

NSPc1 polycomb protein complex binds and cross-talks to lncRNAs in glioma H4 cells

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Abstract. Recently, emerging evidence shows that a number of long non-coding RNAs (lncRNAs) recruit polycomb group (PcG) proteins to specific chromatin loci to silence relevant gene expression. In the present study, we provided evidence that lncRNA candidates, selected by bioinformatic analysis and nervous system polycomb 1 (NSPc1), a key polycomb repressive complex 1 (PRC1) member, were highly expressed in glioma H4 cells in contrast to that noted in non-cancerous cells. RNA binding protein immunoprecipitation (RIP) assays demonstrated that metastasis associated lung adenocarcinoma transcript 1 (MALAT1), SOX2 overlapping transcript (SOX2OT) and maternally expressed 3 (MEG3) among the 8 candidates bound to the NSPc1 protein complex in glioma H4 cells. Furthermore, overexpression of NSPc1 caused a decrease in the expression of MALAT1 and MEG3 and increased expression of SOX2OT, while NSPc1 downregulation caused the levels of all three genes to increase. Meanwhile, suppression of the expression of MALAT1 increased the expression levels of mRNA and protein of NSPc1, whereas downregulation of the expression of SOX2OT decreased NSPc1 expression. Moreover, a significant decrease in cell growth and increased cell apoptosis were observed in the transfected H4 cells by MTT assay and flow cytometric analysis. The results showed that the reduced co-expression between NSPc1 and MALAT1/SOX2OT decreased the proliferation and promoted the death of H4 cells

more obviously than the respectively decrease in expression of NSPc1, MALAT1 and SOX2OT. Remarkably, the influence of a simultaneously decreased expression of NSPc1 and SOX2OT on promoting cell apoptosis was more obvious than the total effect of the separate downregulation of NSPc1 and SOX2OT on accelerating cell death. However, that impact was partially counteracted in the silencing of the co-expression of MALAT1 and NSPc1. Furthermore, they cooperated to affect transcription of p21 and OCT4. Briefly, these data suggest NSPc1 polycomb protein complex binding and cross-talk to lncRNAs in glioma H4 cells, offering new insight into the important function of polycomb protein complex and lncRNA interactions in glioma cells and provide a novel view of potential biomarkers and targets for the diagnosis and therapy of glioma.

Introduction

Glioblastomas are malignant tumors with a high incidence and high mortality rate among all brain tumors with an absence of precise clinical classification biomarkers and effective therapeutic methods. Glioblastomas are associated with poor prognosis and a median patient survival of approximately 15 months (1,2). Therefore, finding effective target genes and specific biomarkers for glioma diagnosis and proliferation block is becoming a 'hotspot' of research.

Long non-coding RNAs (lncRNAs) are defined as a novel class of RNAs which have been a 'hotspot' for research (3,4). Although they lack transcript protein potential, a growing number of lncRNAs have been reported to play a vital role in physiological progresses such as imprinting control, cell differentiation and tumorigenesis (5,6) and lncRNAs are expressed in embryonic stem cells (7), brain tissues (8) and differentiated neurons (9). More significantly, lncRNAs are involved in several diseases and some are the key factors for the evaluation of prognosis (10). However, currently, little is known concerning the related mechanisms of lncRNAs in gliomas.

Polycomb group (PcG) proteins were first found in *Drosophila melanogaster* and these proteins which implement transcriptional silencing are divided into two main family complexes, called polycomb repressive complex 1 (PRC1) and polycomb repressive complex 2 (PRC2) (11), taking crucial

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part in embryonic stem cell stage, determination of cell fate and tumorigenesis (12). Nervous system polycomb 1 (NSPc1), a key member of mouse PRC1, shares high homology with PcG protein Bmi-1. Human NSPc1 gene encodes a 29-kDa nuclear localized protein containing an N-terminal RING finger domain. Previous research pointed out that NSPc1 is highly expressed in the early developing nervous system and is involved in the differentiation of neural crest cells (13,14). The expression of NSPc1 is significantly altered in different grade malignant gliomas and is related with the maintenance of the stemness of cancer stem cells (15). NSPc1 downregulates the cyclin-dependent kinase (CDK) inhibitor p21^{Waf1/Cip1} via the retinoid acid response element (RARE element) resulting in promotion of tumor cell proliferation and cell cycle transition (16). In addition, NSPc1 directed by EZH2, a member of PRC2, mediated histone 2A (H2A) ubiquitination and DNA methylation (17), which means that they are interdependent in specific gene silencing in cancer cells.

Markedly, most PcGs lack the capability of combining proteins due to their own structure. Thus, they must target corresponding genomic loci by some cofactors. As reported, lncRNAs recruit several PRC2 members to specific genes (18), thus, regulating relevant signaling pathways (19). For instance, metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) is among the earliest identified lncRNAs and is also named as nuclear-enriched abundant transcript 2 (NEAT2) (20). MALAT1 has been considered to be correlated with growth and metastasis of various types of cancer cells. In renal cancer, MALAT1 was found to regulate downstream efforts via EZH2-promoting methylation of histone H3 lysine 27 (H3K27), leading to cancer progression and invasion (21). Additionally, MALAT1 was found to recruit EZH2 to suppress the tumor suppressor PCDH10 contributing to gastric cancer proliferation and metastasis (22). Nevertheless, the relationship between PRC1 members and lncRNAs has rarely been reported to date. Therefore, the main focus of our research was the relationship between PRC1 member NSPc1 and lncRNAs.

In the present study, we screened possible lncRNAs which may bind to PRC1 member NSPc1 among numerous candidates and examined potential correlations between NSPc1 and MALAT1/SOX2OT, indicating a cross-talk and functional interaction between the NSPc1 protein complex and MALAT1/SOX2OT in glioma cells. These findings may provide novel biomarker complexes to the clinical treatment of glioma.

