Thrombospondin 2 Potentiates Notch3/Jagged1 Signaling*

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He Meng1, Xiaojie Zhang1, Kurt D. Hankenson5, and Michael M. Wang*1

From the Departments of 4Neurology and 4Molecular and Integrative Physiology, University of Michigan, Ann Arbor, Michigan 48109-5622 and the 5Department of Animal Biology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104-4539

Extracellular thrombospondins (TSP or THBS) and the Notch family of transmembrane receptors share a role in multiple, overlapping cellular functions and participate in developmental signaling and pathological reactions to tissue injury. We demonstrate that TSP2, but not TSP1, enhances the potency of Notch3 signal transduction. In addition, TSP2 reduces cancer cell proliferation in a Notch-ligand dependent fashion. The loss of TSP2 in knock-out mice reduces Notch target gene expression. TSP2 binds directly to Notch3 and Jagged1. TSP1 also binds to Notch3 and Jagged1; however, only TSP2 augments the interaction between Notch3 and Jagged1. These studies demonstrate that the diverse functions of TSP2 may also include a role as an intermediary protein that facilitates transcellular receptor-ligand interactions.

Thrombospondin 2 (TSP2), a matricellular glycoprotein, participates in multiple roles including bone growth, cell adhesion, extracellular matrix modeling, inflammatory responses, and developmental and pathological angiogenesis (1, 2). Like other secreted proteins, TSP2 contains a series of protein binding motifs, including an NH2-terminal heparin binding domain, a collagen-like oligomerization motif (von Willebrand factor-like domain), type I–III TSP repeats, and a globular carboxyl-terminal domain (3). Each of these protein modules has unique functions that are mediated by distinct receptors that stimulate multiple cellular pathways (reviewed in Refs. 2, 4–6). In addition, TSP2 binds to extracellular proteins such as matrix metalloproteinases and facilitates their clearance (7).

The Notch signaling pathway is an evolutionarily conserved, intercellular signaling system that plays an essential role in development of multiple organ systems (8). The four mammalian Notch gene products all appear to signal in a similar fashion. Each Notch receptor is a large transmembrane protein with complex extra- and intracellular domains. The ectodomain is composed of a tandem array of over 34 EGF-like repeats and three cysteine-rich Lin12 repeats. Individual EGF-like repeats include six highly conserved cysteine residues, which participate in the formation of disulfide bonds and promote maintenance of protein conformation. Notch receptors are processed (called S1 processing) within the secretory pathway into a non-covalently linked heterodimer composed of the extracellular NH2-terminal domain and a membrane spanning/intracellular COOH-terminal domain (reviewed by numerous authors, including Refs. 8 and 9). Canonical Notch ligands (Jagged1, Jagged2, Delta1 (DLL1), DLL3, and DLL4) (10), and DNER (11) bind to Notch and trigger two additional proteolytic cleavages. A second cleavage (S2) is regulated by ligand binding and involves a tumor necrosis factor α-converting enzyme (TACE (12)). The third cleavage (S3) is mediated by a presenilin-dependent γ-secretase (13, 14), which severs the membrane spanning domain from the intracellular domain. This series of proteolytic events allows migration of the intracellular domain into the nucleus, which results in the expression of target genes, including the Hairy Enhancer of Split (HES), HES-related (HRT), and Deltex genes (8, 15). This classical pathway of Notch signaling is non-cell autonomous, requiring membrane-bound ligand expression from an adjacent cell to communicate with the Notch-expressing cell.

In humans, the Notch pathway plays an important role in homeostatic function and development. Mutations in Notch3 cause the autosomal dominant genetic disease CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (16)), a systemic arteriopathy which results in stroke, migraine, and vascular dementia. Multiorgan defects are a significant feature of Alagille syndrome, a developmental disorder, which is caused by mutations in the Notch ligand Jagged1 (17, 18) or by mutations in Notch2 (19). Notch signaling also plays an important role in cancer. Activating mutations in Notch1 cause a subset of aggressive lymphomas whose growth are dependent on cell autonomous constitutive activation of the Notch pathway (20). In addition, a series of recent studies have demonstrated gene amplification and protein overexpression of Notch3 in lung (21) and ovarian (22) cancer cells; Notch3 activation in these cells has been hypothesized to drive cancer cell growth and has been proposed as a novel molecular target for tumor therapy. Thus, Notch participates in a number of human disorders, and protein regulators of Notch activity have potential clinical relevance.

