

MNX1 reduces sensitivity to anoikis by activating TrkB in human glioma cells

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Abstract. Glioma is the most common type of malignant intracranial tumor in adults and is associated with the highest mortality rate. Although surgery, radiotherapy, chemotherapy and other treatment methods have progressed, the median survival of patients with glioma is only 14-15 months. Glioma cells are able to penetrate along blood vessels and invade into the surrounding normal brain tissue so that an overall resection of the tumor cannot be performed. In the process of metastasis, the resistance of cancer cells to anoikis has an important role. When tumor cells escape from their original environment, anoikis resistance aids their survival. In the present study, reverse transcription-semi-quantitative polymerase chain reaction (RT-sqPCR), RT-quantitative PCR and western blotting demonstrated that the transcription factor, motor neuron and pancreas homeobox 1 (MNX1), was ectopically expressed in glioma cells compared with normal HUVEC-C human umbilical vein endothelial cells. Furthermore, its expression was higher in more malignant glioma cell lines (T98G and M059K) compared with the less malignant glioma cell line (U-87 MG) and normal HUVEC-C cells. An adhesion assay using fibronectin demonstrated that MNX1 and tyrosine kinase receptor B (TrkB) overexpression in HUVEC-C and U-87 MG cells reduced adhesion and forced them to suspend. Additionally, MNX1 and TrkB overexpression was demonstrated to increase the ability of cells to bypass anoikis. MNX1 and TrkB knockdown increased adhesion and promoted apoptosis after suspension. It was further demonstrated that MNX1 functioned as a transcription factor binding in the upstream regulatory region of TrkB to activate its expression. The results of the present study suggested that MNX1 may suppress the adhesion and apoptosis rates of tumor cells

by activating TrkB. The results of the present study suggest that MNX1 may represent a novel therapeutic target for the treatment of gliomas.

Introduction

Glioma is a common tumor of the central nervous system that is derived from glial cells. Classification of glioma is primarily based on the tissue source, tumor site and invasive ability. Astrocytoma is histologically close to astrocytes. According to the classification criteria of the World Health Organization, astrocytoma may be divided into grades I-IV. Grade I and II astrocytomas are low-grade gliomas with a weak degree of malignancy, while grade III and IV astrocytomas are classified as malignant gliomas. Grade III astrocytoma is also termed anaplastic astrocytoma and grade IV astrocytoma is usually glioblastoma (GBM), which has the strongest invasive potential.

Unfortunately, GBM is the most common type of glioma. Its annual incidence rate is 3.19/10,000 in the United States (1,2). Despite advances in the equipment and technology used for surgery and radiation therapy, the recurrence of glioma cannot be prevented. Glioma cells are highly invasive and infiltrate through normal tissue gaps to metastasize (3). Targeted therapy is the next frontier in glioma treatment.

Anoikis is a type of programmed cell death that is induced following disengagement between cells and the extracellular matrix (ECM) (4). Anoikis has important roles in development, organization balance, disease occurrence and tumor metastasis (5). Anoikis prevents epithelial cells from cloning in other sites. Cells that adhere to ECM proteins do not undergo anoikis and resistance to anoikis is essential for successful tumor metastasis. Once the adhesion of tumor cells to the surrounding cells and ECM is reduced, they escape from primary foci. During the process of transfer and final colonization, tumor cells must be able to survive at ectopic sites (6), and resistance to anoikis aids this process. In both normal and tumor cells, studies have demonstrated that ECM is an inhibitor of anoikis, and integrins have a profound impact on cell survival (7,8). The most important factors involved in the transduction pathways regulated by integrins include focal adhesion kinase, integrin-linked kinase, tyrosine kinase, phosphatidylinositol 3-kinase (PI3K), extracellular

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signal-regulated kinase and the connexin Src homology 2 domain-containing-transforming protein 1 (9).

Motor neuron and pancreas homeobox (MNX1) is a homologous box transcription factor that promotes motor neuron differentiation and pancreatic development. MNX1 knockout in mice indicated that only the early expression of MNX1 is associated with the formation of pancreatic buds, and MNX1 is expressed in late β cells and associated with β cell maturation (10). MNX1 has the ability to induce endocrine progenitor cells to differentiate into β cells and inhibit their ability to differentiate into α cells, thereby maintaining the balance between α and β cells (11). In addition, the association between MNX1 gene mutations and Currarino syndrome has also been demonstrated. The Currarino syndrome phenotype caused by a MNX1 mutations was demonstrated to be a result of haploinsufficiency (12). A recent study also indicated that MNX1 may be an oncogene in prostate cancer (13).

Tyrosine kinase receptors (Trks) are neurotrophin (NT) receptors that are encoded by proto-oncogenes, which are key regulators of cell proliferation, differentiation and apoptosis signaling pathways (14). The TrkB signal transduction pathway has an important role in tumor progression and may lead to the malignant transformation of fibroblasts (15). Brain-derived neurotrophic factor (BDNF) is a ligand of TrkB, and the BDNF/TrkB ligand-receptor interaction induces receptor dimerization. The receptor Trk region activates the autophosphorylation of tyrosine residues in this region, and phosphorylation of Y484 and Y785 in the surrounding area also occurs. This phosphorylation subsequently triggers the binding of the receptor to the SHC, which is the protein adapter of the phosphotyrosine-binding region binding to the phospholipase C (PLC) γ protein, and promotes signal transmission. Downstream signaling pathways include the Ras/mitogen-activated protein kinase pathway, PI3K/3-phosphoinositide dependent protein kinase 1/Akt pathway, increased Ca^{2+} release and activation of the PLC γ pathway (16).

The present study demonstrated that MNX1 was ectopically expressed in glioma cells and glioma cell lines with a higher malignancy were associated with higher MNX1 expression compared with a glioma cell line with lower malignancy. Furthermore, MNX1 contributed to cell adhesion and reduced the level of anoikis during detachment from adhesion. The expression pattern of TrkB in glioma cell lines was similar to that of MNX1, and TrkB expression was altered with the regulation of MNX1. It was also confirmed that MNX1 was able to bind directly to the TrkB gene and regulate its expression. Thus, TrkB, as a downstream signaling pathway of MNX1, may increase the ability of glioma cells to escape anoikis.