Materials and methods

Bioinformatic analysis. The scores of NSPc1-lncRNA interactions were evaluated by bioinformatic analysis through the bioinformatics tool lncPro (online server: <http://bioinfo.bjmu.edu.cn/lncpro>) (23). We selected the lncRNAs closely related in carcinogenesis or multipotency of glioma. Complexes were downloaded from the Protein DataBank (PDB) database (<http://www.pdb.org>) by encoding RNA and protein sequences into numeric vectors. Then the scores for each lncRNA-NSPc1 pair was used to measure the interactions between them.

Cell cultures. Glioma H4 cells were purchased from the Cell Culture Center of the Chinese Academy of Medical Science

(Beijing, China). HK-2 cells were obtained from the American Type Cell Culture Collection (ATCC; Manassas, VA, USA; <https://www.atcc.org/>). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) at 37°C in 5% CO₂.

RNA binding protein immunoprecipitation (RIP). RIP was performed to characterize the NSPc1 protein complex-associated RNAs following the manufacturer's instructions (EMD Millipore, Billerica, MA, USA) with some modifications. Briefly, typically one nuclear RIP reaction using one antibody required 50 µl of chromatin from ~1.0 × 10⁶ cells. The optimization for shearing cross-linked RNA condition was 24 sec on, 30 sec off for 14 cycles at 4°C. lncRNA-NSPc1 immunoprecipitation was performed with anti-NSPc1 monoclonal antibody (m-NSPc1) and anti-NSPc1 polyclonal antibody (poly-NSPc1) both obtained from our laboratory, anti-EZH2 antibody (cat. no. 07-689; EMD Millipore) as non-anti-NSPc1 control and normal mouse IgG (cat. no. 17-371; EMD Millipore) as RIP efficiency control. m-NSPc1 (2 µg), poly-NSPc1 (2 µg), anti-EZH2 (1 µg) and normal mouse IgG (1 µg) were added to each reaction. The co-precipitated lncRNAs were detected by qRT-PCR. Total RNAs (input controls), non-anti-NSPc1 control were assayed to demonstrate endogenous expression of 8 lncRNAs, efficiency of the reaction and specificity of RNA binding to NSPc1.

Isolation of RNA and real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Total RNA isolated from cell cultures were routinely used for qRT-PCR. Total RNA was prepared by TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). TRIzol extractions were conducted according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). For qRT-PCR, the cDNAs were synthesized using PrimeScript™ RT Master Mix (DRR820A; Takara Bio Inc., Shiga, Japan) with total RNA as templates. All the primers of interest were designed and verified through PubMed (data not shown). qRT-PCR was performed using an StepOnePlus™ System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and SYBR Premix Ex Taq™ II (DRR820A; Takara Bio Inc.) according to the manufacturer's instructions. The amplification conditions were 95°C for 30 sec, as well as 40 cycles at 95°C for 5 sec and 60°C for 60 sec. The fold relative enrichment was quantified together with normalization by the largest CT level for RIP, and by the GAPDH level for transfection. Results were analyzed by 2^{-ΔΔC_q} method (24). All qRT-PCR analyses were performed in triplicate.

Cell transfection. H4 cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). The plasmid pCDEF-NSPc1 and small interfering RNAs (siRNAs) targeting NSPc1 were constructed by our team as in a previous study (15). One pair of siRNAs against MALAT1 and SOX2OT and the scramble were constructed by Shanghai GenePharma Co., Ltd. (Shanghai, China) as siRNA1-MALAT1 (sense, GAGGUGUAAAGGGAUUU AUTT and antisense, AUAAAUCCCUUUACACCUCTT);

siRNA1-SOX2OT (sense, GGAGUCCAGUCAACUUCAUTT and antisense, AUGAAGUUGACUGGACUCCTT) and negative control (sense, UUCUCCGAACGUGUCACGUTT and antisense, ACGUGACACGUUCGGAGAATT). The other pairs for each were from articles reported and chemically synthesized by Shanghai GenePharma Co., Ltd., as siRNA2-MALAT1 (sense, GGGCUUCUCUUAACAUAUU AUU and antisense, UAAAUGUUAAGAGAAGCCCUU) and siRNA2-SOX2OT (sense, GGAGAUUGUGACCUGGCU UTT and antisense, AAGCCAGGUCACAAUCUCCTT).

Western blot analysis. H4 cells were transfected separately with siRNA-MALAT1, siRNA-SOX2OT and the negative control. The cells were collected using RIPA Lysis Buffer (cat. no. P0013B; Beyotime Institute of Biotechnology, Nanjing, China) for western blot analysis after 72 h of transfection. Protein concentrations were determined using a BCA Protein Assay reagents (cat. no. P0009; Beyotime Institute of Biotechnology), equal amounts of protein (20 µg) were loaded per lane and resolved on 10% SDS-PAGE gels, and then transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore). The membranes were blocked with 5% skimmed milk in 1% Tween-20 in Tris-based saline (TBST) and then incubated with primary antibodies (NSPc1, diluted at 1:5,000 was laboratory prepared and β-actin diluted at 1:6,000 was from Bioworld, Inc., St. Louis Park, MN, USA), respectively at 4°C overnight. After washing with TBST, the membranes were incubated with HRP-conjugated secondary antibody diluted at 1:6,000 (cat. no. A0216; Bomeike, Tianjin, China) for 1 h at room temperature and the immuno-reactive bands were visualized using ECL Western Blot Detection reagents (EMD Millipore), normalized to β-actin and quantified by ImageJ system (version 1.47v; NIH; National Institutes of Health, Bethesda, MD, USA).

