Reciprocal Regulation of Syndecan-2 and Notch Signaling in Vascular Smooth Muscle Cells*§

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Background: The interaction of endothelial and smooth muscle cells is critical for blood vessel formation.

Results: Endothelial cells induce syndecan-2 expression in smooth muscle cells through Notch signaling, and syndecan-2 acts as a Notch coreceptor.

Conclusion: Notch signaling and syndecan-2 cooperate to govern smooth muscle cell differentiation.

Significance: Interaction of syndecan-2 and Notch signaling is a novel strategy for the regulation of smooth muscle cell differentiation.

Vascular cell interactions mediated through cell surface receptors play a critical role in the assembly and maintenance of blood vessels. These signaling interactions transmit important information that alters cell function through changes in protein dynamics and gene expression. Here, we identify syndecan-2 (SDC2) as a gene whose expression is induced in smooth muscle cells upon physical contact with endothelial cells. Syndecan-2 is a heparan sulfate proteoglycan that is known to be important for developmental processes, including angiogenesis. Our results show that endothelial cells induce mRNA expression of syndecan-2 in smooth muscle cells by activating Notch receptor signaling. Both NOTCH2 and NOTCH3 contribute to the increased expression of syndecan-2 and are themselves sufficient to promote its expression independent of endothelial cells. Syndecan family members serve as coreceptors for signaling molecules, and interestingly, our data show that syndecan-2 regulates Notch signaling and physically interacts with NOTCH3. Notch activity is attenuated in smooth muscle cells made deficient in syndecan-2, and this specifically prevents expression of the differentiation marker smooth muscle α-actin. These results show a novel mechanism in which Notch receptors control their own activity by inducing the expression of syndecan-2, which then acts to propagate Notch signaling by direct receptor interaction.

Proper communication between endothelial and smooth muscle cells is fundamental for the formation and function of the vasculature (1, 2). Defective interactions between these cells during development lead to vascular malformations (3), whereas endothelial dysfunction in mature blood vessels causes smooth muscle abnormalities associated with vascular disease (4). Despite the obvious importance of these interactions within blood vessels, the exact signaling mechanisms that facilitate cell-cell communication remain largely undefined. One signaling pathway that has garnered much attention for facilitating vascular cell interactions is the Notch pathway (5, 6). Notch proteins are evolutionarily conserved and critical for cell fate determination and differentiation (7, 8). Four Notch receptors are present in mammals, Notch1–4, and their activation is triggered by the interaction with membrane-bound ligands (Jagged1 and Delta-like-1/3/4). Upon binding, receptors undergo cleavage events that release a Notch intracellular domain (NICD), which translocates to the nucleus and binds with the transcription factor CSL (CBF1/RBP-Jκ, Su(H), and Lag-1) to regulate downstream gene expression, most notably members of the Hes (hairy/enhancer of split) and Hrt (hairy-related, also referred to as Hey, CHF, and HESR) families (9). A host of studies have examined Notch signaling in the vasculature and shown it to be important for angiogenic remodeling, arterial/venous specification, and tip cell differentiation (10). One particularly relevant study demonstrated that endothelial expressed Jagged1 is essential for vascular smooth muscle differentiation (11). Data from our own laboratory have shown that NOTCH3 expression is induced in smooth muscle cells when cocultured with endothelial cells (12). We further demonstrated that differentiation of smooth muscle cells by endothelial cells was dependent upon NOTCH3. In addition to NOTCH3, in this study, we show syndecan-2 expression is induced by cocultured endothelial cells.

Like the Notch family, syndecans are evolutionarily conserved transmembrane proteins that have been implicated in regulating a broad range of development and disease processes (13, 14). Syndecan-2 is widely expressed in developing mesenchymal tissues, including cells surrounding blood vessels (14, 15). Syndecan-2, which then acts to propagate Notch signaling by direct receptor interaction.

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The abbreviations used are: NICD, Notch intracellular domain; HAoSMC, human aortic smooth muscle cell; HDFN, human dermal neonatal fibroblast; HUVEC, human umbilical vein endothelial cell; DAPT, N-(3,5-difluorophenyl)acetyl-L-alanyl-2-phenylglycine 1,1-dimethyl ethyl ester; qPCR, quantitative real-time PCR.

