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Netrin-4 Promotes Glioblastoma Cell Proliferation through Integrin β_4 Signaling[1,2]

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Abstract

Netrin-4 is a laminin-related secreted molecule originally found to have roles in neuronal axon migration. Recent studies have indicated that netrin-4 also participates in the development of nonneural tissues and modulates tumor cell proliferation and tumor metastasis. Here we have explored the functions and molecular mechanisms of netrin-4 in glioblastoma multiforme. The suppression of netrin-4 expression in glioblastoma cell lines significantly reduced cell proliferation and motility and increased serum deprivation–induced apoptosis. Using tandem affinity purification combined with protein identification by mass spectrometry, we found that integrin β_4 interacts with netrin-4 and that it mediates mitogenic effects as well as AKT and mammalian target of rapamycin phosphorylation induced by netrin-4. Interestingly, netrin-4 acted as an inhibitor of cell proliferation in integrin β_4–silenced glioblastoma cells, and high concentrations of netrin-4 reduced cell proliferation. The negative effects of netrin-4 on proliferation were mediated by UNC5B. Analysis of more than 400 primary tumors from The Cancer Genome Atlas repository revealed that the expression of netrin-4 is significantly downregulated in glioblastoma and that the reduced expression is linked to poor patient survival time. The expression of integrin β_4 is increased in glioblastoma, and it predicts poor patient survival time. Current results illustrate a novel mechanism for glioma progression, where glioma cells reduce netrin-4 expression to decrease its inhibitory effects. In parallel, the expression of integrin β_4 is upregulated to sensitize the cells to low concentrations of netrin-4 for maintaining cell proliferation.

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Abbreviations: AKT, serine/threonine-protein kinase akt (protein kinase B); DCC, netrin receptor deleted in colorectal cancer; GBM, glioblastoma multiforme; ITGB4, integrin β_4; mTOR, mammalian target of rapamycin; NTN, netrin; Q-RT-PCR, quantitative real-time polymerase chain reaction; TAP-TAG, tandem affinity purification; TCGA, The Cancer Genome Atlas; UNC5, netrin receptor uncoordinated 5

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[2]This article refers to supplementary materials, which are designated by Figures W1 to W8 and Supplemental Data Set and are available online at www.neoplasia.com.
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Introduction

Netrins (NTNs) are laminin-related secreted molecules with roles in embryogenesis and tumor development. So far, five mammalian members have been identified in this family: netrin-1, -3, -4, -G1, and -G2 [1].

Netrin-1 (NTN1) has been defined as a neuronal guidance cue displaying both attractive and repulsive roles for neuronal cells and axons. Both netrin receptor deleted in colorectal cancer (DCC) and netrin receptor uncoordinated 5 (UNC5s) are major receptors mediating the attractive and repulsive effects of NTN1 [1,2]. Similar to the dual role in axon guidance, the biphasic function of NTN1 appears in the development of the vasculature, reflected by contrasting results in various studies [3–6]. NTN1 can also modulate the development of many other tissues such as the mammary gland, the pancreas, and the lung [7–9]. During tumor progression, NTN1 acts as a survival factor for numerous types of tumor cells during tumor progression through a "dependence-receptor" mechanism [10].

Another member of the NTN family, netrin-4 (NTN4), is expressed throughout the central nervous system (CNS) and brings beneficial effects for the neuronal development [11–13]. Recent studies have illustrated that NTN4 participates in the development of nonneural tissues by modulating the adhesion, migration, proliferation, and apoptosis of endothelial cells [5,14–17]. NTN4 can also act as a regulator of tumor cell proliferation, apoptosis, angiogenesis, and metastasis [15,18,19]. However, the results of the effects of NTN4 have been discordant in the regulation of tumor progression. NTN4 can also act as an inhibitor of both tumor growth and angiogenesis with relatively high concentrations of NTN4 [16,18,19]. NTN4 has also beneficial effects for tumor cells by promoting tumor cell proliferation, angiogenesis, and lymphangiogenesis at relatively low concentrations [5,15,19]. The expression of NTN4 is upregulated in the effusions or invading edge of solid tumor compared with corresponding tumor core [20,21], suggesting that NTN4 may have roles in tumor cell migration and invasion.

Several molecules have been identified to interact or form complex with NTN4, such as neogenin, UNC5B, UNC5D, laminin γ chain, and integrin $\alpha_6\beta_1$, integrin $\alpha_3\beta_1$, integrin $\alpha_2\beta_1$ [12,16,17,22–24]. However, the roles of these interactions are still under discussion, and the biologic functions mediated by these molecules remain largely unclear.