Recent work has demonstrated that several matrix proteins are capable of altering Notch signaling. These include CCN/NOV3 (23) and MAGP proteins (24), which are capable of acti-
vating Notch signaling independent of canonical ligands. Other experiments have identified TSP1, a protein closely related to TSP2, as a binding partner of a soluble splice form of Jagged1 (25), but a direct role for any TSP in Notch signaling has yet to be elucidated. In the following experiments, we investigated the functional interaction between TSP2 and Notch3; TSP2 was chosen for analysis because of our interest in Notch3 signal modification in vascular smooth muscle, which also synthesizes TSP2. We demonstrate that TSP2 enhances Notch3-dependent signaling. In addition to showing this functional interaction, we also demonstrate that TSP2 physically interacts with both Notch3 and Jagged1, suggesting a novel intermediary function for TSP2 in cell-cell communication.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—293A (Qbiogene), L fibroblast lines, and A2780 were propagated in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). H460 cells were propagated in RPMI1640 with 10% fetal bovine serum (Invitrogen). Co-cultures were all performed in Dulbecco’s modified Eagle’s medium or RPMI complete media, unless otherwise indicated. For transfections (performed in triplicate), cells were grown in 12-well or 24-well plates and transfected using Lipofectamine 2000 (Invitrogen) using the manufacturer’s protocol. For coculture experiments, Notch expressing cells were first transfected on plates; after an overnight incubation, DNA was removed by washing cells with media, and Notch expressing cells were overlaid with Notch ligand expressing cells for 12–24 h. Luciferase assays were performed using a dual luciferase kit. The ratio of firefly (Notch target Hes-luciferase promoter) to Renilla luciferase (encoded by phRG-TK, Promega) was calculated to obtain relative Notch activity levels. Where indicated, we added purified recombinant TSP1 or TSP2 (R&D Systems; >90% per manufacturer; protein appears as a single band on SDS-PAGE) directly to the culture medium. In some experiments, cells were immuno-stained with TRA1–85 (70 ng/ml), which reacts against human (but not mouse) CD147. Unless noted, each experiment was repeated at least three times.

**Western Blots**—Cells were washed with PBS, harvested, and sonicated in lysis buffer (50 mM Tris-HCl, pH 8, 200 mM NaCl, 0.5% Nonidet-40, protease inhibitor mixture (Pierce)). Cell lysates were cleared by centrifugation at 14,000 × g for 10 min at 4 °C. The supernatants were used for Western blot analysis. Notch3 was detected using a 1:1000 dilution of mouse monoclonal antibody (M-01 from Abnova) against the NH2-terminus of Notch3 or polyclonal antibodies against the COOH terminus (Santa Cruz). Myc (clone 9E10; Developmental Studies Hybridoma Bank, Iowa City, IA) and hemagglutinin monoclonal antibodies have been described and were used at 10–50 ng/ml. The TSP2 monoclonal antibody CA1 (BD Biosciences) was used at 50 ng/ml. IRDye-labeled secondary antibodies (Rockland, 1:10,000) were used on membranes that were analyzed with an Odyssey imaging system (LI-COR Biosciences).

**Coimmunoprecipitation**—293A cells grown in 6-well plates were transfected with expression constructs encoding proteins of interest for 48 h. The protocol was performed essentially as described (26). Transfected cells were washed with PBS (pH 7.4) and lysed in 0.3 ml/well of modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Na3VO4, 1 mM sodium fluoride) containing protease inhibitors. Lysates were centrifuged at 14,000 × g for 20 min and supernatants were then mixed with 1 μg of antibody. After 4 h at 4 °C, 40 μl of packed protein G-agarose (Upstate) was added and mixed for 4 h. Immunoprecipitates were then washed five times with ice-cold PBS (pH 7.4) and finally resuspended in 30 μl of 2× sample buffer. Proteins were eluted by boiling the beads for 5 min prior to analysis by SDS-PAGE and Western blotting.

