

The Role of NG2 Proteoglycan in Glioma

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Abstract

Neuron glia antigen-2 (NG2), also known as chondroitin sulphate proteoglycan 4, or melanoma-associated chondroitin sulfate proteoglycan) is a type-1 membrane protein expressed by many central nervous system (CNS) cells during development and differentiation and plays a critical role in proliferation and angiogenesis. 'NG2' often references either the protein itself or the highly proliferative and undifferentiated glial cells expressing high levels of NG2 protein. NG2 glia represent the fourth major type of neuroglia in the mammalian nervous system and are classified as oligodendrocyte progenitor cells by virtue of their committed oligodendrocyte generation in developing and adult brain. Here, we discuss NG2 glial cells as well as NG2 protein and its expression and role with regards to CNS neoplasms as well as its potential as a therapeutic target for treating childhood CNS cancers.

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Introduction

Neuron glia antigen-2 (NG2) glia are chondroitin sulfate proteoglycan 4 protein, expressing cells abundantly present in the developing brain as well as in the adult central nervous system (CNS). NG2 glia actively proliferate and differentiate into mature oligodendrocytes, thus have been characterized as oligodendrocyte progenitor cells (OPCs). NG2 expressing OPCs have diverse functions that include physiologic support of neurons and synaptic signaling with NG2 protein being an important player to execute these functions in healthy brain as well as in brain injury repair and regeneration. Additionally, NG2 protein has also been found to play a critical role in tumorigenesis and tumor progression. Since NG2 expressing OPCs have been identified as the cell of origin in gliomas, it is important to explore the role of NG2 in gliomagenesis. Here, we will first review the characteristics of NG2 protein and NG2 expressing OPCs and then discuss the role of NG2 protein in relation to gliomas and the possibility of using NG2 as a therapeutic target.

NG2 Protein

NG2 protein, encoded by the chondroitin sulfate proteoglycan 4 gene, is highly expressed in developing and adult CNS [1]. In extra neural tissues,

NG2 was originally thought to be expressed during development in progenitor cells like mesenchymal stem cells, chondroblasts, osteoblasts, immature keratinocytes, muscle progenitors, and melanocytes [2]. Subsequent studies supported the presence of NG2 in various post natal tissues that include bone marrow smooth muscle, interfollicular epidermis in skin, musculoskeletal junctions, pancreas, lungs, eyes, heart, and kidneys [3–6]. However, the widespread NG2 expression in extra neural tissues during development in the undifferentiated cell state is highly down-regulated during differentiation [7]. Pericytes that ensheath endothelial layer of blood vessels also express NG2 [8]. In

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pericytes, NG2 expression is important for pericyte localization to endothelial layer and interaction with endothelial cells [8,9]. NG2 deficiency during early development results in loss of pericyte-endothelial association and defective formation of basement membranes in blood vessels [10]. Below, we will further discuss the role of NG2 expression in microvasculature associated-pericytes in relation to CNS tumors [11]. In addition, NG2 expression in postnatal state is associated with response to injury-induced inflammation and certain pathological conditions including CNS tumors, soft tissue sarcomas, and melanomas [12–14].

The expression of NG2 is tightly regulated by a 1,585 base pair promoter region upstream of translation initiation site [13]. NG2 promoter contains binding sites for p300 and CREB binding protein which function as co-activators to regulate gene expression [13]. At the transcription level, we have shown that NG2 mRNA is targeted and regulated by microRNA (miR129-2), which binds 3'UTR of NG2 mRNA [15]. Targeting miR129-2 provides potential targeting avenues for regulating NG2 in glioma which is further elaborated in this review.

