

RNA interference targeting NRP-1 inhibits human glioma cell proliferation and enhances cell apoptosis

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Abstract. Neuropilin-1 (NRP-1) is a non-tyrosine kinase receptor for vascular endothelial growth factor (VEGF) that was recently found to play a role in tumor functions. Previous studies demonstrated that NRP-1 was overexpressed in a number of human tumors, including glioblastoma (GBM). However, the role of NRP-1 in glioma progression has yet to be adequately elucidated. Thus, we examined the expression of NRP-1 in human glioma cell lines using Western blotting, and cell cycle distribution and proliferation by transfection of the U373 cell line with NRP-1 short interference RNA (siRNA). Results showed NRP-1 siRNA to significantly reduce NRP-1 gene expression, decrease *in vitro* cell proliferation and induce cell apoptosis in cultured glioma cells, along with the accumulation of cells in the G1 phase and a decrease in cells in the S phase. Our results further revealed that NRP-1 knockdown decreased the expression levels of Bcl-2 family proteins and deactivated extracellular signal-regulated kinase (ERK) and c-Jun-N-terminal kinase (JNK)/mitogen-activated protein kinase (MAPK) signaling pathways, closely associated with cancer progression. Thus, our results provide a molecular mechanism for the effect of NRP-1 in tumors, rendering NRP-1 an attractive candidate as a therapeutic target in certain types of cancer, such as GBM.

Introduction

Tumors of glial origin, such as glioblastoma multiforme (GBM), constitute the majority of primary brain tumors. Every year tens of thousands of patients are diagnosed with GBM multiforme, and the prognosis of these patients remains extremely poor (1-3). GBM is the most common and aggressive brain tumor in adults. Patients with GBM have an average life expectancy of less than a year, even with aggressive

treatments including surgical resection, chemotherapy and radiation therapy (1,4,5). Glioma remains one of the most malignant types of human cancer (1,6). The main reason for the high mortality rate is that these high-grade gliomas recur following surgical resection with standard chemo- and radiation therapies (1). In addition, GBM is called a 'diffuse' glioma since it readily disperses beyond the tumor margins and into the brain parenchyma (4). Therefore, therapies that effectively target invasive glioma cells may significantly improve therapeutic outcome (7,8).

Neuropilin-1 (NRP-1) is a type I transmembrane glycoprotein and a coreceptor for two extracellular ligands, semaphorins/collapsins and vascular endothelial growth factor (VEGF) (9,10). In endothelial cells, NRP1 enhances VEGFR-2-mediated VEGF functions, including cell migration and angiogenesis (11,12). Additionally, genetic studies have provided strong evidence that NRP-1 is required for vascular morphogenesis. Loss of NRP-1 function results in vascular remodeling and branching defects (13). Previous studies reported that NRP-1 is overexpressed in a variety of cancer cells, and the overexpression of NRP-1 enhances tumor angiogenesis and tumor growth *in vivo* (5,11,14). In contrast, the inhibition of NRP-1 suppresses cell survival and migration (11). However, the functional significance of NRP-1 on tumor cells has yet to be fully elucidated.

Thus, we attempted to further elucidate the potential therapeutic value of NRP-1 for glioma. Moreover, we analyzed the phenotypes of cultured glioma cancer cells following RNA interference (RNAi)-mediated NRP-1 knockdown.

Materials and methods

Materials. Antibodies for Western blotting were as follows: anti-NRP-1 (A-12), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and anti-Bcl-2-associated death promoter (BAD) (C-17) were purchased from Santa Cruz Biotechnology, (Santa Cruz, CA, USA). Rabbit anti-Bcl-2, rabbit anti-p-BAD (Ser-112), rabbit anti-extracellular signal-regulated kinase (ERK)1/2 and specific anti-phospho-ERK 1/2 (p42/44) (Thr202/Tyr204), rabbit anti-c-Jun-N-terminal kinase (JNK) and specific anti-JNK (Thr183/Tyr185) were purchased from Cell Signaling Technology (Beverly, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA, USA).