Cell proliferation and apoptosis. Briefly, the MTT assay (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was applied to evaluate the cell growth state. H4 cells were transfected with negative control, siRNA-NSPc1, siRNA-MALAT1 and siRNA-SOX2OT and co-transfected with siRNA-NSPc1 and siRNA-MALAT1, siRNA-NSPc1 and siRNA-SOX2OT in a 96-well plate. The MTT assay was carried out following the manufacturer's protocol. Absorbance values (OD) were determined with an enzyme-linked immunosorbent detector after 24, 48, 72 and 96 h of transfection, respectively.

Apoptosis of H4 cells was determined by dual staining with FITC-Annexin V (BD Biosciences, Franklin Lakes, NJ, USA) and propidium iodide (PI; Sigma-Aldrich; Merck KGaA). H4 cells cultured in a 6-well plate were transfected as mentioned in the MTT assay. According to the manufacturer's protocol, after a 24-h transfection, H4 cells were analyzed with a flow cytometry system (BD FACSCalibur™ flow cytometer; BD Biosciences) equipped with CellQuest software (BD Biosciences).

Statistical analysis. Results are expressed as the mean ± SEM. Statistical analyses were performed using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). The significance of the differences between various groups was analyzed using one-way analysis of variance (ANOVA). Multiple comparison between the groups was performed using

Table I. The interaction scores evaluated by bioinformatic analysis.

Protein	lncRNA	Interaction score
NSPc1/PCGF1	SOX2OT	82.3497
gi 13436326	MALAT1	76.9802
gb AAH04952.1	APTR	75.095
	ANRIL	71.9274
	MEG3	68.4526
	HOTAIR	61.5625
	H19	58.8719
	HCG4	55.2284
	FAL1	44.1196
	CRNDE	28.8089

The total score for each lncRNA-NSPc1 interaction is 100.

Student-Newman-Keuls (SNK) method. $P < 0.05$ was considered to indicate a statistically significant result.

Results

The prediction of *in vivo* NSPc1-lncRNA interactions by bioinformatic analysis. To determine the possible lncRNAs that bind to NSPc1 by bioinformatics, the top 7 high scoring lncRNAs were chosen as candidates to be investigated (Table I). Moreover, ADAMTS9-AS2 (25) reported currently to be highly expressed in gliomas was also selected.

Differential expression of endogenous lncRNAs and NSPc1 between glioma and non-cancerous cells. Images of normal cell morphology of HK-2 cells and H4 cells in the same culture condition are shown in Fig. 1A. Expression levels of MALAT1, HOTAIR, SOX2OT, H19, ANRIL and NSPc1 were higher in the H4 cells than levels in the HK-2 cells (Fig. 1B-D), while expression levels of ADAMTS9-AS2 and APTR were lower in the H4 cells compared to that in the HK-2 cells (Fig. 1B).

Identification of lncRNAs which bind to the NSPc1 protein complex in H4 cells. To investigate the potential interaction between endogenous NSPc1 protein complex and candidate lncRNAs, RIP-NSPc1 assays were applied in H4 cells. SOX2OT and ADAMTS9-AS2 in the anti-NSPc1 monoclonal antibody (m-NSPc1) group and MEG3 in the anti-NSPc1 polyclonal antibody (poly-NSPc1) group were preferentially enriched compared with anti-EZH2 (non-anti-NSPc1 control) (Fig. 2A). These results indicated that SOX2OT, ADAMTS9-AS2 and MEG3 may have an *in vivo* interaction with NSPc1. In addition, although the enrichment of MALAT1 in the m-NSPc1 group was lower than that in the anti-EZH2 control, in poly-NSPc1 group was higher than anti-EZH2 (Fig. 2A), which also indicated that MALAT1 has the possibility to bind to NSPc1. Furthermore, the enrichment of MEG3 and SOX2OT was higher in both the poly-NSPc1 and m-NSPc1 groups. Remarkably, the endogenous expression of MALAT1 was prominently high in 10% input (Fig. 2B).

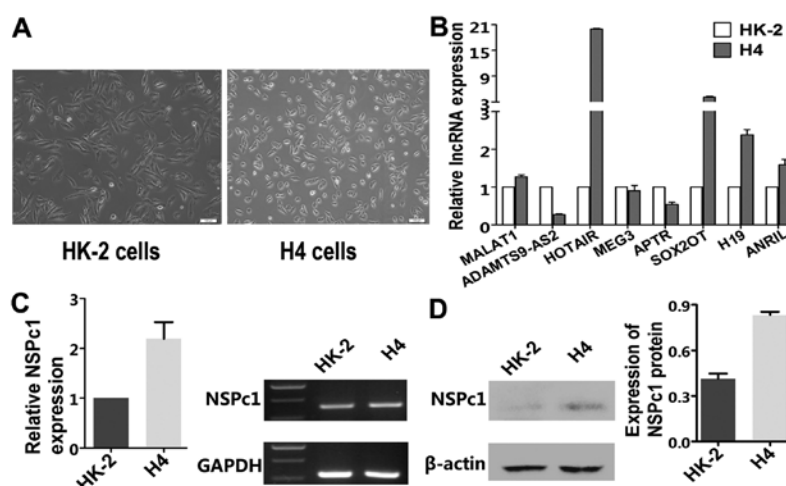


Figure 1. Expression of lncRNA candidates in glioma H4 cells compared with non-cancerous HK-2 cells. (A) Images of normal cell morphology of HK-2 and H4 cells in the same culture condition were taken at x100 magnification. (B) Analysis of expression levels of all candidate lncRNAs were performed by qRT-PCR. The order of the examination was randomized and the following assay order was consistent with this. Expression of each lncRNA was calculated by the $2^{-\Delta\Delta C_q}$ method with the expression of GAPDH as the internal control. (C and D) The mRNA and protein levels of NSPc1 were detected by qRT-PCR and western blotting in glioma and non-cancerous cells. mRNA expression was normalized to GAPDH using the $2^{-\Delta\Delta C_q}$ method. Moreover, their qRT-PCR products were removed for electrophoresis in 2% agarose gel. β -actin was used as internal control in western blot analysis. The expression of NSPc1 was higher in H4 cells than that noted in the HK-2 cells. lncRNAs, long non-coding RNAs; NSPc1, nervous system polycomb 1.

