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Overexpression of the Long Non-coding RNA MEG3 Impairs In vitro Glioma Cell Proliferation

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ABSTRACT

Gliomas are the most common type of primary brain tumor in the central nervous system of adults. Maternally Expressed Gene 3 (MEG3) is an imprinted gene located at 14q32 that encodes a non-coding RNA (ncRNA) associated with tumorigenesis. However, little is known about whether and how MEG3 regulates glioma development. In the present study we assayed the expression of MEG3 in glioma tissue samples by real-time polymerase chain reaction assay, and defined the biological functions and target genes by CCK-8 assay, flow cytometry, and RNA immunoprecipitation. We first demonstrated that MEG3 expression was markedly decreased in glioma tissues compared with adjacent normal tissues. Moreover, ectopic expression of MEG3 inhibited cell proliferation and promoted cell apoptosis in U251 and U87 MG human glioma cell lines. We further verified that MEG3 was associated with p53 and that this association was required for p53 activation. These data suggest an important role of MEG3 in the molecular etiology of glioma and implicate the potential application of MEG3 in glioma therapy. J. Cell. Biochem. 113: 1868–1874, 2012.

KEY WORDS: MEG3; p53; GLIOMAS; TUMORIGENESIS

Gliomas are the most common type of primary brain tumor in the central nervous system of adults [Sathornsumetee and Rich, 2006]. Despite progress in brain tumor therapy, the prognosis for patients with malignant glioma remains grim. The median survival of patients with glioblastoma multiforme, the most common grade of malignant glioma, is 10–12 months [Ehtesham et al., 2005]. Conventional therapy of surgery, radiation, and chemotherapy is largely palliative. The advances in suitable therapy for the purpose of increasing survival rate have been limited because the pathophysiological mechanisms causing this are not known. Therefore, revealing the molecular mechanism for the glioma development is indispensable for developing effective therapy.

Recently, large-scale complementary DNA cloning projects have identified that majority of the mammalian genome is transcribed, although only minority of these transcripts represent protein-coding genes [Kawaji et al., 2011]. The function of these transcripts remains obscure and their relevance to disease is undefined [Braconi et al., 2011]. The involvement of non-coding genes in tumor pathogenesis and growth is less well characterized. Long non-coding RNAs (LncRNAs) have been implicated as having a functional role in carcinogenesis or cancer growth, such as HOTAIR, MALAT-1, H19, and Maternally Expressed Gene 3 (MEG3) [Matouk et al., 2010; Zhang et al., 2010; Benetatos et al., 2011; Braconi et al., 2011; Kogo et al., 2011; Lai et al., 2011]. Kogo et al. [2011] demonstrated that HOTAIR expression levels were higher in cancerous tissues than corresponding noncancerous tissues and high HOTAIR expression tightly correlated with the presence of liver metastasis. Moreover, patients with high HOTAIR expression had a relatively poorer prognosis. They further revealed a close correlation between expression of HOTAIR and members of the PRC2 complex (SUZ12, EZH2, and H3K27me3).

MEG3 is an imprinted gene with maternal expression which encodes a ncRNA [Zhang et al., 2010]. MEG3 plays a role in cell proliferation control by interacting with cyclic AMP, p53, murine double minute 2 (MDM2), and growth differentiation factor 15 (GDF15) [Benetatos et al., 2011]. MEG3 expression is under epigenetic control, and aberrant CpG methylation has been observed in several types of cancer. MEG3 represents a tumor suppressor gene located in chromosome 14q32 and its association with tumorigenesis is growing every day [Benetatos et al., 2011]. Zhang et al. found that MEG3 RNA expression was lost in the majority of clinically non-functioning human pituitary tumors and other cancer cell lines,

Abbreviations: lncRNA, long non-coding RNA; MEG3, Maternally Expressed Gene 3; RIP, RNA immunoprecipitation.

Conflicts of interest: None.

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and it suppressed cancer cell growth, stimulated p53-mediated transcriptional activation, and selectively activated p53 target genes [Zhang et al., 2003, 2010; Zhou et al., 2007]. MEG3 is highly expressed in the normal human brain [Zhang et al., 2003]. However, little is known about whether MEG3 expression levels are abnormal, and whether and how MEG3 regulates glioma development.