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Cell culture. Human glioma cell lines U373MG, U87MG, LN319, U118MG and U251MG were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM supplemented with 10% FBS and 100 U/ml penicillin/streptomycin. The cells were incubated at 37°C with humidified air containing 5% CO₂.

siRNA transfection. The siRNA sequences for the target gene NRP-1 were developed and designed using the Ambion siRNA web design tool. The two target sequences utilized were NRP-1 siRNA1, 5'-AAGCTCTGGGCATGGAATCAG-3' and NRP-1 siRNA2, 5'-AAAGCCCCGGGTACCTTACAT-3'. In brief, cells were transfected with 100 nM of the indicated siRNA or a control siRNA (con siRNA, Invitrogen Inc., Carlsbad, CA, USA) using the Lipofectamine 2000 (Invitrogen Inc.), according to the manufacturer's instructions. After 24 h, siRNAs were removed and the cells were maintained in medium containing 10% FBS for an additional 48 h. The cells were harvested 48 h following transfection and used for the experiments.

Reverse transcriptase-polymerase chain reaction (RT-PCR). At 48 h following transfection, total RNA was extracted from the parental, con siRNA, NRP-1 siRNA1 and NRP-1 siRNA2 transfected U373MG glioma cells with the TRIzol reagent according to the manufacturer's instructions (Invitrogen Inc.). First strand cDNAs were synthesized by M-MLV reverse transcriptase (Fermentas, Lithuania) and subjected to PCR by specific primers for human NRP-1 (sense, 5'-CGTGGAAGTCTTCGATGGAG-3'; antisense, 5'-AAGAAATGGCCCTGAAGACA-3'). DNA was amplified using the following conditions: 95°C for 1 min, followed by 35 cycles of 95°C for 30 sec, 60°C for 30 sec and 72°C for 1 min. NRP-1 gene expression was normalized using internal control primers for the GAPDH gene, 5'-CCTTCATTGACCTCAACTAC-3' (sense) and 5'-GATGATGTTCTGGAGTGCC-3' (antisense). PCR products were separated in a 1.2% agarose gel and visualized by ethidium bromide staining. The experiments were repeated three times with similar results.

Western blotting. At 48 h following transfection, U373MG cells from parental, con siRNA, NRP-1 siRNA1 and NRP-1 siRNA2 were lysed directly on the culture dishes using lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.02% NaN₃, 1% Triton X-100, 1 mM PMSF, 1 µg/ml aprotinin and 1 µg/ml leupeptin). The protein concentration was determined by the Bradford assay kit (Bio-Rad, Hercules, CA, USA). Cell lysates containing equal amounts of protein were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (8-10%) and were then transferred to PVDF membranes. The membranes were initially blocked with 5% non-fat dry milk in Tris-buffered saline (TBS) for 1 h at room temperature and then incubated with the primary antibodies at 4°C overnight and HRP-conjugated secondary antibodies for 2 h at room temperature. Protein bands were then developed with enhanced chemiluminescence (ECL) detection reagents (Amersham Biosciences, Piscataway, NJ, USA). All analyses were performed in triplicate with similar results.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. U373MG cells were seeded in 96-well plates at a density of 1x10⁴ cells/well in 100 µl medium, and incubated in a 37°C humidified incubator for attachment. An MTT assay (Sigma, St. Louis, MO, USA) was performed according to the manufacturer's instructions. Briefly, cells were transfected with con siRNA and NRP-1 siRNA2 after 24, 48 and 72 h at 37°C in a 5% CO₂ and 95% air-humidified environment. Then 20 µl of 5 mg/ml MTT dissolved in phosphate-buffered saline (PBS) was added to each well. Cells were incubated for another 4 h, followed by the addition of 150 µl dimethyl sulfoxide (DMSO), and were agitated for 15 min to dissolve the formazan dye crystals. Absorbance was measured at 570 nm using a microplate reader. The cell survival rate was determined as: cell proliferation rate = (mean absorbance from transfected group/mean absorbance from parental group) x 100%. Each assay was performed in triplicate and the experiment was repeated on at least three separate occasions.

Cell apoptosis analysis. At 48 h following transfection, U373MG cells from parental, con siRNA and NRP-1 siRNA2 were trypsinized, collected, washed and then stained with Annexin V-FITC (BD Biosciences, San Diego, CA, USA) and propidium iodide (PI, BD Biosciences) for 10 min at 4°C according to the manufacturer's instructions. Apoptotic cells were determined by a FACScan flow cytometer (Becton-Dickinson, San Jose, CA, USA).

