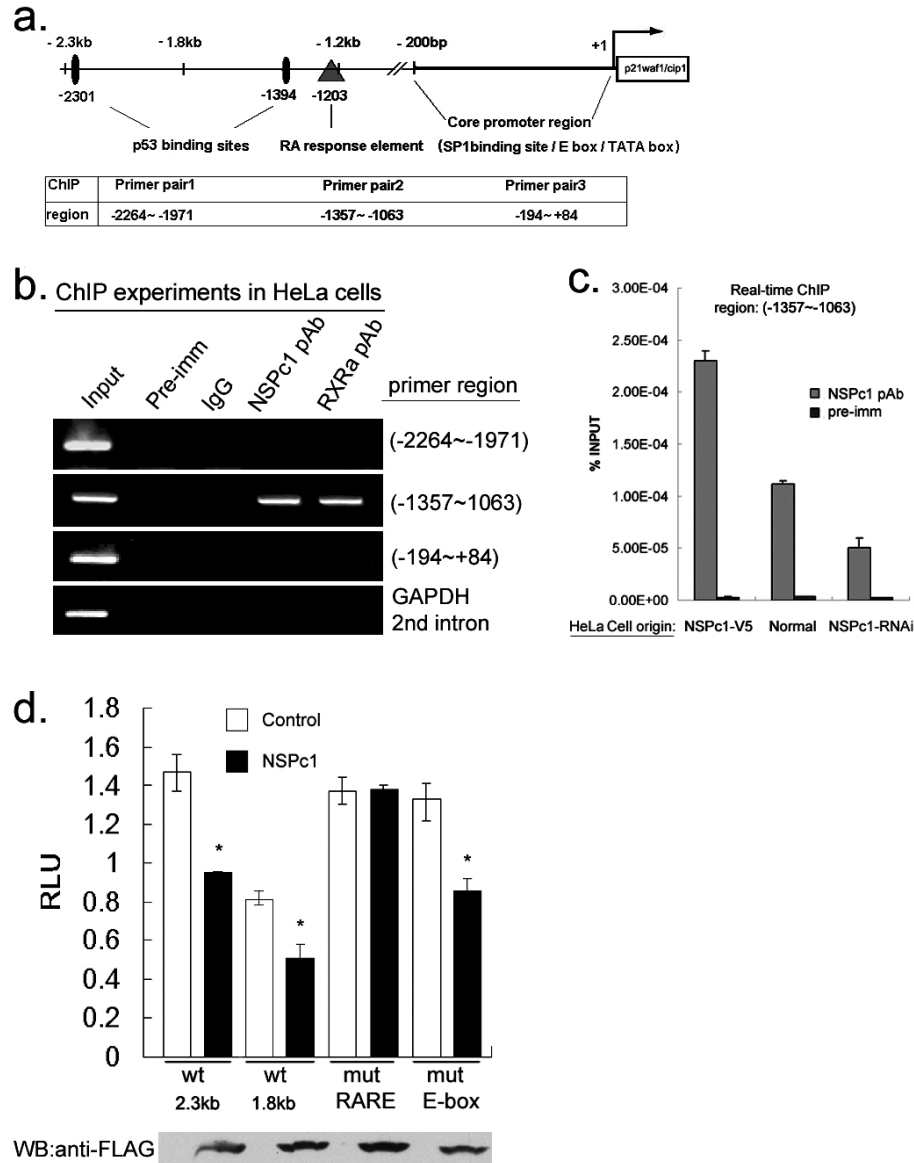


Figure 5. NSPc1 binds directly to the p21 promoter via the RA response element. (a) Up: schematic depiction of the human p21 promoter illustrating regions bound by some of the major regulatory factors, the arrow represents the transcription start site at +1; Down: table of the relative location of the three primer pairs used in ChIP assays. (b) ChIP assays in ordinary HeLa cells. Three different regions of p21-promoter [as shown in (a)] were amplified after chromatin immunoprecipitation from HeLa cell lysates using polyclonal anti-RXR α antibody (Santa Cruz), anti-NSPc1 antisera, pre-immune antisera or mouse IgG (as a negative control). Primers to the second intron of GAPDH were used as the negative control. (c) Real-time ChIP in the ordinary HeLa cells and stable HeLa pools. PCR primers were designed for amplification of the region (-1357 to -1063). Amount of immunoprecipitated DNA was quantified by Absolute Quantitative PCR. Error bars, SDs. (d) Repression activity assays of NSPc1 on the mutated p21 promoters. COS-7 cells were serially co-transfected with equal amounts of the control plasmid pcDNA6-FLAG or pcDNA6-FLAG-NSPc1 (300 ng/well), the various pGL3-p21-promoter constructs (200 ng/well) as reporter plasmid and the internal control plasmid pRL-TK (100 ng/well). Abbreviations: wt: wild type; mut, mutated; RARE, RA responsive element; RLU, relative luciferase unit; CON, control (pcDNA6-FLAG-empty vector). Error bars, SDs. * $P < 0.01$ versus control.