NG2 protein is a membrane spanning proteoglycan with a molecular weight of 252 kDa in its native form and 300 kDa in glycosylated state. NG2 consists of a large extracellular domain with 2,225 amino acids that makes up for 95% of the protein, a transmembrane domain with 25 amino acids, and a short cytoplasmic tail of 76 amino acids [2,16] (Figure 1). These domains facilitate the interaction of NG2 with extracellular and intracellular ligands to activate signaling events that are mediated through focal adhesion kinase and MAP kinase pathways and regulate important cellular functions such as cell proliferation, migration, invasion, cytoskeletal reorganization, survival, chemoresistance, and modulation of neuronal network [2,17]. In NG2 dependent signal

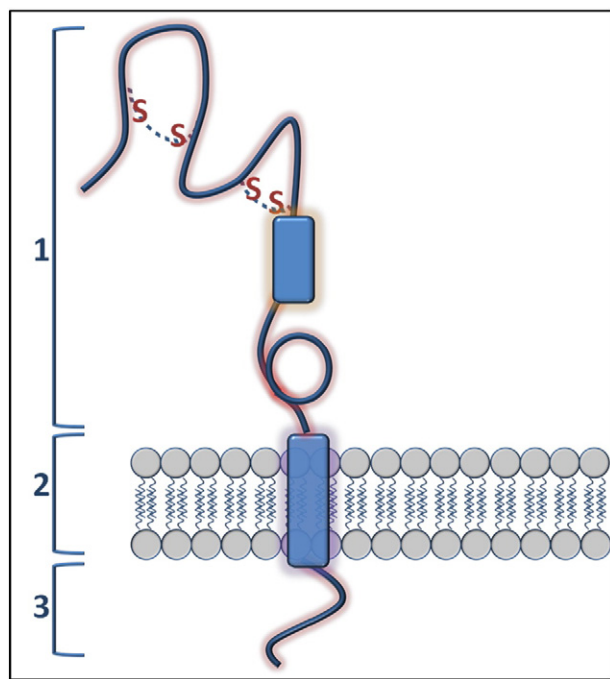


Figure 1. The structure of NG2 protein. NG2 protein is a transmembrane protein that consists of a (1) large transmembrane domain consisting of 2,225 amino acids, (2) a small transmembrane domain (25 amino acids) and (3) a short cytoplasmic c-terminal domain consisting of 76 amino acids. The extracellular domain consists of the n-terminal, disulfide bonds, and a CSPG repeat domain.

transduction, NG2 functions as a co-receptor in conjunction with PDGFR alpha for receptor tyrosine kinase PDGF to activate focal adhesion kinase and MAP kinase pathways [18–20]. The intracellular or cytoplasmic domain of NG2 contains binding sites for multi-PZD domain protein 1 (MUPP1), which facilitates the physical interaction of NG2 with the key structural and/or signaling components in the cytoplasm [21]. The cytoplasmic domain also contains binding sites for synaptic protein GRIP1 and syntenin-1 that are important for NG2 mediated cellular migration [22,23]. The two phosphorylation sites on intracellular domain, Thr 2256 and 2314 are phosphorylated by PKC α and ERK, respectively [24]. β 1 integrin-mediated signaling of cell motility and proliferation has been shown to be balanced through interaction with differentially phosphorylated NG2, which results in localization of integrin protein to the cell surface [24]. Cell surface localization is followed by β 1 integrin binding to the NG2 extracellular domain which also contains binding sites for collagens II, V, and VI, galectin, laminin, and tenascin [25]. NG2 binding to these proteins facilitates enhanced cellular adhesion. According to recent studies, activity dependent sequential cleavage of NG2 by α and γ secretase results in releasing of extracellular and intra cellular domains [17]. The cleaved peptides will then become biologically functional molecules regulating neuronal network by bidirectional communication between neurons and oligodendrocyte precursors [17]. In addition, the two conserved N-terminal domains of NG2 extracellular domain (laminin neurexin sex-hormone binding globulin domains) were found to be important for neuromodulation [17].

