RNaseH2A is involved in human gliomagenesis through the regulation of cell proliferation and apoptosis

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Abstract. Mutations in the RNaseH2A gene are involved in Aicardi-Goutieres syndrome, an autosomal recessive neurological dysfunction; however, studies assessing RNaseH2A in relation to glioma are scarce. This study aimed to assess the role of RNaseH2A in glioma and to unveil the underlying mechanisms. RNaseH2A was silenced in glioblastoma cell lines U87 and U251. Gene expression was assessed in the cells transfected with RNaseH2A shRNA or scramble shRNA by microarrays, validated by quantitative real time PCR. Protein expression was evaluated by western blot analysis. Cell proliferation was assessed by the MTT assay; cell cycle distribution and apoptosis were analyzed by flow cytometry. Finally, the effects of RNaseH2A on colony formation and tumorigenicity were assessed *in vitro* and in a mouse xenograft model, respectively. RNaseH2A was successively knocked down in U87 and U251 cells. Notably, RNaseH2A silencing resulted in impaired cell proliferation, with 70.7 and 57.8% reduction in the U87 and U251 cells, respectively, with the cell cycle being blocked in the G0/G1 phase *in vitro*. Meanwhile, clone formation was significantly reduced by RNaseH2A knockdown, which also increased cell apoptosis by approximately 4.5-fold. In nude mice, tumor size was significantly decreased after RNaseH2A knockdown: 219.29±246.43 vs. 1160.26±222.61 mm^3 for the control group; similar findings were obtained for tumor weight (0.261±0.245 and 1.127±0.232 g) in the shRNA and control groups, respectively). In the microarray data, RNaseH2A was shown to modulate several signaling pathways responsible for cell proliferation and apoptosis, such as IL-6 and FAS pathways. RNaseH2A may be involved in human gliomagenesis, likely by regulating signaling pathways responsible for cell proliferation and apoptosis.

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Introduction

Human brain glioma, also known as neuroglioma, exhibits invasive growth without an overt boundary to surrounding tissues; it has unique cell origin, pathological structures, biological features, and clinical symptoms (1-6). Malignant glioma is a tumor with extremely high malignancy and recurrence rate; low total resection rate, high postoperative recurrence and residual lesion recurrence caused by radiation, and chemotherapeutic tolerance constitute the main problems faced by neurosurgeons (7-13). Considering the bottleneck in glioma treatment, dissecting relevant molecular mechanisms and finding tumor-related gene therapy approaches in human glioma attracts increasing attention for improved diagnosis, treatment and prognosis of glioma (6,14-23).

Our laboratory and others have recently screened clinical relevant genes in glioma (24-28); notably, we found a new glioma-expressed gene that plays an important regulatory role in the proliferation, progression and apoptosis of glioma cells. The gene is called ribonuclease H2, subunit A (RNaseH2A) also known as AGS4, JUNB, RNHL, RNHIA and RNaseHI. RNaseH2A (1148 bp), located on chromosome 19 (29,30), is a component of ribonuclease H2 (RNAseH2) and is responsible for its endoribonuclease activity (31-33). RNaseH was discovered and isolated from calf thymus (34,35) and is widely distributed in mammalian cells, yeasts, prokaryotes and virus particles; it catalyzes in-nucleus degradation of RNA in DNA-RNA hybrids and is involved in reverse transcriptase of multifunctional enzymes in retroviruses, playing an important role in various stages of viral genome transcription (36,37). In eubacteria, RNaseH is required for several processes, including removal of RNA primers from Okazaki fragments, transcription of primers required for DNA polymerase I initiated DNA synthesis, and removal of R-ring to provide conditional initiating sites required for irregular DNA synthesis (38). In eukaryotes, RNaseH may play similar roles (39). Recent studies have shown that RNaseH2A mutations lead to Aicardi-Goutieres syndrome, an autosomal recessive neurological dysfunction, which mainly causes microcephaly, mental motor development retardation, cerebral calcification, increased IF-α and leukocytes in cerebrospinal fluid, fever, thrombocytopenia, and hepatitis (40-42). RNaseH2A was proposed as a putative cancer target (31). In

agreement, logistic regression analysis revealed that expression levels of RNaseH2A, among other genes, were positively correlated with aggressive prostate cancer (43). However, studies assessing RNaseH2A in relation to glioma are scarce. Therefore, in the present study, we aimed to assess the role of RNaseH2A in glioma, exploring the underlying mechanisms.

