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Identification and characterization of a truncated isoform of NELL2

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ABSTRACT

NELL2 is a neuron-specific secreted glycoprotein containing an N-terminal thrombospondin I-like domain (TSP-N). In this study, we describe NELL2-Tsp, a novel alternative splice variant of rat NELL2. NELL2-Tsp uses an alternate stop codon resulting in a C-terminal truncated form of NELL2, containing a signal peptide and a TSP-N domain. NELL2-Tsp is a glycosylated protein specifically expressed in brain tissue. NELL2-Tsp and NELL2 are secreted, likely due to the putative signal peptide. However, due to the truncation, the secreted portion of NELL2-Tsp is smaller than that of NELL2. Immunoprecipitation analysis confirmed that NELL2-Tsp was able to associate with NELL2 and with itself. In addition, expression of NELL2-Tsp notably reduced secretion of NELL2 and inhibited NELL2-mediated neurite outgrowth. These results suggest that NELL2-Tsp may act as a negative regulator of wild-type NELL2.

Introduction

Nel, a protein strongly expressed in *n*eural tissues and containing *E*GF-*l*ike domains, was first identified in a chicken embryo-derived cDNA library [1]. Expression of the Nel gene is restricted to neural tissues after hatching, while the mRNA of this gene is found in all tissues during fetal development [1]. Subsequent studies identified a mammalian counterpart of chicken Nel, NELL2, in human and rat tissues [2,3]. NELL2 is strongly expressed in the mammalian brain [3,4] and is neuron-specific within the nervous system [4,5].

Previous studies have reported that different sizes of NELL2 mRNAs were present in developing mouse embryos [3], adult human tissues [2], and the human neuroblastoma IMR32 cell line [6] by Northern blot analysis. The differences in mRNA sizes may be due to alternative splicing. Recently, we reported a novel cytosolic form of rat NELL2 (cNELL2) that is generated by alternative splicing [7]. cNELL2 mRNA has a single deletion of 129 bp, equivalent to exon 3. However, the difference in length of cNELL2 and NELL2 mRNAs are not great enough to be detected by Northern blot analysis. We hypothesized that additional NELL2 splice variants might exist.

In this study, we used 3′ RACE PCR to identify a novel splice variant of NELL2 (NELL2-Tsp). NELL2-Tsp is generated by alternative splicing between exon 9 and exon 21, resulting in a C-terminal truncated variant of NELL2 containing a signal peptide and a TSP-N domain. In addition, Western blot analysis confirmed NELL2-Tsp is a secreted glycoprotein and can self-oligomerize as does wild-type NELL2. Moreover, NELL2-Tsp bound wild-type NELL2 and inhibited the secretion and function of wild-type NELL2. Based on these results, we propose that NELL2-Tsp is a negative regulator of wild-type NELL2.

Materials and methods

Antibodies. Monoclonal antibodies specific for GFP (B-2) and HA (F-7) were obtained from Santa Cruz Biotechnology. A monoclonal antibody specific for Flag (F6531-5MG) and a polyclonal antibody specific for actin (A2066) were purchased from Sigma. An anti-neurofilament monoclonal antibody (SMI-31) was purchased from Sternberger.

3′ Rapid amplification of cDNA ends (3′ RACE). NELL2-Tsp cDNA was obtained using a 3′-RACE System (Takara). Briefly, 1 μg of total RNA from adult male Sprague–Dawley rat (SD, 14-weeks-old) brain was reverse-transcribed using the supplied adapter primer. A supplied universal amplification primer and a specific internal sense primer (5′-TGCCAAGTGGCACAAGCTCTCCTTAGCC-3′) were used for PCR amplification. The PCR products were cloned into a pGEM-T Easy vector (Promega) and sequenced.

RNA extraction and RT-PCR. Total RNA was isolated from tissues of adult male Sprague–Dawley rats (SD, 14-weeks-old) using TRIzol® reagent (Invitrogen) according to the manufacturer's protocol.