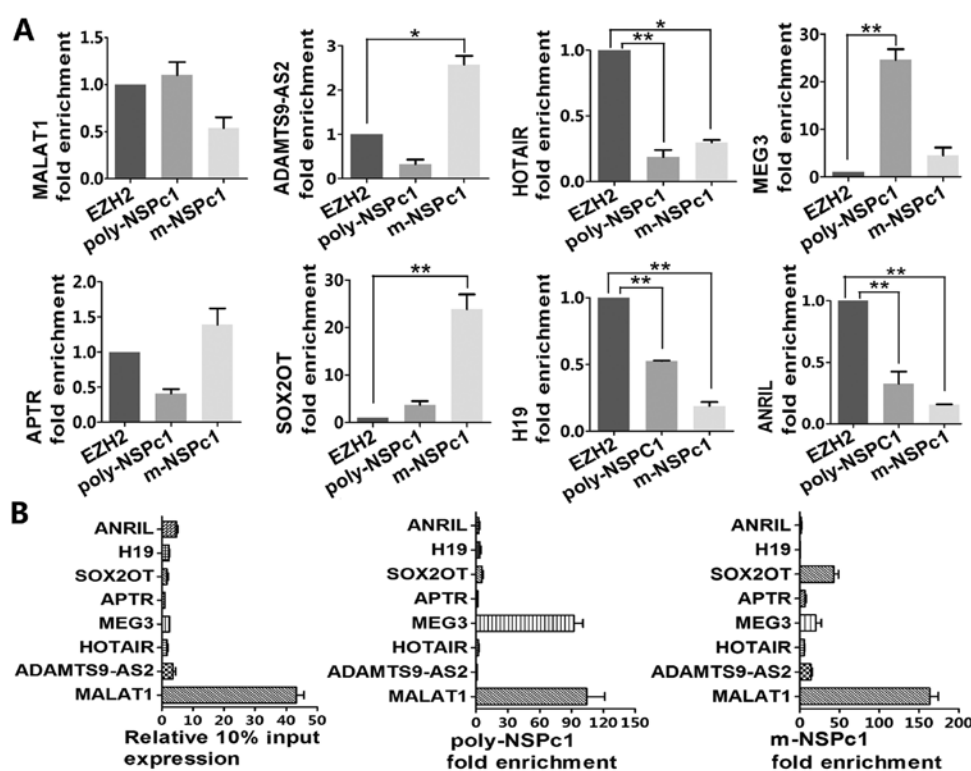


Figure 2. RIP approach for lncRNA-NSPc1 binding profile analysis. (A) Purified RNA immunoprecipitated by the antibodies was then analyzed by qRT-PCR. Verification of RIP enrichment was performed applying the comparative Ct ($\Delta\Delta C_q$) method with m-NSPc1 binding to lncRNA compared to positive control binding lncRNA and poly-NSPc1 binding to lncRNA compared to positive control binding lncRNA. (B) qRT-PCR was performed with eight pairs of primers targeting 10% input total lncRNA, m-NSPc1 combining lncRNA and poly-NSPc1 combining lncRNA. Then, the relative expression of the eight candidates or enrichment folds were calculated by Ct ($\Delta\Delta C_q$) method in each group. All data are presented as the mean \pm SD from three independent experiment. One-way ANOVA, * $P < 0.05$, ** $P < 0.01$. EZH2, anti-EZH2 antibody; m-NSPc1, anti-NSPc1 monoclonal antibody; poly-NSPc1, anti-NSPc1 polyclonal antibody; RIP, RNA binding protein immunoprecipitation; lncRNAs, long non-coding RNAs; NSPc1, nervous system polycomb 1.

Exogenous expression levels of NSPc1 affect the expression levels of MALAT1, SOX2OT and MEG3. To investigate whether the variability of NSPc1 expression influenced the expression of those 8 lncRNAs, the overexpression vector pCDEF-NSPc1

and siRNA-NSPc1 were used. We found that the expression of MALAT1 and MEG3 (Fig. 3A) was decreased by elevated exogenous expression of NSPc1 in H4 cells, and vice versa (Fig. 3B). Expression of SOX2OT was always significantly increased no