**Animals**—Congenic TSP2 knock-out (27) and wild type control mice on a C57Bl6/129/Sv background were maintained under AALAC approved conditions. All animal usage was approved by an institutional animal research review board and performed in accordance with standard animal use methodology. For carotid artery studies, animals were anesthetized with xylazine and ketamine prior to tissue procurement.

**Gene Expression Analysis**—RNA was prepared using the RNaseasy kit (Qiagen) and subjected to quantitative reverse transcriptase-PCR using a Stratagene thermal cycler. Equivalent amounts of RNA were used for control reactions (for 18S rRNA) to ensure equal RNA content. Primers for quantitative reverse transcriptase-PCR were designed using Primerbank online software. The sequences were: mouse Hes1 forward, 5’-CCA GCC AGT GTC AAC ACG A-3’; mouse Hes1 reverse, 5’-AAT GCC GGG AGC TCT TCT TCT-3’; mouse Hey1 forward, 5’-AGG CCC AAC GAG GAG AAA AAC CGA-3’; mouse Hey1 reverse, 5’-GCT GTG TTT CAG GTA GCT GAC-3’; mouse Hey1 forward, 5’-CTG GCT ATG GAC TAT CGG AGT-3’; and mouse Hey1 reverse, 5’-GAC CAG GGG AAC GAG AAG C-3’.

**Binding Assays**—The following purified recombinant proteins were obtained from R&D Systems: recombinant human Notch-3-Fc fusion (first 11 EGF repeats), rat Jagged1-Fc, human full-length TSP1 and TSP2, and control human IgG1 Fc. Proteins (5 μg) were labeled with Alexa700-succinimide (10 μg) in PBS at room temperature for 1 h. Excess label was removed by gel filtration through a size exclusion column equilibrated in Tris buffer (Bio-Rad; 25-kDa cutoff). Labeled proteins were visualized by SDS-PAGE under reducing conditions; the gel was imaged without further processing using a LiCor Odyssey flatbed infrared detector; all proteins were predominantly one band of the expected molecular weight, which was consistent with the manufacturer’s reported purity (>90%).

Target proteins were coated on 96-well flat bottom Nunc ELISA plates at 5 μg/ml in Tris-buffered saline (50 mM Tris, 150 mM NaCl) with 2 mM CaCl2 overnight at 4 °C and blocked with 1% BSA in Tris-buffered saline with 2 mM CaCl2 for 1 h at room temperature. The wells were then incubated with increasing concentrations of Alexa700-succinimide labeled proteins in Tris-buffered saline plus 2 mM CaCl2 and 0.05% Tween 20 at 4 °C overnight. Bound proteins were detected using a LiCor Odyssey flatbed infrared detector and quantified by measuring the density of Alexa700 label adhering to the plates. In some experiments, additional unlabeled proteins were added to wells to test for potential competition or enhancement of binding.
Alexa-labeled ligands have been used before in binding studies of proteins adsorbed to plastic (28).

To validate the specificity and accuracy of this assay, we performed several controls. We showed that only trivial amounts of proteins adsorbed to plastic (28).