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Total RNA samples (1 μg) was reverse-transcribed using 50 pmol random primers, SuperScript™ II Reverse Transcriptase (Invitrogen), and 1 mM dNTPs at 42 °C for 1 h. Forward primer A (5′-TGTCTAGAGCCTGAGCCC-3′) and reverse primer B (5′-CAA TAGCTTGTTTCAGGGTT-3′) were designed for specific detection of NELL2-Tsp cDNA. Rat GAPDH primers were used as an internal control and amplified a 111-bp fragment (5′-AACCTGCCAAGTAT-GATGAC-3′ and 5′-TGTTGAAGTCACAGGAGACA-3′). NELL2-Tsp fragments were amplified by 35 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 30 s (Fig. 1C). GAPDH fragments were amplified under the same conditions except that only 25 cycles were performed and the extension time was 30 s. PCR products were analyzed by agarose gel (1.5%) electrophoresis, purified, ligated into a pGEM-T Easy vector (Promega), and sequenced.

Plasmid construction. Full-length NELL2-Tsp (Accession No.: GQ376510) was cloned into several destination vectors (non-tagged, C-terminal EGFP, C-terminal HA, and C-terminal Flag tagged vectors) using the Gateway Cloning System (Invitrogen). The amino-terminal primer 5′-GGGGACAAGTTTGTACAAAA AAGCAGGCT**CCACC**ATGGAATCCCGGGTATTACTGAGA-3′ was used to introduce an *att*B1 sequence (underlined) followed by a Kozak sequence (bold) upstream of the coding sequences of NELL2-Tsp. Similarly, the carboxy-terminal primer (5′-GGGGACCACTTTGTA CAAGAAAGCTGGGTTGGCTCTAGACAACTTGGCT-3′) was used to introduce an *att*B2 sequence immediately after the last amino acid of the gene. After amplification of full-length NELL2-Tsp, cDNA was cloned into pDONR207 vectors and converted into destination vectors, including pDS_XB-HA, pDs_XB-Flag, pDS_XB-EGFP, and pDS_XB-RFP expression vectors. Full-length NELL2 (Accession No.: AY089719) was cloned as previously described [7].

Cell culture and transfection. COS-7 and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin under a humidifying atmosphere containing 5% CO_2 at 37 °C. Cells were seeded onto poly-L-lysine-coated coverslips for imaging analysis or 60-mm dishes for Western blot analysis. Transfection with expression vectors was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Transfected cells were cultured for an additional 24 h in growth medium before further analyses. When necessary, 5 μg/