Materials and methods

Cell culture. HUVEC-C, U-87 MG, T98G and M059K cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Concerning the U-87 MG cell line, this cell line is not the original GBM cell line established in 1968 at the University of Uppsala, but is most probably also a GBM cell line whose origin is unknown (17). HUVEC-C cells were maintained in EGMTM-2 BulletKitTM (Lonza Group, Ltd., Basel, Switzerland). U-87 MG cells were maintained in Dulbecco's

modified Eagle's/F12 supplemented with 20 ng/ml rh-b-FGF, 20 ng/ml rh-EGF, 2% B27 and 2 mg/l L-glutamine (all Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). T98G and M059K cells were maintained in Eagle's minimum essential medium (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS. Cells were maintained in an incubator at 37°C in a humidified atmosphere containing 5% CO₂.

RNA isolation and cDNA synthesis. For RNA isolation, $\sim 1 \times 10^6$ cells were lysed with 1 ml TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.). Cell lysates were added to 200 μ l chloroform and centrifuged at 12,000 x g for 15 min at 4°C. The upper phase was extracted, added to an equal volume of isopropanol, incubated for 10 min at room temperature and centrifuged at 12,000 x g for 10 min at 4°C. The supernatant was discarded and the precipitate was washed with 75% ethanol and centrifuged at 7,500 x g for 10 min at 4°C. The supernatant was again discarded, the RNA precipitate was allowed to dry and it was subsequently dissolved in RNase-free H₂O.

A PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China) was used to conduct reverse transcription (RT) reactions. cDNA synthesis was performed for 1 h at 42°C and 10 min at 72°C.

Construction of overexpression and knockdown vectors. Total RNA was extracted from HUVEC-C cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcribed according to the aforementioned protocol. HUVEC-C cDNA was used to amplify the full-length MNX1 open reading frame (ORF) and TrkB ORF via polymerase chain reaction (PCR) using Platinum[®] Taq DNA Polymerase (Invitrogen; Thermo Fisher Scientific, Inc.). BamHI and XhoI restriction sites were ligated to both ends of the amplified fragments, which were subsequently cloned into pCDH expression vectors (Addgene, Inc., Cambridge, MA, USA). The sequences of the primers used for amplification of MNX1 ORF were forward, 5'-CGCGGATCCATGGGGGGACTC TCAACAGT-3' and reverse, 5'-CCGCTCGAGCTACTGGGG CGCGGGCTGG-3'. The sequences of the primers used for amplification of TrkB ORF were forward, 5'-CGCGGATCC ATGTCGTCCTGGATAAGGTGGCA-3' and reverse, 5'-CCG CTCGAGCTAGCCTAGAATGTCCAGGTAG-3'. The thermocycling conditions used for PCR were as follows: Initial denaturation for 5 min at 95°C; followed by 35 cycles of 30 sec at 95°C, 30 sec at 55°C and 2 min at 72°C; followed by a final extension for 5 min at 72°C. Empty pCDH vectors were used as a control for overexpression vectors. pLKO.1 vectors containing short hairpin (sh)RNA for MNX1 and TrkB knockdown were purchased from Shenzhen Zhonghong Boyuan Biological Technology Co., Ltd. (Shenzhen, China). The following shRNA sequences were employed, MNX1 shRNA-1, GCC CGACTTCAACTCCCAGGC; MNX1 shRNA-2, GTCCT CGGAGGACGACTCGC; TrkB shRNA-1, TGAAAGATT TCTCATGGTTTG; and TrkB shRNA-2, TGGCGTCTGCGT GGAGGGCGA. Empty pLKO.1 vectors were used as control. HEK293T cells were used for lentiviral packaging. A total of 12 ng target plasmids were transfected into HEK293T cells at a density of 80% in 100 mm dishes. The amount of transfection reagent-polyethylenimine (Polysciences, Inc., Warrington, PA, USA) is one third of the plasmid. Following 24 h, lentiviruses

were collected in order to transfect corresponding target cells. Prior to transfection, a total of 1.2×10^6 cells were incubated for 24 h in six-well plates at 37°C. MNX1 and TrkB pCDH overexpression vectors were used for transfection of HUVECs and U-87 MG cells. MNX1/TrkB shRNA pLKO.1 vectors were used for transfection of T98G and M059K cells. The mass of transfected MNX1 and TrkB pCDH overexpression vectors, as well as MNX1/TrkB shRNA pLKO.1 vectors, were the same.

Adhesion assay. For the adhesion assay 500 μ l fibronectin (10 g/ml; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added to 6-well plates and incubated at 37°C for 24 h. Subsequently, excess liquid was discarded and 500 μ l bovine serum albumin (BSA; 0.2%) (Sangon Biotech, Co., Ltd., Shanghai, China) was added to block each well at room temperature for 1 h. A total of 24 h post-transfection, 1×10^5 transfected cells were placed into each well and incubated for 30 min. Medium was subsequently discarded and 5% (w/v) crystal violet was used to stain adhered cells for 10 min at room temperature, and the results were observed using a light microscope (magnification, x100).

Cell death detection ELISA. A total of 24 post-transfection, 1×10^5 cells were seeded in normal and low-attachment surface 24-well plates. Following incubation for 24 h at 37°C, a Cell Death Detection ELISA PLUS kit (cat. no. 11774425001; Sigma Aldrich; Merck KGaA, Darmstadt, Germany) was used to detect apoptosis, according to the manufacturer's protocol.

RT-semi-quantitative (sq)PCR and RT-qPCR analyses. RT-sqPCR was performed with Platinum[®] Taq DNA Polymerase (Invitrogen; Thermo Fisher Scientific, Inc.). Cycling conditions were as follows: Initial denaturation for 5 min at 95°C, followed by 32 cycles of 30 sec at 95°C, 30 sec at 57°C and 1 min at 72°C; followed by a final extension for 5 min at 72°C. Primer sequences used for RT-sqPCR were as follows: GAPDH forward, 5'-GATTCACCCATGGC AAATTC-3' and reverse, 5'-GTCATGAGTCCTTCCACG ATAC-3'; MNX1 forward, 5'-CTAAGATGCCCGACTTCA ACT-3' and reverse, 5'-CTTCTGTTTCTCCGCTTCCT-3'; TrkB forward, 5'-GACACCACGAACAGAAGTAATG-3' and reverse, 5'-CTGCTCAGGACAGAGGTTATAG-3'. DNA products were run on a 1.5% agarose gel and then visualized using 0.5 μ g/ml ethidium bromide. ImageJ 1.8.0 software (National Institutes of Health, Bethesda, MD, USA) was used for densitometry.