Cell lysates were analyzed for firefly luciferase activity normalized to Renilla luciferase activity, which reflects potency of Notch signaling (y axis). Jagged1 and Delta1 both activated Notch signaling in H460 cells. The transfection of TSP2 stimulated a significant increase in Notch activity, whereas all ligand dependent activity was blocked by γ-secretase inhibitor (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) (10 μM). B, immunoblotting for Notch3 ectodomain demonstrates that TSP2 transfection of H460 cells does not enhance the levels of Notch3 expression; the upper band corresponds to full-length, unprocessed Notch3; the lower band corresponds to processed Notch3 ectodomain. C, TSP2 (recombinant from R&D Systems; >90% pure) applied to the cell culture media also enhanced Notch3 signaling in H460 cells. Increasing doses of TSP2 were added to cocultures of Hes1-luciferase-transfected H460 cells and ligand expressing cells (shaded bars). The concentrations of TSP2 are indicated on the x axis (ng/ml). y axis shows relative Notch reporter activity normalized to Renilla luciferase. The minimal dose of TSP2 tested for significant activation of Notch3 was 50 ng/ml. Peak stimulation was noted at 100 ng/ml. D, TSP2 modulation of Notch requires the extracellular domain. The intracellular domain (ICD) of Notch3 was cotransfected into H460 cells without coculturing with ligand producing cells. The ICD was able to activate Notch signaling; however, cotransfection of TSP2 did not stimulate further activation by ICD. * denotes statistical significance (p < 0.05) of TSP2 compared with control. # denotes significance (p < 0.05) of Jagged1 or Delta1 compared with L cell controls. ** in indicates significant increases in Notch3 responses over 50 ng/ml dose (p < 0.01). Representative experiments are shown, and each experiment was repeated three or more times.

FIGURE 1. Functional modulation of Notch signaling in H460 lung cancer cells. A, Notch3 activation was measured in a coculture system. Notch3 expressing H460 cells were transfected with the Notch reporter Hes1-luciferase and either a mouse TSP2 expression construct or blank vector pcDNA3.1. Cells were then cocultured with L cells (negative control), Jagged1 expressers, or Delta1 expressers (in an L cell background). Cell lysates were analyzed for firefly luciferase activity normalized to Renilla luciferase activity, which reflects potency of Notch signaling (y axis). Jagged1 and Delta1 both activated Notch signaling in H460 cells.

RESULTS

TSP2 Modulates Notch3 Signaling—We investigated Notch3 signaling in the H460 lung cancer cell line, which selectively expresses Notch3 but not Notch1, -2, and -4 (29). To quantify the level of Notch3 activation, H460 cells were transfected with Hes1-luciferase (30). The Notch3 pathway was stimulated by coculturing reporter-transfected H460 cells with well established stable fibroblast cell lines expressing empty vector, Jagged1, or Delta1 cDNA (31). As expected, coculture with ligand-expressing cells significantly increased Hes1-luciferase activity in H460 cells (Fig. 1A, black bars). TSP2 cDNA cotransfection further increased Notch3 activity in the presence of Jagged1 and Delta1 (Fig. 1A, white bars). To verify that TSP2 stimulates Notch3 signaling through classical γ-secretase-activated intracellular processing, we applied (N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester) to cocultures; this γ-secretase inhibitor reduced all Hes1-luciferase activity to baseline, including ligand-stimulated function, regardless of whether TSP2 was expressed. Processed Notch3 levels were not increased in the presence of transfected TSP2 (Fig. 1B); thus, the enhancement of Notch3 activation by TSP2 does not involve up-regulation of receptor levels (transfection efficiencies for H460 cells ranged from 40 to 60%). Ligand-stimulated Hes1-luciferase reporter activity was also significantly amplified with recombinant TSP2 protein, demonstrating that the stimulatory effect of TSP2 does not require co-expression within the Notch3 producing cell. The minimal tested dose required for significant stimulation of Notch3 was 50 ng/ml of TSP2. The maximal response to TSP2 was seen at 100 ng/ml (Fig. 1C).

TSP2 can activate multiple intracellular signaling pathways that are independent of Notch. It is, in theory, possible that TSP2 could potentiate the transcriptional activity of the intracellular fragment of Notch3 through an intracellular pathway rather than through extracellular interactions. However, TSP2 cotransfection was unable to potentiate the action of the isolated Notch3 intracellular fragment (32) (Fig. 1D). Thus, the enhancement of Notch3 activity by TSP2 requires the presence of the Notch3 extracellular domain.