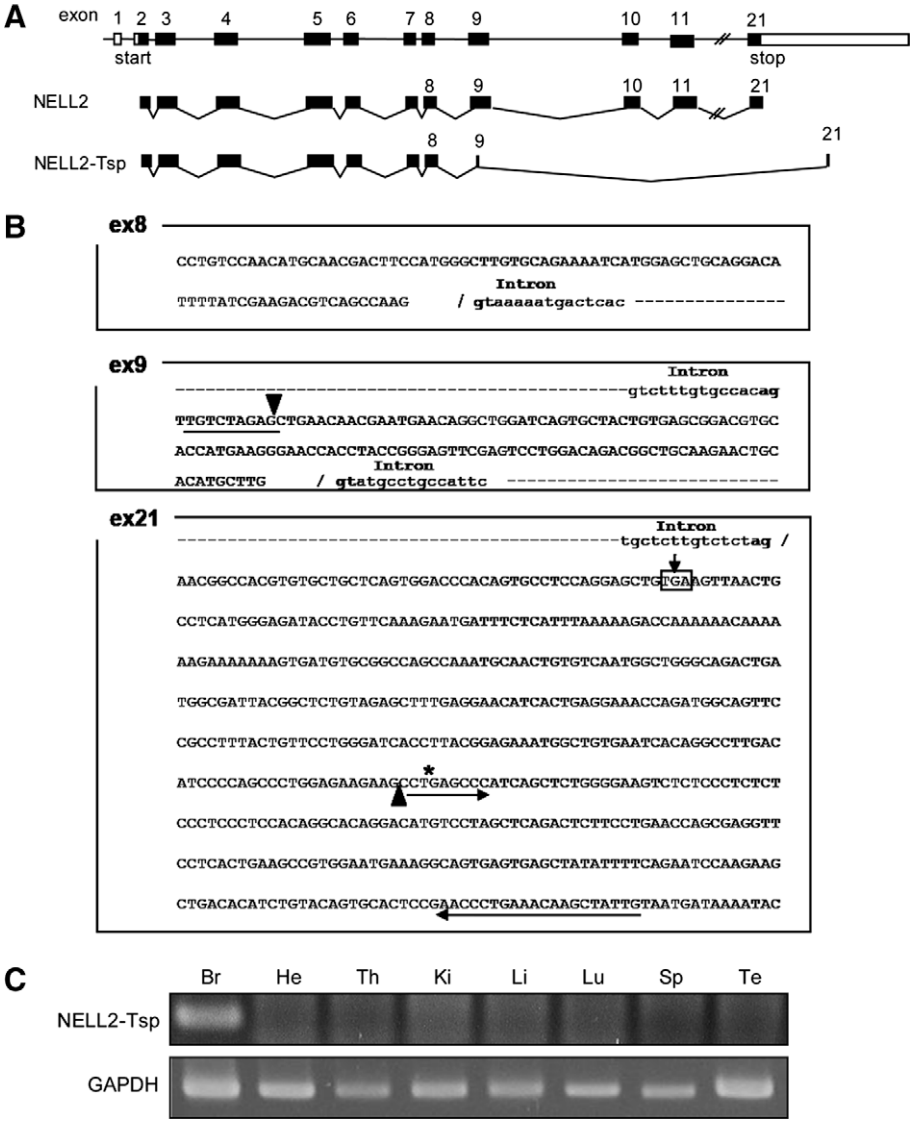

Fig. 1. Identification of NELL2-Tsp. (A) Schematic representation of wild-type NELL2 and the splice variant NELL2-Tsp. NELL2-Tsp is generated by alternative splicing between intermediate sites of exon 9 and exon 21, resulting in a C-terminal truncated form of the NELL2 protein. (B) Sequences of exon 8, exon 9, and exon 21 of NELL2. Capital letters and small letters represent exon and intron sequences, respectively. Black arrowheads indicate the splicing sites of NELL2-Tsp between exon 9 and exon 21. The box and asterisk in exon 21 indicate the stop codons for NELL2 and NELL2-Tsp, respectively. (C) Expression analysis of NELL2-Tsp in various tissues. Specific RT-PCR primers for NELL2-Tsp are indicated by arrows in (B). Br, brain; He, heart; Th, thymus; Ki, kidney; Li, liver; Lu, lung; Sp, spleen; Te, testis.

ml tunicamycin (Sigma) was treated into culture medium to deglycosylation assay.

Western blot analysis. Transfected cells were incubated for 2 h in serum-free DMEM. Subsequently, the medium was collected and proteins were precipitated with 10% trichloroacetic acid. Samples were centrifuged at 20,000*g*. Pellets were washed twice with ice-cold acetone. Proteins were separated by SDS–PAGE in 10% gels and blotted onto PVDF membranes. Blots were blocked with 5% skim-milk in TBST (20 mM Tris-buffered saline and 0.05% Tween 20, pH 7.5) at room temperature for 20 min and incubated overnight at 4 °C with anti-GFP antibody (1:1000), anti-HA antibody (1:1000), or anti-FLAG antibody (1:1000). Blots were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG. Immunoreactivity was visualized using enhanced chemiluminescence (ECL, Amersham).

Immunoprecipitation. Immunoprecipitation was performed 24 h after transfection. Transfected cells were harvested in PBS and centrifuged for 5 min at 300*g*. The cells were lysed on ice for 30 min in cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1 mM PMSF, and 1% NP-40) containing a protease-inhibitor cocktail (Sigma). Whole-cell lysates were incubated on ice for 30 min and centrifuged at 20,000*g* for 20 min at 4 °C. Supernatant fractions were collected and incubated with 1 μg anti-HA antibody for 1 h at 4 °C with gentle rotation. The immune complexes were incubated with 20 μl protein G agarose beads (Santa Cruz Biotechnology) that were pre-washed and suspended in 100 ml cold lysis buffer for 1 h at 4 °C with gentle rotation. The samples were washed three times in cold lysis buffer and eluted in SDS–PAGE sample buffer. Immunoprecipitated proteins were detected by immunoblotting using an anti-GFP antibody.