Cell cycle analysis. At 48 h following transfection, cells from parental, con siRNA and NRP-1 siRNA2 were collected by trypsinization and centrifugation, washed twice with cold PBS, and fixed with cold 70% ethanol at 4°C overnight. The fixed cells were collected, washed twice with PBS and suspended in PBS containing 10 µg/ml PI (Sigma-Aldrich, St. Louis, MO, USA) and 100 µg/ml RNase A (Invitrogen Inc.), and incubated at 37°C for at least 30 min avoiding light to eliminate the intracellular RNA. Cell cycle distribution was determined using a FACScan flow cytometer (Becton-Dickinson).

Statistical analysis. Experiments were performed in duplicate or triplicate. The results were presented as the mean ± standard deviation (SD). The data were analyzed using one-way analysis of variance (ANOVA) to determine where differences among groups existed. P<0.05 was considered to be statistically significant, and this was indicated by asterisks in the figures.

Results

NRP-1 expression in different glioma cell lines. We examined the expression of NRP-1 in human glioma cell lines by Western blot analyses. As shown in Fig. 1, NRP-1 protein was detected at various levels in all of the glioma cell lines examined. It should be noted that there was no markedly detectable expression of NRP-1 in the U251MG cell lines.

Endogenous NRP-1 expression was suppressed by RNAi. First, RT-PCR was performed to measure the NRP-1 mRNA levels from the parental, con siRNA, NRP-1 siRNA1 and NRP-1 siRNA2 transfected glioma U373MG cells for 48 h. As shown in Fig. 2A, the expression of NRP-1 was suppressed by approxi-

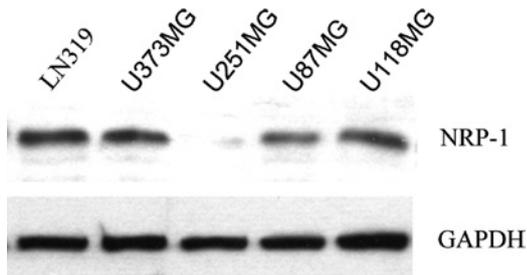


Figure 1. Neuropilin-1 (NRP-1) expression in the glioma cell line U373MG. Western blot analysis of NRP-1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in LN319, U373MG, U251MG, U87MG and U118MG cell lines.

mately 50 and 85%, respectively, compared with the parental and the con siRNA group. However, the siRNA oligonucleotides did not cause a non-specific inhibition of gene expression, as shown by the expression of GAPDH. In addition, the NRP-1 protein levels were also significantly decreased, consistent with the pattern of the transcript levels, and as shown in Fig. 2B, the expression of NRP-1 was reduced by approximately 47 and 77% when treated with NRP-1 siRNA1 and NRP-1 siRNA2 for 48 h, respectively. These results indicated that NRP-1 expression was significantly inhibited by siRNA.

Effect of NRP-1 siRNA on the proliferation and cell cycle of U373MG. To explore whether the down-regulation of NRP-1 expression in glioma cells affected cell proliferation and cell cycle distribution, the U373 cell line was transfected with or without NRP-1 siRNA. As shown in Fig. 3A, cell proliferation was significantly suppressed by NRP-1 siRNA2 in U373MG cells as compared with the other two groups following transfection at 48 and 72 h. However, no significant change was found between the parental and con siRNA groups.

To determine whether the cell proliferation inhibition of the glioma cells we observed by the RNAi-mediated inhibition of NRP-1 was caused by the disruption of the cell cycle transition with delay in mitotic entry, we further determined the cell cycle distribution by analyzing the DNA contents of cell populations. As shown in Fig. 3B, a marked increase in the G1 population with a concomitantly significant decrease in the S population was observed after U373MG cells were transfected with NRP-1 siRNA2 for 48 h. These results indicate that the siRNA-mediated inhibition of NRP-1 significantly suppressed cell proliferation and induced cell cycle arrest at the G1 phase of the glioma cell.

RNAi-mediated inhibition of NRP-1 enhances cell apoptosis of U373MG. We further determined the effect of the siRNA-mediated inhibition of NRP-1 on the cell apoptosis of glioma cells 48 h following transfection. Apoptosis was measured with an Annexin V/FITC kit according to the manufacturer's instructions. As shown in Fig. 4A, NRP-1 siRNA2, but not con siRNA, increased cell apoptosis in U373MG cells compared to the parental cells. Cell apoptosis in NRP-1 siRNA2-infected cells was almost 2-fold greater compared to that of the controls (Fig. 4A), suggesting a marked increase in cell apoptosis by the down-regulation of NRP-1 by RNAi.

