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LRRC4 Inhibits the Proliferation of Human Glioma Cells by Modulating the Expression of STMN1 and Microtubule Polymerization

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ABSTRACT

LRRC4 is a tumor suppressor of glioma, and it is epigenetically inactivated commonly in glioma. Our previous study has shown that induction of LRRC4 expression inhibits the proliferation of glioma cells. However, little is known about the mechanisms underlying the action of LRRC4 in glioma cells. We employed two-dimensional fluorescence differential gel electrophoresis (2-D DIGE) and MALDI –TOF/TOF-MS/MS to identify 11 differentially expressed proteins, including the significantly down-regulated STMN1 expression in the LRRC4-expressing U251 glioma cells. The levels of STMN1 expression appeared to be positively associated with the pathogenic degrees of human glioma. Furthermore, induction of LRRC4 over-expression inhibited the STMN1 expression and U251 cell proliferation in vitro, and the glioma growth in vivo. In addition, induction of LRRC4 or knockdown of STMN1 expression induced cell cycle arrest in U251 cells, which was associated with modulating the p21, cyclin D1, and cyclin B expression, and the ERK phosphorylation, and inhibiting the CDK5 and cdc2 kinase activities, but increasing the microtubulin polymerization in U251 cells. LRRC4, at least partially by down-regulating the STMN1expression, acts as a major glioma suppressor, induces cell cycle arrest and modulates the dynamic process of microtubulin, leading to the inhibition of glioma cell proliferation and growth. Potentially, modulation of LRRC4 or STMN1 expression may be useful for design of new therapies for the intervention of glioma. J. Cell. Biochem. 112: 3621–3629, 2011.

KEY WORDS: GLIOMA CELLS; LRRC4; STMN1; PROLIFERATION; CELL CYCLING; MICROTUBIN POLYMERIZATION

Leucine-rich repeat C4 (LRRC4) is an important regulator of cell growth, and differentiation in the nervous system and its expression is relatively high and preferable in the brain tissues (Wang et al., 2002; Zhang et al., 2005a; Wu et al., 2006a; Wu et al., 2006b).

Previous studies have shown that LRRC4 is a critical tumor suppressor in regulating the development of malignant gliomas, as its expression is down-regulated and even absent in different grades of human astrocytoma (Wang et al., 2002; Zhang et al., 2005a; Wu et al., 2006a, 2008a). The absent or low expression of LRRC4 in glioma is associated with the high degree of methylation in the LRRC4 promoter (Zhang et al., 2008). Conversely, induction of LRRC4 expression inhibits the proliferation and invasiveness of glioma cells in vitro and suppresses the tumorigenesis of glioblastoma cells in vivo (Zhang et al., 2005a; Wu et al., 2006a; Wu et al., 2008b). The inhibitory effect of LRRC4 on the proliferation, migration, and angiogenesis of glioblastoma is likely related to the down-regulation of pleiotropic cytokine expression and responses, and the SDF-1α/CXCR4-mediated ERK1/2 and Akt signaling pathways (Wu et al., 2008a). However, whether other molecular mechanisms could be involved in the LRRC4-mediated inhibition on the glioma tumorigenesis and cell proliferation remains to be determined.

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Oncoprotein 18/stathmin 1 (STMN1) is a mitotic regulator, and plays an important role in the maintenance of cell biological characteristics by regulating rapid microtubule remodeling (Lin et al., 2009). STMN1 is critical for neoplastic transformation, and high levels of STMN1 are indeed expressed in tumors (Polzin et al., 2004). Furthermore, STMN1 regulates cell cycling, and its expression is associated with specific phases of cell cycling and is negatively regulated by p53 activation and $p21^{waf}$ (Ahn et al., 1999; Johnsen et al., 2000). In addition, STMN1 is a phosphorylation-regulated microtubule destabilizer that functions to modulate microtubule stability (Steinman et al., 2000; Mistry and Atweh, 2002). It is possible that LRRC4 may regulate the STMN1expression and phosphorylation, leading to the inhibition of glioma cell proliferation.

In this study, we first screened the LRRC4-regulated differentially expressing proteins by 2D-DIGE and MALDI-TOF mass spectrometry, and found that LRRC4 down-regulated the STMN1 expression in glioma cells. Subsequently, we characterized that LRRC4 and STMN1 regulated the proliferation and cell cycling of glioma cells, and found that LRRC4 inhibited the U251 cell proliferation and induced glioma cell cycle arrest by down-regulating the STMN1 expression and increasing microtubule polymerization.

MATERIAL AND METHODS

CELLS CULTURE AND TISSUE SAMPLES

Human glioma cell line, U251, was obtained from the Cell Center of Peking Union Medical College in China. U251-LRRC4 cells that had been transfected with LRRC4 and stably expressed LRRC4 were previously established in our laboratory. U251, and U251-LRRC4 cells were maintained in DMEM with 10% fetal calf serum (FCS) and standard antibiotics at 37°C in an atmosphere of 5% CO_2 and 95% air.

A total of 20 primary brain tissue samples were obtained from glioma patients and other non-glioma patients when they were subjected to brain surgery at Xiangya Hospital (Hunan, the People's Republic of China). There were four human non-tumor brain tissue samples and 16 glioma tissue samples, including four of each astrocytoma grade I, II, III, or glioblastoma multiforme (grade IV). Individual tumor tissues were graded by two pathologists in a blinded fashion, according to the revised classification of the World Health Organization (Louis et al., 2007). Informed consent was obtained from individual patients, and experimental protocols were evaluated and approved by the Institute Review Broad of Xiangya Hospital.