Immunocytochemistry. For immunocytochemistry experiments, 24-well microplates containing coverslips were seeded with HiB5 cells prepared in DMEM supplemented with 10% FBS (30,000 cells per coverslip). After 24 h, cells were incubated with recombinant adenovirus for 48 h in serum-free DMEM and fixed with 4% paraformaldehyde in PBS. Fixed cells on coverslips were stained with anti-neurofilament antibody (1:200 in 3% BSA). Coverslips were incubated for 1 h at room temperature and washed three times with PBS. Subsequently, coverslips were treated with cy3-conjugated anti-mouse IgG (Jackson Lab) diluted 1:500 in 3% BSA for 1 h at room temperature and washed three times with PBS. Coverslips were mounted and analyzed by confocal microscopy.

Construction and infection of recombinant adenoviruses (rAV). Adenoviruses were generated using Gateway® recombination technology (Invitrogen) according to the manufacturer's instructions. Briefly, the NELL2 and NELL2-Tsp ORF clones in Gateway® entry vectors (pENTR™207) were transferred into rAV vectors (pAd/CMV/V5-DEST) using the ViraPower™ Adenoviral Gateway® Expression Kit and the LR Clonase™II enzyme mix (Invitrogen). The reaction mixtures were transformed into DH5™ chemically competent *Escherichia coli* to select for expression clones. Pac I-digested vectors were used to transfect HEK293A cells to produce rAV stocks. rAV vectors were amplified by infecting HEK293A producer cells with crude viral lysates. HiB5 cells were infected with rAV-NELL2, rAV-NELL2-Tsp, or rAV-GFP constructs. In some experiments, two constructs were used simultaneously.

Results

Identification of a novel alternative splice variant of the rat NELL2 gene

Using 3′ RACE PCR analysis of the NELL2 gene isolated from rat brain, we identified a novel NELL2 transcript, alternatively spliced between exon 9 and exon 21 (Fig. 1A). This splice variant used an alternative stop codon in exon 21, which resulted in a 3′-deletion of 1674-bp from wild-type NELL2. Fig. 1A shows the genomic structure of the rat NELL2 gene and the splicing events necessary for the generation of both NELL2 transcripts. The novel splice variant was named NELL2-Tsp because the terminal amino acids are those of the N-terminal thrombospondin-1-like (TSP-N) domain of the NELL2 protein (Fig. 2B).

Alignment of rat genomic DNA (chromosome 7 genomic contig NW_047784) and NELL2 cDNA sequences identified flanking nucleotides at convergent points in exon 8, exon 9, and exon 21. This suggests that NELL2-Tsp is generated by additional alternative splicing between an internal region of exon 9 and an internal region of exon 21 (Fig. 1B). Next, RT-PCR analyses were performed using RNA samples from eight different tissues to determine NELL2-Tsp expression patterns (Fig. 1C). To specifically amplify NELL2-Tsp, we designed a NELL2-Tsp-specific forward hybrid primer corresponding to the exon 9/exon 21 junction (primer A, Fig. 1B). As shown in Fig. 1C, NELL2-Tsp (110 bp) was specifically expressed in brain tissue. However, previous studies, including our own, using RT-PCR or Northern blot analysis demonstrated that NELL2 was strongly expressed in brain, thymus, and testis [2–7].

NELL2-Tsp is a secreted glycoprotein

Based on the amino acid sequence, several protein motifs are likely present in NELL2, including a signal peptide, a TSP-N domain, five cysteine-rich von Villebrand factor C (CR) domains, and six EGF-like domains [2,3,8]. Of these motifs, NELL2-Tsp contains the signal peptide and the TSP-N domain (Fig. 2A). A previous report indicated that NELL2 has eight N-linked glycosylation sites [8]. Based on the sequence of NELL2-Tsp, there are two potential sites for N-linked glycosylation (Asn-Xaa-The/Ser, Xaa represents any residue other than Pro) within the TSP-N domain of the truncated protein (Fig. 2B).

Western blot analysis with anti-Flag antibody detected an approximately 130-kDa NELL2-Flag protein and an approximately 30-kDa NELL2-Tsp-Flag protein in HEK293T cells transfected with the corresponding cDNA (Fig. 2C). The molecular masses of NELL2-Flag and NELL2-Tsp were reduced to 90 and 27 kDa, respectively, by treatment with tunicamycin, a glycosylation-disrupting drug. NELL2 was heavily glycosylated with an additional 40-kDa N-linked carbohydrate moiety, as previously reported [3,8]. NELL2-Tsp was also glycosylated with an additional 3-kDa N-linked carbohydrate moiety.