The Bcl-2 family acts to suppress or delay the induction of apoptosis in a number of systems, and the Bcl-2-associated death promoter (BAD) protein is a pro-apoptotic member of the Bcl-2 gene family, which is involved in initiating apoptosis (15,16). Since cell apoptosis was increased by NRP-1 knockdown in U373MG cells, we investigated whether Bcl-2 family proteins were involved in this process. To clarify the molecular mechanism of NRP-1 siRNA on cell apoptosis, the immunoblot analysis of the Bcl-2, BAD and p-BAD Ser112 proteins showed that levels of Bcl-2 and p-BAD Ser112

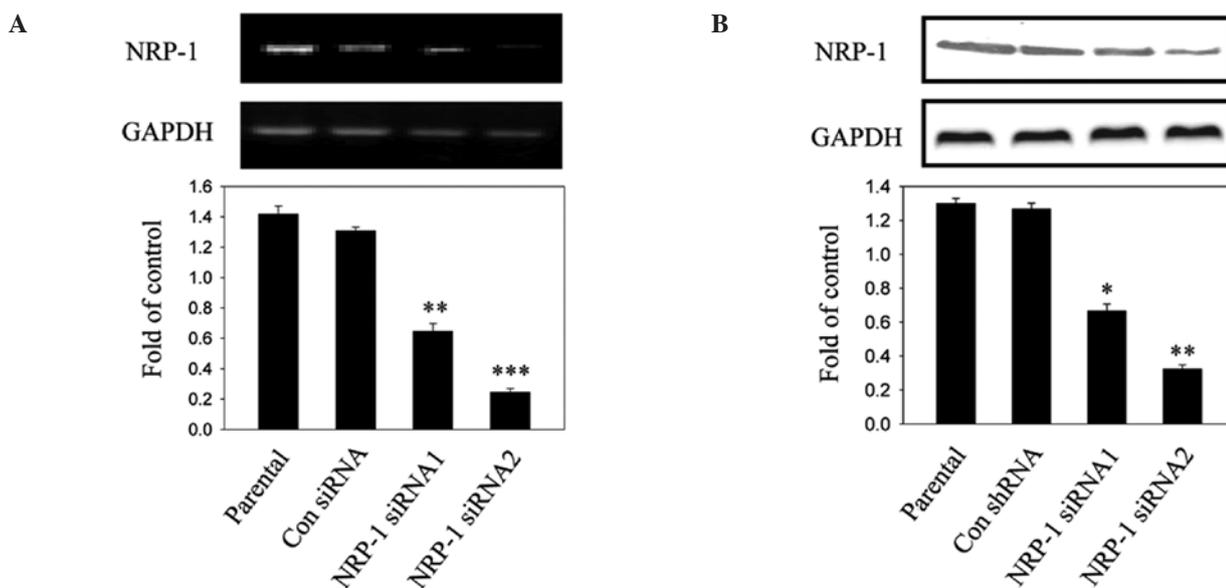


Figure 2. Suppression of neuropilin-1 (NRP-1) expression by siRNA in U373MG cells. (A) The expression of NRP-1 mRNA was examined by reverse transcriptase-polymerase chain reaction (RT-PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control. (B) The protein levels were analysed by Western blot analysis. NRP-1 DNA and protein band quantification were obtained by densitometric analysis of the DNA and protein band area. The protein product quantified was relative to the internal control GAPDH. Experiments were performed at least two times with consistent and repeatable results. Each bar is the mean \pm SD. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$.