TWO DIMENSIONAL DIFFERENTIAL GEL ELECTROPHORESIS (2-D DIGE), IN-GEL DIGESTION, AND PROTEIN IDENTIFICATION

The LRRC4-induced differential expression of proteins in glioma cells was characterized by 2-D DIGE analysis, as described previously (Cilia et al., 2009). Briefly, U251 cells were transfected with vehicle plasmid and used as mock control. The U251-mock and U251-LRRC4 cells were harvested, and the proteins in cell lysates were extracted. Subsequently, these lysate proteins were treated using the ReadyPrep 2D Clean-up kit, according the manufacturers' instruction (Bio-Rad). The lysate proteins were re-suspended in lysis buffer (8 M urea, 4% w/v CHAPS, 30 mM Tris-Cl, pH 8.5), and determined for their protein concentrations using BCA (Pirece, Beijing, China). These proteins were labeled with 400 pmol of fluorescence dye per 100 μg of lysates proteins using the DIGE labeling solution (GE Healthcare). A total of 20 μg of proteins from each group was mixed with the same volume of DIGE 2× buffer (8 M urea, 4% w/v CHAPS, 2% w/v DTT, 2% v/v Pharmalytes 3–10 for IEF), and 20 μg of individual samples were diluted in rehydration solution (8 M urea, 0.5% w/v CHAPS, 0.2% w/v DTT, and 0.2% v/v Pharmalyte pH 3-10) and loaded on IPG strips (18 cm, pH 3–10, non-linear, GE Healthcare) for 2-D gel electrophoresis. Fluorescence images were acquired using the Ettan DIGE imager (GE Healthcare), and the DIGE gels were analyzed using the DIA module of the Decyder software (Version 6.5, GE healthcare).

To prepare gels for capturing the spots of interest, 500 ~ 1,000 μg of proteins were subjected to 2-D DIGE on IPG strips and stained with Coomassie Brilliant Blue. The protein spots of interest were excised and destained with 25 mM ammonium bicarbonate/50% acetonitrile (CAN), followed by in-gel digestion with 0.01 μg/μl trypsin (Promega, USA) in 25 mM ammonium bicarbonate for 15 h at 37°C. The hydrolysates were collected, and the tryptic peptides were extracted from the gel pieces sequentially with 5% TFA at 40°C for 1 h, and with 2.5% TFA, 50% ACN at 30°C for 1 h. The extracts were pooled, lyophilized, and stored at −20°C until use. Gel pieces from a "blank" region and from BSA molecular mass marker were used as negative and positive controls, respectively.

The peptide mixtures were re-dissolved in 0.5% of TFA, and 1 μl of peptide solution was mixed with equal volume of matrix (4-hydroxy-alpha-cyanocinnamic acid, HCCA in 30% ACN/0.1% TFA), followed by spotting on the target plate. Individual protein peptides were identified by MALDI-TOF mass spectrometry on a 4700 proteomics analyzer (Applied Biosystems, Foster City, CA). Mass spectrum was used to interrogate human protein sequences in the SWISS-PROT database using the MASCOT database search algorithms (version 1.9).

IMMUNOBLOT ANALYSIS

Total cell extracts (30–50 μg) were resolved on a 10% SDS polyacrylamide gel electropheresis and transferred to nitrocellulose membranes. After being blocked with 5% fat-free milk, the membranes were incubated with anti-ACAT2 polyclonal antibodies (1:500), anti-ARHGDIA monoclonal antibody (1:500), anti-TPT1 monoclonal antibody (1:500, Abnova, Taiwan), anti-S100A11 polyclonal antibodies (1:500, ProteinTech Group), anti-STMN1 polyclonal antibodies (1:500, Abcam, UK), anti-ERP19 polyclonal antibodies (1:500, Santa Cruz Biotechnology, Santa Curz), anti-HSP27 polyclonal antibodies (1:1,000, Boster, China), or anti-β-actin (1:2,000, Sigma, St Louis), respectively. After washing, the bound antibodies were detected with horseradish peroxidase (HRP)-conjugated optimal secondary antibody (diluted 1:10,000, Santa Cruz Biotechnology) for 1 h at room temperature. Immunoreactive complexes were visualized using ECL reagents (Santa Cruz Biotechnology). The relative levels of target proteins to control β-actin were determined using Quantity One 462 analysis software (BIO-RAD).

Additional immunoblot assays were characterized for the relative levels of p21, ERK, cyclin D1, cyclin B, and GAPDH expression, and pERK and pStath1 phosphorylation in different U251 cells using anti-p21 monoclonal antibody (1:1,000, Sigma), anti-ERK polyclonal antibodies (1:1,000, Abcam), anti-cyclinD1 polyclonal antibodies (1:1,000), anti-cyclin B polyclonal antibodies (1:1,000, Santa Cruz Biotechnology), anti-GAPDH polyclonal antibodies (1:2,000), anti-pERK polyclonal antibodies (1:500, Cell Signaling), and anti-pStath 1 polyclonal antibodies (1:500, Abcam), respectively.

IMMUNOHISTOCHEMISTRY STAINING

The expression of STNM1 and LRRC4 in different human glioma and non-tumor brain tissue sections were characterized by immunohistochemistry (IHC) analysis using anti-STMN1 polyclonal antibodies (1:200, Abcam), anti-LRRC4 monoclonal antibody (1:2,000, Proteintech Group, China), and the standard HRP-streptavidin staining, as described previously (Fan et al., 2006). The expression levels of individual target proteins were analyzed using the GSM-2000P pathology image analysis system (Heima, Zhuhai, China).

REAL-TIME QUANTIFICATION PCR

U251 and U251-LRRC4 cells were cultured in 6-well plates overnight and transfected with 50 nM of control shRNA or STMN1-specific shRNA (Santa Cruz Biotechnology) using Lipofectamine 2000 (Invitrogen, Carlsbad) for 48 h. The relative levels of STMN1 expression were determined by quantitative real-time PCR. Briefly, total RNA was extracted from different groups of cells with the Trizol reagent, according to the manufacturer's instructions (Invitrogen and reversely transcribed into cDNA using the reverse transcription system kit (Promega) in a 20 μl reaction containing 2 μl of 10 × reverse transcription buffer, 4 μl of 10 mM $MgCl_2$, 2 μl of 10 mM dNTP mixture, 0.5 μl of recombinant RNasin® ribonuclease inhibitor, 0.6 μl of AMV reverse transcriptase, 1 μl of oligo(dT)15 primer, 1 μg of total RNA, and nuclease-free water. The relative levels of STMN1 mRNA transcripts to control β-actin were determined by quantitative real-time PCR, using a SYBR-green PCR kit (TaKaRa China) and specific primers on an iCycler IQ multicolor detection system (BioRad, Hercules, CA). The sequences of the primers were forward 5′-CTCGGACTGAGCAGGACTTTC-3′ and reverse 5′- ATTCTTTTGACCGAGGGCTG-3′ for STMN1; forward 5′-TTCCAGCCTTCCTTCCTGGG-3′ and reverse 5′- TTGCGCTCAGGA GGAGCAAT -3′ for human β-actin, respectively. The relative levels of STMN1 mRNA transcripts were normalized to control β-actin and quantified by the $\Delta\Delta C_T$ method.