RT-qPCR was performed using SYBR-Green qPCR Master Mix (Takara Biotechnology Co., Ltd.). The thermocycling conditions used were as follows: Initial denaturation for 3 min at 95°C; followed by 15 sec at 95°C, 15 sec at 57°C and 20 sec at 72°C; followed by a final extension for 3 min at 72°C. The sequences of primers used for RT-qPCR were as follows: MNX1 forward, 5'-CGAGACCCAGGTGAAGAT TT-3' and reverse, 5'-CTTCTGTTTCTCCGCTTCCT-3'; TrkB forward, 5'-GGACACCACGAACAGAAGTAA-3' and reverse, 5'-CAATCACCACACAGCATAGA-3'; and GAPDH forward, 5'-GGTGTGAACCATGAGAAGTATGA-3' and reverse, 5'-GAGTCCTTCCACGATACCAAAG-3'. Protein levels were normalized against GAPDH, and the $2^{-\Delta\Delta C_t}$ method was used for protein quantification (18).

Protein extraction and western blot analysis. For protein extraction, 2×10^6 cells were incubated in lysis buffer that was composed of 100 μ l 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl, 0.1% SDS, 1% NP-40 and 100 μ g/ml phenylmethylsulfonyl fluoride. Lysis was performed on ice and lysates were subsequently denatured at 100°C for 10 min. A Pierce BCA Protein assay kit (Invitrogen; Thermo Fisher Scientific, Inc.) was used to measure the protein concentration and 20 μ g protein was loaded into each well. Proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked in 5% (w/v) milk for 1 h at room temperature. The following primary antibodies were employed: Rabbit MNX1 polyclonal antibody in 5% (w/v) milk (cat. no. SAB2101494; 1:2,000; Sigma-Aldrich; Merck KGaA), rabbit TrkB polyclonal antibody in 5% (w/v) milk (cat. no. ab18987; 1:2,000; Abcam, Cambridge, UK) and mouse GAPDH monoclonal antibody in 5% (w/v) milk (cat. no. ab8245; 1:5,000; Abcam). The primary antibodies were incubated with the membranes at 4°C overnight. Goat anti-rabbit IgG H&L in 3% (w/v) BSA (cat. no. ab6721; 1:2,000; Abcam) and goat anti-mouse IgG H&L in 5% (w/v) milk (cat. no. ab6789; 1:2,000; Abcam) horseradish peroxidase (HRP)-conjugated antibodies were used as secondary antibodies. The secondary antibodies were incubated with the blots at room temperature for 4 h. Bands were visualized with Chemiluminescent HRP Substrate (EMD Millipore, Billerica, MA, USA) and images were captured by X-OMAT BT Film (Carestream Health, Inc., Rochester, NY, USA).

Chromatin immunoprecipitation (ChIP). The DNA products following RT-qPCR and RT-sqPCR analysis were subjected to ChIP analysis. The MNX1 binding motif was predicted using the JASPAR database (<http://jaspar.genereg.net/>). Following the identification of DNase I hypersensitive sites using the Encyclopedia of DNA Elements at the University of California, Santa Cruz database (<http://genome.ucsc.edu/ENCODE/>), ChIP primers were designed as follows: TrkB-1F, 5'-TTC CACCTCCCAGGTTCAAG-3' and TrkB-1R, 5'-GTCAAG AGATCGAGACCATCC-3'; TrkB-2F, 5'-ATTCTGTTC CTCCACTGAC-3' and TrkB-2R, 5'-GAGGTAAGAGGA GAAAAGACC-3'; TrkB-3F, 5'-TAAAGGAACTCCAGC AGAAC-3' and TrkB-3R, 5'-TGGATTGTGGTGTATGGTT TC-3'; TrkB-4F, 5'-TCTTCACAAAGAACCAGCTC-3' and TrkB-4R 5'-CCACCTTAAGAAATCAGAGG-3'; TrkB-5F, 5'-ACTGCGGTGTATTTTCCCCGTT-3' and TrkB-5R, 5'-TAGGGACAAATTAGGCGATCCG-3'; TrkB-6F, 5'-GAC AGTCTCTCCCTAACAT-3' and TrkB-6R, 5'-CACTCA GAACCTCTCCTCTT-3'; TrkB-7F, 5'-TTGACCCTACTT TCTTCCAC-3' and TrkB-7R, 5'-AAACTCCACACTAGG GAAGTGG-3'; TrkB-8F, 5'-CCAGTGAGCAAACCTTCAG GA-3' and TrkB-8R 5'-AGGGTGCTTTTCTCCGAAAG-3'. A Pierce Agarose ChIP kit (Thermo Fisher Scientific, Inc.) was used to conduct ChIP according to the manufacturer's protocol. During the process, the MNX1 primary antibody (cat. no. SAB2101494; Sigma-Aldrich; Merck KGaA) was used at a 1:100 dilution.

Luciferase reporter gene technology. T98G and M059K cells (1×10^5) were plated in 24-well plates and tested at a cell density of ~80% after approximately 24 h. Fragments of