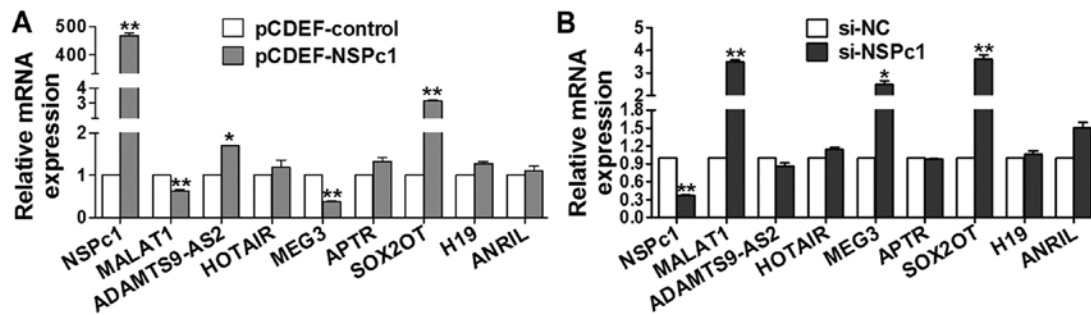


Figure 3. Expression profiles of lncRNA candidates in H4 cells following alteration of the expression of NSPc1. (A and B) H4 cells were transfected with pCDEF-NSPc1 or si-NSPc1 separately, and the measurement of the endogenous expression of NSPc1 by qRT-PCR was used to confirm the upregulation and downregulation efficiency. GAPDH was used as the internal control. Results were analyzed by the $2^{-\Delta\Delta C_t}$ method. One-sample Student's t-test, * $P<0.05$, ** $P<0.01$. si-NC, siRNA negative control; lncRNAs, long non-coding RNAs; NSPc1, nervous system polycomb 1.

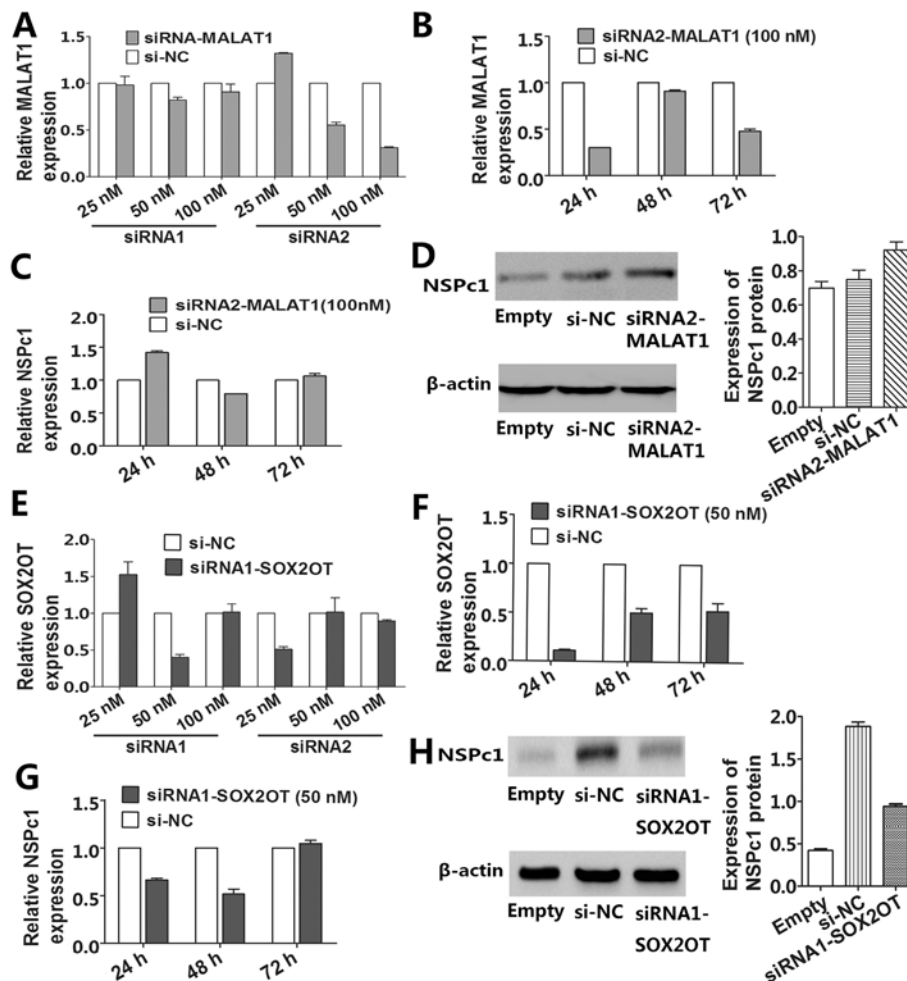


Figure 4. Expression change of NSPc1 was investigated following knockdown of MALAT1 and SOX2OT by siRNAs. (A and E) The knockdown efficiency was primarily investigated according to concentration gradient of siRNAs by qRT-PCR. GAPDH was used as the loading control. (B and F) We further examined the expression of MALAT1 and SOX2OT, respectively, after knockdown with the respective siRNA after 24, 48 and 72 h and (C and G) the influence of the knockdown of MALAT1 or SOX2OT on NSPc1 expression after 24, 48 and 72 h in optimum efficiency by qRT-PCR. (D and H) Western blot analysis was employed to verify the protein expression of NSPc1 following MALAT1 and SOX2OT knockdown separately after 72 h in H4 cells. β -actin was used as internal control. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; NSPc1, nervous system polycomb 1; MALAT1, metastasis associated lung adenocarcinoma transcript 1; SOX2OT, SOX2 overlapping transcript; MEG3, maternally expressed 3.

matter how the expression of NSPc1 was altered while the other 5 lncRNAs exhibited no significant changes (Fig. 3).