TUMOR FORMATION ASSAY IN NUDE MICE

Groups of male BALB/c nude mice at eight weeks of age were inoculated subcutaneously with U251-mock or U251-LRRC4 cells ($n = 5$, per group). The formation and growth of human glioma in the recipients were monitored twice per week, and the tumor volumes were estimated by measuring two dimensions of the tumors using a digital caliper in a blinded manner. The mice were sacrificed on day 56 post inoculation. Their tumors were dissected out and weighed. The experimental protocols were approved by the Animal Research Protection Committee of the Xiangya Hospital.

CELL PROLIFERATION ASSAY

The effect of LRRC4 and STMN1 expression on the proliferation of glioma cells was determined by MTT assays. U251-mock and U251-LRRC4 cells (1×10^4 cells/well) were transfected in sextuplet with control siRNA or the STMN1-specific siRNA for 24, 48, and 72 h, respectively. During the last 4-h culture, the cells were exposed to 5 mg/ml of MTT in complete DMEM medium, and the formed formazan in DMSO was measured at an absorbance of 570 mm on an ELX-800 ELISA plate reader (Bio-Tek Instruments, Winooski).

CELL CYCLE ANALYSIS

The effect of LRRC4 and STMN1 expression on the cell cycling was determined by cell cycling assay. Briefly, U251-mock and U251-LRRC4 cells were cultured in 10-cm dishes, and when they reached ~80% confluence, the cells were harvested and fixed in 70% cold ethanol overnight at 4°C. Subsequently, the cells were digested with 1 μg/ml of RNase and stained with 10 μg/ml of propidium iodide (PI, Boehringer Mannheim) for 30 min at room temperature, followed by flow cytometry analysis on a FACScan flow cytometer using CellQuest software (Becton Dickinson, San Jose, CA).

KINASE ACTIVITY ASSAY

The kinase activities of CDK and cdc2 in different cells were determined using the Cell CDK5/P25 colorimetry assay (GENMED, Shanghai, China) and the MESACUP cdc2 Kinasc Assay Kit (code No. 5235, MBL, Japan), according to the manufacturers' instructions.

EXTRACTION OF SOLUBLE AND POLYMERIZED MICROTUBULIN

The soluble and polymerized microtubulins were extracted from different cells, as described previously (Chen et al., 2003). Briefly, U251-mock and U251-LRRC4 cells were harvested and lysed in the microtubule-stabilization buffer (MT-SB, 0.1 M Pipes, pH 6.9, 2 M glycerol, 1.5 mM $MgCl_2$, 2 mM EGTA, 0.5% Triton X-100, and protease inhibitors) containing 4 M Taxol for 20 min on ice to maintain the microtubule stability throughout the isolation. The cell lysates were centrifuged at 20,000*g* for 45 min, and the supernatant containing soluble tubulins were collected. The pellets containing polymerized tubulins were washed with MTSB and then denatured in Laemmli buffer.

CONFOCAL MICROSCOPE ANALYSIS

U251-mock, U251-LRRC4, the control shRNA, or the STMN1 specific shRNA-transfected U251 cells were cultured overnight on glass slides, fixed with 3.7% buffered-formaldehyde, permeabilized with 0.2% Triton-X100, and blocked with 3% BSA. The cells were incubated with rabbit anti-tubulin antibody (1:200, Sigma) in PBS overnight at 4°C, and the bound antibodies were detected with CY3-conjugated anti-rabbit IgG (1:200) at 37°C for 1 h. After washing, the microtubule status was examined under a confocal microscope (Olympus, Japan).

STATISTICAL ANALYSIS

Data are expressed as mean ± SD or representative images from different groups. The difference among different groups was

analyzed by one-way ANONA or Student's *t*-test using the statistical package SPSS 13.0 (SPSS, Inc., Chicago, IL). A *P*-value of <0.05 was considered statistically significant.

RESULTS

IDENTIFICATION AND VALIDATION OF DIFFERENTIALLY EXPRESSED PROTEINS REGULATED BY LRRC4

After the 2-D DIGE, the Cy2, Cy3, and Cy5 channels of individual gels were imaged and analyzed using the DeCyder 5.0 software. As shown in Figure 1A, there were 14 matched protein spots with significant difference in the signal intensity between the control U251-mock and U251-LRRC4 cells, suggesting that those proteins were differentially expressed. Eight out of 14 proteins were significantly up-regulated in the U251-LRRC4 cells as the ratio of the protein in U251-LRRC4 to that in U251-mock was ≥ 2 ($P \leq 0.05$), while six out of 14 proteins were significantly down-regulated as the ratio of the protein in U251-LRRC4 to that in U251-mock was ≤ 0.05 ($P \leq 0.05$). Further MALDI-TOF/TOF analysis of eleven differentially expressed proteins revealed that they were different proteins (Table I), and were functionally involved in metabolism (4), proliferation (4), signal transduction (1), structural protein (1), translation (1), and chaperone (1) (Fig. 1B, analyzed using the Swiss-Prot database annotation).

To verify their differential expression, the U251-mock and U251-LRRC4 cell lysates were analyzed by immunoblot assays using the available antibodies against translationally-controlled 1 (TPT1), Rho GDP dissociation inhibitor alpha (ARHGDIA), heat shock protein 27 (HSP27), S100 calcium binding protein A11(S100A11), chain A, endoplasmic reticulum protein Rp19 O95881 (ERP19), STMN1, and acetyl-coenzyme A acetyltransferase 2 (ACTA2), respectively. As shown in Figure 1C, the relative levels of TPT1, ARHGDIA, and HSP27 expression to control β-actin were up-regulated in U251-LRRC4 cells, as compared with that in U251-mock cells. In contrast, the relative levels of S100A11, Chain A, ERP19, STMN1, and ACTA2 expression to control β-actin were down-regulated in U251-LRRC4 cells, as compared with that in U251-mock cells, particularly for the STMN1 expression. These two independent lines of evidence demonstrated that the expression of these proteins was regulated by LRRC4 in glioma cells.