(Figure 2d and g and Figure 3a and b). Since CDK inhibitor p21 negatively affects cell cycle progression (35–39), the regulation of p21 would account for a possible molecular mechanism through which NSPc1 controls tumor cell cycle and cell proliferation. While the knockdown of NSPc1 is not sufficient to abrogate the colony formation ability of tumor cells although it decreases cell growth (Figure 2d and g). So further studies such as selective over-expression or knock-out of NSPc1 in animal models are needed to prove the pathogenetic role of NSPc1 in tumorigenesis.

p21 was originally characterized as an important effector in p53-mediated cell cycle arrest (35–39). The expression of this gene was mainly regulated in the transcription level (31). Previous studies indicated that many transcription factors other than p53 also can regulate p21 during cell differentiation and growth arrest, such as c-Myc (32,40,41) and RA receptors (34,42). Here we firstly demonstrated that NSPc1 represses p21 transcription as c-Myc does but Bmi-1 has no effect on p21 transcription (Figure 4a and b). It agrees with our previous view that the transcriptional repression process

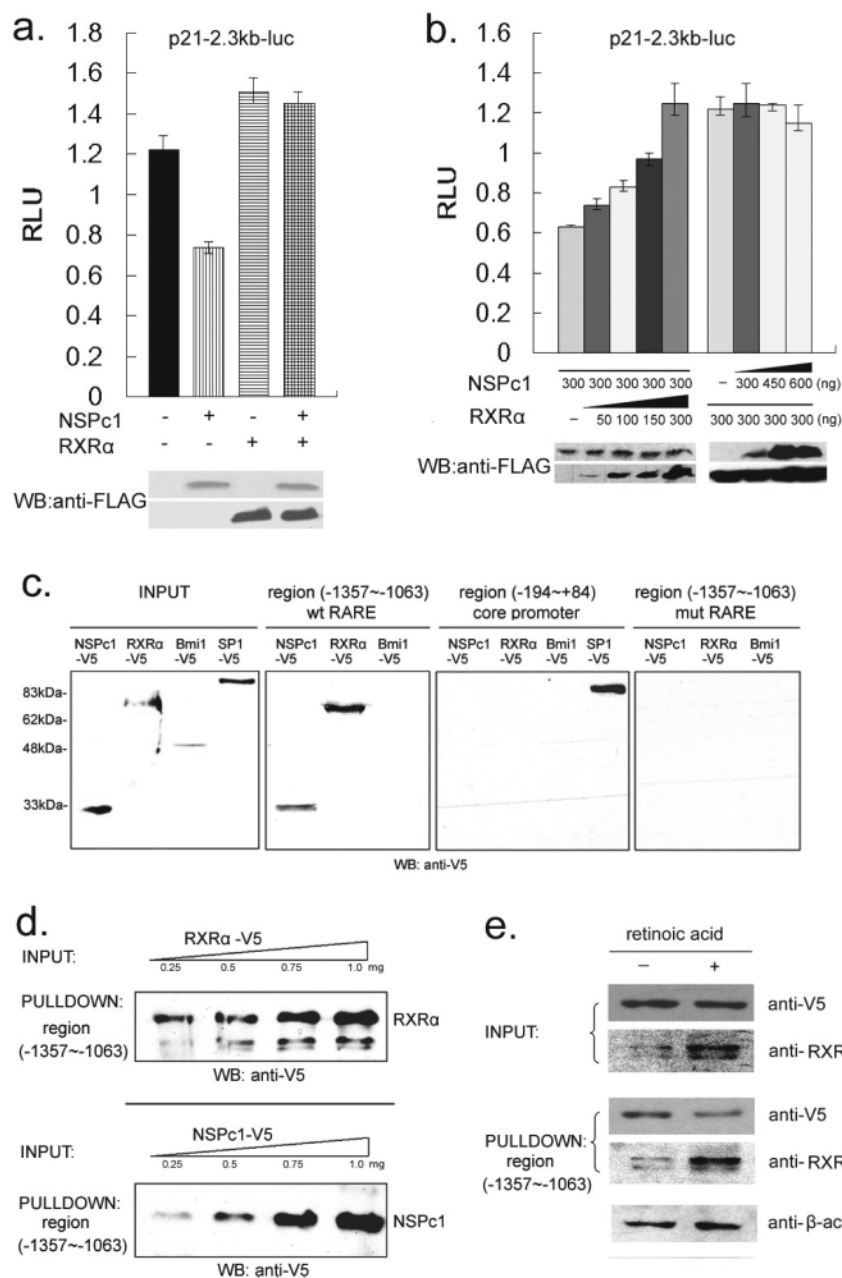


Figure 6. The RARE element within the p21 promoter recruits both NSPc1 and RXRα. (a) Exogenous expression of RXRα deprives the repression effect of NSPc1 on the p21 promoter. COS-7 cells were serially co-transfected with equal amounts of the pcDNA6-FLAG-empty, pcDNA6-FLAG-NSPc1 or/and pcDNA6-FLAG-RXRα plasmid (300 ng/well) together with the reporter plasmid and pRL-TK plasmid. (b) Over-expression of NSPc1 could not resume the repression effect of the p21-promoter once a certain amount of exogenous RXRα existed. COS-7 cells were