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15. Syndecan-2 knockdown in zebrafish leads to defective angiogenic sprouting (16), and syndecan-2 inactivation in microvascular endothelial cells causes impairments in capillary tube-like structures (17, 18). In addition to regulating angiogenesis, functional studies have demonstrated a role in left-right axis formation (19) and promotion of membrane protrusions and migration (20–22). Syndecan-2 is a heparan sulfate proteoglycan with glycosaminoglycan chains attached to the extraacellular domain (ectodomain) of the protein (23). The glycosaminoglycan ectodomains of syndecan family members are known to control both cell-matrix and cell-cell interactions and serve as a coreceptor for growth factor PDGF, FGF, and VEGF signaling (13, 14) and TGFβ signaling (24). In this work, we describe the interaction of two cell surface signaling mediators, NOTCH3 and syndecan-2. We show that syndecan-2 expression is induced in smooth muscle cells by coculturing with endothelial cells, and this induction relies on Notch signaling. Furthermore, we demonstrate that syndecan-2 augments Notch activity and directly binds to the NOTCH3 receptor. These data highlight the importance of crosstalk between individual signaling pathways in governing cell communication within the vasculature.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary cultures of human aortic smooth muscle cells (HAoSMCs) and human coronary artery smooth muscle cells were purchased from Lonza and grown in DMEM (Mediatech, Inc.) supplemented with 10% FBS (HyClone), 2 mM glutamine, 1 mM sodium pyruvate, and 100 units/ml penicillin/streptomycin. Human dermal neonatal fibroblasts (HDFNs) were purchased from Cascade Biological and cultured in DMEM supplemented as described above with 5% FBS. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza and grown in EBM-2 supplemented with the BulletKit components as recommended by the manufacturer. Primary cells between passages 6 and 9 were used for all experiments. Human hepatoblastoma (HepG2) cells and human adenocarcinoma (HeLa) cells were purchased from American Type Culture Collection and cultured in DMEM supplemented as indicated with 10% FBS. For virus production, TN-293 cells were purchased from Stratagene and cultured in 10% DMEM as described above. All cultures were maintained in humidified 5% CO2 at 37 °C. For coculture, 6 × 10⁴ mural cells were plated in 12-well plates, and after adhesion, 6 × 10⁴ HUVECs were added. To separate endothelial cells from fibroblasts and smooth muscle cells, anti-PECAM1-conjugated Dynabeads (Invitrogen) were used according to the manufacturer’s instructions. All cell coculture experiments, unless indicated, were performed in medium consisting of EBM-2 supplemented with all BulletKit components except FBS, VEGF, and basic FGF. This medium was supplemented with 1% FBS and 30 ng/ml VEGF-A165 (PeproTech). N-[3-(3,5-Difluorophenyl)acetyl]-l-alanyl-2-phenyglycine 1,1-dimethyl ethyl ester (DAPT; Calbiochem) was added to specified wells at the time of plating. Transwell experiments were performed as described previously (12). Transwell inserts (12-well type, Corning Costar) with 0.4-μm pores were coated with 50 μg/ml rat tail collagen I (BD Biosciences). 2 × 10⁴ HDFNs or HUVECs were first plated on the outside of the polycarbonate membrane of the Transwell inserts. After cell adherence, the Transwell inserts were inverted and reinserted onto 12-well plates, and 2 × 10⁴ HDFNs or HUVECs were plated on the top surface of the insert and cultured in a final volume of 1.3 ml of medium (0.3 ml in the insert and 1 ml in the well). Following incubation for 48 h, cells grown on the top side of the inserts were harvested by trypsinization and processed for quantitative real-time PCR (qPCR).