To test whether TSP2 activates Notch signaling in additional cell lines, we examined A2780 ovarian cancer cells that express Notch3 (22). TSP2 enhanced A2780 Notch3 function in coculture assays (Fig. 2). TSP2 cotransfected with Hes1-luciferase increased Jagged1 and Delta1 stimulated activity (Fig. 2A). Recombinant TSP2, added to cocultures of A2780 and ligand expressing cells, also potentiated ligand-
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A

FIGURE 2. Effect of TSP2 on Notch signaling in A2780 ovarian cancer cells. Coculture experiments were performed as described in the legend to Fig. 1, using luciferase reporter-transfected A2780 cells (with and without TSP2 expression vector) combined with control, Jagged1, or Delta1 expressing mouse fibroblasts. A, TSP2 or empty vector was introduced into A2780 cells by cotransfection. B, TSP2 (200 ng/ml) or PBS were added to the culture media. y axis values represent the ratio of Hes1-luciferase expression to constitutive Renilla luciferase expression. * denotes significant (p < 0.05) effect of TSP2. # denotes significance (p < 0.05) of Jagged1 or Delta1 compared with L cell controls. Experiments were repeated three times.

B

FIGURE 3. Relative effects of TSP1 and TSP2 on Notch signaling. Coculture experiments were performed as described in the legend to Fig. 1, using Hes1-luciferase reporter-transfected H460 (A and C) or A2780 (B and D) cells and Jagged1 or Delta1 expressing mouse fibroblasts (compared with control L cells). Cells were cocultured with serum overnight. In A and C, cells were washed and incubated with serum-free Dulbecco’s modified Eagle’s medium. TSP1 (100 ng/ml), TSP2 (100 ng/ml), or PBS were added to the media for 12 h prior to luciferase analysis. In B and D, cells were cotransfected with TSP1 or TSP2 CDNA (or blank vector) prior to coculture with L, Jagged1, or Delta1 expressing cells under serum-free conditions; luciferase assays were performed the next day. * denotes significant (p < 0.05) effect of TSP2 compared with TSP1 and PBS controls. # denotes significance (p < 0.05) of Jagged1 or Delta1 compared with L cell controls. Experiments were repeated three times or more.

dependent Notch3 responses (Fig. 2B). Thus, TSP2 regulates Notch3 activity in two independent Notch3-expressing cell lines.

TSP1 Does Not Enhance Notch3 Signaling—TSP1 is closely related to TSP2. We therefore tested whether TSP1 could also enhance Notch3 signaling using the coculture assay described above. Because TSP1 is found in significant quantities in serum, we performed coculture experiments in serum-free media and supplemented recombinant TSP1 or TSP2 into the media. In contrast to TSP2, TSP1 failed to augment Notch3 signaling in H460 cells (Fig. 3A). Similar findings were observed when we used A2870 cells (Fig. 3B) cocultured in serum-free conditions.

We also cotransfected TSP1 or TSP2 cDNA with the Hes1-luciferase construct to determine whether TSP1 coexpressed with Notch3 could potentiate Notch3 signaling (Fig. 3, C and D). Unlike TSP2, TSP1 failed to enhance Notch signaling, confirming that the Notch-enhancing ability is specific for TSP2 under serum-free conditions. Collectively, we conclude from these experiments that TSP1 does not have Notch3 enhancing properties and that TSP2 activation of Notch3 does not require coactivating serum factors.

Notch3 Target Gene Expression in TSP2 Knock-out Animals—Notch3 is selectively expressed in arterial smooth muscle cells (33, 34), and inactivation of the murine Notch3 gene results in decreased expression of the Notch target genes Hes1 and Hes5 (35). Thus, Notch3 is a major transcriptional regulator for the canonical Notch targets in arterial tissues in vivo. The levels of Hes1, Hes5, and Hey1 gene expression were significantly reduced in TSP2 knockouts, indicating that TSP2 plays a role in Notch3-stimulated gene expression in vivo (Fig. 4).