STMN1 is a critical regulator of the cell proliferation and cycling, and is associated with the tumorigenesis of glioma. Next, we investigated the expression pattern of STMN1 and LRRC4 in different grades of human glioma tissues by IHC. Interestingly, while very low levels of STMN1 expression was detected in human non-tumor brain tissues, the levels of STMN1 expression were significantly up-regulated in glioma tissues and their expression levels appeared to be associated with the pathogenic degrees of human glioma (Fig. 1D and E). In contrast, high levels of LRRC4 expression were detected in human non-tumor brain tissues and the levels of LRRC4 expression in glioma tissues were significantly reduced, which appeared to be negatively correlated with the pathogenic degrees of glioma in these patients. The negative association in the levels of LRRC4 with STMN1 suggests that they have opposite roles in the tumorigenesis of human glioma.

LRRC4 INHIBITS THE EXPRESSION OF STMN1 AND THE GROWTH OF GLIOMA CELLS IN VIVO

To test the effect of LRRC4 on the expression of STMN1, U251-mock and U251-LRRC4 cells were transfected with control siRNA or the STMN1-specific siRNA, respectively, and the relative levels of STMN1 expression were characterized by quantitative RT-PCR (Fig. 2B). The levels of STMN1 mRNA transcripts in U251-LRRC4 cells were significantly reduced, as compared with that in U251-mock cells. Furthermore, the levels of STMN1 mRNA transcripts in the U251-mock or U251 LRRC4 cells that had been transfected with the STMN1-specific siRNA, but not with the control siRNA, were dramatically reduced ($P < 0.01$). Next, we examined the effect of STMN1 knockdown on the proliferation of glioma cells by MTT assays (Fig. 2A). Knockdown of STMN1 expression significantly inhibited the proliferation of U251-mock and U251-LRRC4 cells in vitro. Similarly, the reduced STMN1 expression inhibited the growth of inoculated glioma tumors in vivo, accompanied by significantly reduced tumor weights (Fig. 2C and D). Further characterization of STMN1 expression in the inoculated tumors demonstrated significantly lower levels of STMN1 expression in U251-LRRC4 tumors (Fig. 2E). However, we did not find that LRRC4 directly interacted with STMN1 by co-immunoprecipitation, immunostaining, and confocal microscopy analysis (data not shown). Apparently, LRRC4 inhibited the expression of STMN1, which contributed to the inhibition of human glioma cell proliferation.

THE IMPACT OF LRRC4 AND STMN1 ON GLIOMA CELL CYCLING

We next examined whether tumorigenicity inhibition of LRRC4 could be mediated by STMN1-induced cell cycle arrest in glioma cells. To determine the impact of STMN1 on the cell cycling, U251-mock and U251 LRRC4 cells were transfected with, or without, the STMN1-specific shRNA and control shRNA (sh Con) and their cell cycling was examined for measuring the DNA content by flow cytometry analysis. As shown in Figure 3A, the frequency of G0/G1 phase of U251-LRRC4 cells were significantly higher than that U251-mock, indicating that induction of LRRC4 expression induced U251 cell cycle arrest at G0/G1 phase (Fig. 3A). Furthermore, transfection with control, siRNA did not alter the cell cycling in U251-mock transfection with the STMN1-specific siRNA, reduced the frequency of G0/G1 phrase, but increased the percentage of S phase in U251-mock cells, suggesting that knockdown of STMN1 expression induced U251 cell cycle arrest at S phase. However, knockdown of STMN1 expression in U251-LRRC4 cells did not alter the cell cycling as compared with that of U251-LRRC4 cells. Evidentially, the frequency of each phase of the STMN1-specific shRNA-transfected U251-LRRC4 was similar to that of U251-LRRC4 cells, and those cells remained cell cycle arrest at G0/G1 phrase.

To understand the mechanisms underlying the impact of LRRC4 over-expression and STMN1-silencing on glioma cell cycling, we characterized the expression and phosphorylation of cell cycle-related molecules by Western blot assays. We found that the levels of p21 expression were up-regulated, while the levels of cyclin D1 and cyclin B expression and ERK and STMN1 phsphorylation were down-regulated, accompanied by significantly reduced levels of the CDK5 and cdc2 kinase activities in U251-LRRC4 cells, as compared with that in U251-mock cells (Fig. 3B, C). These data clearly