co-transfected with the reporter plasmid, pRL-TK, pcDNA6-FLAG-NSPc1 or pcDNA6-FLAG-RXRα together with increasing amounts of pcDNA6-FLAG-RXRα or pcDNA6-FLAG-NSPc1 as shown. Error bars, SDs. Abbreviations: RLU, relative luciferase unit. (c) Binding ability of NSPc1-V5 and RXRα-V5 on the region (-1357 to -1063) of the p21 promoter was dependent on RARE element. Different biotinylated DNA probes (abbreviated as wt RARE, core promoter, and mut RARE, respectively) representing the regions (-1357 to -1063 and -194 to +84) of the p21 promoter were used to recruit the specific binding proteins when incubated with NSPc1-V5, RXRα-V5, Bmi1-V5 (negative control), or SP1-V5 (positive control) over-expressed COS7 extracts. Binding samples were detected by western blot with V5 monoclonal antibody. Abbreviations: wt, wild type; mut, mutated. (d) Dose-dependent binding effect of NSPc1-V5 and RXRα-V5 on the region (-1357 to -1063) of the p21 promoter. 0.25, 0.5, 0.75 and 1.0 mg of NSPc1-V5 and RXRα-V5 over-expressed COS7 extracts were used as the input. (e) Up-regulated endogenous RXRα competed the RARE binding sites of the p21 promoter probe (-1357 to -1063) with over-expressed NSPc1-V5. NSPc1-V5 over-expressed COS-7 extracts before or after retinoic acid (10^{-7} M) induction for 3 days as the input samples.

of NSPc1 may involve different mechanisms from Bmi-1 (13). We suppose here that NSPc1 is an important transcriptional regulator of p21, whereas Bmi-1 is a key upstream factor of the p16ARF/p19INK4a pathway (9,43). Moreover,

we showed the independence of p53 protein activity in NSPc1-mediated repression of p21 *in vivo* (Figures 4d and 5d), and the NSPc1-mediated repression has a crosstalking with the RA signaling pathway (Figures 5d and 6a, b).