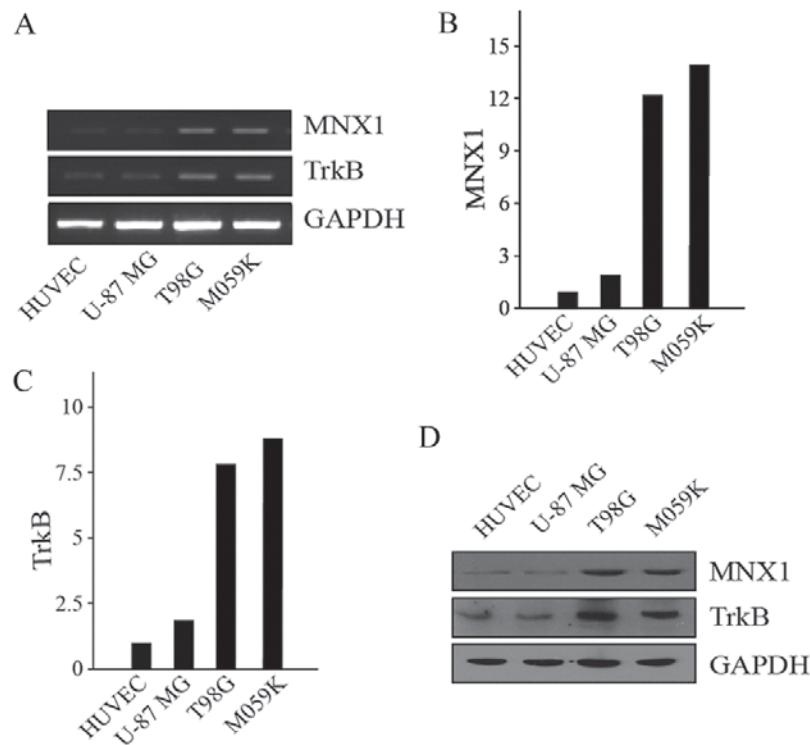


Figure 1. Expression of MNX1 and TrkB in normal human HUVEC-C cells and glioma cell lines. (A) Representative gel presenting mRNA expression of MNX1 and TrkB in HUVEC-C, U-87 MG, T98G and M059K cells. Quantified mRNA levels of (B) MNX1 and (C) TrkB in HUVEC-C, U-87 MG, T98G and M059K cells were determined via reverse transcription-quantitative polymerase chain reaction. (D) Protein levels of MNX1 and TrkB were measured by western blotting in HUVEC-C, U-87 MG, T98G and M059K cells. $P < 0.05$ vs. HUVEC-C cells. MNX1, motor neuron and pancreas homeobox 1; TrkB, tyrosine kinase receptor B; HUVECs, human umbilical vein endothelial cells; NS, not significant.

different lengths upstream of TrkB were cloned into pGL3 vectors containing firefly luciferase (Promega Corporation), which were considered to be the target plasmids. The primers used to generate each of the 7 fragments were as follows: Position -2,665 bp to position -2,368 bp, 5'-TTCCACCTC CCAGTTCAAG-3'; -2,665 bp to position -2368 bp, 5'-TCA ACGTTGTATGTTTTA-3'; -2,665 bp to position -2,368 bp, 5'-GGTCAGCAGATTGAGACCAT-3'; -2,665 bp to position -2,368 bp, 5'-TGGAGGTTCTAAAGGAACTCCA-3'; -2,368 bp reverse primer, 5'-TATAGCACTAAATTCCTG TATCAG-3'; -3,425 bp to position -2,666 bp, 5'-TCAACG TTGTATGTTTTA-3'; -4,216 bp to position -2,666 bp, 5'-GGT CAGCAGATTGAGACCAT-3'; -5,002 bp to position -2,666 bp, 5'-TGGAGGTTCTAAAGGAACTCCA-3'; -2,666 bp reverse primer 5'-ACACTGAATATTCATAG-3'. The cells in each well were co-transfected with 800 ng target plasmid and 15 ng control *Renilla reniformis* luciferase plasmid (pRL-TK; cat. no. E2231; Promega Corporation) using 1 μ l Lipofectamine® (Invitrogen; Thermo Fisher Scientific, Inc.). After 24 h, the Dual-Luciferase® Reporter assay system was used to measure the luciferase activity in a Promega GloMax 20/20 Luminometer following cell lysis (all Promega Corporation). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

Statistical analysis. Statistical analyses were performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). Data are presented as the mean + standard deviation for continuous data. One-way analysis of variance followed by Dunnett's post-hoc test was performed for multiple

comparisons. All experimental groups were compared with the control groups. $P < 0.05$ was considered to indicate a statistically significant difference. Each experiment was performed in triplicate.

Results

Positive association between MNX1 and TrkB expression in normal human HUVEC-C cells and glioma cell lines. The present study investigated the expression of MNX1 in the HUVEC-C normal human cell line, U-87 MG (low malignancy) human glioma cell line, and T98G and M059K (high malignancy) human glioma cell lines. RT-sqPCR (Fig. 1A) and RT-qPCR (Fig. 1B and C) were used to detect the expression of MNX1 and TrkB at the transcriptional level. Western blotting was performed to detect the expression of MNX1 and TrkB at the protein level (Fig. 1D). The results demonstrated that the expression of MNX1 was higher at both the transcriptional and translational levels in high malignancy T98G and M059K cells compared with HUVEC-C and low malignancy U-87 MG cells (Fig. 1A-D).

It has been reported that mature BDNF and its receptor, TrkB, are upregulated in human glioma tissues (19); however, its specific mechanism of action has not been clearly explained. Thus, the present study also measured the expression of TrkB in the aforementioned cell lines. The results demonstrated that TrkB expression was similar to that of MNX1; TrkB levels were higher in T98G and M059K cells compared with HUVEC-C and U-87 MG cells at the transcriptional and translational levels (Fig. 1A, C and D).