TSP2 Potentiates Notch3 Inhibition of Cell Proliferation—Lung (29), ovarian (22), and breast (36) cancer cell lines exhibit cell autonomous Notch3-dependent cell growth, but the influence of Notch ligands on cancer cells has not been characterized. We investigated in a coculture system whether non-cell autonomous Notch3 activation could modulate cell growth, and whether TSP2 could further amplify ligand-dependent Notch3 signaling effects (Fig. 5). H460 cells were cocultured with control, Jagged1, or Delta1 expressing fibroblasts to stimulate Notch signaling. We stained cocultured cells with the pan-human specific antibody TRA1–85, which specifically labels Notch3 expressing cancer cells but not ligand expressing mouse cells. Quantitation of TRA1–85 positive cancer cells demonstrated decreases in cancer cell number with Jagged1 or Delta1 cocultures. Although we were somewhat surprised by the inhibition of proliferation by non-cell autonomous Notch signaling, recent studies have demonstrated
both pro- and anti-proliferative effects of Notch (37–39). Transfection of H460 cells with TSP2 cDNA further reduced cell growth in the Jagged1 cocultures, but did not affect cancer cells cocultured with control cells that did not express ligands. These experiments demonstrate that ligand-stimulated Notch3 suppression of cancer cell proliferation is amplified by TSP2.

TSP2 and Notch3 Directly Interact—We examined whether TSP2 forms complexes with Notch3 in cells (Fig. 6). Notch3 and TSP2 cDNAs were transfected into 293 cells and protein complexes were immunoprecipitated using Notch3 antibodies to pull down Notch3 full-length protein (Fig. 6A). Immunoprecipitates were analyzed by Western blotting to test for association of cognate proteins. Our results demonstrate that TSP2 binds to Notch3 in cells. Similar results were obtained using isolated hemagglutinin-tagged Notch3 ectodomain and TSP2 (not shown) showing that TSP2 interacts with Notch3 via Notch3 EGF-like repeats. Thrombomodulin and fibulin, which also contain EGF-like repeats, did not interact with Notch3 in co-immunoprecipitation assays, suggesting that the Notch3-TSP2 interaction was specific (not shown).

To test whether the binding of TSP2 to Notch3 occurred on cells, we applied recombinant TSP2 to the culture media of H460 cells and then immunoprecipitated Notch3. TSP2 coprecipitated with Notch3 (Fig. 6B) but did not associate with immunoprecipitates generated with control antibodies. This experiment suggests that TSP2 binds directly to Notch at the surface of cells.

To confirm that Notch3 and TSP2 were capable of binding directly, purified, recombinant TSP2 was immobilized on plastic plates. Notch3 ectodomain (expressed as an Fc fusion to the first 11 EGF-like repeats of Notch3 and labeled with Alexa700-succinimide) was applied to TSP2-coated plates. Significant amounts of Notch3 bound to TSP2-coated plates (Fig. 6C) with an EC50 of 2 μg/ml. Only trace amounts of Fc protein bound to TSP2-coated plates (and were subtracted from the Notch3 value to generate data displayed in Fig. 6C). Taken together, the coimmunoprecipitation and in vitro binding studies demonstrate that TSP2 and Notch3 bind specifically and directly.

In addition, we tested whether purified Notch3 and TSP1 bind in vitro. Using plate binding assays, we showed that Notch3 binds to TSP1-coated plates (Fig. 6D). Thus, although only TSP2 activated Notch3 signaling, TSP1 and TSP2 both bind to Notch3 in vitro.