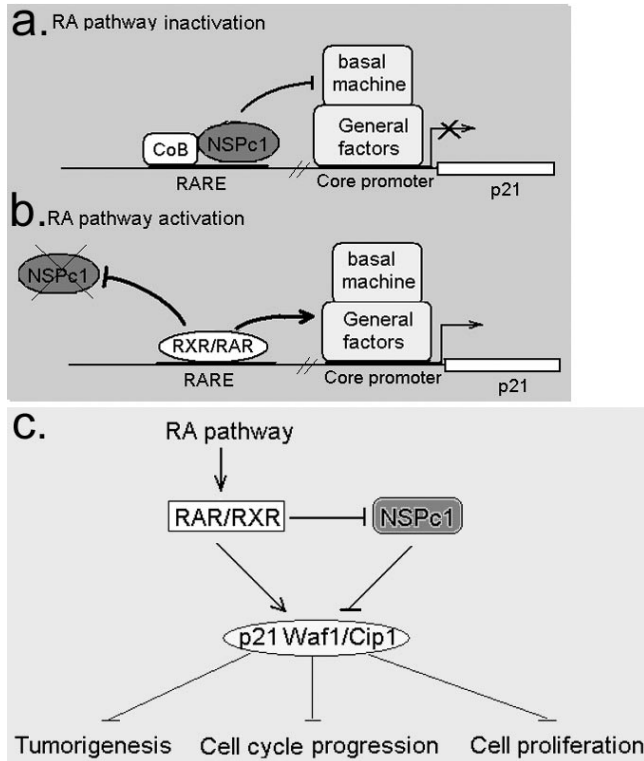


Figure 7. A regulation model of NSPc1/RA receptors in the p21 pathway. (a) Regulatory model: NSPc1 represses p21 expression via RARE element. Note, NSPc1 needs co-binding factor (CoB) to facilitate its localization on RARE element. (b) When the RA signaling pathway is activated, RXRs/RARs compete with NSPc1 for binding RARE element on the p21 promoter, and then transcription is activated. (c) Signal pathway model shows connections between NSPc1, RA and p21 pathways.

In *Drosophila*, polycomb proteins regulate gene expression by forming multiprotein complexes at specific chromosomal sites called polycomb response elements (PREs) (44,45), but until now no exact ‘mammalian PRE’ consensus sequence has been reported. It was difficult for us to decide exactly which promoter region of p21 should be used in the ChIP experiments. However, recent studies showed that the mammalian polycomb protein Bmi-1 can bind to a certain site (around the transcription start site) in the proximal promoter region of its target gene (46). So in the NSPc1-ChIP assays, three representative promoter regions were selected (Figure 5a and b). The selected proximal region (–194 to +84) of the p21 promoter represents the core promoter region, which includes the TATA box, the SP1/SP3 binding sites, the E-boxes and the transcription start site. We observed that the repression of p21 by NSPc1 does not involve disturbing the basal transcription machine, for there was no positive signal from this core promoter region in the ChIP assay (Figure 5b). The second selected distal region (–2264 to –1971), which does not contain any typical binding site, represents a random or non-specific region of the p21 promoter. The third selected region (–1357 to –1063) includes a typical RA response element, based on the reports from Djabali’s lab that the polycomb group protein M33 is able to control the accessibility of the RA response elements in the vicinity of the Hox genes (47,48). In our ChIP assay,

a positive signal was obtained from the region (–1357 to –1063) (Figure 5b) and it was confirmed by using real-time ChIP in our stable HeLa cell pools (Figure 5c). This indicated that a native NSPc1 protein complex is formed in the vicinity of the RARE region *in vivo*. Further mutational analysis of the p21 promoter revealed that the RARE sequence, which is capable of forming a complex with RA receptors, was critical for the repression function of NSPc1, but neither the p53 binding site nor the p53 protein was needed for the repression by NSPc1 (Figure 5d). Thus, a relationship between the polycomb group protein NSPc1 and the RA signaling pathway was revealed: the RXR α antagonizes the repression of NSPc1 on the p21 promoter, while NSPc1 does not affect exogenous RXR α targeting of RARE element (Figure 6a and b). Furthermore, DNA pulldown assays sequentially verified the binding ability of NSPc1 dependent on the RARE element of the p21 promoter and a competitive binding relationship between NSPc1 and RXR α both *in vitro* (Figure 6c and d) and *in vivo* (Figure 6e). These outcomes raise some interesting issues with respect to the roles of both polycomb members and the RA regulatory mechanisms. We propose that once the excessive RAR/RXR(s) dimer(s) occupy the RA response element in the p21 promoter, NSPc1 can no longer target and repress p21 as usual. Thus the RARE region may serve as a switch of p21 expression, which is controlled by the balance of polycomb repressions and the RA signaling pathways.