Our data demonstrated that RNaseH2A silencing altered cell proliferation and clone formation and enhanced apoptosis in the glioma cells *in vitro*. Meanwhile, RNaseH2A-knockdown xenografted glioma cells were less tumorigenic in a nude mouse model. Moreover, microarray data confirmed that RNaseH2A regulated genes that are frequently involved in multiple important cellular regulatory processes, including focal adhesion, cancer, p53 signaling, and cell cycle. Our findings provide a strong basis for finding new gene-based therapeutic approaches against human glioma.

Materials and methods

Cell culture. The human glioblastoma (GBM) cell lines U87, U251, A373 and A172 were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin at 37˚C in 5% CO_2.

RNaseH2A knockdown in GBM cells. For RNaseH2A silencing, U85 and U251 cells were transfected with the pGCSIL-GFP plasmid containing RNaseH2A-specific shRNA, or non-effective shRNA control fragments (GeneChem, Montreal, QC, Canada).

Gene expression microarray analyses. Total RNA was extracted from the U87 cells (transfected with NC or RNaseH2A shRNA) using an RNeasy Mini-kit (Qiagen, Valencia, CA, USA), and quantified on a Nanodrop ND-1000 spectrophotometer (NanoDrop Technology). Samples with adequate RNA quality index (>7) were used for the microarray analyses.

Genome-wide transcriptome profiles were assessed by expression microarrays (GeneChip PrimeView human, Affymetrix) and validated by quantitative real-time PCR. Microarray data and fully detailed experimental procedures are published online at Gene Expression Omnibus (GEO, NCBI) (http://www.ncbi.nlm.nih.gov/geoprofiles/). Differentially expressed genes were identified using 1.5- or 2-fold change as cutoff values. Gene ontology enrichment analysis was carried out using David Functional Annotation Resources 6.7 (http://david.abcc.ncifcrf.gov/) and the KEGG pathway analysis.

Cell proliferation, apoptosis, and cell cycle analysis. GBM cells were seeded onto 96-well plates at $2x10^3$ cells/well for culture. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay 96 h after transfection with NC or RNaseH2A shRNA. Briefly, 20 μl MTT solution (5 mg/ml) was added to each well for 4 h at 37˚C. After the medium was aspirated, 100 μl dimethyl sulfoxide (DMSO) was added. Optical density was measured at 492 nm on a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Viability index was calculated as experimental OD value/control OD value. Three independent experiments were performed in quadruplicate.

Table I. List of primers.

Gene	Sequence
RNaseH2A-F	AAGACCCTATTGGAGAGCGAG
RNaseH2A-R	AGTTCAGGTTGTATTTGACCCG
GAPDH-F	TGACTTCAACAGCGACACCCA
GAPDH-R	CACCCTGTTGCTGTAGCCAAA
MDM2-F	GAATCATCGGACTCAGGTACATC
MDM2-R	TCTGTCTCACTAATTGCTCTCCT
SMAD3-F	GACTACAGCCATTCCATCC
SMAD3-R	CAGGTCCAAGTTATTATGTGC
FGF2-F	ATCAAAGGAGTGTGTGCTAACC
FGF2-R	ACTGCCCAGTTCGTTTCAGTG
SMAD2-F	AGAGGGAAACAAGAACAGG
SMAD2-R	ATGCTCTGGCGTCTACTG
IGF1R-F	TGCGTGAGAGGATTGAGTTTC
IGF1R-R	CTTATTGGCGTTGAGGTATGC
IL6-F	CAAATTCGGTACATCCTCG
IL6-R	CTCTGGCTTGTTCCTCACTA
IL8-F	TGGCAGCCTTCCTGATTT
IL8-R	AACCCTCTGCACCCAGTT
FAS-F	ACACTCACCAGCAACACCAA
FAS-R	CTTCCTTTCTCTTCACCCAAACA
BIRC5-F	ACCGCATCTCTACATTCAAG
BIRC5-R	CAAGTCTGGCTCGTTCTC
TGFBR2-F	GTGCCAACAACATCAACC
TGFBR2-R	GACTGCCACTGTCTCAAACT

For cell cycle analysis, the cells were stained with propidium iodide (PI) solution (50 μg/ml) and analyzed on a FACSCalibur flow cytometer 24 h after transfection.