Since NELL2-Tsp retained the secretion signal peptide, it could be also secreted like the NELL2 protein. The intracellular distribution of NELL2-Tsp in COS-7 cells was determined by tagging it with EGFP at the carboxy-termini. NELL2-Tsp-EGFP was distributed to distinct subcellular structures, including the endoplasmic reticulum and Golgi apparatus, in a similar fashion to other secreted proteins (Supplementary Fig. 1). Western blot analysis of secreted proteins confirmed the presence of both NELL2-Tsp and NELL2 (Fig. 2D). However, NELL2-Tsp was found at lower levels than NELL2 in the culture medium (Fig. 2D and E).

NELL2-Tsp inhibits secretion of NELL2

NELL2 proteins associate intermolecularly to form homotrimers through their coiled-coil region or CR domains [8]. Since NELL2-Tsp has a partial coiled-coil region (Fig. 2B), we examined whether NELL2-Tsp can interact with wild-type NELL2. Confocal imaging analysis showed that NELL2-Tsp and NELL2 were mainly co-localized to intracellular substructures of COS-7 cells (Fig. 3A). Immunoprecipitation analysis also showed that NELL2-Tsp could interact with NELL2-Tsp and wild-type NELL2 (Fig. 3B).

Since NELL2-Tsp was mainly restricted to intracellular regions and secreted into the culture medium at low levels (Fig. 2D and E),