However, there are still some pivotal questions to be answered: for example, whether NSPc1 can directly bind to the RARE region? Although Mel-18 (a mammalian *Psc* homologue) has been demonstrated to bind DNA *in vitro* (11), most polycomb proteins cannot bind to DNA directly. *In vitro* EMSA assay showed that recombinant NSPc1 protein alone could not bind to the RARE region of the p21 promoter (data not shown), which indicated other factors are needed for targeting. So which protein recruits NSPc1 to the RARE region in the p21 promoter remains an intriguing and unresolved question. We speculate that some unknown co-binding factors (CoB) may form complexes with NSPc1 *in vivo* and facilitate NSPc1 to recognize and target the RARE element (Figure 7a and b). It could be very interesting to identify the NSPc1 involved protein complex that binds the RARE element.

Taken together, current data support the hypothesis that NSPc1 has a positive role in promoting tumor cell cycle transition and cell proliferation, at least partially through down regulating the CDK inhibitor p21 via the RARE element. Furthermore, our results not only give a possible working model for the ‘mammalian PREs’, but connect transcriptional repression by mammalian PcGs with p21 and the RA signaling pathways as well (Figure 7c).

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Conflict of interest statement. None declared.

REFERENCES

- Jacobs,J.J. and van Lohuizen,M. (1999) Cellular memory of transcriptional states by Polycomb-group proteins. *Semin Cell. Dev. Biol.*, **10**, 227–235.
- Pirrotta,V. (1997) Chromatin-silencing mechanisms in *Drosophila* maintain patterns of gene expression. *Trends Genet.*, **13**, 314–318.
- van Lohuizen,M., Frasch,M., Wientjens,E. and Berns,A. (1991) Sequence similarity between the mammalian bmi-1 proto-oncogene and the *Drosophila* regulatory genes Psc and Su(z)2. *Nature*, **353**, 353–355.
- Nunes,M., Blanc,I., Maes,J., Fellous,M., Robert,B. and McElreavey,K. (2001) NSPc1, a novel mammalian Polycomb gene, is expressed in neural crest-derived structures of the peripheral nervous system. *Mech. Dev.*, **102**, 219–222.
- Akasaka,T., Takahashi,N., Suzuki,M., Koseki,H., Bodmer,R. and Koga,H. (2002) MBLR, a new RING finger protein resembling mammalian Polycomb gene products, is regulated by cell cycle-dependent phosphorylation. *Genes Cells*, **7**, 835–850.
- Brunk,B.P., Martin,E.C. and Adler,P.N. (1991) *Drosophila* genes Posterior Sex Combs and Suppressor two of zeste encode proteins with homology to the murine bmi-1 oncogene. *Nature*, **353**, 351–353.
- Ishida,A., Asano,H., Hasegawa,M., Koseki,H., Ono,T., Yoshida,M.C., Taniguchi,M. and Kanno,M. (1993) Cloning and chromosome mapping of the human Mel-18 gene which encodes a DNA-binding protein with a new 'RING-finger' motif. *Gene*, **129**, 249–255.
- Jacobs,J.J. and van Lohuizen,M. (2002) Polycomb repression: from cellular memory to cellular proliferation and cancer. *Biochim. Biophys. Acta*, **1602**, 151–161.
- Jacobs,J.J., Kieboom,K., Marino,S., DePinho,R.A. and van Lohuizen,M. (1999) The oncogene and Polycomb-group gene bmi-1 regulates cell proliferation and senescence through the ink4a locus. *Nature*, **397**, 164–168.
- Molofsky,A.V., Pardoll,R., Iwashita,T., Park,I.K., Clarke,M.F. and Morrison,S.J. (2003) Bmi-1 dependence distinguishes neural stem cell self-renewal from progenitor proliferation. *Nature*, **425**, 962–967.
- Kanno,M., Hasegawa,M., Ishida,A., Isono,K. and Taniguchi,M. (1995) mel-18, a Polycomb group-related mammalian gene, encodes a transcriptional negative regulator with tumor suppressive activity. *EMBO J.*, **14**, 5672–5678.
- Tetsu,O., Ishihara,H., Kanno,R., Kamiyasu,M., Inoue,H., Tokuhisa,T., Taniguchi,M. and Kanno,M. (1998) mel-18 negatively regulates cell cycle progression upon B cell antigen receptor stimulation through a cascade leading to c-myc/cdc25. *Immunity*, **9**, 439–448.
- Gong,Y., Wang,X., Liu,J., Shi,L., Yin,B., Peng,X., Qiang,B. and Yuan,J. (2005) NSPc1, a mainly nuclear localized protein of novel PcG family members, has a transcription repression activity related to its PKC phosphorylation site at S183. *FEBS Lett.*, **579**, 115–121.
- Gieseg,M.A., Man,M.Z., Gorski,N.A., Madore,S.J., Kaldjian,E.P. and Leopold,W.R. (2004) The influence of tumor size and environment on gene expression in commonly used human tumor lines. *BMC Cancer*, **4**, 35.
- Carson,J.P., Zhang,N., Frampton,G.M., Gerry,N.P., Lenburg,M.E. and Christman,M.F. (2004) Pharmacogenomic identification of targets for adjuvant therapy with the topoisomerase poison camptothecin. *Cancer Res.*, **64**, 2096–2104.