Apoptosis was assessed using a fluorescein isothiocyanate (FITC) Annexin V staining kit (Life Technologies, Grand Island, NY, USA) followed by fluorescence-activated cell sorter (FACS) analysis according to the manufacturer's instructions.

Colony formation assay. Following treatment, adherent cells were trypsinized and counted to determine viability. Then, 800 viable cells were seeded into each well of a 6-well plate (in triplicate). Cells were allowed to adhere and grow for 10-14 days. To visualize colonies, media were removed, and cells were fixed in paraformaldehyde for 30 min and stained with Giemsa solution (ECM 550, Chemicon). Finally, colonies were counted; data are presented as mean colony number ± SEM from at least three independent experiments.

RT-PCR and quantitative real-time PCR. RNA extraction was performed with TRIzol (Invitrogen Life Technologies, Carlsbad, CA, USA); cDNA was synthesized using RT-Phusion kit (Thermo Scientific, Waltham, MA, USA). Gene-specific mRNA levels were quantified by standard RT-PCR or quantitative PCR (qPCR), using the $\Delta\Delta$Ct method, as previously described (44). Primer sequences are listed in Table I.

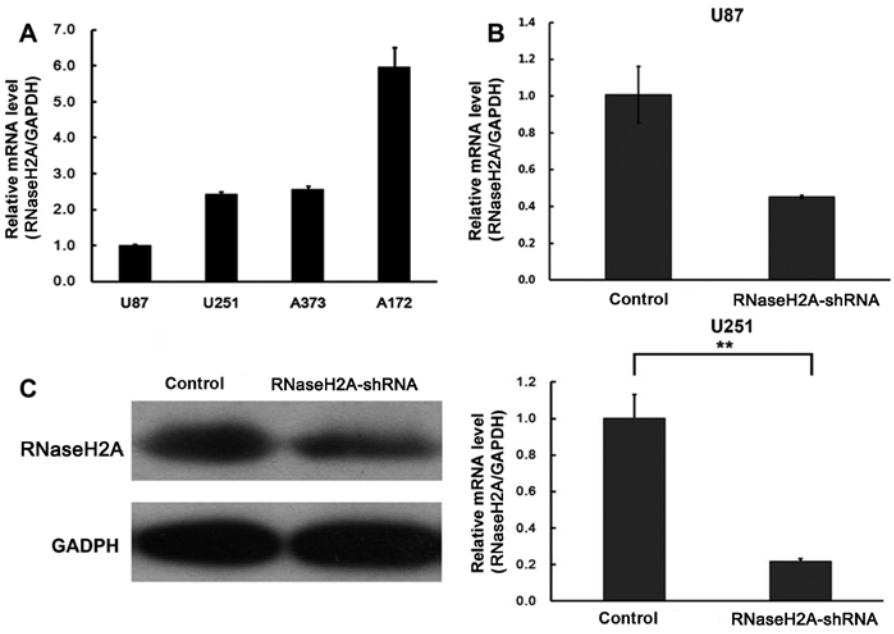

Figure 1. Gene expression levels of RNaseH2A in human glioblastoma cells silenced or not for RNaseH2A. (A) RNaseH2A mRNA in different glioma cell lines, assessed by qRT-PCR. (B) Silencing efficacy of RNaseH2A shRNA in the U87 cells, as assessed by qRT-PCR. (C) Silencing efficacy of RNaseH2A shRNA in U251 cells, as assessed by qRT-PCR. Data are represented as the mean ± SD. Each data point was determined from at least three replicates; *P<0.05 or **P<0.01, significant difference between the RNaseH2A shRNA and the shRNA control groups.

Western blot analysis. After treatment, the cells were harvested and lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) that contained a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 4˚C. Forty micrograms of protein from each lysate were fractionated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). After blocking with 5% nonfat milk in PBS-Tween-20 for 1 h at room temperature, the membranes were blotted with the appropriate primary antibody. Primary antibodies against RNaseH2A (1:1,000; Proteintech), IL-6 (1:2,000; Abcam), FAS (1:1,000; Abcam), IGTA2 (1:10,000; Abcam), GAPDH (1:20,000; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) were incubated at 4˚C overnight. After washing four times with TBST, the membranes were incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc.) for 2 h. The proteins were visualized using enhanced chemiluminescence (ECL; Beyotime Institute of Biotechnology, Nantong, China).