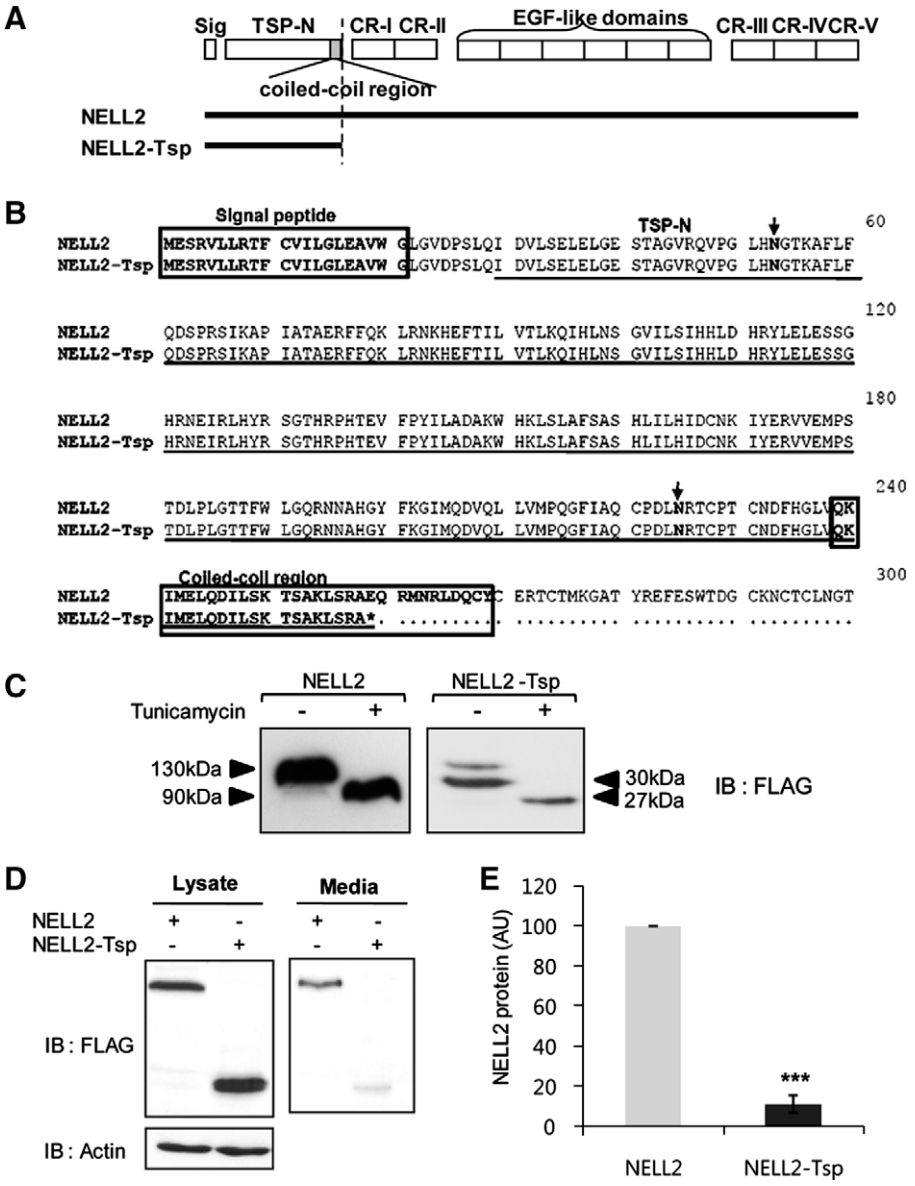

Fig. 2. NELL2-Tsp is a secreted glycoprotein. (A,B) Amino acid sequences of NELL2 and NELL2-Tsp. The signal peptide and coiled-coil region of NELL2 are indicated by a box and the TSP-N domain is underlined. The asterisk indicates the termination site of NELL2-Tsp, and the two arrows indicate the N-glycosylation sites of NELL2-Tsp, as predicted by software available online from the Swiss Institute for Experimental Cancer Research (http://ulrec3.unil.ch/software/COILS_form.html). (C) After C-terminal Flag-tagged NELL2 or NELL2-Tsp was transiently transfected into COS-7 cells, cells were treated with 1 μg/ml tunicamycin. Treatment with tunicamycin clearly showed NELL2 and NELL2-Tsp are both glycosylated proteins. (D) HEK293T cells were transiently transfected with NELL2-Flag or NELL2-Tsp-Flag. The cell lysates and culture medium from the transfected cells were immunoblotted with Flag antibody. Actin was used as the internal control. (E) Relative amounts of secreted NELL2 and NELL2-Tsp were determined by Western blot in the medium of the each transfected cells. ($^{***}p < 0.001$, ANOVA, $n = 4$).

we next examined the effect of NELL2-Tsp on NELL2 secretion. As shown in Fig. 3C, when cells were co-transfected with NELL2 and increasing amounts of NELL2-Tsp cDNA, secreted NELL2 was dramatically decreased in a dose-dependent manner. These data suggested that NELL2-Tsp acts as a negative regulator of NELL2 secretion.

NELL2-Tsp negatively regulates NELL2-mediated neuronal differentiation

NELL2 has an autonomous function to promote differentiation of NELL2-expressing neuronal progenitor cells [8]. To determine whether ectopic expression of NELL2 or NELL2-Tsp promote differentiation of neuronal progenitor cells, adenoviruses expressing NELL2, NELL2-Tsp, or GFP (rAV-NELL2, rAV-NELL2-Tsp, and rAV-GFP) were constructed. HiB5 cells, a hippocampal progenitor cell line, were infected by the recombinant adenoviruses. Cells showing neurite outgrowth were examined. rAV-NELL2 expression induced neurite outgrowth in HiB5 cells in a dose-dependent manner, while rAV-NELL2-Tsp or control virus (rAV-GFP) did not induce neurite outgrowth (Fig. 4A and C). However, rAV-NELL2-mediated neurite outgrowth was dramatically decreased by co-infection with rAV-NELL2-Tsp (Fig. 4B and C). Taken together, these data suggest NELL2-Tsp can suppress NELL2 secretion via intermolecular interactions, and can inhibit the function of NELL2 in neural differentiation.

Discussion

NELL2 is a secreted protein [4] that promotes neuronal survival and differentiation [8,9,12]. Previous studies have described differ-