Interaction between TSP2 and Jagged1—TSP2 does not activate Notch3 by itself; rather, it potentiates signaling in the presence of ligands. It is possible that TSP2 could simultaneously bind Notch and ligand to facilitate binding. Therefore, we investigated whether TSP2 could also bind to Jagged1, which structurally resembles Notch proteins and has been shown previously to interact with TSP1 (25). TSP2 and Jagged1 were coexpressed and pulled down with monoclonal antibodies. As in previous experiments, binding was determined by detection of the reciprocal protein by Western blotting. The interaction was dependent on coexpression of the two proteins (Fig. 7A). To determine whether Jagged1 could bind to TSP2 added to the cell culture media, we incubated recombinant TSP2 with Jagged1 expressing fibroblasts. We detected TSP2 protein with immunoprecipitated Jagged1 (Fig. 7B), indicating that the TSP2/Jagged1 interaction could take place on the cell surface. In addition, we confirmed that TSP2 binds Jagged1 directly in an in vitro binding assay (Fig. 7C). Previous studies demonstrated that Jagged1 interacts with TSP1 in yeast (25). We performed additional studies that demonstrate that Jagged1 binds to TSP1 in direct binding assays (Fig. 7D).
To test whether TSP2 could affect binding between Jagged1 and Notch3, we applied unlabeled TSP2 to in vitro binding reactions. Total Jagged1 binding to the Notch3 ectodomain was significantly enhanced by the addition of unlabeled recombinant TSP2 (Fig. 7E). TSP2 did not change the binding affinity between Notch3 and Jagged1 (calculated EC_{50} was 2 ng/ml for both). Significantly, although TSP1 bound Notch3 and Jagged in vitro, TSP1 did not alter the affinity of the amount of binding between Notch3 and Jagged1. The specific ability of TSP2 to enhance binding of the ligand and its receptor is consistent with its role in stimulating the overall levels of Notch signaling that was demonstrated in Fig. 1.

**DISCUSSION**

Modifiers of Notch identified to date principally act on intracellular signaling components. However, the immense size of the Notch extracellular domain has suggested that non-ligand extracellular binding proteins modify Notch responses. Our experiments demonstrate for the first time that the matricellular protein TSP2 modifies Notch signaling potency and binds to Notch3 and Jagged1.

We show a significant effect of TSP2 on Notch3 signaling in cell culture models. First, we show that TSP2 quantitatively increases Notch signaling potency. A second novel observation is that at the cellular level, TSP2 and Notch3 signaling synergize to control cell proliferation. Examination of TSP2 knock-out mice demonstrates that TSP2 expressed at physiological levels indeed appears to be an important modulator of Notch3 signaling in the vasculature.

Our studies indicate a novel cooperative role for a matricellular protein in Notch signaling. A growing list of extracellular matrix proteins, including MAGP (24), CCN/NOV3 (23), and contactin (40), activate Notch signaling independent of classical ligands (Jagged and Delta families of proteins). In contrast to these proteins, TSP2 is not sufficient for Notch3 signaling, because stimulation of Notch3 signaling only occurs when cells are cocultured with Jagged1 or Delta1 producing cells. We are not certain that this represents a truly distinctive characteristic of TSP2, because the experimental paradigms used here were not the same as in prior studies, but it is intriguing to note additional differences between TSP2 and previously identified factors. MAGP activation of Notch1, for example, only activates Notch when coexpressed in the Notch expressing cell (24), whereas TSP2 amplifies Notch3 activity regardless of whether it is transfected into the Notch3 expressing cell or provided exogenously.

We show that TSP2 binds to both Notch3 and Jagged1 directly. It is therefore reasonable to hypothesize that TSP2 stabilizes the interaction between these two molecules and improves signaling by facilitating their physical association. This is supported by the finding that TSP2 in vitro is capable of increasing the total levels of Jagged1 binding to Notch3, although the affinity does not change. Certainly, the multivalent properties of trimeric TSP2 support the possibility that it could enhance the multimerization of protein complexes involving Notch, which may enhance signaling. TSP2 may also facilitate signaling by bridging Notch to ligands. If true, it is conceivable that TSP2 could potentiate Notch3 signaling in cells separated by large distances, and, in some cases, by virtue of its size, may be an important adaptor required for signaling when the intercellular space exceeds the combined lengths of the Notch/ligand proteins.

TSP2 activates cellular pathways through other well studied receptors such as heparan sulfate proteoglycans, integrins, CD36, CD47, and LRP1, which play critical roles in an array of processes that widely overlap with Notch function. Identification of other cell surface factors that bind to TSP2 significantly increases the complexity of regulation of Notch signaling, which up until now has been shown to be regulated principally...
by intracellular factors. Further work will be needed to define receptors involved in this process.