In vivo GBM xenograft studies. All mouse care and experiments were carried out with approval of the Institutional Animal Care and Use Committee at the Beijing Tian Tan Hospital, Capital Medical University. Subcutaneous xenograft models were performed by injecting U87 cells transfected with NC or RNaseH2A shRNA as previously described (45).

Statistical analysis. Results are representative of 3 independent experiments. Data are expressed as the mean ± standard deviation (SD). Student's t-test was employed to compare groups. P<0.05 was considered statistically significant. SPSS 10.0 software (SPSS, Chicago, IL, USA) was used for statistical analyses.

Results

The RNaseH2A gene is expressed in GBM cells. Four GBM cell lines, including U87, U251, A373 and A172 were assessed for RNaseH2A expression by RT-PCR. RNaseH2A was detected in all of the cell lines (Fig. 1A).

RNaseH2A silencing suppresses glioma cell proliferation in vitro and in vivo. Two cell lines (U87 and U251) were selected for RNA interference experiments, to assess RNaseH2A function in glioma cells. As shown in Fig. 1B and C, RNaseH2A was successfully silenced in both cell lines. RNaseH2A mRNA expression was reduced to a greater extent in the U251 cell line.

Cell proliferation analysis was performed in the two glioma cell lines (U87 and U251) transfected with RNaseH2A-shRNA. Both glioma cell lines showed significantly reduced viability after RNaseH2A silencing (Fig. 2A and B). Five days after shRNA transfection, cell viability was reduced by 70.7 and 57.8% in the U87 and U251 cells, respectively. In accordance, colony formation was also significantly decreased (P<0.05) in the U87 cells after RNaseH2A knockdown, compared with the control values (Fig. 2C). Furthermore, xenografted tumor growth in the RNaseH2A-shRNA group was markedly reduced compared with that of the normal control group *in vivo* (Fig. 2D-F). Indeed, 34 days after U87 cell injection, tumor volumes were 219.29±246.43 and 1160.26±222.61 mm^3 in the RNaseH2A-shRNA and normal control groups, respectively; tumor weights of 1.127±0.232 and 0.261±0.245 g were obtained from the control and RNaseH2A-silenced