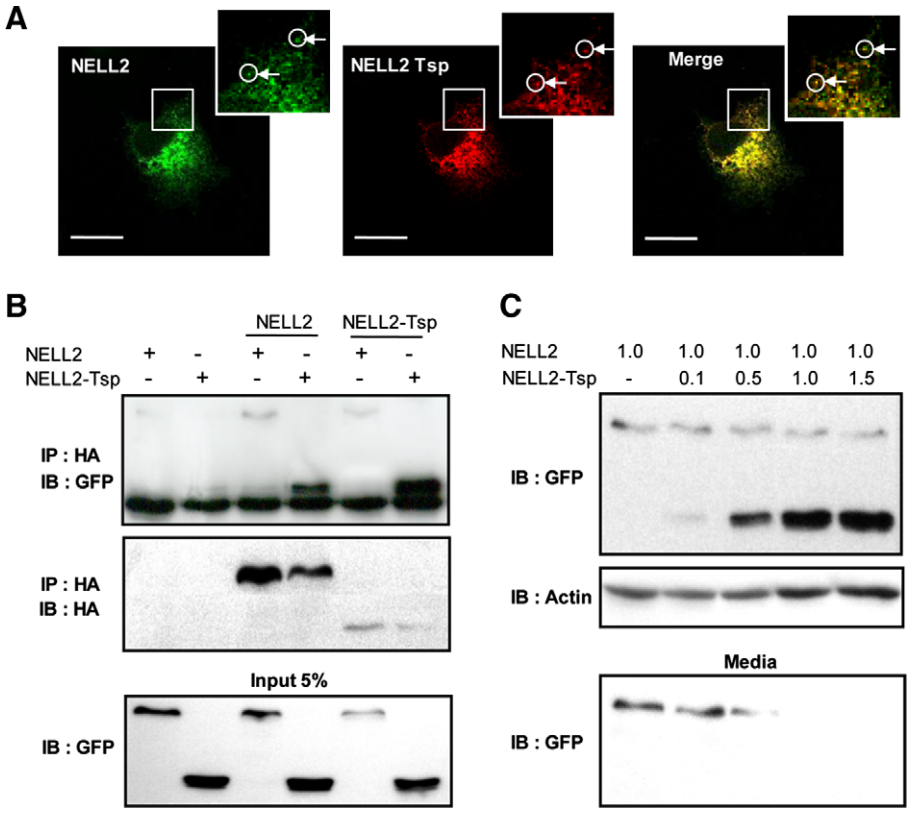

Fig. 3. NELL2-Tsp inhibits secretion of NELL2. (A) COS-7 cells were transiently co-transfected with NELL2-Tsp-GFP (green) and NELL2-Tsp-RFP (red). Arrows indicate vesicles containing both NELL2-GFP and NELL2-Tsp-RFP. Scale bar represents 20 μm. (B) NELL2-Tsp can associate with NELL2 as demonstrated by immunoprecipitation assays. Lysates were immunoprecipitated (IP) with anti-HA antibody and then immunoblotted (IB) with an anti-GFP antibody. NELL2-GFP and NELL2-Tsp-GFP were immunoprecipitated by anti-HA and were visualized by immunoblotting with an anti-GFP antibody. Membrane were then reprobed with anti-HA antibody. Five percent of input cell lysates were also analyzed with anti-GFP antibody. (C) Secretion of NELL2 is inhibited by NELL2-Tsp. HEK293T cells were co-transfected with 1 μg NELL2-GFP vector and varying concentrations of NELL2-Tsp-GFP vector. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