In the study, we demonstrated that MEG3 expression was markedly decreased in glioma tissues compared with adjacent normal tissues. Moreover, ectopic expression of MEG3 inhibited cell proliferation and promoted cell apoptosis in U251 and U87 MG human glioma cell lines. We further verified that MEG3 was associated with p53 and this association was required for the regulation of p53 activation.

MATERIALS AND METHODS

TUMOR TISSUES AND CELL LINES

Human glioma U251 and U87 MG cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in DMEM medium containing 10% FBS (GIBCO, Carlsbad, CA) and cultured at 37°C with 5% CO_2.

Seventeen specimens of glioma tissue and their adjacent benign tissues were collected at The Fourth Affiliated Hospital of Harbin Medical University (Table I). Written informed consent for the biological studies was obtained from the patients involved in the study or from their parents/guardians. The study was approved by the Ethics Committee of the Harbin Medical University. The histopathologic diagnoses were determined using WHO criteria and evaluated by the hospital's pathologist using both morphologic criteria and immunocytochemistry, including primary grade pilocytic astrocytomas (WHO I), grade II astrocytoma (WHO II) and grade III anaplastic astrocytomas (WHO III).

REAL-TIME POLYMERASE CHAIN (PCR)

Total RNA was extracted from 17 pairs of glioma tissues by using Trizol reagent (Invitrogen, Carlsbad, CA), and the reverse-transcription reactions were performed using MEG3-specific primers and an M-MLV Reverse Transcriptase kit (Invitrogen). Real-time PCR was performed using a standard SYBR Green PCR kit (Toyobo, Osaka, Japan) and a Rotor-Gene RG-3000A (Corbett Life Science, New South Wales, Australia) according to the instructions from the respective manufacturer. β-actin was used as references for lncRNAs. Each sample was analyzed in triplicate. Bars represent the ratio between expression in adjacent normal tissues and glioma tissues (log scale).

CELL PROLIFERATION ASSAY

Cell proliferation assays were performed with a Cell Counting Kit-8 (Dojindo, Japan) [Qin et al., 2010]. U251 or U87 MG cells were plated in 24-well plates in triplicate at about 5×10^4 cells per well. Then cells were treated with pcDNA-MEG3 and the numbers of cells per well were measured by the absorbance (450 nm) of reduced WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-isulfo-phen-yl)-2H-tetrazolium, monosodium salt) at the indicated time points. Plasmid pcDNA-MEG3 was constructed by introducing a *BamHI-EcoRI* fragment containing the MEG3 cDNA into the same sites in pcDNA3.1.

FLOW CYTOMETRIC ANALYSIS

Glioma cells transfected with MEG3 (4×10^5) were plated in 6-well plates and apoptosis inducers A (Apopisa)and B (Apobid) (1:1,000, Beyotime, China) were added to the culture. After 48-h incubation, the cultures were stained with annexin V-fluorescein isothiocyanate and apoptosis rates were analyzed using a flow cytometer (FACSCalibur, BD Biosciences). Flow cytometry was performed, and percentages of cells in G0-G1, S, and G2 phase were determined using CellQuest (BD Biosciences).

RNA IMMUNOPRECIPITATION

RNA immunoprecipitation (RIP) was performed using the Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) according to the manufacturer's instructions. The p53 antibody used for RIP is ab2433 (Abcam, Cambridge, MA). The coprecipitated RNAs were detected by reverse transcription PCR. The prime for

TABLE I. Characteristics of Patient Tissues Used in This Study

Sample designation	Age	Sex	Histology type	WHO Grade
1	22	M	Arachnoid Cyst	0
2	47	M	Arachnoid Cyst	0
3	26	M	Pilocytic Astrocytomas	I
4	33	M	Pilocytic Astrocytomas	I
5	45	F	Pilocytic Astrocytomas	I
6	19	M	Pilocytic Astrocytomas	I
7	16	M	Pilocytic Astrocytomas	I
8	24	F	Diffuse Astrocytomas	II
9	20	M	Diffuse Astrocytomas	II
10	30	F	Diffuse Astrocytomas	I
11	66	F	Diffuse Astrocytomas	II
12	36	M	Diffuse Astrocytomas	II
13	41	F	Diffuse Astrocytomas	II
14	20	F	Diffuse Astrocytomas	II
15	20	M	Anaplastic Astrocytoma	III
16	35	M	Anaplastic Astrocytoma	III
17	51	F	Anaplastic Astrocytoma	III