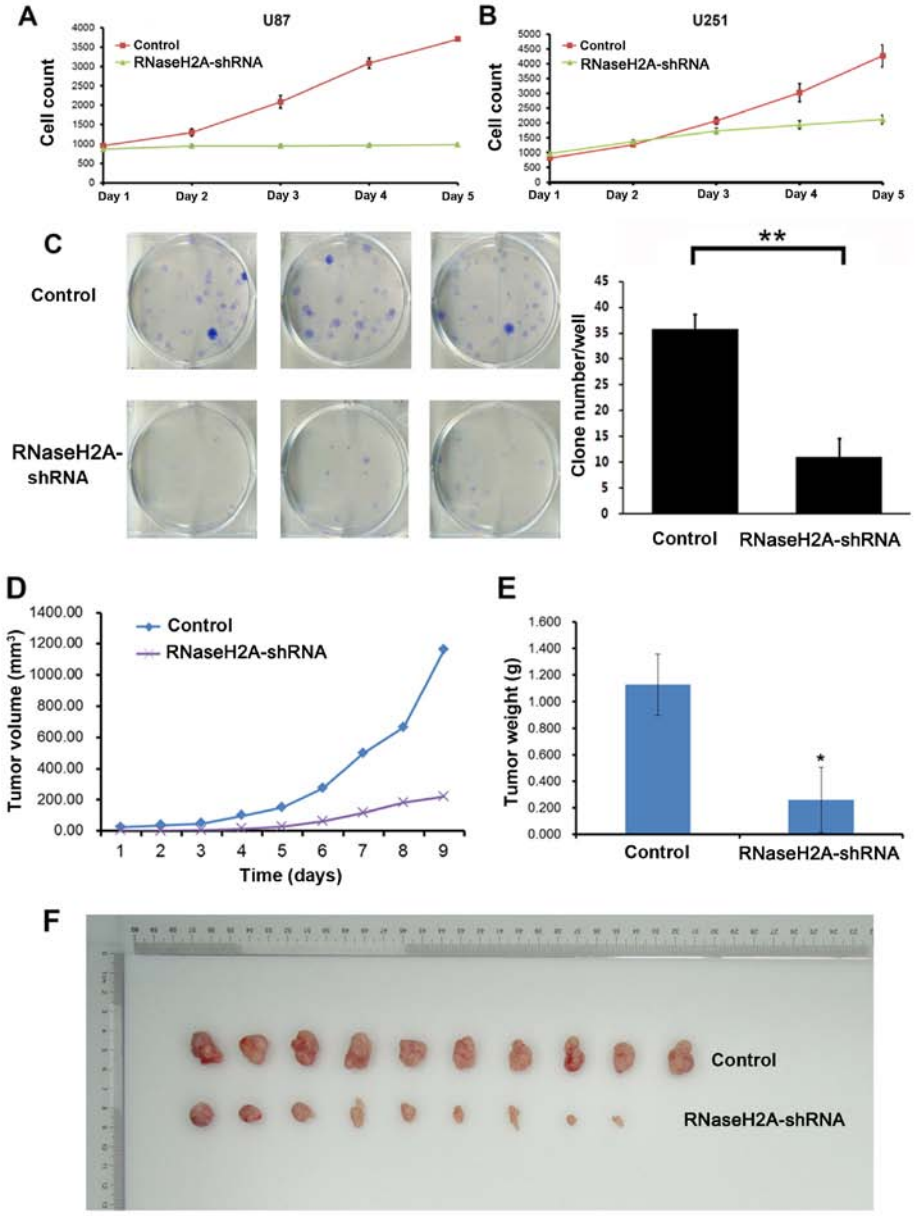

Figure 2. Effects of RNaseH2A silencing on glioma cell proliferation *in vitro* and *in vivo*. RNaseH2A silencing inhibits the growth of (A) U87 and (B) U251 glioma cells. (C) RNaseH2A silencing reduces colony formation of U87 cells. (D-F) RNaseH2A silencing inhibits tumor growth *in vivo*. shRNA control- and RNaseH2A shRNA-transfected U87 cells were injected subcutaneously into nude mice. Tumor volumes were calculated daily. Mice were then sacrificed and tumors dissected, photographed and weighed. Quantitative data are represented as the mean ± SD. Each data point was determined from at least three replicates; *P<0.05 or **P<0.01, significant difference between the RNaseH2A shRNA and the shRNA control groups (n=10).

cells, respectively (P<0.01). These findings demonstrated that RNaseH2A was required for glioma cell proliferation, both *in vitro* and *in vivo*.

RNaseH2A silencing causes cell cycle arrest in the G0/G1 phase and apoptosis in glioma cells. To explore the mechanism underlying the observed impaired growth of glioma cells after RNaseH2A silencing, cell cycle distribution was assessed by flow cytometry. RNaseH2A silencing resulted in an increased percentage of U87 cells in the G0/G1 phase, with 75.14±1.004 and 61.51±0.43% obtained respectively, in the RNaseH2A knockdown and control groups, respectively (P<0.01, Fig. 3A).

The effect of RNaseH2A on apoptosis in glioma cells (Fig. 3B) was also investigated. As shown in Fig. 3B, RNaseH2A knockdown induced cell apoptosis by 4.5-fold compared to the normal control cells.

RNaseH2A silencing induces differential expression of genes involved in multiple cell signaling pathways. To further explore the biological significance of RNaseH2A in glioma cells, gene expression profiles of U87 cells transfected with or without RNaseH2A shRNA were assessed. As shown in the heat map (Fig. 4A), gene expression profiles in the NC and RNaseH2A shRNA groups were remarkably different. A total of 821 upregulated and 941 downregulated genes were found in the RNaseH2A shRNA-transfected U87 cells. GO and KEGG pathway analysis revealed that the differentially expressed genes are frequently involved in multiple important cellular