NG2 Glia Cells

Neuroglia, also known as glia, are non-neuronal cells derived from ectoderm during embryonic development [26]. Glia are present throughout the mammalian nervous system and maintain homeostasis, form myelin, and provide support and protection for neurons [27–29]. CNS glial cells are categorized into three types: astrocytes, oligodendrocytes, and microglial cells [26]. More recently, a fourth major glial cell has been identified, expressing NG2 as an integral membrane chondroitin sulphate proteoglycan [30,31]. The NG2 glial cells are generated from neural stem cells through glial restricted progenitors [32] and are present in large numbers throughout the developing and mature CNS. In the developing murine brain, NG2 glial cells emerge in three different regional waves at different times [33]. The first wave to emerge is in the ventral medial ganglionic eminence at embryonic day (ED) 12.5 and subsequently migrate dorsally to cerebral cortex and proliferate throughout the cortex [33]. The second wave appears around ED 16 in the lateral ganglionic eminence and migrate to telencephalon. Cells generated during these two waves disappear over time, but the NG2 glia formed during the third wave of formation in the cerebral cortex at post natal day 0 survive and expand throughout the brain [33]. This proliferation and differentiation of NG2 glial cells is regulated by Sonic-Hedgehog signaling, helix-loop-helix, HMG domain transcription factors, and epigenetic mechanisms regulating the expression of cell cycle genes Cdc2 and methylation enzymes Dnmt1 [34–36]. In adult rat brain, NG2 glia are mainly found in the corpus callosum and in the gray matter regions [37].

The original observation that NG2 glial cells give rise to mature oligodendrocytes in the CNS led to an initial designation of NG2-glia cells as OPCs. However, subsequent research described NG2 glia as bi-potential oligodendrocyte-type 2 astrocyte progenitors capable of forming oligodendrocytes and type 2 astrocytes. These type 2

astrocytes exist in limited numbers in CNS, express ganglioside marker A2B5 and lack astrocyte protein GFAP, and Ran2 [38,39]. Based on these observations, NG2 glia cells have more recently been referred to as polydendrocytes [31]. Although some studies suggest the possibility of NG2-glial generation of neurons [40,41], further studies in support of these observations are warranted.

Microenvironment within the brain seems to contribute to the ability of NG2 glial cells to form oligodendrocytes or astrocytes [38]. Fate mapping of NG2 glia has been achieved using NG2creBAC:ZEG double transgenic mice [38]. In these mice, active Cre resulted in the constitutively expressed EGFP under the NG2 promoter. Fate mapping showed that NG2 cells to be able to give rise to oligodendrocytes in both gray and white matter of the brain and spinal cord [38,42,43]. Furthermore, in the gray matter of the ventral forebrain and spinal cord, NG2 expressing glial cells give rise to protoplasmic astrocytes. Protoplasmic astrocytes are defined by being predominantly present in white matter, having fewer glial filaments, and exhibiting irregular contours when compared to fibrous astrocytes [38,44].

NG2 OPCs have been reported as the glioma precursor cells in adult gliomas [45–47]. The failure of OPCs to express asymmetric levels of NG2 during mitotic division constitutes an important step in glioma formation [47] (Figure 2). In healthy OPCs, only one daughter cell inherits NG2 expression while the NG2 expression in the second daughter cell is silenced [47]. This segregated NG2 expression is accompanied by co-expression of trophic factors including platelet-derived growth factors (PDGF) in the pertinent daughter cell. PDGF signaling contributes to glioma tissue remodeling, which is known to be important for transformation of adjacent glial cells in the local tumor microenvironment [46]. Symmetric segregation of NG2 in glioma precursors result in an increased population of uncommitted NG2 glioma precursor cells within the tumor, which facilitates EGF-dependent proliferation and self-renewal.

Role of NG2 Protein in Glioma

High NG2 expression is found in human adult glioma and is associated with aggressive disease course and poorer survival. Gliomas are malignant tumors arising from glial cells and include astrocytoma (arising from astrocytes), oligodendroglioma (originating from oligodendrocytes or OPCs), and oligoastrocytoma (with mixed glial