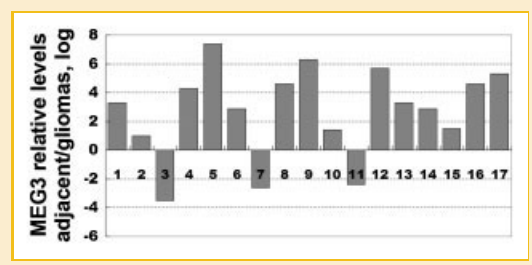

Fig. 1. Deregulated expression of the long non-coding RNA MEG3 in gliomas. MEG3 expression was assessed by real time PCR using SYBR Green in human glioma tissues and adjacent normal tissues. RNA was extracted with Trizol from human glioma and adjacent tissues. MEG3 expression was assessed by real time PCR and normalized to β-actin. Bars represent the ratio between expression in adjacent normal and in glioma tissues (log scale).

detecting MEG3 as follow: Sense, CTGCCCATCTACACCTCACG; Antisense, CTCTCCGCCGTCTGCGCTAGGGGCT. Total RNAs (tRNA) and controls were also assayed to demonstrate that the detected signals were from RNAs specifically binding to p53.

LUCIFERASE REPORTER ASSAYS

For analysis of luciferase reporters, cells were plated in 6-well plates, and transfected MEG3 and p53-Luc using the Lipofectamine 2000. Luciferase activity was determined using the Luciferase assay