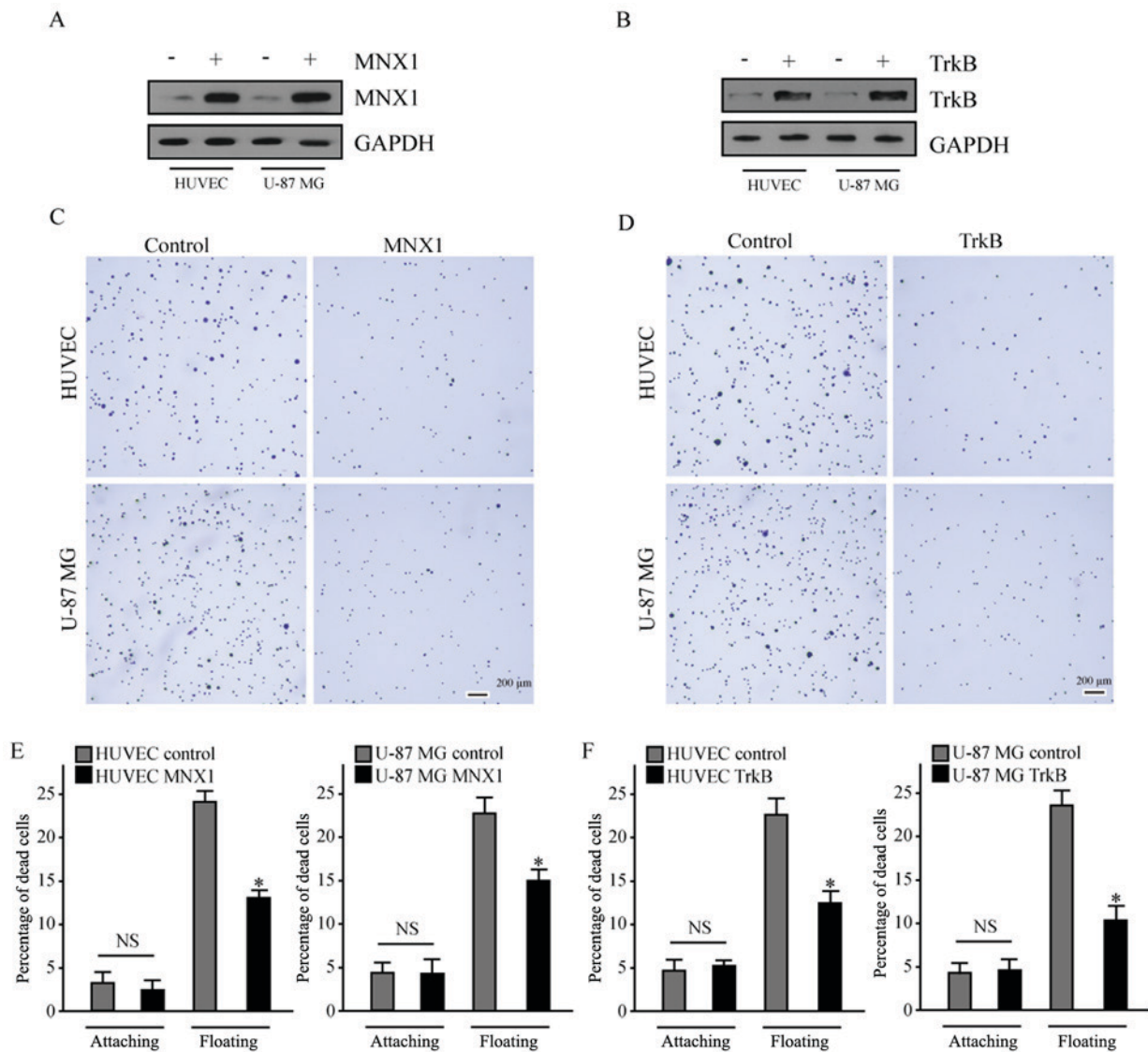


Figure 2. MNX1 and TrkB promote cell detachment and anchorage independence. Western blot analysis confirmed that (A) MNX1 and (B) TrkB were successfully overexpressed in HUVEC-C and U-87 MG cells. Overexpression of (C) MNX1 and (D) TrkB reduced the adhesion ability of HUVEC-C and U-87 MG cells. Scale bars, 200 μ m. Resistance to anoikis was increased in suspended HUVEC-C and U-87 MG cells following overexpression of (E) MNX1 and (F) TrkB. * $P < 0.05$ vs. control. MNX1, motor neuron and pancreas homeobox 1; TrkB, tyrosine kinase receptor B; HUVECs, human umbilical vein endothelial cells; NS, not significant; attaching, cells attached to the extracellular matrix; floating, cells suspended without the extracellular matrix; A405 nm, Absorbance in 405 nm; A490 nm, Absorbance in 490 nm.

MNX1 and TrkB promote cell detachment and anchorage independence. Western blot analysis confirmed that MNX1 and TrkB were successfully overexpressed in the HUVEC-C and U-87 MG cells (Fig. 2A and B). Fibronectin is an important constituent of the ECM, therefore, the present study employed cell culture dishes that were coated with fibronectin to detect changes in adhesion between cells and the ECM. This method is termed the adhesion assay. The results of the adhesion assay demonstrated that MNX1 and TrkB overexpression led to reduced adhesion between cells and fibronectin compared with the respective control groups (Fig. 2C and D), which is considered to be an early event in tumor cell metastasis (20).

TrkB has an important role in resistance to anoikis (21). The current study aimed to determine whether MNX1 and TrkB may increase the ability of HUVEC-C and U-87 MG cells to bypass anoikis. Cells in the control and experimental groups were cultured in the normal adherent state and

low-attachment plates for 24 h. A Roche Cell Death Detection ELISA kit was used to detect cell apoptosis levels. The results demonstrated that MNX1 and TrkB overexpression both reduced the mortality of cells in suspension culture, compared with the control group (Fig. 2E and F).

Knockdown of MNX1 and TrkB increases cell adhesion and induces anoikis in highly malignant glioma cell lines. To further verify the role of MNX1 and TrkB in the development of glioma, shRNA was employed in the current study to knock down their expression in T98G and M059K cells. To prevent off-target effects, two shRNAs each were designed for MNX1 and TrkB. Western blot analysis demonstrated that MNX1 and TrkB were successfully downregulated in T98G and M059K cells following transfection with shRNA (Fig. 3A and B). Furthermore, downregulation of MNX1 and TrkB markedly increased the adhesion of T98G and M059K