cell origin) [48]. The most common and malignant form of glioma, Glioblastoma multiforme (GBM) is an astrocytoma, which is highly invasive and the invasion and migration of this tumor into the CNS involves the interaction of tumor cells with the host's cells and extracellular matrix molecules. Analysis of mRNA data of human GBM samples using The Cancer Genome Atlas revealed that NG2 is one of the highly upregulated proteoglycans [49]. NG2 expression in GBM increases the invasive and migratory capabilities of glioma cells by facilitating interactions with extracellular matrix proteins such as collagen VI and laminin 2 [25,50]. NG2 interaction with collagen is facilitated at the nonglobular domain which is modified with glycosaminoglycan chains [50,51]. However, modification does not play a significant role in the collagen binding of NG2. Given the relatively low quality of collagen in the brain parenchyma, it is plausible that NG2 may facilitate the cellular migration by binding the vascular associated collagen VI [52]. These proteins are important for cell adhesion and motility and thus play an important role in progression of neoplasia. Indeed, B28 rat glioma cells expressing mutant NG2 protein lacked collagen-binding sites and exhibited retardation of tumor cell migration [50]. This property of NG2 protein may contribute to the highly proliferative and infiltrative nature of diffused pontine gliomas [15]. NG2 expression in pericytes and the basement membrane components of tumor vasculature facilitates angiogenesis and thus tumor growth by sequestering angiostatin, which is known to inhibit neovascularization [53]. In glioma murine models, NG2 expression drives increased vascular leakiness, vasogenic edema, tumor volumes, and necrosis resulting in dysregulation of the host-derived tumor vasculature [54].

While the expression and role of NG2 in adult gliomas is well established, little is known about role of NG2 expression in pediatric brain tumors. A study by Chekenya and colleagues (2002) reported the overexpression of NG2 in pediatric brain tumors that included two medulloblastomas, which is the most common type of pediatric malignant brain tumor occurring in the cerebellum and one pilocytic astrocytoma, which is a benign low-grade tumor [55]. However, NG2 expression was not detected in the cell culture models of medulloblastoma [56,57]. Recently, differential expression of NG2 was reported in a cohort of 57 various pediatric brain tumor samples obtained at biopsy [57]. High NG2 expression was detected in all dysembryoplastic neuroepithelial (DNETs) tumors, and two of the fourteen

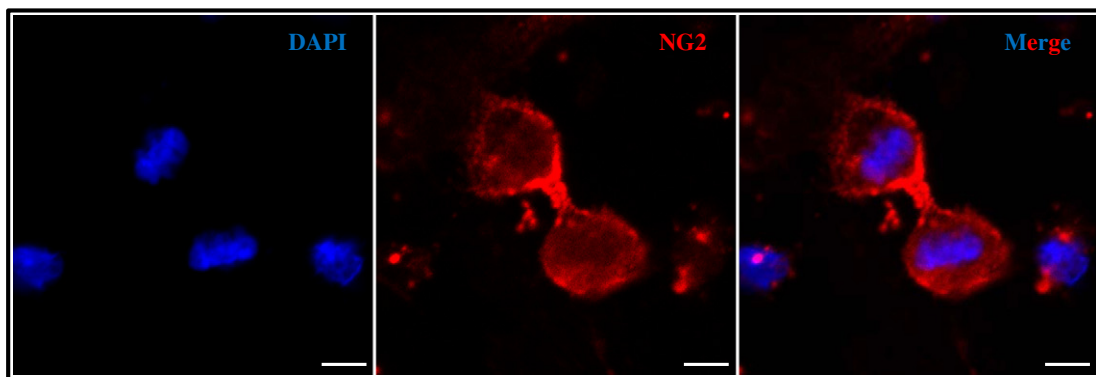


Figure 2. Aberrant symmetric segregation of NG2 in mouse glioma cells. Mouse glioma neurospheres were expanded in culture and assessed for NG2 expression. Immunocytochemical assays using NG2 antibody (red) and DAPI (blue) nuclear staining showed symmetric NG2 expression in both daughter cells resulting in continuous post mitotic NG2 expression which may contribute to tumorigenicity of these cells. Scale bar = 5 μ m.