Proliferation of Notch3-expressing H460 cells is inhibited by TSP2 only in the presence of Notch ligand. Taking quantitative gene expression and in vitro luciferase reporter experiments into consideration, we propose that TSP2 specifically activates Notch signaling to further suppress cell proliferation. The requirement for both Notch ligand and TSP2 together strongly implies that the two proteins’ functions are integrated. The apparent preferential effect of TSP2 for Jagged in H460 cell proliferation studies may be due to cell type-specific differences in response to different Notch ligands or, more likely, the relative variability of proliferation assays compared with luciferase reporter experiments.

These cell proliferation results appear to contrast previous studies in which Notch3 inhibition using a dominant negative ectodomain and γ-secretase inhibitors have been shown to inhibit lung (29, 41), breast (36), and ovarian cancer cell proliferation (22). However, our studies involve a different experimental paradigm than those used in previous studies, in which cell autonomous growth (single cell types grown in culture) was examined (21) (22). In contrast to prior studies, we examined the role of Notch3 signaling in cocultures, in which Notch3 signaling is stimulated by cells presenting ligand, which may be more relevant to specific conditions in vivo. An alternative explanation for the antiproliferative effects seen in our studies is that the effects of Notch3 may be highly dosage dependent such that only a narrow range of Notch signaling results in growth promotion. Divergent effects of Notch have been noted in other systems, including a vast literature on tumor angiogenesis (10), and could be a consequence of unrecognized differences in experimental protocols.

Our results suggest that the role of Notch3 in cancer cells is complex and may be context dependent. For example, Notch3 overexpression may provide a growth advantage for tumor cells in the absence of ligand stimulation by non-tumor cells (cell autonomous signaling); such conditions may occur deep within the bed of a tumor. However, if tumor cells are admixed with ligand expressing cells, growth may, in fact, be inhibited through a non-cell autonomous mechanism; potential sources of ligand-stimulated Notch3 activity could come from invading endothelial cells and from fibroblasts and other stromal cells at the tumor margins, which may also express TSP2 (42). It is noteworthy that TSP2 expression in tumors has been associated with decreased vascularity, progression, and metastasis (42–44); the inhibitory role of TSP2 in tumors is thought to be related to its antiangiogenic function; however, our studies suggest that it may also have direct actions on tumor cells through Notch. Further studies will be required to investigate whether TSP2 or proteolytically derived fragments of TSP2 could be useful against Notch expressing tumors.

TSP1 and TSP2 share significant sequence homology, and we show here for the first time that both proteins bind to Notch3
and Jagged1. Thus, it is somewhat surprising that TSP1 does not enhance Notch3 signaling. The physiological functions of TSP1 and TSP2 are similar, but not identical (e.g., sensitivity to heparin inhibition (45) and activation of transforming growth factor-β (46, 47)); differences between the respective knock-out mouse phenotypes have been thought to arise from differential temporal and spatial gene expression. Our studies suggest that some of the differences in development features of the knock-outs may also arise through differential actions of the TSP isoforms on Notch, which have significant impact on development in multiple organ systems.

The absence of Notch enhancing function of TSP1 supports the possibility that TSP1 binds in a different manner compared with TSP2. In support of this, we found that TSP1 binds to both Notch3 and Jagged1 in pairwise interaction assays, but TSP1 did not enhance the binding of Notch3 and Jagged1. These studies thus imply important functional roles of structural differences between TSP1 and TSP2, which include relative divergence in the NH₂-terminal domain primary sequence (only 32% identity), an unpaired cysteine (Cys₉⁹²) in TSP1, and the possible additional calcium ion bound to the lectin-like domain of TSP1 (recently discussed by Ref. 3).

Based on significant sequence homology, it is probable that physical and functional interactions may exist between other TSPs (TSP3–5) and Notch (1, 2, and 4) proteins; delineation of the spectrum of interactions between TSP and Notch family members will require further experimental verification. Given the intricate reciprocal relationships between TSP proteins, the extracellular matrix, membrane-bound receptors, and the small but growing number of secreted proteins that modify Notch signaling, our study supports the concept that cell-associated extracellular factors may play a role in modulating Notch signaling.

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