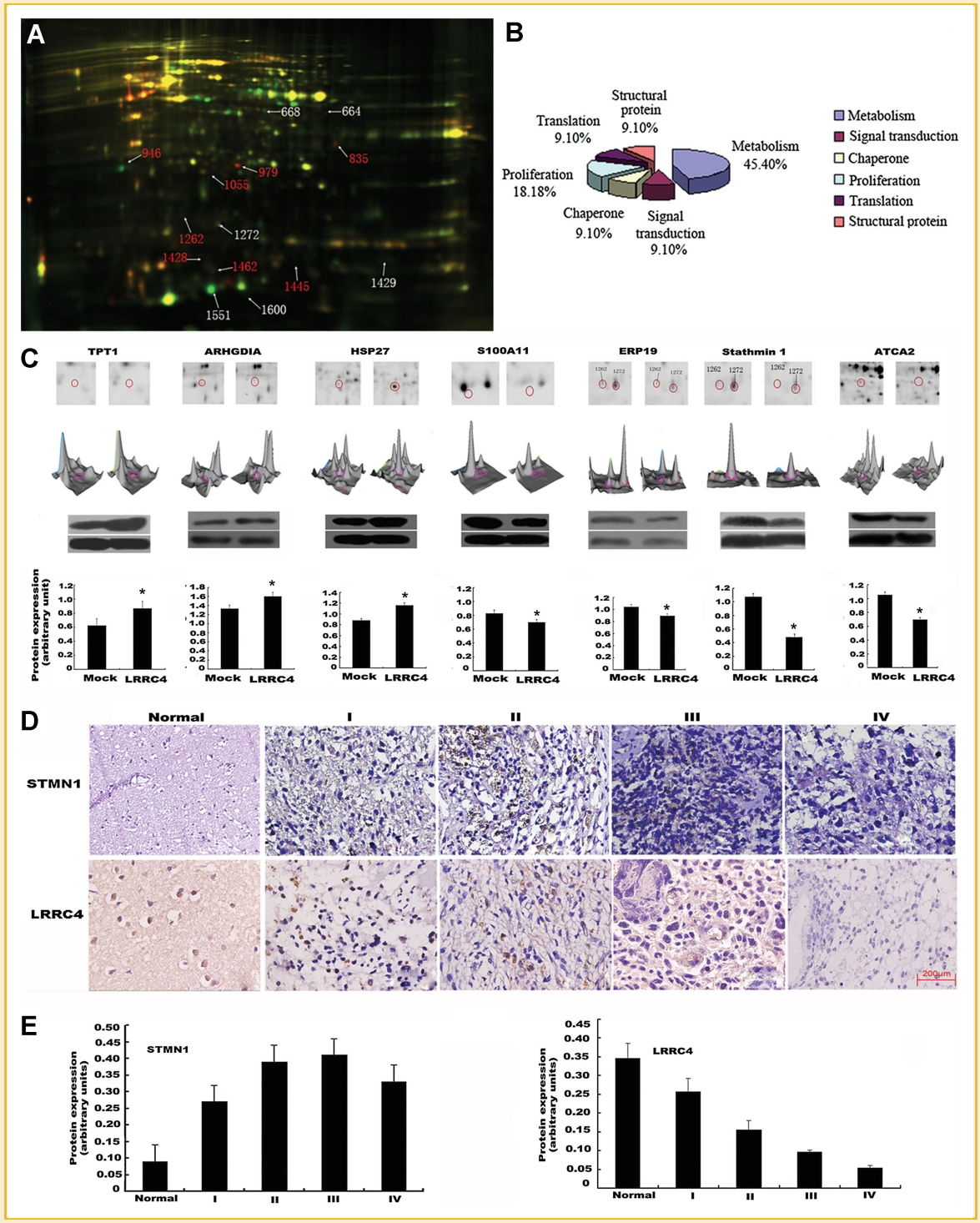

Fig. 1. Characterization of the differentially expressing proteins regulated by LRRC4 in glioma cells. A: The 2D-DIGE analysis of the differentially expressing proteins regulated by LRRC4 in glioma cells. U251-mock, U251-LRRC4 cell lysates, and internal standard proteins were treated with the ReadyPrep 2D reagents and labeled with Cy3, Cy5, and Cy2, respectively. Subsequently, these proteins were subjected to 2-D electrophoresis. Data shown are the representative images from three separate experiments. The red spots represent the up-regulated proteins, and the green spots are the down-regulated proteins, while the blue spots are internal standard proteins. B: Functional analysis of the LRRC4-regulating differentially expressing proteins. All of the differentially expressing proteins were analyzed using the Swiss-Prot database annotation. Data shown are percentages of proteins in each category. C: Analysis of the seven differentially expressing proteins. The top row of images: DIGE gel maps of the differentially expressing protein spots corresponding to TPT1, ARHDD2A, HSP27, S100A11, ERP19, STMN1, and ATCA2, respectively. The second row of images: Three-dimensional peak maps of the differentially expressing protein spots. The third row of images: Western blotting validations of the differentially expressing proteins, including TPT1, ARHDD2A, HSP27, S100A11, ERP19, STMN1, and ATCA2 (up panel) and control β-actin (down panel). The fourth row of panels: Quantitative analysis of these differentially expressing proteins from three separate experiments. $^{*}P < 0.05$, determined by Students' t test. Mock: U251 cells transfected with control plasmid; LRRC4: U251-LRRC4 cells. D: Immunohistochemistry analysis of STMN1 (upper) and LRRC4 (down) expression in different grades of glioma tissue samples (brown color for positive cells). E: Quantitative analysis of STMN1 and LRRC4 expression in glioma tissue samples using the GSM-2000P pathology image analysis system (Heima Zhuhai, China). Data are expressed as mean ± SD of the gray values for each protein tested in different grades of human glioma tissue samples. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

TABLE I. Differentially Expressed Proteins Identified by MALDI TOF/TOF

Spot no.	Accession no.	Protein name	Function
Up-regulated protein			
835	gi|31645	glyceraldehyde-3-phosphate dehydrogenase	Metabolism
946	gi|4757768	Rho GDP dissociation inhibitor (ARHGDIA) alpha	Signal transduction
979	gi|662841	heat shock protein 27	Chaperone
1445	gi|4507669	translationally-controlled 1	Translation
1462	gi|24987402	chain A, solution structure of recombinant human brain-type fatty acid binding protein	Metabolism
Down-regulated protein			
664	gi|17834080	haymaker protein	Structural protein
668	gi|148539872	acetyl-Coenzyme A acetyltransferase 2	Metabolism
1262	gi|51247348	chain A, endoplasmic reticulum protein Rp19 O95881	Metabolism
1272	gi|122890670	stathmin 1	Proliferation
1429	gi|5542165	chain A, human platelet profilin complexed with an L-Pro10-iodotyrosine peptide	Metabolism
1600	gi|5032057	S100 calcium binding protein A11	Proliferation

demonstrated that induction of LRRC4 over-expression enhanced the p21 expression, but inhibited the cyclinD1 expression, and ERK and STMN1 phosphorylation as well as CDK5 and cdc2 kinase activities in glioma cells, which contributed to cell cycle arrest at G0/G1 phase in glioma cells. Interestingly, knockdown of STMN1 expression also inhibited the expression of cyclinD1 and cyclinB, but increased the expression of p21 in glioma cells. Apparently, LRRC4 over-expression and STMN1-silencing induce cell cycle arrest by different mechanisms in glioma cells in vitro.

LRRC4 OVER-EXPRESSION OR STMN1 SILENCING ENHANCES THE MICROTUBULE POLYMERIZATION IN GLIOMA CELLS.