- Sultmann,H., von Heydebreck,A., Huber,W., Kuner,R., Buness,A., Vogt,M., Gunawan,B., Vingron,M., Fuzesi,L. and Poustka,A. (2005) Gene expression in kidney cancer is associated with cytogenetic abnormalities, metastasis formation, and patient survival. *Clin. Cancer Res.*, **11**, 646–655.
- Pines,J. (1999) Four-dimensional control of the cell cycle. *Nature Cell Biol.*, **1**, E73–E79.
- Sherr,C.J. and Roberts,J.M. (1999) CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.*, **13**, 1501–1512.
- Ogryzko,V.V., Wong,P. and Howard,B.H. (1997) WAF1 retards S-phase progression primarily by inhibition of cyclin-dependent kinases. *Mol. Cell Biol.*, **17**, 4877–4882.
- Dulic,V., Stein,G.H., Far,D.F. and Reed,S.I. (1998) Nuclear accumulation of p21Cip1 at the onset of mitosis: a role at the G₂/M-phase transition. *Mol. Cell Biol.*, **18**, 546–557.
- Barboule,N., Lafon,C., Chadebecq,P., Vidal,S. and Valette,A. (1999) Involvement of p21 in the PKC-induced regulation of the G₂/M cell cycle transition. *FEBS Lett.*, **444**, 32–37.
- Chen,Y.Q., Cipriano,S.C., Arenkiel,J.M. and Miller,F.R. (1995) Tumor suppression by p21WAF1. *Cancer Res.*, **55**, 4536–4539.
- Cardinali,M., Jakus,J., Shah,S., Ensley,J.F., Robbins,K.C. and Yeudall,W.A. (1998) p21(WAF1/Cip1) retards the growth of human squamous cell carcinomas *in vivo*. *Oral Oncol.*, **34**, 211–218.
- Gil,J., Bernard,D. and Peters,G. (2005) Role of polycomb group proteins in stem cell self-renewal and cancer. *DNA Cell Biol.*, **24**, 117–125.
- Pasini,D., Bracken,A.P. and Helin,K. (2004) Polycomb group proteins in cell cycle progression and cancer. *Cell Cycle*, **3**, 396–400.
- Bernard,D., Martinez-Leal,J.F., Rizzo,S., Martinez,D., Hudson,D., Visakorpi,T., Peters,G., Carnero,A., Beach,D. and Gil,J. (2005) CBX7 controls the growth of normal and tumor-derived prostate cells by repressing the Ink4a/Arf locus. *Oncogene*, **24**, 5543–5551.
- Mosmann,T. (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J. Immunol. Meth.*, **65**, 55–63.
- Liu,Y., Encinas,M., Comella,J.X., Aldea,M. and Gallego,C. (2004) Basic helix-loop-helix proteins bind to TrkB and p21(Cip1) promoters linking differentiation and cell cycle arrest in neuroblastoma cells. *Mol. Cell Biol.*, **24**, 2662–2672.
- Christova,R. and Oelgeschlager,T. (2002) Association of human TFIID-promoter complexes with silenced mitotic chromatin *in vivo*. *Nature Cell Biol.*, **4**, 79–82.
- Hata,A., Seoane,J., Lagna,G., Montalvo,E., Hemmati-Brivanlou,A. and Massague,J. (2000) OAZ uses distinct DNA- and protein-binding zinc fingers in separate BMP-Smad and Olf signaling pathways. *Cell*, **100**, 229–240.
- Gartel,A.L. and Radhakrishnan,S.K. (2005) Lost in transcription: p21 repression, mechanisms, and consequences. *Cancer Res.*, **65**, 3980–3985.
- Mitchell,K.O. and El-Deiry,W.S. (1999) Overexpression of c-Myc inhibits p21WAF1/CIP1 expression and induces S-phase entry in 12-O-tetradecanoylphorbol-13-acetate (TPA)-sensitive human cancer cells. *Cell Growth Differ.*, **10**, 223–230.
- Kessis,T.D., Slebos,R.J., Nelson,W.G., Kastan,M.B., Plunkett,B.S., Han,S.M., Lorincz,A.T., Hedrick,L. and Cho,K.R. (1993) Human papillomavirus 16 E6 expression disrupts the p53-mediated cellular response to DNA damage. *Proc. Natl Acad. Sci. USA*, **90**, 3988–3992.
- Liu,M., Iavarone,A. and Freedman,L.P. (1996) Transcriptional activation of the human p21(WAF1/CIP1) gene by retinoic acid receptor. Correlation with retinoid induction of U937 cell differentiation. *J. Biol. Chem.*, **271**, 31723–31728.
- Harper,J.W., Adami,G.R., Wei,N., Keyomarsi,K. and Elledge,S.J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805–816.
- Xiong,Y., Hannon,G.J., Zhang,H., Casso,D., Kobayashi,R. and Beach,D. (1993) p21 is a universal inhibitor of cyclin kinases. *Nature*, **366**, 701–704.
- el-Deiry,W.S., Harper,J.W., O'Connor,P.M., Velculescu,V.E., Canman,C.E., Jackman,J., Pietenpol,J.A., Burrell,M., Hill,D.E., Wang,Y. et al. (1994) WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.*, **54**, 1169–1174.
- el-Deiry,W.S., Tokino,T., Velculescu,V.E., Levy,D.B., Parsons,R., Trent,J.M., Lin,D., Mercer,W.E., Kinzler,K.W. and Vogelstein,B. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.
- Dulic,V., Kaufmann,W.K., Wilson,S.J., Tlsty,T.D., Lees,E., Harper,J.W., Elledge,S.J. and Reed,S.I. (1994) p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell*, **76**, 1013–1023.
- Claassen,G.F. and Hann,S.R. (2000) A role for transcriptional repression of p21CIP1 by c-Myc in overcoming transforming growth factor beta -induced cell-cycle arrest. *Proc. Natl Acad. Sci. USA*, **97**, 9498–9503.