Knockdown of MALAT1 and SOX2OT affects the expression of NSPc1. MALAT1 and SOX2OT were chosen for subsequent

research as the P-value of their expression alterations with NSPc1 variation were much less than the other candidates. The optimum transfection efficiency of the silencing of siRNA2-MALAT1 (Fig. 4A and B) and siRNA1-SOX2OT (Fig. 4E and F) were optimum at downregulating their target genes 24 h post-DNA

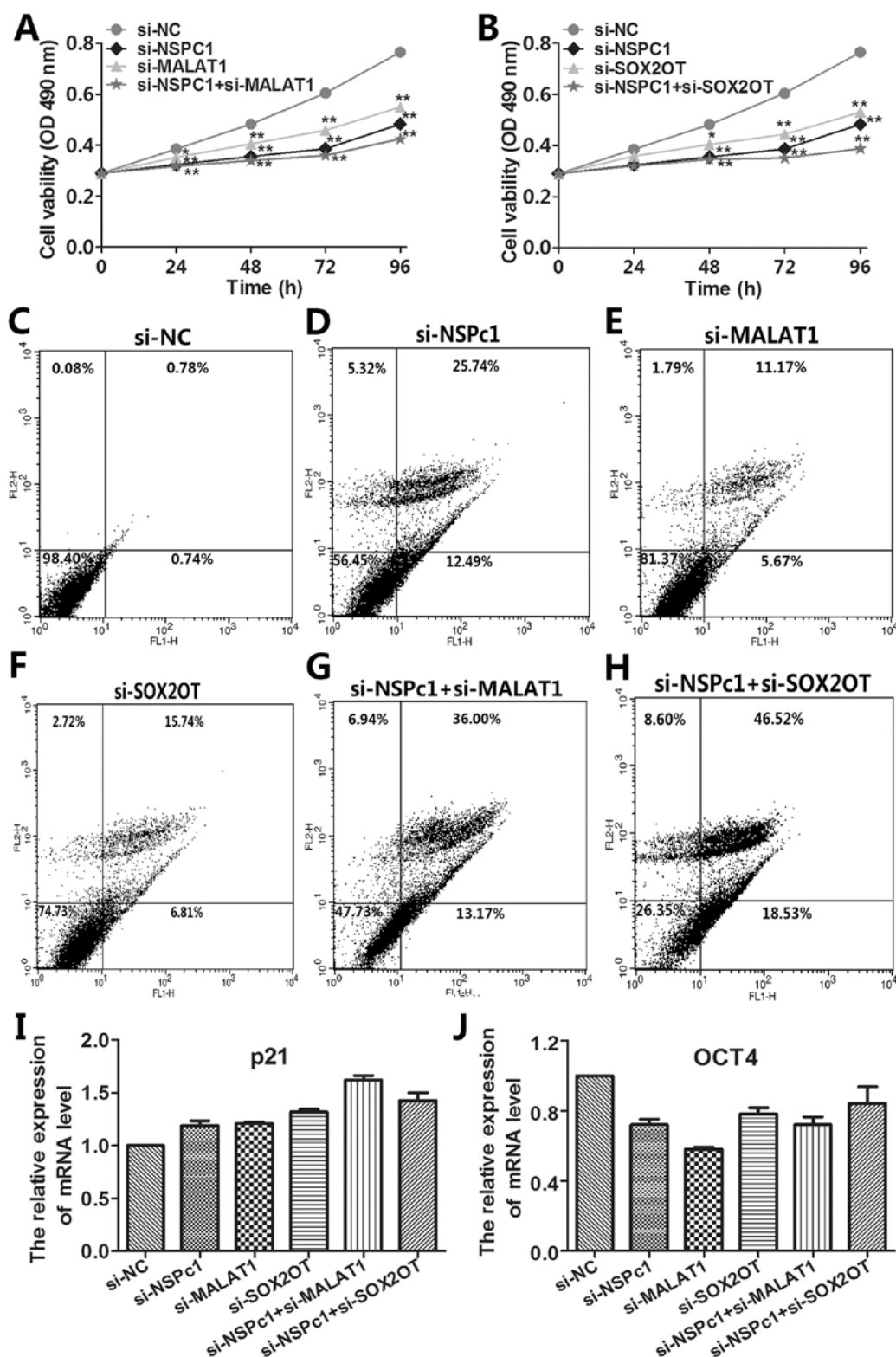


Figure 5. Effects of the knockdown of NSPC1, MALAT1, SOX2OT, NSPC1 plus MALAT1, NSPC1 plus SOX2OT on H4 cell proliferation and apoptosis *in vivo* and the effects of the knockdown of the expression of NSPC1, MALAT1, SOX2OT and co-suppression of NSPC1 and MALAT1, NSPC1 and SOX2OT on the expression of p21 and OCT4. (A and B) MTT assay was applied to determine H4 cell viability in the si-NC, si-NSPC1, si-MALAT1, si-SOX2OT, si-NSPC1+si-MALAT1 and si-NSPC1+si-SOX2OT transfected cells. One-way ANOVA, * $P < 0.05$, ** $P < 0.01$. (C-H) Representative flow cytometric analysis were performed to evaluate the H4 cell apoptosis 24 h following transfection. (I and J) The knockdown transfection was used to explore the influence on the expression levels of p21 and OCT4. MALAT1, metastasis-associated lung adenocarcinoma transcript 1; NSPC1, nervous system polycomb 1.

addition (Fig. 4B and F). Above all, the RNA level of NSPC1 increased after 24 and 72 h of transfection and the protein level of NSPC1 increased after 72 h of transfection in response to

the decrease in MALAT1 (Fig. 4C and D), whereas both the RNA and protein levels of NSPC1 were increased following the decrease in SOX2OT (Fig. 4G and H) after 72 h of transfection.