STMN1 is a critical regulator of the microtubule polymerization (Lin et al., 2009). Finally, we analyzed the effect of LRRC4 over-expression on the levels of polymerized tubulin by Western blot and immunofluorescence assays (Fig. 4). In comparison with that in 251-mock cells, the levels of polymerized tubulin markedly increased, while the levels of soluble tubulin decreased in the U251-LRRC4

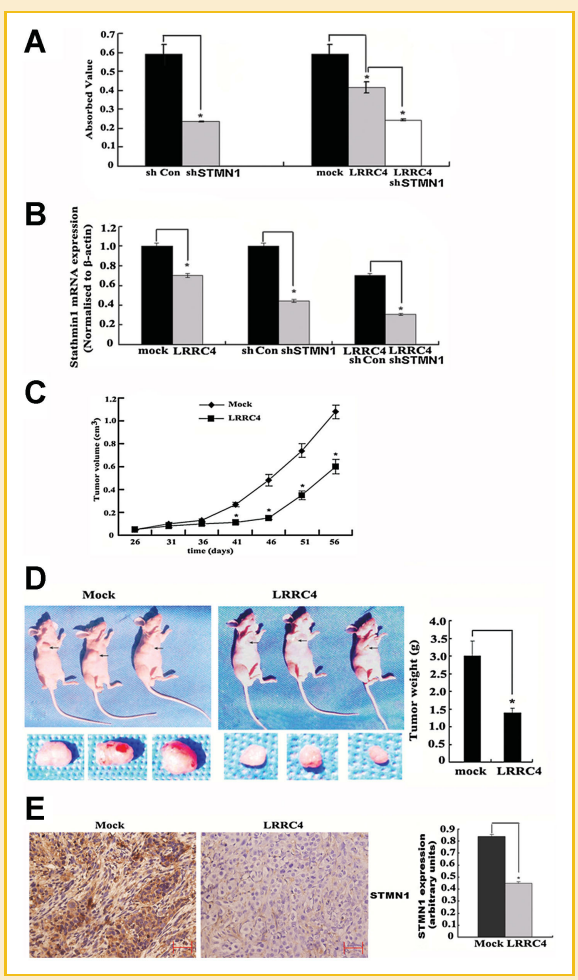

Fig. 2. LRRC4 inhibits the STMN1 expression and glioma cell proliferation in vitro and glioma growth in vivo. A: Analysis of the relative levels of STMN1 mRNA transcripts. U251-mock and U251-LRRC4 cells were transfected with, or without, control or the STMN1-psecific shRNA, respectively, and the relative levels of STMN1 mRNA transcripts to control β-actin were determined by quantitative RT-PCR. Data are expressed as mean ± SD of each group of cells from three independent experiments. Sh Con: U251-mock cells transfected with control shRNA; shSTMN1: U251-mock cells transfected with the STMN1 shRNA; Mock: U251-mock cells; LRRC4: U251-LRRC4 cells; RRC4/shSTMN1: U251-LRRC4 cells transfected with the STMN1-specific shRNA. $^{*}P < 0.05$, determined by Students' *t* test. B: Proliferation of glioma cells. The impact of LRRC4 and STMN1 expression on the proliferation of U251 cells was determined by MTT assay. Data are expressed as mean ± SD of each group of cells from three independent experiments. $^{*}P < 0.05$, determined by Students' *t* test. C: Effect of LRRC4 expression on the growth of implanted glioma cells in vivo. Groups of BALB/c mice were subcutaneously inoculated with U251-mock or U251-LRRC4 cells, respectively (n = 5 per group). The growth of glioma was monitored as the indicated time points and the volumes of tumors were estimated by the formula of $4/3\pi ab^2$, where "a" is the length of the tumor and "b" is the width of the tumor. Data are expressed as mean ± SD of each group of tumors. $^{*}P < 0.05$ versus the volume at the corresponding time point. D: Quantitative measurement of tumor weights. At the end of the experiment, the mice were sacrificed, and individual tumors were dissected out and weighed. Data are representative images of mice and tumors or expressed as mean ± SD of each group of tumors. $^{*}P < 0.05$ versus the U251-mock tumor group. E: Immunochemistry analysis of STMN1 expression in tumor tissues. The collected tumor tissues were sectioned at 5 μM and stained with anti-STMN1 (brown color), followed by quantitative analysis of the STMN1 expression. Data shown are representative images from the U251-mock tumors and U251-LRRC4 tumors, respectively and expressed as mean ± SD of the values from each group of five mice. $^{*}P < 0.05$ versus the U251-mock control tumors. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