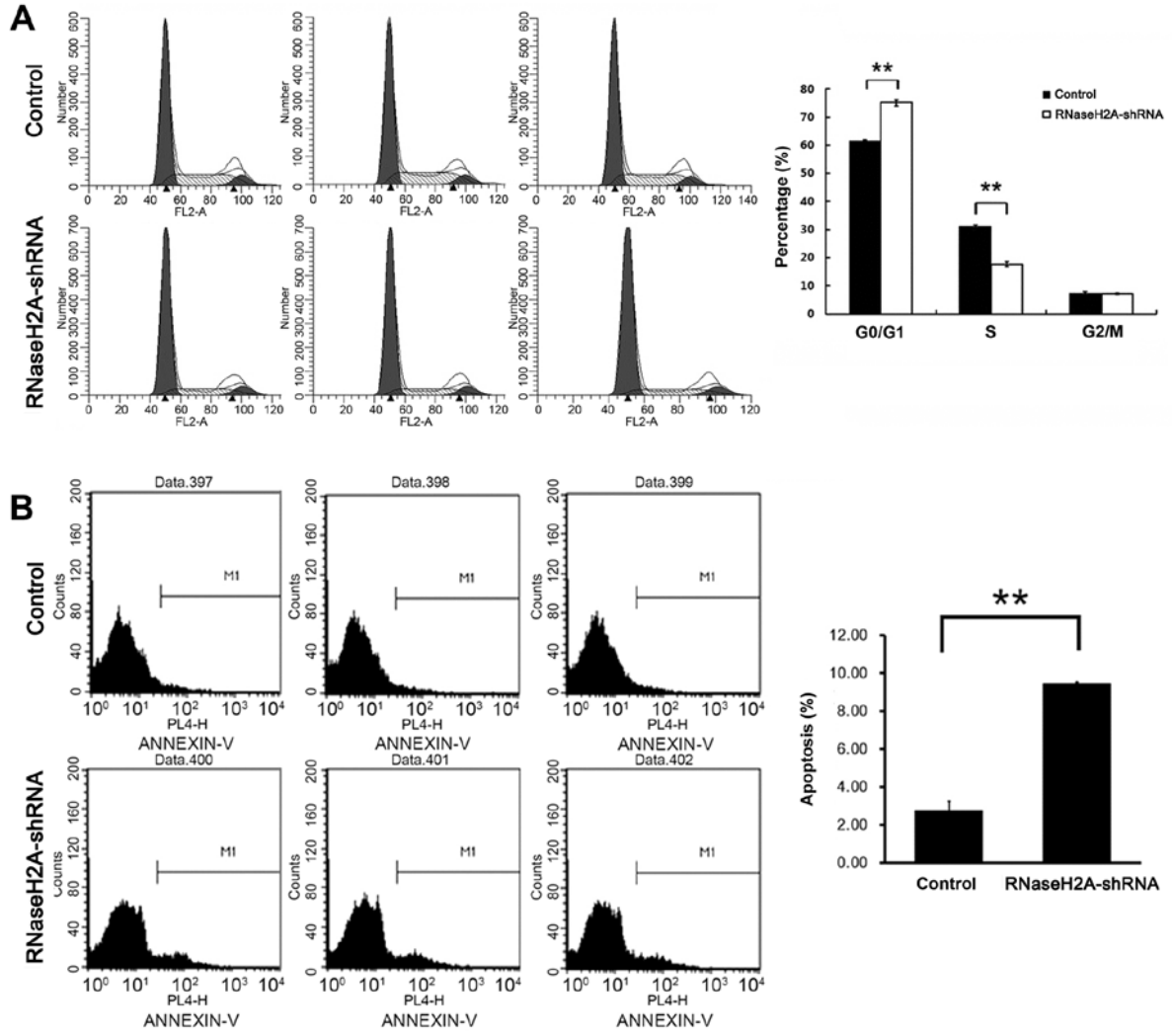

Figure 3. RNaseH2A knockdown alters glioma cell cycle progression and apoptosis. (A) Effects of RNaseH2A knockdown on (A) cell cycle progression and (B) apoptosis. Quantitative data are expressed as mean ± SD. Three representative flow-cytograms for the control and RNaseH2A groups, respectively, are presented for cell cycle distribution and apoptosis assays. Each data point was determined from at least three replicates; $^{*}P<0.05$ or $^{**}P<0.01$, significant difference between the RNaseH2A shRNA and the shRNA control groups.

regulatory processes, including focal adhesion, cancer, p53 signaling, and cell cycle (Fig. 4B). Therefore, our data suggest that RNaseH2A shRNA affects various genes involved in important biological processes.

To validate the microarray data, qRT-PCR and western blot analysis were carried out. Protein and gene expression levels were consistent with the microarray data. For instance, IL-6 mRNA levels were decreased significantly in the RNaseH2A shRNA group compared to the control group, while FAS mRNA amounts were increased significantly in the RNaseH2A shRNA group compared to the control group ($P<0.001$); western blotting yielded similar results. These findings indicated that IL-6 and FAS might be suitable RNaseH2A target genes (Fig. 4C and D).