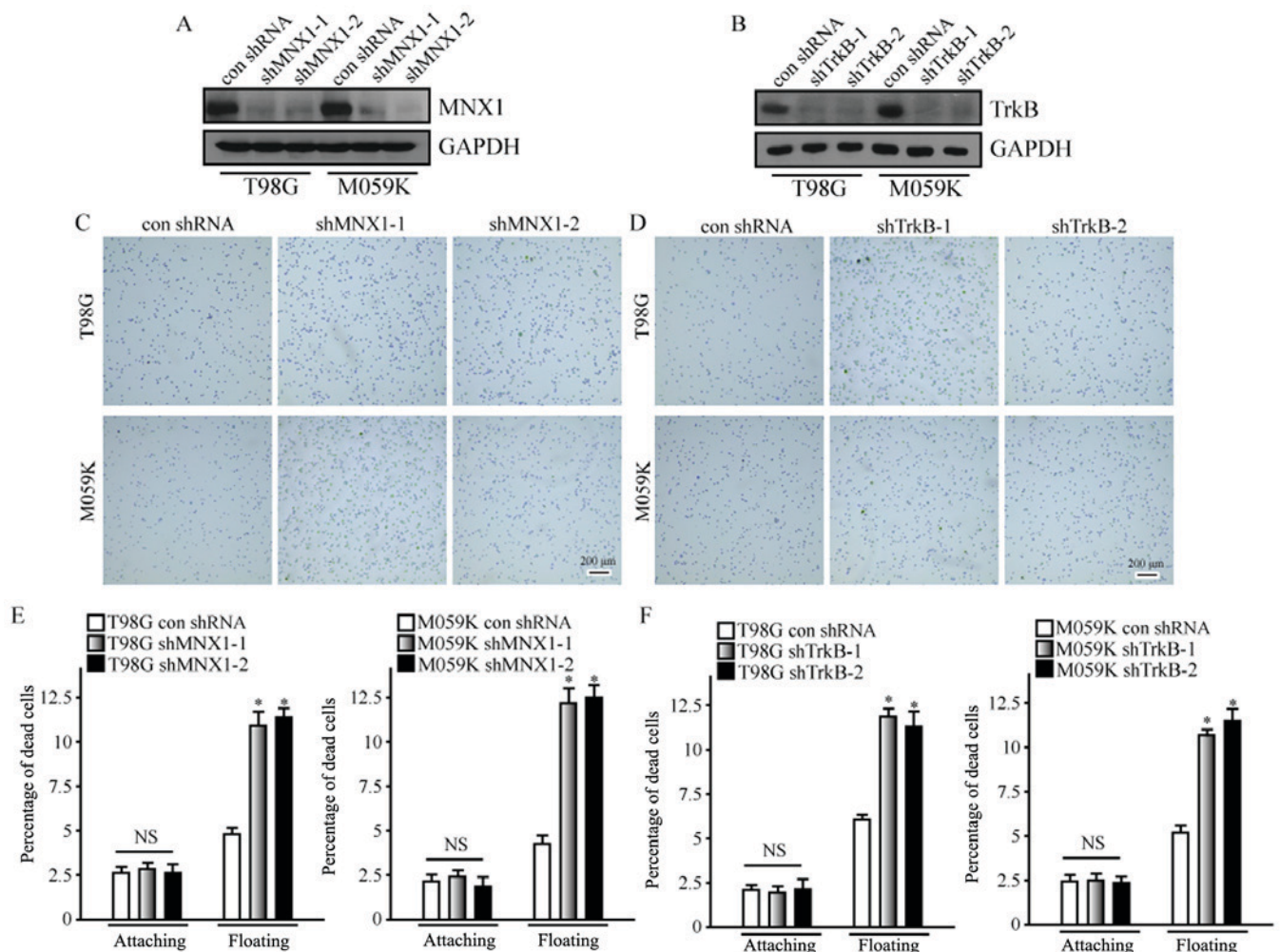


Figure 3. Knockdown of MNX1 and TrkB increases cell adhesion and induces anoikis in highly malignant glioma cell lines. Western blot analysis confirmed successful knockdown of (A) MNX1 and (B) TrkB in T98G and M059K cells. Knockdown of (C) MNX1 and (D) TrkB increased the adhesion ability of T98G and M059K cells. Scale bars, 200 μ m. The ratio of suspended T98G and M059K cells undergoing anoikis was increased following knockdown of (E) MNX1 and (F) TrkB. * $P < 0.05$ vs. con shRNA. MNX1, motor neuron and pancreas homeobox 1; TrkB, tyrosine kinase receptor B; shRNA, short hairpin RNA; con shRNA, control shRNA; NS, not significant; attaching, cells attached to the extracellular matrix; floating, cells suspended without the extracellular matrix; A405 nm, absorbance at 405 nm; A490 nm, absorbance at 490 nm.

cells to fibronectin (Fig. 3C and D); this was consistent with the above experimental results in HUVEC-C and U-87 MG cells, and confirmed that MNX1 and TrkB may allow cells to reduce their association with the ECM. Additionally, downregulation of MNX1 and TrkB led to increases in the apoptosis of T98G and M059K cells in suspension (Fig. 3E and F), further indicating that MNX1 and TrkB may contribute to anoikis resistance in cells.

MNX1 confers anoikis resistance through TrkB. To verify the association between MNX1 and TrkB, a series of experiments were performed. Western blot analysis demonstrated that overexpression of MNX1 and TrkB in HUVEC-C and U-87 MG cells both led to the upregulation of TrkB (Fig. 4A), and decreased levels of apoptosis in cells in suspension culture were also observed (Fig. 4B). However, when cells were co-transfected with MNX1 overexpression vector and TrkB shRNA-1 to restore normal TrkB levels (Fig. 4A), the anoikis levels of cells were also partially restored (Fig. 4B). Similarly, knockdown of MNX1 and TrkB was performed in T98G and M059K cells to verify these results. Western blot

analysis and apoptosis analysis demonstrated that knockdown of MNX1 resulted reduced TrkB protein expression levels and increased apoptosis in suspension culture (Fig. 4C and D). Furthermore, overexpression of TrkB combined with MNX1 knockdown restored the levels of TrkB protein and anoikis (Fig. 4C and D). These data indicate that MNX1 may act by regulating the expression of the downstream TrkB gene.

MNX1 binds directly to TrkB to regulate its expression. The aforementioned experiments clearly demonstrated an association between MNX1 and TrkB, but the specific mechanism was yet to be established. For example, it was unclear whether MNX1 binds directly to TrkB as a transcription factor to regulate its transcription. Initially, the MNX1 binding motif was predicted using the JASPAR database (Fig. 5A) and a putative MNX1 binding site was identified in the TrkB gene (Fig. 5B). DNase I hypersensitive sites were analyzed using the Encyclopedia of DNA Elements at the UCSC database. Subsequently, ChIP was performed to analyze whether MNX1 binds to TrkB. In combination with the analysis of binding sites and DNase I hypersensitive sites, ChIP primers were designed for eight