Decrease in the co-expression of NSPc1 and MALAT1/SOX2OT inhibits H4 cell growth and promotes apoptosis more significantly than the other single transfections in vitro. The proliferation and apoptosis of H4 cells after transfection are shown in Fig. 5. MTT assay showed that the knockdown of expression of NSPc1, MALAT1, SOX2OT and knockdown of the co-expression of NSPc1 and MALAT1, NSPc1 and SOX2OT decreased the cell proliferation compared with the negative control. Above all, the silenced co-expression of NSPc1 and MALAT1, NSPc1 and SOX2OT blocked the growth of H4 cells more significantly than the single downregulation of the expression of NSPc1, MALAT1 and SOX2OT (Fig. 5A and B). Furthermore, the cell apoptotic rate following the simultaneous knockdown of the expression of NSPc1 and SOX2OT (65.05%) was higher than the sum of those following single downregulation of NSPc1 and SOX2OT (38.23±22.55%). Whereas the cell apoptotic rate following the decrease in the co-expression of NSPc1 and MALAT1 (49.17%) was not higher than the sum of those following the respectively descending expression of NSPc1 and MALAT1 (38.23±16.84%). In addition, flow cytometric analysis indicated that the decreased expression of NSPc1, MALAT1, SOX2OT and co-expression of NSPc1 and MALAT1, NSPc1 and SOX2OT induced more cell apoptosis than the negative control. The coordinate knockdown of NSPc1 and MALAT1, NSPc1 and SOX2OT with siRNAs promoted more H4 cell death compared with the other single transfections (Fig. 5C-H).

Decreased expression of NSPc1, MALAT1, SOX2OT and co-expression of NSPc1 and MALAT1, NSPc1 and SOX2OT affect the expression of p21 and OCT4. As shown in Fig. 5I and J, the expression of p21 was increased and the expression of OCT4 was decreased following the decreased expression of NSPc1, MALAT1, SOX2OT, NSPc1+MALAT1 and NSPc1+SOX2OT ($P<0.01$). Moreover, the decrease in co-expression of NSPc1 and MALAT1 caused the expression of p21 to be more highly and significantly increased when compared to the other knockdown groups (Fig. 5I). Also, silencing of the expression of MALAT1 significantly decreased the expression of OCT4 to a greater degree than the other knockdown groups (Fig. 5J).

Discussion

Accumulating evidence indicates that long non-coding RNAs (lncRNAs) are engaged in different pathologic processes including gliomagenesis, and the polycomb group (PcG) members play a vital role in cellular life span and stimulate the growth and promotion of many cancer types (26). Links between PcG complexes and lncRNAs have long been proposed. Several lncRNAs which have probable interaction with PcG members have been suggested. CBX7 of polycomb repressive complex 1 (PRC1) (27) and SUZ1 of polycomb repressive complex 2 (PRC2) (28) associate with lncRNA ANRIL to repress the tumor suppressor INK4b/ARF/INK4a locus and control senescence in cancer tissues. lncRNA FAL1 combines with PRC1 core protein, BMI1 to regulate the transcription of various genes such as CDKN1A (29). HOTAIR recruits EZH2, SU12 and EED, which is a PRC2 member, to enhance methylation of histone H3 lysine 27

(H3K27) and accelerated metastasis of breast cancer by silencing metastasis-suppressing genes (30). We used the latest RNA binding protein immunoprecipitation (RIP) technique to identify the most possible lncRNAs which may interplay with nervous system polycomb 1 (NSPc1) protein complex. Among eight candidates, we calculated a high score by bioinformatics. Yet, one limitation included the lack of direct binding data from the RNA pull-down assay which should further be researched in future studies. In summary, we identified the probable interactions with NSPc1 protein complex in the aspect of expression and function in H4 cells.

In addition to metastasis-associated lung adenocarcinoma transcript 1 (MALAT1)-EZH2 and NSPc1-EZH2 interaction mentioned above (16,19,20), NSPc1 recruitment is the downstream event of EZH2-driven H3K27 methylation during tumorigenesis (16) and MALAT1 regulates downstream factors via EZH2-mediated H3K27 in renal cancer tissue (19). This research suggested that MALAT1-EZH2 interaction and NSPc1-EZH2 combination, which indicates a novel correlation of MALAT1-NSPc1. As expected, among the 8 candidates, our RNA immunoprecipitation results indicated the possible binding relationship between MALAT1 and NSPc1 protein complex in H4 cells. Next, we identified that the expression of MALAT1 as measured by qRT-PCR was contrary to the variation of NSPc1 expression regulated by pCDEF-NSPc1 and siRNA-NSPc1 separately in H4 cells. Furthermore, the decreased expression of MALAT1 increased gene and protein levels of NSPc1 in H4 cells. Thus, MALAT1 is negatively regulated by NSPc1 in H4 cells. However, more research should be conducted to explore the regulatory mechanism of the NSPc1/PRC1/MALAT1 complex model in the future.

The overexpression of MALAT1 has been shown to act as a tumor-promotor factor in colorectal (31), lung (32) and gastric cancer (33). The expression of NSPc1 is significantly increased in high grade malignant gliomas (15). We identified that expression levels of MALAT1 and NSPc1 were higher in glioma cells than levels in non-cancerous cells by qRT-PCR. Furthermore, it was observed that inhibition of the respective expression levels of NSPc1 and MALAT1 or the suppression of the co-expression of these was able to retard H4 cell growth and promote their apoptosis. Noticeably, the decreased co-expression of NSPc1 and MALAT1 had a more significant impact on survival and apoptosis than separately silenced expression. However, the cell apoptosis rate caused by the downregulation of the co-expression of NSPc1 and MALAT1 was not higher than the total cell apoptosis caused by a separate decrease in expression of MALAT1 and NSPc1. We speculated that the primary cause of this was that in the situation of the co-transfection of NSPc1 and MALAT1, upon the silencing of the expression of MALAT1, the endogenous expression of NSPc1 increased due to the negative regulation of MALAT1. At the same time, the expression of NSPc1 was knocked down by siRNA based on the basic condition of MALAT1-mediated increase of the endogenous expression of NSPc1. As a result, the downregulation efficiency of NSPc1 in the single NSPc1 transfection group was much higher than that in the co-transfection group, leading to the phenomenon that the decreased co-expression effect on promoting cell apoptosis was partly neutralized by the negative cross-talk

between them. In summary, these data demonstrated that the NSPc1/PRC1/MALAT1 functional interaction may regulate glioma cell proliferation and apoptosis to a certain extent, which may play important roles as biomarkers for glioma H4 cells. Moreover, it is inappropriate to apply co-downregulation of NSPc1 and MALAT1 strategy to the gene therapy in glioma H4 cells. However, the conclusion, to some degree, lacks generalized applicability for the target therapy for all types of glioma cells because the only one type of glioma cells was used. Therefore, additional glioma cell lines and animal models will be utilized for further research in order to confirm the biomarkers for brain tumors. Furthermore, the definite molecular mechanisms need further investigation.