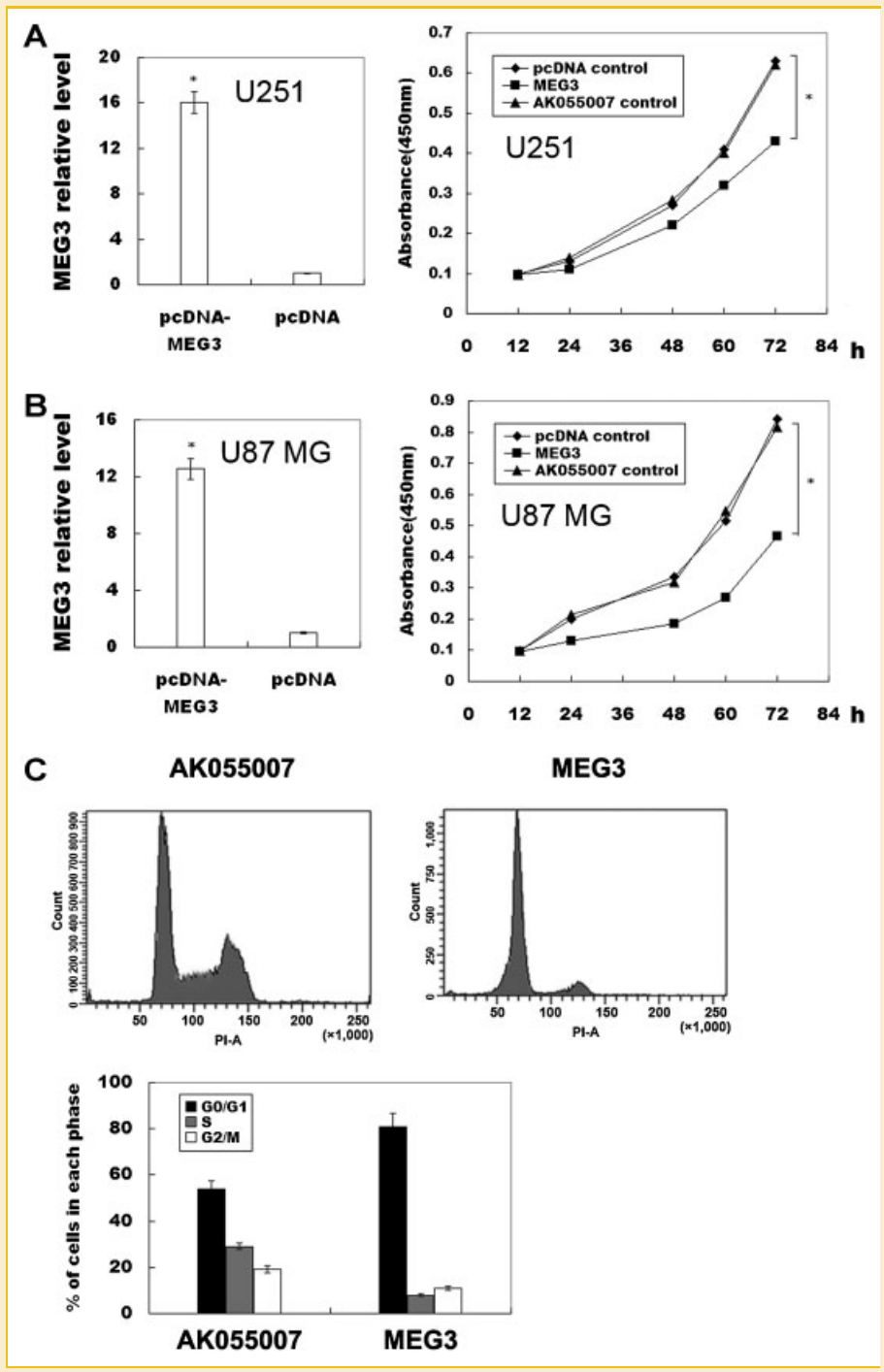

Fig. 2. MEG3 inhibited glioma cell proliferation. A: U251 cells were transfected with MEG3, and at the indicated time points, the numbers of cells per well were measured by the absorbance (450 nm) of reduced WST-8. B: U87 MG cells were transfected with MEG3, and at the indicated time points, the numbers of cells per well were measured by the absorbance (450 nm) of reduced WST-8. The results show data from at least three independent experiments, expressed as the mean ± SD. $^{*}P < 0.05$. C: U251 cells were overexpressed with MEG3, and cell cycle profiles were determined by FACS analysis after 48 h. The data are from one of three independent experiments. LncRNA AK055007 was an lncRNA control.

system (Promega) according to the manufacturer's instructions and normalized for pRL-TK (Promega).

STATISTICAL ANALYSIS

All data are expressed as mean ± standard deviation (SD) from at least three separate experiments. The differences between groups were analyzed using Student's *t*-test. The difference was deemed statistically significant at $P < 0.05$.

RESULTS

DEREGULATED EXPRESSION OF THE LONG NON-CODING RNA MEG3 IN GLIOMAS

We firstly examined MEG3 expression levels in glioma tissue samples and adjacent normal tissues using quantitative real-time PCR. Figure 1 showed that the MEG3 levels were downregulated in most gliomas tissues compared with normal tissues. MEG3 was remarkably reduced in expression in 82% of gliomas in comparison with adjacent normal tissue (Fig. 1). These data indicate that abnormal MEG3 expression may be related to glioma development.

MEG3 INHIBITED GLIOMA CELL PROLIFERATION AND PROMOTED CELL APOPTOSIS

To study the role of MEG3 in cells growth, the glioma cell lines treated with MEG3 were analyzed. The MEG3 levels were significantly increased in U251 cells transfected with MEG3, and upregulated MEG3 significantly inhibited U251 cells growth compared with control (Fig. 2A). Similarly, upregulated MEG3 also significantly suppressed U87 MG cells proliferation (Fig. 2B). MEG3 overexpression resulted in an obvious accumulation of U251 cells in G0/G1 phase (Fig. 2C). Meanwhile, Figure 3A showed that overexpression of MEG3 promoted U251 cells apoptosis. Overexpression of MEG3 also increased cell apoptosis in U87 MG cells (Fig. 3B). These data suggest that MEG3 negatively regulates glioma cells growth.

ASSOCIATION OF MEG3 AND p53

To understand the molecular mechanism by which MEG3 suppresses glioma cell growth, we examined whether MEG3 can affect p53 functions because the tumor suppressor p53 potently inhibits cell growth by inducing block of proliferation or by activating cell death programs [Kim et al., 2010]. Recent studies demonstrated that lncRNAs have been shown to physically associate with p53, suggesting that lncRNAs may play an important role in regulating cell growth by p53 pathway [Huarte et al., 2010]. Therefore, we performed RIP with an antibody against p53 from nuclear extracts of U251 and U87 MG cells. We found a significant enrichment of MEG3 with the p53 antibody (Fig. 4A) compared with the IgG control antibody. To further confirm the association between MEG3 and p53 we performed RNA pull-down. Figure 4B showed a significant enrichment of p53 with the MEG3 RNA compared with the negative control RNA. These data suggest the association of MEG3 and p53.