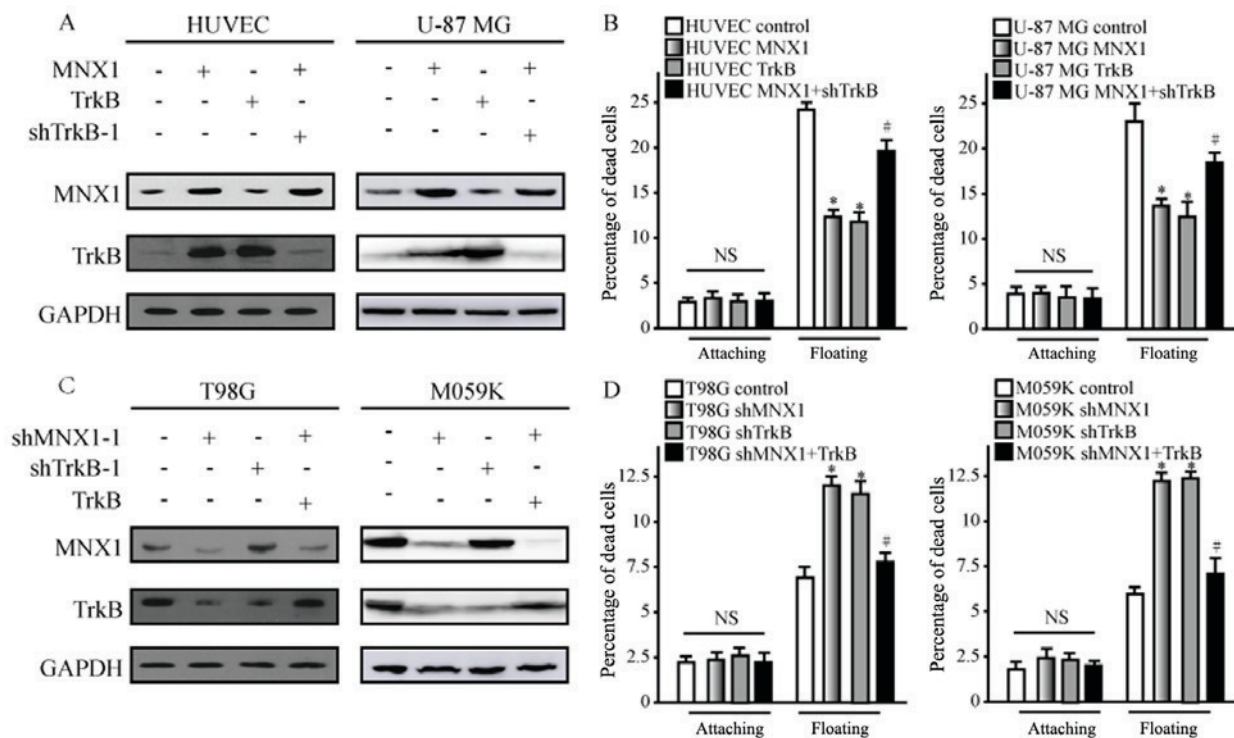


Figure 4. MNX1 confers anoikis resistance through TrkB. HUVECs were transfected with MNX1 or TrkB overexpression vector, or a combination of MNX1 overexpression vector and shTrkB-1. (A) Western blot analysis was performed on HUVEC-C and U-87 MG cells to determine the effects on MNX1 and TrkB protein expression. (B) Anoikis was measured in attached and suspended U-87 MG cells and HUVECs following transfection. T98G cells were transfected with shMNX1-1, shTrkB-1 or a combination of shMNX1-1 and TrkB overexpression vector. (C) Western blot analysis was performed on T98G and M059K cells to determine the effects on MNX1 and TrkB protein expression. (D) Anoikis was measured in attached and suspended T98G and M059K cells following transfection. For anoikis, the rate of cell death was assessed after 24 h. * $P < 0.05$ vs. control; ** $P < 0.05$ vs. MNX1 or shMNX1-1 group. MNX1, motor neuron and pancreas homeobox 1; TrkB, tyrosine kinase receptor B; HUVECs, human umbilical vein endothelial cells; sh, short hairpin RNA; NS, not significant; attaching, cells attached to the extracellular matrix; floating, cells suspended without the extracellular matrix; A405 nm, absorbance at 405 nm; A490 nm, absorbance at 490 nm.

regions close to the transcriptional start site of TrkB (R1-8; Fig. 5B). The results of ChIP analysis demonstrated that R4 of TrkB was pulled down by Pierce Protein A PLUS Agarose binding with the MNX1 antibody following overexpression of MNX1 in HUVEC-C and U-87 MG cells, indicating that MNX1 was bound to the R4 site of TrkB by RT-qPCR (Fig. 5C) and RT-sqPCR (Fig. 5D). A luciferase assay was subsequently performed. Fragments of different lengths upstream of TrkB were cloned into a vector containing a luciferase reporter. For this experiment, T98G and M059K cells that highly express MNX1 were employed to verify the above results. The results demonstrated that the activity of the luciferase reporter containing the -2665 to -2368 bp fragment was high compared with a number of the other fragments (Fig. 5E). Furthermore, the position of this fragment coincided with the position of R4. The data described here indicated that MNX1 binds directly upstream of the TrkB gene to regulate its transcription.

Discussion

Glioma is associated with the highest incidence of intracranial malignant tumors, accounting for ~45% of all cases (22). The treatment of glioma has always been a difficult problem in neurosurgery; ~77% of patients succumb to glioma within 1 year following diagnosis (23). Clinical investigations as well as experiments on animals and cells have demonstrated the poor prognosis associated with glioma, and uncontrollable

cell proliferation, reduced apoptosis, tumor cell invasiveness, neovascularization and other biological behaviors are reported to contribute to the difficulty of treating glioma (24,25). With the application and development of molecular biology in tumor research in recent years, the molecular mechanisms and apoptosis of glioma have been investigated extensively. Due to its high degree of hereditary and pathological heterogeneity, even within the same tumor sample, single molecular abnormalities are not common, and malignant gliomas usually exhibit multiple gene mutations and molecular changes (26,27). Therefore, it is difficult to target and identify a single gene responsible for glioma. In recent years, the Cancer Genome Atlas has identified three core signaling pathways in malignant glioma: Trk/Ras/PI3K, p53 and retinoblastoma protein signal transduction pathways (28). In addition, other typical signaling pathways, such as the angiogenic pathway, have also been indicated to be important for the development of gliomas (29,30).