41. Gartel,A.L., Ye,X., Goufman,E., Shianov,P., Hay,N., Najmabadi,F. and Tyner,A.L. (2001) Myc represses the p21(WAF1/CIP1) promoter and interacts with Sp1/Sp3. *Proc. Natl Acad. Sci. USA*, **98**, 4510–4515.
42. Casini,T. and Pelicci,P.G. (1999) A function of p21 during promyelocytic leukemia cell differentiation independent of CDK inhibition and cell cycle arrest. *Oncogene*, **18**, 3235–3243.
43. Jacobs,J.J., Scheijen,B., Voncken,J.W., Kieboom,K., Berns,A. and van Lohuizen,M. (1999) Bmi-1 collaborates with c-Myc in tumorigenesis by inhibiting c-Myc-induced apoptosis via INK4a/ARF. *Genes Dev.*, **13**, 2678–2690.
44. Strutt,H. and Paro,R. (1997) The polycomb group protein complex of *Drosophila melanogaster* has different compositions at different target genes. *Mol. Cell. Biol.*, **17**, 6773–6783.
45. Orlando,V., Jane,E.P., Chinwalla,V., Harte,P.J. and Paro,R. (1998) Binding of trithorax and Polycomb proteins to the bithorax complex: dynamic changes during early *Drosophila* embryogenesis. *EMBO J.*, **17**, 5141–5150.
46. Cao,R., Tsukada,Y. and Zhang,Y. (2005) Role of Bmi-1 and Ring1A in H2A ubiquitylation and Hox gene silencing. *Mol. Cell*, **20**, 845–854.
47. Core,N., Bel,S., Gaunt,S.J., Aurrand-Lions,M., Pearce,J., Fisher,A. and Djabali,M. (1997) Altered cellular proliferation and mesoderm patterning in Polycomb-M33-deficient mice. *Development*, **124**, 721–729.
48. Bel-Vialar,S., Core,N., Terranova,R., Goudot,V., Boned,A. and Djabali,M. (2000) Altered retinoic acid sensitivity and temporal expression of Hox genes in polycomb-M33-deficient mice. *Dev. Biol.*, **224**, 238–249.