Then we investigated whether MEG3 regulates p53 activation. The MEG3 levels of glioma tissues were significantly lower than that of normal tissue samples (Fig. 5A). Importantly, p53 levels were

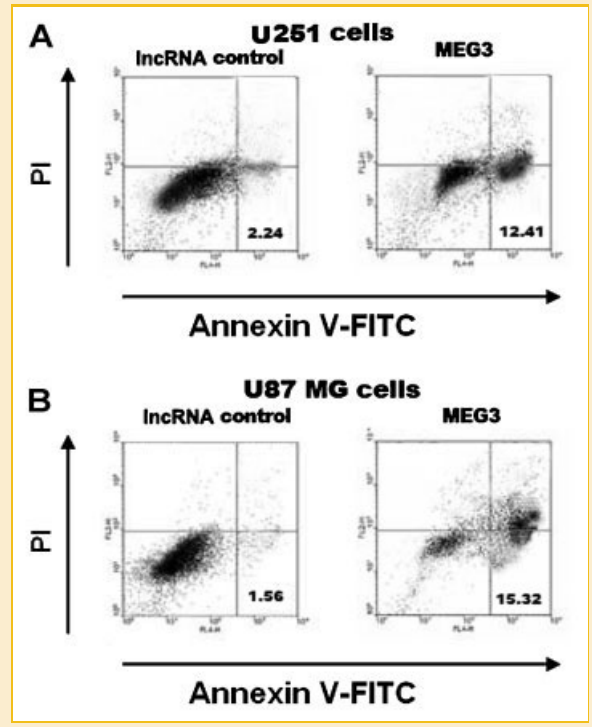

Fig. 3. MEG3 promoted gliomas cell apoptosis. A: U251 cells were treated with MEG3 and apoptosis inducers, and apoptosis was detected using the flow cytometry. Overexpression of MEG3 promoted cell apoptosis in U251 cells. B: U87 MG cells were treated with MEG3 and apoptosis inducers, and apoptosis was detected using the flow cytometry. Overexpression of MEG3 promoted cell apoptosis in U87 MG cells. LncRNA AK055007 was an lncRNA control.

decreased in glioma tissues that showed significantly decreased MEG3 expression. Glioma cells were cotransfected with the p53-responsive reporter plasmid and MEG3. Figure 5B showed that cells transfected with MEG3 significantly increased p53 activity. Additionally, we also found that MEG3 overexpression increased caspase 8/3 and TP53 mRNA levels in U251 cells (Fig. 5C). Previous studies have shown that p53, EGFR, and PTEN are markers of diagnosis and prognosis in patients with glioma [Sung et al., 2000; Yakut et al., 2007]. However, we did not find the correlation of MEG3 with EGFR and PTEN by RIP and real-time PCR analysis (data now shown). These data confirm that downregulated MEG3 is tumor suppressor gene by regulating p53 activation in glioma.

DISCUSSION

It was determined that at least 90% of the genome is actively transcribed with the advent of whole genome and transcriptome sequencing technologies [Gibb et al., 2011]. The human transcriptome was found to be more complex than a collection of protein-coding genes and their splice variants; showing extensive antisense and ncRNA expression [Washietl et al., 2005; Kapranov et al., 2007; Gibb et al., 2011]. Although initially argued to be spurious transcriptional noise, recent evidence suggests that the transcriptional noise of the genome may play a major biological role in