Discussion

RNaseH2A was expressed in all glioma cells assessed. Notably, RNaseH2A knockdown by shRNA silencing resulted in reduced proliferation and viability of the tumor cells, which were blocked in the G0/G1 phase. In addition, RNaseH2A-knockdown cells showed increased apoptosis *in vitro*. In agreement, clone formation (*in vitro*) and tumor growth (*in vivo*) were significantly decreased after RNaseH2A silencing. Gene-chip based transcriptome assay indicated that RNaseH2A plays an important role in several signaling pathways involved in cell proliferation, including the IL-6 and FAS pathways.

Gene dysregulation is a hallmark of tumor genesis and progression (46-51), with post-transcriptional regulation of messenger RNA constituting an important step. Ribonucleases (RNases) catalyze RNA breakdown, thus influencing mRNA turnover and gene expression; their dysfunction is linked to various types of tumors. For instance, failure to recruit PARN, a poly A ribonuclease, has been observed in malignant glioma (52,53). In addition, reduction and/or depletion of XRN1, a 5'-3' exonuclease which initiates mRNA decay, is implicated in primary osteogenic sarcoma and its derived cell lines (54). Furthermore, the gene encoding truncated RNase L is positively correlated with hereditary

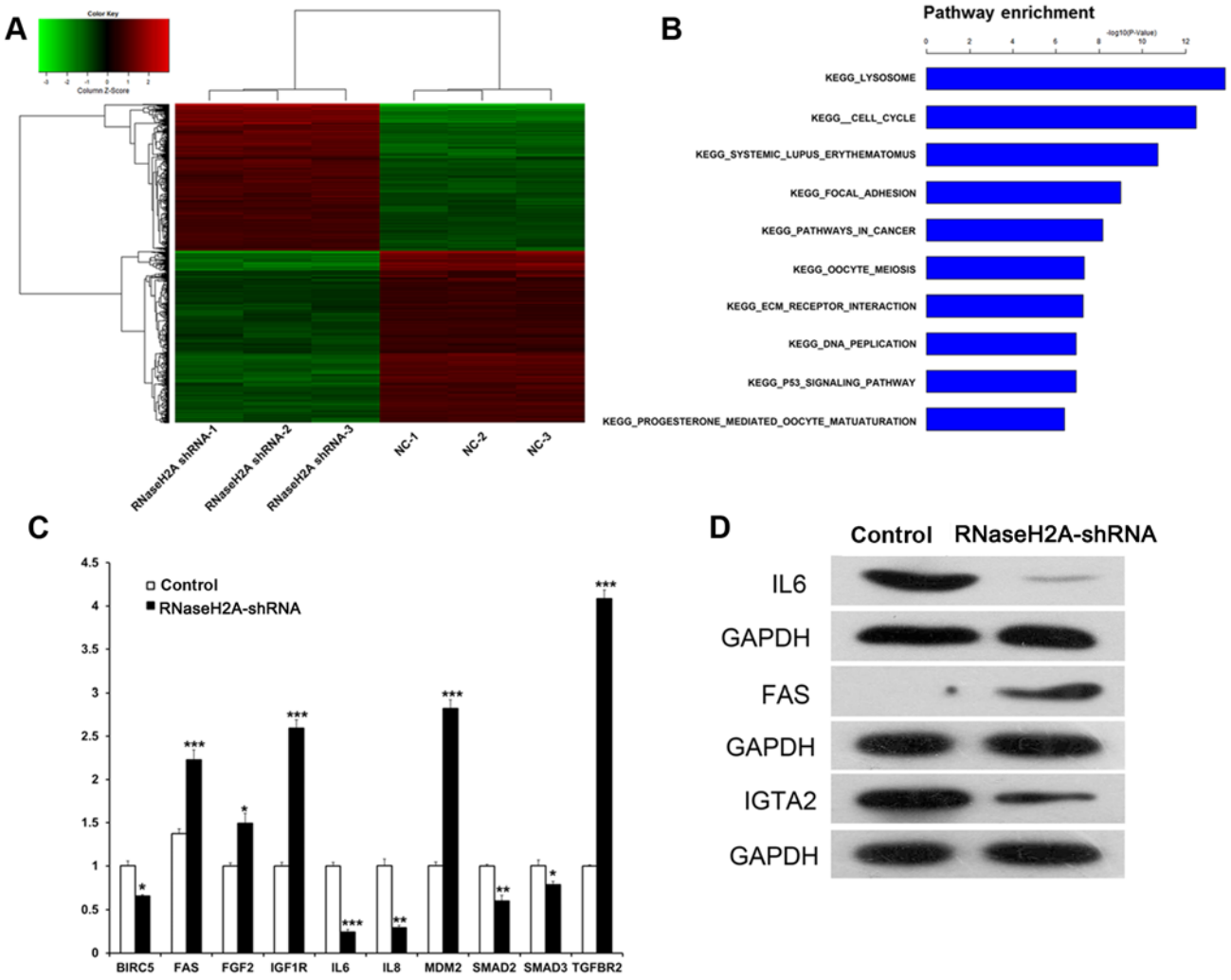

Figure 4. RNaseH2A knockdown alters mRNA and protein levels of several genes involved in multiple cellular signaling pathways. (A) Heat map showing differentially expressed genes after RNaseH2A silencing. Rows and columns represent genes and samples, respectively. The intensity of each color denotes the standardized ratio between each value and the average methylation/expression of the gene across all samples. Red and green indicate increased and decreased transcript levels, respectively. (B) KEGG pathway analysis of differentially expressed genes (cutoff of 1.5- or 2-fold). (C) Validation of microarray results by qPCR. (D) Validation of microarray results by western blot analysis are represented as the mean ± SD; *P<0.05, **P<0.01 or ***P<0.001, significant difference between the RNaseH2A shRNA and the shRNA control groups.

prostate cancer (55), while moderated reduction in enzyme activity is related to a higher risk of prostate and colorectal cancers (56) as well as pancreatic carcinoma (57-59). IRE1, a transmembrane endoribonuclease found in the endoplasmic reticulum (60), can act as a tumor suppressor, deciding the fate of cancer cells (61). RNases from the miRNA pathway, including Drosha, Dicer, and Ago2, are also implicated in tumor biology. For instance, elevated expression of Drosha was found in esophageal cancers (62), with its suppression leading to decreased cancer cell proliferation; in addition, elevated mRNA levels and genomic copy numbers of Drosha were found in clinical cervical squamous cell carcinoma samples and derived cell lines (63). Several studies have reported overexpression of Dicer in multiple cancers, including salivary gland, lung, prostate, and ovary carcinomas, as well as Burkitt's lymphoma; Ago2-overexpression was also found in these cancers (64-68).