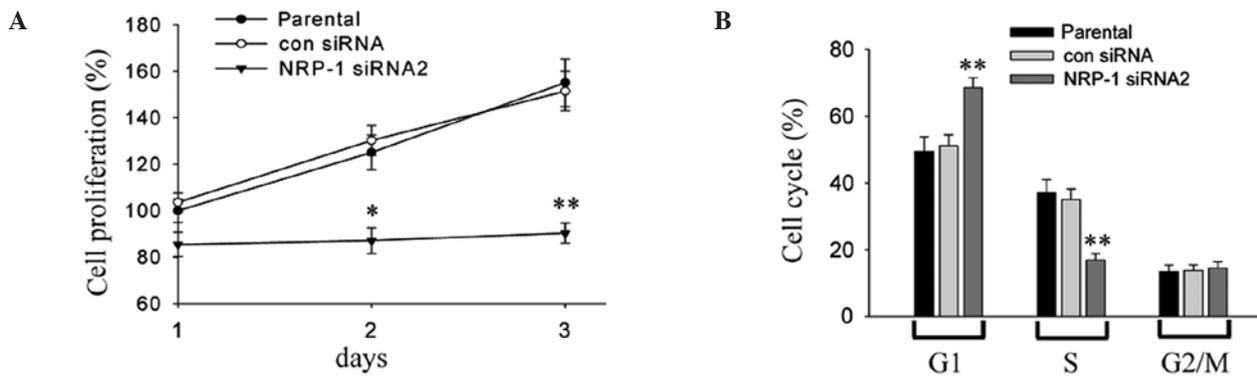


Figure 3. Short interference RNA (siRNA)-mediated inhibition of neuropilin-1 (NRP-1) blocks cell proliferation and induces G1 phase accumulation. (A) U373MG cells were transiently transfected with NRP-1 siRNA for 24, 48 and 72 h respectively, and a MTT assay was performed to assess cell proliferation. (B) Cells were collected 48 h following siRNA transfection at 100 nM and subsequently assayed for their DNA content by flow cytometry. Each bar is the mean \pm SD. * $P < 0.05$ and ** $P < 0.01$. All of the experiments were repeated at least three times.

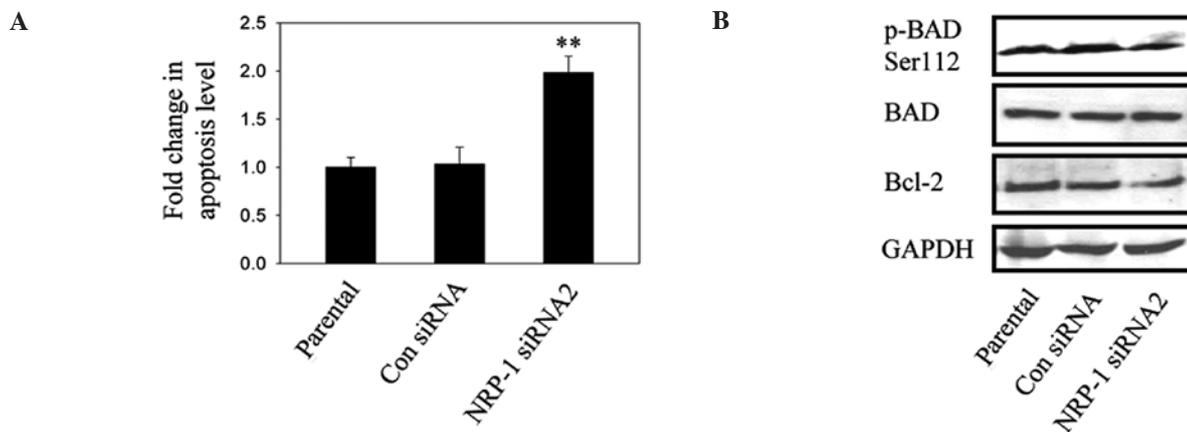


Figure 4. RNAi-mediated inhibition of neuropilin-1 (NRP-1) enhances cell apoptosis and suppresses Bcl-2 family protein expression. (A) Cells were harvested 48 h following short interference RNA (siRNA) transfection at 100 nM and cell apoptosis was evaluated. The apoptotic index was determined as the ratio of apoptotic cell number to total cell number. Each bar is the mean \pm SD. ** $P < 0.01$. (B) Western blot analysis shows the expression of p-BAD Ser12, BAD and Bcl-2 with specific antibody, respectively. All of the experiments were repeated independently at least two times.

slightly decreased in the NRP-1 siRNA2 transfected cells, compared with the other two groups; whereas no significant differences were found among the parental, con siRNA and NRP-1 siRNA2 groups (Fig. 4B).

RNAi-mediated inhibition of NRP-1 decreases phosphorylation of ERK1/2 and JNK. The MAPK signaling pathway plays a key role in regulating a number of cellular activities (16-18). Stress-activated protein kinase/JNK and ERK are two major pathways of MAPK. Generally, the JNK promotes inflammation, apoptosis, growth, differentiation and oncogenic transformation, whereas ERK is involved in growth, differentiation and development (19-21). Therefore, we analyzed the effect of NRP-1 siRNA on the MAPK signaling pathway.

As shown in Fig. 5, there was a marked decrease in the expression of p-ERK1/2 and p-JNK by NRP-1 knockdown in the U373MG cells, compared with that of the remaining two groups. However, the levels of total ERK1/2 and JNK did not alter in either group. These findings suggest that inhibition of NRP-1 by siRNA blocked the phosphorylation of ERK1/2 and JNK.