medulloblastoma samples studied [57]. Other pediatric tumors including astrocytoma, pilocytic astrocytoma, ependymoma that arise from ependymal cells lining the ventricles and spinal cord, and supratentorial primitive neuroectodermal tumor, showed varied NG2 expression levels. We have recently shown NG2 expression to be associated with childhood diffuse intrinsic pontine glioma (DIPG) that occurs exclusively in children. DIPG is the most aggressive and infiltrative form of tumor accounting for 10% to 20% of pediatric CNS tumors. In a cohort of 50 samples obtained at postmortem, NG2 expression was detected in 78% of DIPG specimens tested [15] (Figure 3). In accordance with the observation made by Sugiarto and colleagues (2011), primary human and murine DIPG cells exhibited aberrant symmetric NG2 expression, indicating their pluripotency and tumor stem cell-like properties [15]. Moreover, NG2-expressing primary DIPG cells showed co-expression of oligodendrocyte (Olig2) and astrocyte (GFAP) cell markers denoting their pluripotent potential, thought to be caused by excessive and dysregulated NG2 expression in mitotic cells. Although these studies established NG2 expression in various types of pediatric brain tumors, additional studies are required to elucidate the role of NG2 expression in pediatric brain tumors.

NG2 as a Potential Target for Diagnosis and Therapy

The ever-emerging role of NG2 in promoting angiogenesis, tumor infiltration, and expansion warrants studies investigating its role as a target for cancer treatment. Despite NG2 overexpression in a wide variety of tumors such as melanomas, breast, head and neck carcinomas, mesotheliomas, and brain tumors, recognition of NG2's potential therapeutic value has not been fully utilized [58]. NG2 targeting has been achieved at the expression level using RNA interference (RNAi) and at the protein level using NG2 binding antibodies or peptides [59–63]. Such targeting of NG2 at both mRNA and protein levels have successfully resulted in tumor cell apoptosis, reduced angiogenesis, and reduced tumor cell invasion [60,61,64]. In this section, we will review few strategies used to target NG2 expressing cancer cells.

Targeting of NG2 using RNAi can be achieved using siRNA, shRNA, or miRNA constructs. RNAi is achieved by using small non-coding double stranded RNA molecules resulting in degradation of target mRNA and subsequent down-regulation of target protein [59]. For example, siRNA mediated NG2 targeting alleviate

chemoresistance and render cancerous cells to cytotoxic treatment in vitro [59]. shRNA mediated NG2 knockdown result in normalized vasculature, reduced tumor growth, and edema in GBM xenograft mice [60] and reduced tumor proliferation and increased necrosis in a murine model of melanoma [60]. Complimentary in vitro and in vivo studies using shRNA-mediated NG2 knockdown resulted in enhanced chemotherapy response via mitigation of $\beta 1$ integrin signaling and increased tumor cell response to cytotoxic treatment [64]. miR129-2 is an endogenously expressed microRNA targeting NG2 mRNA. In DIPGs, miR129-2 is down-regulated by epigenetic (hypermethylation) regulation. We have shown that miR129-2 regulation is restored in vitro and in vivo by using either hypomethylating drug (5-azacytidine) or lentivirus-mediated miR129-2 transduction [15]. In both cases, NG2 protein down-regulation resulted in reduced cellular migration [15]. However, the limitations associated with any RNA interference based approach such as off target effects and need for an efficient delivery system, making it as a less favorable candidate for human use.

Immunotherapeutic approaches using the large extracellular domain of the NG2 protein as a tumor antigen have been also explored [65]. GBMs, for example, have been effectively treated in vivo using an intralesional adoptive cellular immunotherapy approach where NG2-binding monoclonal antibodies (mAb9.2.27) combined with natural killer cells were used [61,62]. Dual targeting of NG2 and GD3A in neoplastic GBM astrocytes was also achieved using the Map-Zap saporin immunotargeting system [66]. We have recently targeted NG2 using manganese-containing Prussian blue nanoparticles coated with anti-NG2 antibodies and used for fluorescent and MRI imaging of glioma cells [67]. NG2 specific monoclonal antibody also conferred anti-tumor effect in in vitro and murine in vivo preclinical models of triple negative breast cancer by inhibiting signaling pathways crucial for cell survival, proliferation, and metastasis [68]. Metastatic melanoma cells were targeted in vitro using NG2 antibodies which caused loss of NG2 interaction with extracellular matrix components and resulted in blockade of signal transduction pathways crucial for melanoma metastasis [69]. A single-chain antibody scFv-FcC21 that specifically binds to human NG2 was shown to be useful in inhibiting tumor growth and improving survival in melanoma cell derived lung metastases mouse model [70]. Although antibody-mediated mechanism of NG2 targeting seems feasible, the associated disadvantages such as non-specific antibody toxicity and antibody size (limiting diffusion across within the tumor or