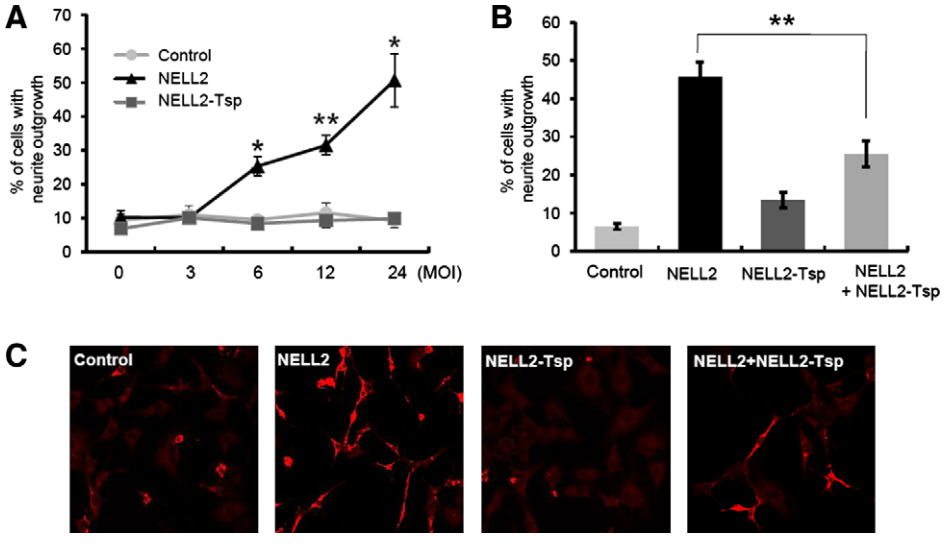

Fig. 4. NELL2-mediated neuronal differentiation is hindered by NELL2-Tsp. (A) NELL2 expression increased neurite outgrowth in HiB5 cells in a dose-dependent manner. After 1 day in culture, cells were treated with the recombinant adenoviruses rAV-GFP, rAV-NELL2-Tsp, or rAV-NELL2 for 48 h in serum-free media. Neurite outgrowth was quantified by counting cells positive for a neurofilament marker protein. (B) Expression of NELL2-Tsp decreased neurite outgrowth in NELL2-overexpressing cells. Cells were treated with recombinant adenoviruses (rAV-GFP, rAV-NELL2-Tsp, or rAV-NELL2-Tsp) and rAV-NELL2 at an MOI of 24 for 48 h in serum-free DMEM. Data are presented as means ± SE percentage of control values. ($^{*}p < 0.05$; $^{**}p < 0.01$, ANOVA, $n = 4$). (C) The expression of neurofilament protein is not observed in rAV-GFP or rAV-NELL2-Tsp infected cells. Scale bar represents 50 μm.

ent sizes of NELL2 transcripts in mouse brain tissue and several cancer cell lines [3,4,6]. These data implied that other isoforms of the NELL2 protein could be produced by alternative splicing. In this study, we described a novel, truncated, alternative splice variant of rat NELL2, NELL2-Tsp. Expression of NELL2-Tsp mRNA was specifically detected in brain tissue, in which NELL2 mRNA is also highly expressed [2,4,5,10] (Fig. 1C).

NELL2 contains a signal peptide, a TSP-N domain, six EGF-like domains, and five CR domains [3]. On the other hand, NELL2-Tsp also contains a signal peptide and a TSP-N domain, but lacks the EGF-like domains and CR domains. Interestingly, only a small amount of secreted NELL2-Tsp was detected, as compared to wild-type NELL2 (Fig. 3B and C). Additionally, a mutant lacking all EGF-like domains was also secreted at lower levels than wild-type NELL2 (data not shown). Taken together, these data suggest that EGF-like domains could be involved in the secretion mechanism of NELL2.

Three of the six EGF-like domains of NELL2 contain the residues required for Ca^{2+} binding [11,13,14], suggesting that NELL2 may act as a Ca^{2+}-dependent signaling molecule. Additionally, NELL2-deficient mice showed increased long-term potentiation in hippocampal dentate gyrus cells [15]. *In vivo* inhibition of NELL2 synthesis caused decreased expression of Ca^{2+}-binding proteins involved in the transport and release of synaptic vesicles in the hypothalamus [5]. Although additional studies are needed to clarify the role of EGF-like domains, these domains seem to be involved in the Ca^{2+}-dependent function or the secretion mechanism of NELL2. However, NELL2-Tsp cannot act as a Ca^{2+}-dependent signaling molecule since it lacks the EGF-like domains.

The protein domains of NELL2 are very similar to that of the homotrimeric glycoprotein TSP-1 [16]. TSP-1 interacts with various receptors, cytokines, proteases, and extracellular molecules. The TSP-N domain of TSP-1 can interact with integrin receptors, sulfated glycolipids, decorin, and heparin [17–20]. Since the TSP-N domain of NELL2 also has heparin binding activity [3], secreted NELL2, like TSP-1, may have several binding partners. This hypothesis has not yet been confirmed. In this study, we demonstrated that the TSP-N domain is important for intermolecular binding of NELL2 or NELL2-Tsp. Additionally, NELL2-Tsp inhibited secretion of wild-type NELL2 and NELL2-mediated neurite outgrowth in a dose-dependent manner (Figs. 3C and 4B). However, we cannot exclude the possibility that secreted NELL2-Tsp has other physiological functions due to interactions with other binding partners simply because NELL2-Tsp has only a single TSP-N domain. Details regarding the physiological role of NELL2-Tsp by itself remain to be elucidated.

In summary, our findings suggest that NELL2-Tsp, a novel alternatively-spliced NELL2, is specifically expressed in brain tissue and could be a negative regulator involved in the secretion and function of wild-type NELL2.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.11.092.

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