RNaseH2A is a component of the heterotrimeric type II ribonuclease H enzyme (RNAseH2), which provides the main ribonuclease H activity (31-33). Consistent with our findings, a previous study indicated that RNaseH2A is a putative anticancer drug target in transformed stem cells (31). However, how RNaseH2A is involved in tumor genesis and progression remains unclear. In this study, gene silencing and transcriptome profiling were combined to assess downstream signaling pathways mediating the effect of RNaseH2A. Multiple genes involved in important cellular processes, including focal adhesion, cancer, p53 signaling, and cell cycle, were differentially expressed after RNaseH2A silencing. Further analysis demonstrated that IL-6 was downregulated after RNaseH2A knockdown. GBM cells produced IL-6 *in vitro* and *in vivo* (69,70), and Goswami *et al* reported that IL-6 mediated autocrine growth promotion in the human GBM multiforme cell line U87MG (71); in addition, targeting IL-6Rα or IL-6 expression in GSCs impaired cell growth and increased the survival of mice bearing intracranial human glioma xenografts (71), in agreement with our findings of decreased xenograft growth after RNaseH2A silencing. We also found that FAS expression was upregulated after RNaseH2A silencing, which is consistent with the enhanced

apoptosis described above after RNaseH2A silencing. FAS receptor is a death receptor on the surface of cells that leads to programmed cell death (72). Activation of FAS was shown to initiate apoptosis in different glioma cell lines (73). Fas-ligand, which is expressed in GBM cell lines and primary astrocytic brain tumors (74), can lead to >90% inhibition of clonal tumor cell growth in high grade gliomas *ex vivo* (73). Thus, silencing of RNaseH2A may inhibit proliferation and promote apoptosis in glioma cells, via downregulation of IL-6 and upregulation of FAS simultaneously.

In summary, we unveiled a possible mechanism by which RNaseH2A contributes to glioma cell proliferation. Indeed, RNaseH2A silencing resulted in growth arrest and apoptosis in glioma cells, possibly through IL-6 and FAS regulation. These findings indicate that RNaseH2A upregulation may contribute to gliomagenesis and progression, via modulation of factors involved in cell growth and apoptosis. Therefore, RNaseH2A should be considered as a potential molecular target for glioma diagnosis and treatment.

Acknowledgements

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