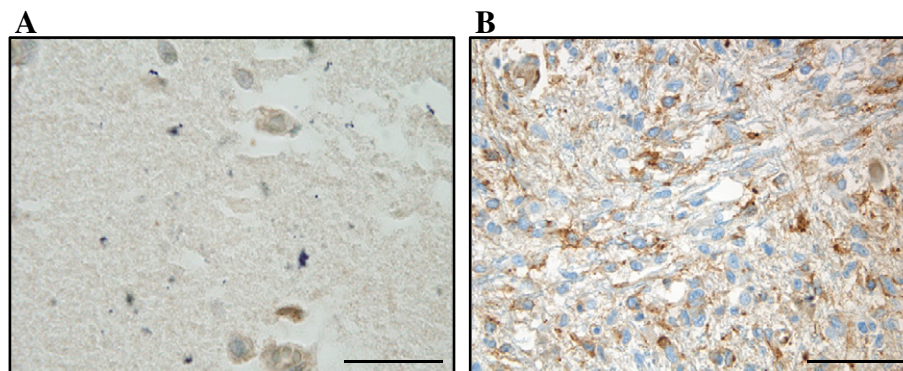


Figure 3. NG2 expression in pediatric diffuse intrinsic pontine glioma. Tissue sections representing healthy (A) or tumor (B) brain specimens obtained at autopsy from patients with DIPG. Immunohistochemical assays indicated high expression levels of NG2 (brown) in tumor sample when compared to control specimen. Scale bar = 20 μm .

across the blood brain barrier) have dampened enthusiasm for translational application of antibodies. As a remedy to the relatively large size of anti-NG2 antibody, two short peptides (TAASGVSRSMH and LTLRWVGLMS) have been designed and shown to specifically target NG2 protein [63,71]. These deca peptides have been used for drug delivery to tumor sites in melanoma xenografts [63]. Additionally, NG2 binding peptides provide the feasibility to explore downstream effectors and molecular mechanisms that can be targeted to design anti-cancer therapies.

As discussed above, NG2 expression is limited to OPCs and as expected a developing child's brain contains a larger number of NG2 expressing OP cells. One critical question in targeting NG2 in pediatric gliomas is the potential of such approach in destroying healthy OPCs that are important for the developing brain of a child. However, a limited number of studies indicate the differential expression of NG2 isoforms in adult GBM when compared to fetal and healthy adult brain [72]. Specifically, Girolamo and colleagues documented 48 immunologically distinct NG2 isoforms, 14 of which were present in the fetal and neoplastic cerebral sections and absent in the adult brains [72]. Further characterization of these isoforms is warranted using more sensitive platforms such as mass spectroscopy to ensure the specific expression of these isoforms in various tissue and tumor types.

Conclusion

Despite the fact that NG2 is being widely used as a potential targeting molecule in in vivo and in vitro pre-clinical studies, substantial evidence has not yet been established for NG2's utility in clinical studies and in treating human disease. Due to its role in maintaining a pluripotent pool of tumor cells, and its role in tumor migration and infiltration, NG2 provides multiple avenues for developing therapeutics. Moreover, the large extracellular domain of NG2 provides an excellent antigen repertoire for immunotherapeutic interventions. As such, further research is warranted to define the role and expression regulation of NG2 in CNS cancers.

Competing Interests

The authors declare no potential conflicts of interest.

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Authors' Contributions

SY and JN assisted in manuscript preparation. EH and RP assisted by editing and providing scientific input.

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