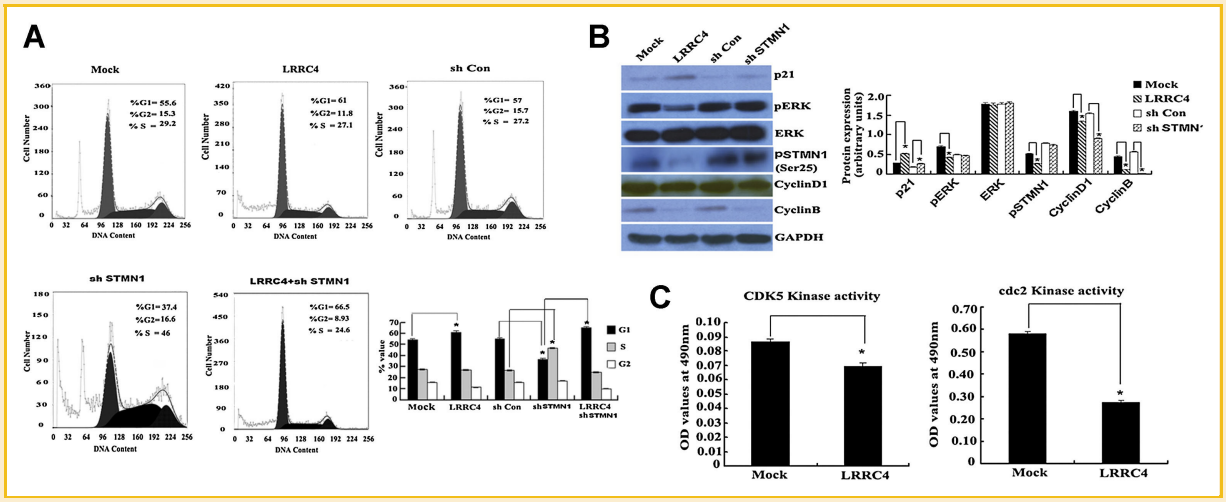

Fig. 3. Induction of LRRC4 or knockdown of STMN1 expression induces glioma cell cycle arrest in vitro. A: Flow cytometry analysis of cell cycling. U251-mock and U251-LRRC4 cells were transfected with, or without, control shRNA or the STMN1-specific shRNA for 48 h, respectively and their cell cycle distributions were analyzed by flow cytometry analysis. Data shown are representative histograms from each group of cells and are expressed as mean ± SD of each phase of cells from three separate experiments. Mock: U251-mock cells; LRRC4: U251-LRRC4 cells; sh Con: U251-mock cells transfected with control shRNA; shSTMN1: U251-mock cells transfected with the STMN1-specific shRNA; LRRC4 + shSTMN1: U251-LRRC4 cells transfected with the STMN1-specific shRNA. $^{*}P < 0.05$. B: Western blot analysis of the expression and phosphorylation of cell cycle-related molecules. Data shown are representative images of different proteins (left) and quantitative analysis by densimetric scanning (right). $^{*}P < 0.05$ versus controls. C: LRRC4 down-regulates the CDK5 and cdc2 kinase activity. The activities of CDK5 and cdc2 kinases in the cell lysates were determined by enzymatic assays, and data are expressed as mean ± SD of each group of cells from three separate experiments. $^{*}P < 0.05$ versus control U251-mock cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

cells (Fig. 4A). In parallel, the intensity of anti-tubulin staining in the U251-LRRC4 cells and the STMN1-silencing U251-mock cells dramatically increased, as compared with that in the control U251-mock cells or the control shRNA-transfected U251-mock cells (Fig. 4B). Therefore, these two independent lines of data demonstrated that induction of LRRC4 over-expression or knockdown of STMN1 expression increased the microtubule polymerization in glioma cells in vitro.

DISCUSSION

LRRC4 has been identified as a tumor suppressor of glioma (Wu et al., 2006a), and loss of LRRC4 expression is a common event associated with brain tumors, especially in glioma (Wang et al., 2002). To explore the mechanisms by which LRRC4 regulates the pathogenic process of glioma, we analyzed the proteomic profiles of U251-mock and U251-LRRC4 cells using the 2D-DIGE method. We found that 14 proteins were differentially expressed between U251-mock and U251-LRRC4 cells, and eight out of 14 proteins were significantly up-regulated, while six out of 14 proteins were significantly down-regulated in U251-LRRC4 cells. Furthermore, 11 out of them were identified by MADLE-TOF-MS/MS technique, and they were functionally involved in metabolism, proliferation, signal transduction, structural protein, translation, and chaperone. Further validation by Western blot assays revealed that the expression of TPT1, ARHGDIA, and HSP27 was up-regulated, while S100A11, Chain A, ERP19, STMN1, and ACTA2 were down-regualted in U251-LRRC4 cells. These data suggest that LRRC4 regulates the expression of those proteins in glioma cells in vitro. Given that LRRC4 acts as a negative regulator of the development and progression of glioma, it is possible that LRRC4, through regulating the expression of these proteins, inhibits the growth of glioma cells in vivo.

STMN1 is an important regulator of the maintenance of cell biological behaviors through integrating and relying on different intracellular and extracellular signals (Sellin et al., 2008; Holmfeldt et al., 2009). STMN1 can regulate the microtubule dynamics by interacting with tubulin and directly participates in controlling the cell-cycle progression (Budhachandra et al., 2008; Lin et al., 2009). In this study, we found that high levels of STMN1 were expressed in glioma tissues, determined by immunochemistry staining (Fig. 1D). Furthermore, induction of LRRC4 over-expression inhibited the expression of STMN1 in glioma cells in vitro and in vivo, which were associated with significant inhibition of glioma cell proliferation in vitro and growth in vivo. Interestingly, knockdown of STMN1 expression also significantly inhibited the proliferation of glioma cells in vitro. However, we did not find that LRRC4 directly interacted with STMN1 protein in vitro, determined by co-immunoprecipitation and confocal microscopy (data not shown). Given that STMN1 is an important regulator of the proliferation and cell cycling of many types of cells, it is possible that LRRC4 inhibited the expression of STMN1, through some unknown mechanisms, leading to inhibition of glioma cell proliferation. We are interested in further investigating the mechanisms by which LRRC4 regulates the expression of STMN1 in glioma cells.

Further analysis revealed that induction of LRRC4 over-expression induced cell cycle arrest at G0/G1 phase in U251 cells, and knockdown of STMN1 expression in the LRRC4-over-expressing glioma cells did not alter the frequency of different phases of cells, while knockdown of STMN1 expression induced cell cycle