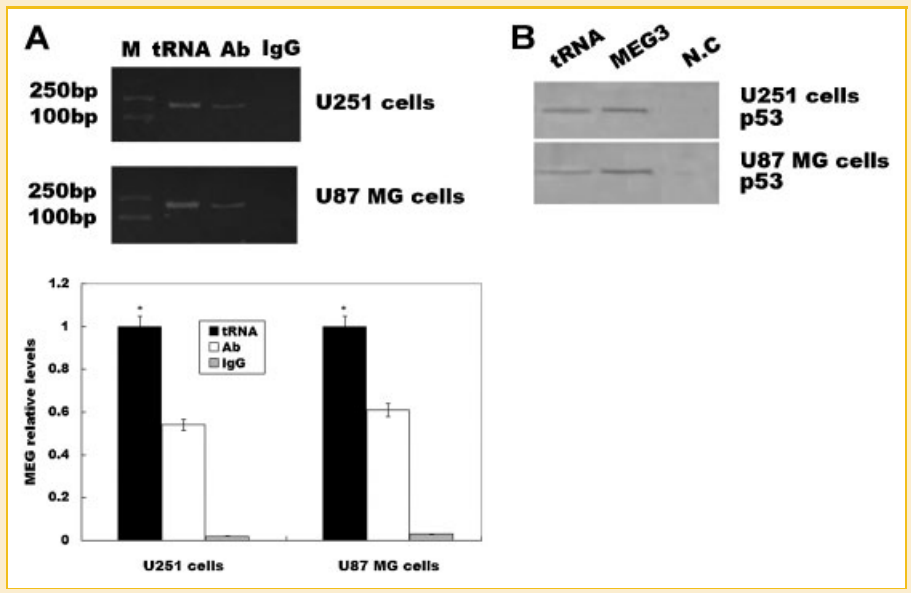

Fig. 4. Association of MEG3 and p53. A: RIP were performed using the p53 antibody to immunoprecipitate MEG3 and a primer to detect MEG3 (up, U251 cells; down, U87 MG cells). M, Marker; tRNA, total RNA; Ab, p53 antibody; IgG, an antibody control. $^{*}P < 0.05$. B: RNA pull-down was performed using RNA to immunoprecipitate p53, and a p53 antibody to detect the association of MEG3 with p53.