Glioblastoma multiforme (GBM) is the most common malignant tumor of CNS [25]. NTN4 is strongly expressed by astrocytes [11] and astrocyte stem cells [12]. White matter–invading glioblastoma cells express more NTN4 than tumor cores do [20]. However, the functions and molecular mechanisms of NTN4 in glioblastoma need more elucidation. We have explored here the biologic functions of NTN4 in glioblastoma cell lines and analyzed the potential underlying molecular mechanisms for growth modulation.

Materials and Methods

Cell Lines and Reagents

293FT cells (Invitrogen Life Technology, Carlsbad, CA), Astrocytes (Lonza, Switzerland), U251MG cells (Health Science Research Resources Bank, Osaka, Japan), and U87MG and U373MG (American Type Culture Collection, Rockville, MD) were cultured according to the supplier's instructions. Cell migration and proliferation assays were performed by using recombinant NTN4 (R&D Systems, Minneapolis, MN) as modulator.

The following primary antibodies were used: anti-HA.11 from Covance, Princeton, NJ; anti-NTN4 from R&D Systems; anti-ITGB4 from Sigma-Aldrich, St Louis, MO; anti–phospho-AKT (Ser473), anti–phospho-p44/43MAPK (ERK1/2) (Thr202/Tyr204), and anti–phospho-mammalian target of rapamycin (mTOR; Ser2448) from Cell Signaling (Danvers, MA); and anti-BrdU and anti–β-tubulin from Santa Cruz Biotechnology (Santa Cruz, CA).

Bromodeoxyuridine Incorporation Assay

Cells were cultured on a 96-well plate (PerkinElmer, Waltham, MA) with the indicated medium. Subsequently, the medium was replaced to medium with 5-bromo-2-deoxyuridine (BrdU) and incubated for 70 (serum containing) or 120 minutes (serum starvation). After incubation, the BrdU labeling medium was removed, and the cells were washed with phosphate-buffered saline (PBS) twice for 5 minutes each. The cells were then fixed with 70% ethanol containing glycine at −20°C for 30 minutes. Next, the cells were washed three times with PBS and incubated in 2 M HCl at room temperature for 60 minutes. Subsequently, the cells were rinsed and treated with PBS containing 3% bovine serum albumin (BSA) at room temperature for 30 minutes and incubated with anti-BrdU antibodies. The primary antibody was detected by A594 Alexa Fluor secondary antibodies (Invitrogen). After Hoechst staining at 4°C for 15 minutes, cells were washed again. Finally, the images were captured and quantified by using ArrayScan 4.5 high-content-screening system (Cellomics, Pittsburg, PA).

Tandem Affinity Purification and Protein Identification by Mass Spectrometry

For tandem affinity purification (TAP-TAG) and mass spectrometry analysis, we separated and amplified NTN4 full-length sequence to five fragments based on functional domains (Figure W4*B*).

Using the lentivirus system, we successfully obtained U251MG cells expressing FLAG/HA–tagged fragments and full-length NTN4 complementary DNA (cDNA). Immunofluorescence and immunoblot analysis with anti-HA antibody were performed to confirm target sequence expression (Figure W4, *B* and *C*). U251MG cells expressing the indicated protein were lysed in RIPA buffer (50 mM Tris-HCl buffer pH 7.4, containing 150 mM NaCl, 1% NP-40, 0.1% SDS), and lysates were incubated with anti-FLAG M2 Affinity Gel (Sigma-Aldrich) at 4°C overnight. Subsequently, Sepharose beads were washed with RIPA buffer for five times using micro bio-spin column (Bio-Rad, Hercules, CA). FLAG-containing proteins were eluted by incubating the Sepharose beads with 3× FLAG peptide (Sigma-Aldrich) for 30 minutes on ice. Eluates were incubated with anti-HA Affinity Gel (Sigma-Aldrich) at 4°C for 2 hours. Sepharose beads were washed for five times in spin column, and proteins were eluted with Laemmli sample buffer for 5 minutes on ice.

For identification, proteins were separated by SDS-PAGE and visualized by silver staining followed by in-gel digestion with trypsin and liquid chromatography–tandem mass spectrometry analysis of the resulting peptides as previously described [26]. The nonspecifically interacting proteins identified from control samples were excluded from the identification lists of interacting proteins for full-length NTN4 and fragments of it to create the final list of candidate interacting proteins (Supplemental Data Set).

Statistical Analysis

All numerical values represent mean ± SE or SD. Statistical significance was determined with the nonparametric Mann-Whitney *U* test.

Lentiviral transfection, quantitative real-time polymerase chain reaction (Q-RT-PCR), migration assay, apoptosis assay, immunofluorescence, and immunoblot analysis were carried out using standard protocols, and these are described in detail in Supplemental Materials