The association between MNX1 and Currarino syndrome has long been a concern of researchers (31). The role of MNX1 in the development of β cells has also been previously investigated (10). Furthermore, the role of MNX1 in cancer development been investigated recently, and MNX1 was indicated to be an oncogene in prostate cancer (13,32,33). However, to the best of our knowledge, the expression and function of MNX1 in gliomas has not been previously reported. The expression of TrkB has been reported to be closely associated with the invasion and metastasis of tumor cells; and BDNF/TrkB

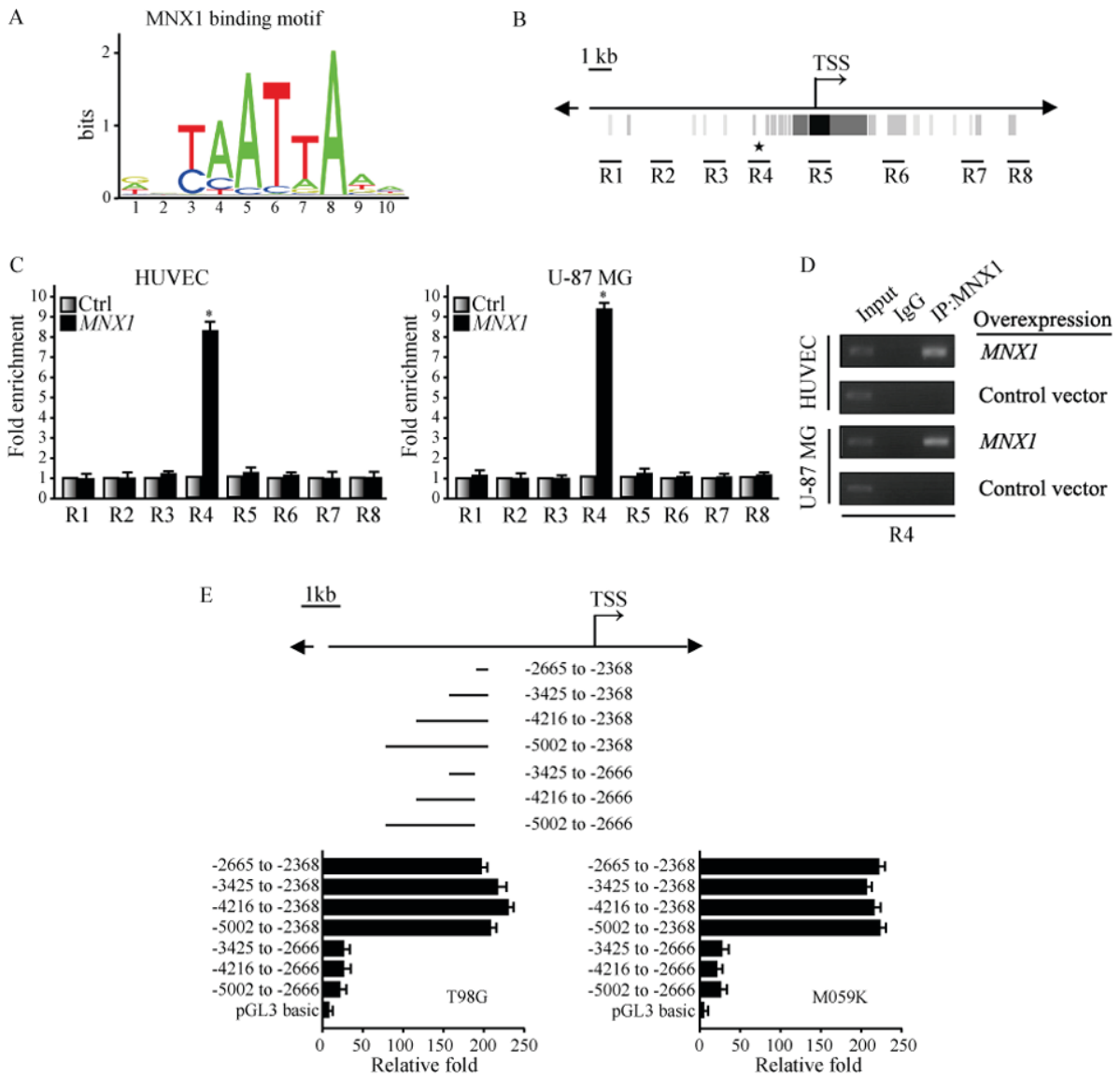


Figure 5. MNX1 binds directly to TrkB to regulate its expression. (A) MNX1 binding motif. (B) Eight regions designed for ChIP are highlighted. (C) ChIP analysis demonstrated that the MNX1 antibody was enriched in R4 of TrkB in HUVEC-C and U-87 MG cells following MNX1 overexpression by RT-quantitative PCR. (D) Representative gel for R4 of TrkB following ChIP analysis of HUVEC-C and U-87 MG cells that overexpressed MNX1 by RT-semi-quantitative PCR. (E) Luciferase reporter studies indicated that the region of TrkB regulation by MNX1 may be located within the -2665 to -2368 bp fragment. *P<0.05 vs. control. MNX1, motor neuron and pancreas homeobox 1; TrkB, tyrosine kinase receptor B; ChIP, chromatin immunoprecipitation; R4, region 4; HUVECs, human umbilical vein endothelial cells; TSS, transcriptional start site; Ctrl, control; RT, reverse transcription; PCR, polymerase chain reaction.

signaling was revealed to suppress apoptosis and enhance angiogenesis (34,35). This provides the basis for growth conditions during the migration of tumor cells to distant sites and continued proliferation. However, the association between TrkB and MNX1 has not been previously investigated.

The present study demonstrated that the expression of MNX1 and TrkB was higher in highly invasive glioma cell lines (T98G and M059K) compared with less invasive glioma cells (U-87 MG) and normal HUVEC-C cells. A positive association was observed between MNX1 and TrkB expression and the degree of malignancy of gliomas, and a series of experiments were performed to verify these results. The results of an adhesion assay demonstrated that MNX1 and TrkB reduced cell adhesion and enhanced the ability to resist anoikis in HUVEC-C and U-87 MG cells. Furthermore, ChIP and luciferase assays demonstrated that MNX1 may be an upstream

gene that regulates TrkB expression. Based on these results, it may be concluded that as a transcription factor, MNX1 may bind directly to TrkB and promote its transcription.

The present study investigated the expression and function of the transcription factor MNX1 in glioma. To the best of our knowledge, the present study is the first to indicate that MNX1 may directly regulate TrkB expression, which may increase their metastatic potential via suppression of anoikis and enhanced adhesion to the ECM. The binding site of MNX1 upstream of the TrkB gene was also identified. However, further validation of pathways downstream of TrkB that affect the sensitivity of cells to anoikis, as regulated by MNX1, require further investigation. The results of the current study may contribute to an improved understanding of the development of gliomas and provide a theoretical basis for future therapeutic strategies utilizing MNX1 as a molecular target.

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Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DZ and LJ conceived and designed the study. LJ, SC, JY, JC and CY performed the experiments and acquired the data. GZ analyzed and interpreted the data. DZ wrote, reviewed and revised the manuscript.

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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