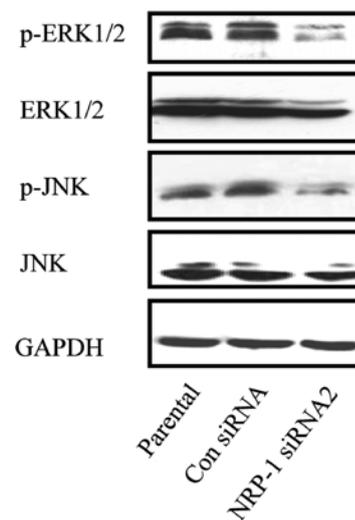


Figure 5. RNAi-mediated inhibition of neuropilin-1 (NRP-1) suppresses mitogen-activated protein kinase (MAPK) signaling in U373MG cells. Total proteins from parental, con short interference RNA (siRNA) and NRP-1 siRNA2 groups were analysed by Western blotting for phosphorylated and total extracellular signal-regulated kinase (ERK)1/2 and c-Jun-N-terminal kinase JNK. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown as a loading control.

Discussion

NRP-1, initially described as an axon guidance factor, was discovered to be a co-receptor for VEGF, stimulating endothelial cell migration, angiogenesis and tumor growth (22,23). The importance of NRP-1 as a functional receptor in neurobiology has been well established, but its role in tumor functions has only recently been identified. Since NRP-1 is present in various types of tumor, and overexpression of NRP-1 promotes tumor cell survival and migration, we investigated whether NRP-1 serves as a potential target for anticancer therapy in glioma. In the present study, the RNAi-mediated inhibition of NRP-1 in glioma cells was found to significantly suppress cell proliferation and induce a cell cycle arrest at the G1 phase. Furthermore, we found that the inhibition of NRP-1 markedly decreased the cell viability, and increased cell apoptosis in glioma cells.

It was previously shown that the blockade of NRP-1 inhibits tumor cell survival and migration (11). However, the mechanism is less understood. Certain recent studies demonstrated that NRP1 inhibition of endothelial cell function and angiogenesis appeared to be partly independent of VEGF. Moreover, no effects of NRP-1 blocking antibody/peptide on tumor cell proliferation were observed (11,13). Thus, we examined the role of NRP-1 in cancer therapy. In the present study, the novel results indicate that NRP-1 is an attractive candidate for a therapeutic target. Western blot analysis data showed that Bcl-2 and p-BAD Ser112 were down-regulated by NRP-1 siRNA, which may be one of the reasons to explain the observed cell cycle arrest and apoptosis.

Another significant finding of the present study is that NRP-1 inhibition disturbed the MAPK signaling pathway by suppressing the phosphorylation of ERK1/2 and JNK. We found that the expression of p-ERK1/2 and p-JNK were significantly suppressed by NRP-1 knockdown in the U373MG cells, compared with that of the other two groups (Fig. 5), whereas total ERK1/2 and JNK protein levels did not change. Our findings were not completely consistent with those of Raskopf *et al* (22), which showed that inhibition of NRP-1 expression led to strong anti-endothelial effects, whereas tumor cells and tumor growth were not affected. Furthermore, inhibition of NRP-1 by siRNA did not affect phosphorylation of AKT, ERK, PI3K or p38 MAPK in a mouse hepatoma cell line, Hepa129. These discrepancies may be due to the different species and cell types used. Significantly, both ERK and JNK are activated by the sequential protein phosphorylation of the MAPK signaling pathway (24). Therefore, our results suggest that NRP-1 inhibition in glioma cells promotes cell apoptosis via suppression of the ERK1/2 and JNK signaling pathways.

In conclusion, we have shown that RNAi significantly reduced gene expression and protein levels of NRP-1. In addition, we found that NRP-1 silencing markedly suppressed *in vitro* cell proliferation and induced cell apoptosis in cultured glioma cells, by down-regulation of Bcl-2 family expression and blocked the mitogen-activated protein kinase (MAPK) signaling pathway. However, this process alone could not clearly elucidate the mechanism we observed. Therefore, further studies are required to explore whether other upstream or downstream signaling molecules were affected by NRP-1

siRNA. These results demonstrate a specific role for NRP-1 in glioma cells. Thus, RNAi of the NRP-1 gene may be an effective therapeutic strategy for human glioma in the future.

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