According to a previous study, Nspc1 is highly expressed in differentiated pluripotency P19 cells and maintains the pluripotency of P19 cells (34). Furthermore, Nspc1 activates the key pluripotent Oct4/Nanog/Sox2 axis in P19 cells and Oct4, Nanog and Sox2 are positively regulated by Nspc1 in P19 cells. SOX2, a master regulator of pluripotency, is embedded within the third intron of an lncRNA known as SOX2 overlapping transcript (SOX2OT) (35). SOX2OT has been suggested to participate in the transcriptional regulation of SOX2 (36). However, the presence of any physical interaction between SOX2OT and SOX2 has been alleged in recent studies that SOX2OT has a positive effect on SOX2 expression via knockdown and overexpression assays (37,38). Also, SOX2OT plays a vital role in regulating and mediating pluripotency and tumorigenesis events, probably by the expression of SOX2 (39). Therefore, in the present study, we confirmed the *in vivo* cooperation between SOX2OT and the NSPc1 protein complex by RIP method. Furthermore, we identified that the expression of SOX2OT was consistently increased no matter how NSPc1 expression was altered whereas the gene and protein expression of NSPc1 was decreased by the downregulation of SOX2OT in H4 cells. Moreover, whether the regulation between NSPc1 and SOX2OT affects transcription of target genes or expression of each will be further studied as a next research step.

In addition, recent studies reported that patients with low expression of SOX2OT had prolonged survival compared with those with a high level of SOX2OT in hepatocellular carcinoma (40) and gastric cancer (41). As in this study, the expression of SOX2OT was much higher in glioma cells than that noted in non-cancerous cells. We also studied the interaction between the NSPc1 protein complex and SOX2OT at the functional level. Our results showed that downregulation of NSPc1 and SOX2OT co-expression reduced the cell growth and increased cell death more obviously compared with that of NSPc1 and SOX2OT alone. Moreover, the cell apoptosis rate following decreased co-expression of NSPc1 and SOX2OT was higher than the sum of that in the respective downregulation of the expression of NSPc1 and SOX2OT. The main reason that we speculated was that in the co-downregulation of SOX2OT and NSPc1 conditions, the endogenous expression of NSPc1 was decreased by the downregulation of SOX2OT. Besides this circumstance, the expression of NSPc1 was knocked down by exogenous siRNA simultaneously. Hence, the downregulation efficiency of NSPc1 in the co-expression group was higher than that in the single NSPc1 silenced group, resulting in the more significant

influence on accelerating cell apoptosis. It is meaningful that this collaborative cross-talk between NSPc1 and SOX2OT could act as a more effective therapeutic complex target for glioma H4 cells. Nevertheless, the data of our research do not represent universal therapeutic complex targets for all glioma cell lines. In addition, the exact biological function of the interaction between the NSPc1 protein complex and SOX2OT has yet remained unknown. Hence, we will conduct in-depth study on this unsolved issue.

It has been reported that p21 is the direct target gene regulated by NSPc1 in tumor cell growth (15) and the pluripotency stem cell marker OCT4 was positively regulated by NSPc1 in P19 cells (33). Thus, we explored the expression influence of p21 and OCT4 following knockdown of the expression of NSPc1, MALAT1, SOX2OT, NSPc1+MALAT1 and NSPc1+SOX2OT. We found that co-knockdown of the expression of NSPc1 and MALAT1 increased the expression of p21 most obviously in glioma H4 cells. Furthermore, silencing of the expression of MALAT1 decreased the expression of OCT4 most significantly in glioma H4 cells. This may provide a new concept in the influence of the lncRNA-NSPc1 complex on regulating target cancer marker genes.

In conclusion, our data provide an initial insight into the potential function of the MALAT1-NSPc1 protein complex and SOX2OT-NSPc1 protein complex binding and crosstalk relationship in H4 glioma cells. In addition, it highlights the negative correlation between MALAT1 and NSPc1 in H4 cells and that NSPc1 is positively regulated by SOX2OT in H4 cells. However, the altered expression of NSPc1 promotes SOX2OT expression increase. In addition, decreased co-expression of NSPc1 and MALAT1, NSPc1 and SOX2OT suppressed the growth of glioma H4 cells and accelerated apoptosis more obviously compared with the respective decline in expression. Furthermore, the co-expression of NSPc1 and SOX2OT induced a higher cell apoptosis compared with the sum of the decreased expression levels of NSPc1 and SOX2OT alone, which may provide a novel complex in gene strategy. Nevertheless, this effect was partially counteracted in the silenced co-expression of MALAT1 and NSPc1 due to the negative correlation between them.

In brief, these findings may provide potential therapeutic gene targets and novel biomarkers for malignant glioma. Moreover, further research, particularly different animal models, must be conducted to explore the detail mechanism of the functional complexes of lncRNAs-PcGs and their molecular mechanisms in glioma.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YW, ZL and HL performed the experiments. HL, JT, YS and YG analyzed and interpreted the data. HL and YG conceived and designed the study. YW and YG were major contributors in writing the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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