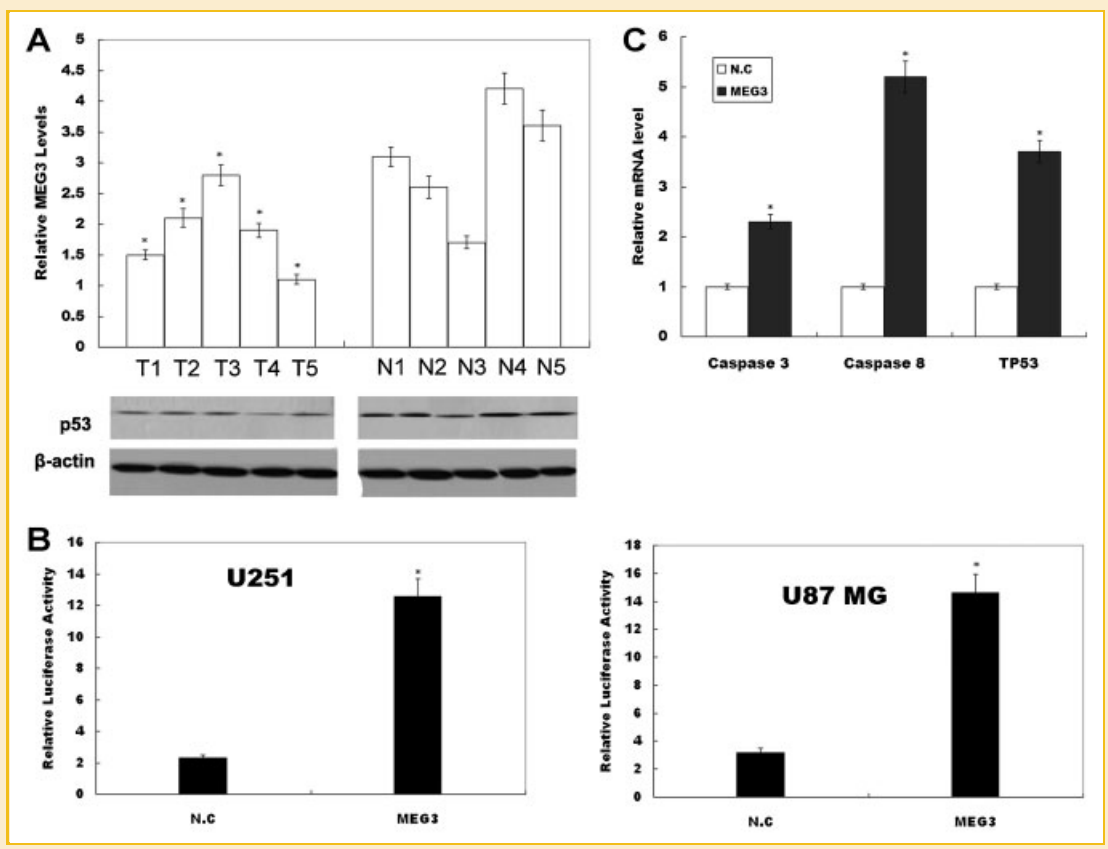

Fig. 5. MEG3 increased p53 activation. A: In five randomly chosen gliomas tissues, MEG3 RNA levels were assayed by realtime PCR and p53 protein expression was examined by western blot analysis. B: Luciferase activity of p53 in U251 or U87 MG cells transfected with MEG3 or N.C. An asterisk indicates significantly different from the N.C ($^{*}P < 0.05$). LncRNA AK055007 was a negative control (N.C). C: MEG3 was overexpressed in U251 cell and caspase 8/3 and TP53 mRNA levels were assayed by real time PCR analysis using SYBR Green. β-actin was used as a reference. Each sample was analyzed in triplicate. The $2^{-\Delta\Delta Ct}$ method was used to quantify the relative gene expression levels. $^{*}P < 0.05$.

cellular development and human diseases [Mercer et al., 2009; Wilusz et al., 2009]. The newly discovered lncRNAs demonstrate developmental and tissue-specific expression patterns, and aberrant regulation in a variety of diseases, including cancer [Guffanti et al., 2009]. However, the function of lncRNAs in tumor pathogenesis and growth is less well characterized.

Recent studies are beginning to unravel their importance in tumorigenesis. Khaitan et al. [2011] reported that lncRNA SPRY4-IT1 is predominantly localized in the cytoplasm of melanoma cells, and SPRY4-IT1 RNAi knockdown resulted in defects in cell growth, differentiation, and higher rates of apoptosis in melanoma cell lines. These data suggest that the higher expression of SPRY4-IT1 may have an important role in the molecular etiology of human melanoma. H19, an imprinted gene, is in fact lncRNA. Loss of imprinting at the H19 locus resulted in high H19 expression in HCC and bladder cancer [Matouk et al., 2007]. H19 has been implicated as having both tumor suppression and oncogenic properties in cancer. It was shown that mice lacking H19 manifested an increased polyp count compared to wild-type by using a mouse model for colorectal cancer [Colnot et al., 2004]. On the contrary, Barsyte-Lovejoy et al. demonstrated that the c-Myc oncogene directly induced the H19 by allele-specific binding to potentiate tumorigenesis [Barsyte-Lovejoy et al., 2006].

Another imprinted gene MEG3 was the first lncRNA proposed to function as a tumor suppressor. Zhang et al. reported MEG3 expression was not detectable in various brain cancers and in a range of human cancer cell lines implicating a potential role of this lncRNA in suppression of cell growth. Moreover, ectopic expression of MEG3 RNA was found to suppress the cell growth, further supporting the role of MEG3 as a tumor suppressor [Zhang et al., 2003; Gibb et al., 2011]. In clinically nonfunctioning pituitary tumors, it was demonstrated that hypermethylation of the MEG3 regulatory region was associated with the loss of MEG3 expression, providing evidence for a mechanism of MEG3 inactivation. However, the role of MEG3 in the glioma progression is unknown. In the present study we evaluated the expression of MEG3 in glioma tissues. We demonstrated that MEG3 expression was markedly decreased in glioma tissues compared with adjacent normal tissues. Moreover, ectopic expression of MEG3 inhibited cell proliferation and resulted in G0/G1 arrest in the human glioma cell lines. We further verified that MEG3 was associated with p53 and that this association was required for p53 activation. It is well known that EGFR oncogene and PTEN/p53 tumor supressor genes were usually altered in glioma specimens [Smith et al., 2001; Zheng et al., 2008]. However, our results did not find that MEG3 RNA was associated with PTEN and EGFR. These data suggest an important role of MEG3 in the molecular etiology of glioma and implicate the potential application of MEG3 in glioma therapy.

CONCLUSION

Our data demonstrate that downregulated MEG3 contributed to glioma cells growth in vitro by regulating p53 activation. Thus, MEG3 may be an important regulator in the development of glioma and implicates the potential application of MEG3 in glioma therapy.

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