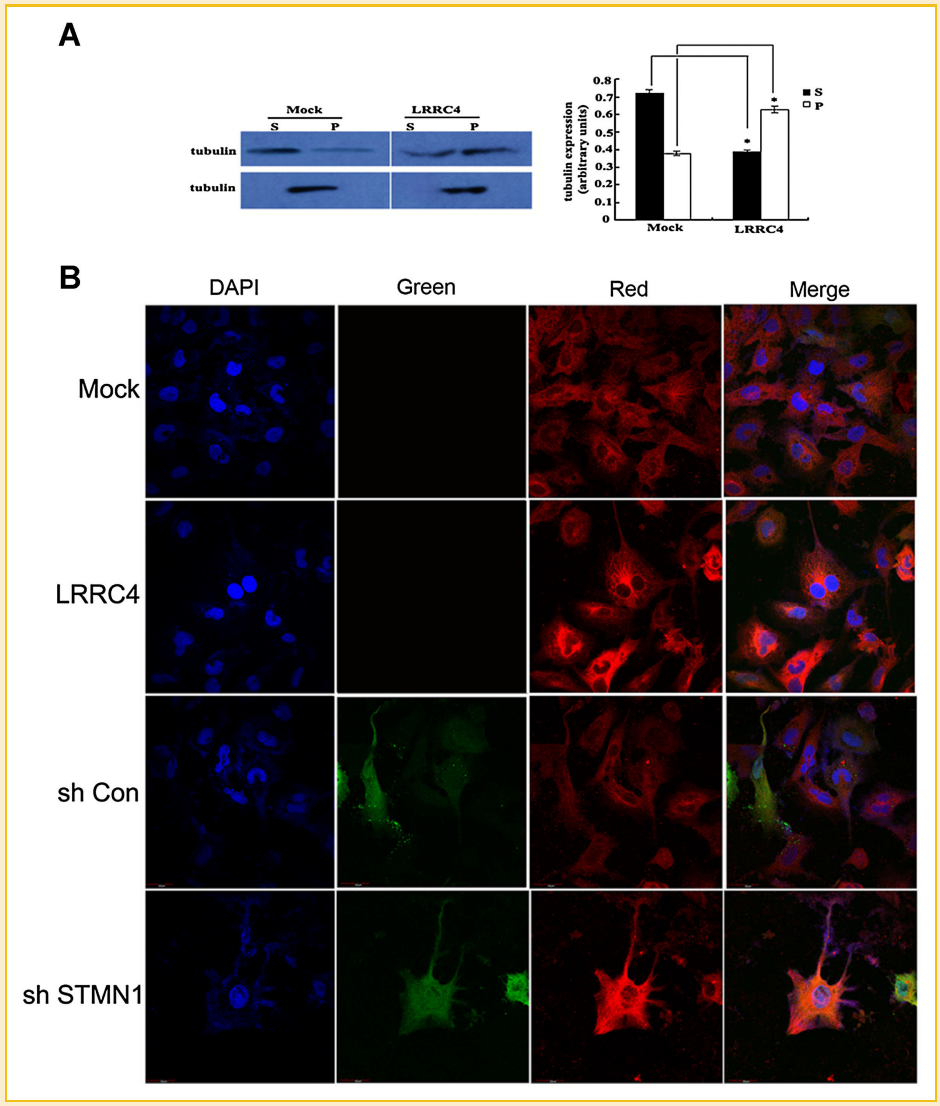

Fig. 4. Induction of LRRC4 or knockdown of STMN1 expression increases the polymerization of microtubelin in U251 cells. A: The levels of soluble and polymerized microtubuline in glioma cells were determined by Western blot assays (left) and quantitatively analyzed by densimetric scanning (right). Data shown are representative images and are expressed as mean ± SD of each group of cells from three independent experiments. $^{*}P < 0.05$ versus control U251-mock cells. B: Immunofluorescent confocal microscope analysis of the microtubulin. U251-mock cells were transfected with control shRNA or the STMN1-specific shRNA, respectively. The levels of tubolin in U251-mock, U251-LRRC4, the control shRNA-transfected U251-mock, and the STMN1-shRNA-transfected U251-mock cells were characterized by staining with Cy3-antibody (red) and costaining with DAPI for the nuclei. Data shown are representative images from each group of cells. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

arrest at S phase in U251 cells. One possible reason is that LRRC4 over-expression up-regulated p21 expression and that knockdown of STMN-1 severely inhibited the cyclinD1 expression. Further analysis of the cell cycling-related molecules revealed that induction of LRRC4 over-expression increased the p21 expression, but decreased the cyclinD1 and cycling B expression, and the ERK and STMN1 phosphorylation, accompanied by decreasing the CDK5 and cdc2 kinase activities in glioma cells, consistent with previous reports (Zhang et al., 2005b; Wu et al., 2006a). The p21 is a universal cell cycle inhibitor, and can directly bind with CDK-Cyclin complex, such as CyclinD-CDK4/5, CyclinE-CDK2, CyclinA-CDK2, and others, inhibiting CDK activation and the G1/S transition (Romanov et al., 2005; Schmider-Ross et al., 2006; Srivastava et al., 2007). A previous study has shown that STMN1 is predominantly phosphorylated by CDK5 at Ser (Hayashi et al., 2006; Lin et al., 2009) and can also be phosphorylated by the cdc2, a crucial kinase that regulates the cell-cycle process (Lin et al., 2009). Therefore, it is possible that LRRC4 expression down-regulated STMN1 expression and phosphorylation, and in turn up-regulated the expression of p21 and down-regulated the cyclin D1 and cyclin B expression, and CDK5 and cdc2 kinase activities, contributing to the LRRC4-mediated cell cycle arrest in glioma cells.

STMN1 has both tubulin sequestering and microtubule depolymerizing activities (Lin et al., 2009). We found that induction of LRRC4 over-expression down-regulated the STMN1 expression and phosphorylation in glioma cells in vitro, which further increased the

microtubule polymerization. Evidentially, significantly higher levels of polymerized microtubulin were detected in U251-LRRC4 cells, as compared with that in U251-mock cells (Fig. 4). Notably, STMN1 can adjust microtubule dynamics by interacting with tubulin, regulating the cell-cycle progression (Lin et al., 2009). Indeed, we found that induction of LRRC4 expression or knockdown of STMN1 expression up-regulated the p21 expression, but inhibited the cyclin D1 and cyclin B expression, leading to glioma cell cycle arrest in vitro. Apparently, LRRC4, at least partially by down-regulating the expression of STMN1, acts as a major glioma suppressor, induces cell cycle arrest, and modulates the dynamic process of microtubule polymerization, leading to the inhibition of glioma cell proliferation and growth.

In summary, our data indicated that the expression of LRRC4 was significantly down-regulated in glioma, and induction of LRRC4 over-expression in glioma cells inhibited the expression of several genes, including the STMN1. Induction of LRRC4 over-expression in U251 cells inhibited the proliferation of glioma cells in vitro and the growth of glioma tumors in vivo. Induction of LRRC4 over-expression or knockdown of STMN1 expression induced cell cycle arrest, which was associated with modulating the expression and phosphorylation of cell cycling regulators, and increased the polymerization of microtubulin in glioma cells in vitro. Apparently, therapeutic induction of LRRC4 or down-regulation of STMN1 expression may be new strategies for the intervention of glioma.

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