qPCR—Total RNA was isolated using TRIzol reagent (Invitrogen) following the manufacturer’s instructions and reverse-transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) to generate cDNA. Real-time PCR was performed using a StepOne PCR system (Applied Biosystems) with SYBR Green and 50 ng of cDNA template. The -fold difference in various transcripts was calculated using the ΔΔCT method with 18 S RNA the internal control. Primer sequences were as follows: syndecan-2, 5′-CTG GCC ACC GAC TAT GAG AA (forward) and 5′-AAA ATC CAC GTG AAA AAG TTG GA (reverse); NOTCH3, 5′-CCT AGA CCT GGT GGA CAA G (forward) and 5′-ACA CAG TCG TAG CTT GGG (reverse); 18 S, 5′-GTT GGT TTT CTT GGG AAC TGA GGC (forward) and 5′-GTC GGC ATC GTT TAT GGT CG (reverse); NOTCH2, 5′-ACA GTT GTG TCT GCT CAC CAG GAT (forward) and 5′-GCC AGG GCT CAG ATT CAC GGT GAT (reverse); HRT3, 5′-CAT ACA ATG TCC TTG TGG AGT ACA CA (forward) and 5′-GCC AGG GCT CGG GCA TCA AAG AA (reverse); PDGF receptor-β, 5′-AGA GCC CAG CCG AGC AA (forward) and 5′-CAT ACA ATG TCC TTG TGG AGT ACA CA (reverse); smooth muscle α-actin, 5′-CAA GTG ATC ACC ATC GGA AAT G (forward) and 5′-GAC CTTAC CCG ATG AA GAA AG (reverse); SM22α, 5′-CAA GCT GGT GAA CAG CCT GTA C (forward) and 5′-GAC CAT GGA GGG TGG GTT CT (reverse); and CNN1, 5′-TGA ACC CCC ACG ACA TTT TT (forward) and 5′-GGG TGG ACT GCA CCT GTG TA (reverse). RNAs from yolk sacs of Notch2 (25) and Notch3 (26) mutant mice were isolated using RNasy mini columns (Qiagen). Mouse syndecan-2 primers were 5′-TCG CCT TTC GGC ATC CT (forward) and 5′-GCC AGA GCT GAA TCC CTC (reverse). Western Blotting—Equivalent amounts of protein were run on 10% SDS-polyacrylamide gels; transferred to Immobilon PVDF membranes (Millipore); and subjected to incubation using primary antibodies to NOTCH3 (sc-5593, Santa Cruz Biotechnology), NOTCH2 (C651.6DbHN, Developmental Studies Hybridoma Bank), β-tubulin 1 (T7816, Sigma), smooth muscle α-actin (1A4, Sigma), and HA tag (sc-7392, Santa Cruz Biotechnology). Secondary antibodies conjugated to HRP (Amersham Biosciences) were used for detection. Protein was detected by enhanced chemiluminescence (Thermo Scientific).

RNA Interference—HAoSMCs were plated in a 12-well plate at 6 × 10⁴ cells/well. After 12 h, the cells were transfected with siRNA using Lipofectamine 2000 (Invitrogen). Efficiency of knockdown was assessed using qPCR (supplemental Fig. 1) and Western blotting (see Fig. 3B). siRNAs were synthesized by IDT as follows: NOTCH3 siRNA sequence, 5′-AAC UGC GAA GUG AAC AUU G (used as described previously (12)); NOTCH2 siRNA sequence, 5′-CCC CCC AUU GUG ACU
UUC CAG CUC A; and syndecan-2 siRNA sequence, 5'-GCU GAC AUC UGA UAA AGA CAU. All siRNAs were transfected at 100 nM. Following transfection, cells were cocultured with HUVECs for 48 h, separated, and collected for qPCR analysis and Western blotting.

Lentivirus Expression—Human NOTCH2 intracellular domain (NICD2) cDNA (a gift from Dr. Igor Prudovsky) was cloned with a HA-tag attached to the 3'-end into pCDF1-MCS2-EF1-copGFP (System Biosciences) using BamHI and EcoRI sites. NOTCH3 intracellular domain (NICD3) and dominant-negative MAML (mastermind-like 1) constructs were made as described previously (12). The human syndecan-2 open reading frame (American Type Culture Collection) was amplified by PCR and cloned into pCDF1-MCS2-EF1-copGFP using XbaI and BglII sites. A HA tag was conjugated to the 3'-end of syndecan-2 by PCR and cloned using XbaI and EcoRI sites. The lentivirus plasmids were transfected into TN-293 cells using Lipofectamine 2000 (Invitrogen), and the viral particles were amplified and purified as described (12). For HDFN and HAoSMC infection, equal volumes of viral particles were diluted in 10% FBS in DMEM and were incubated with cells for 24 h. The efficacy of infection was evaluated using GFP expression and qPCR. Viral particles were titrated to achieve 90–100% infection. Expression of NICD2, NICD3, and syndecan-2 cDNAs was confirmed by qPCR and Western blot analysis.

Plasmid Transfection and Luciferase Assays—A 5×CBF1-luciferase plasmid was generated as described (12). To measure the transcriptional activity, HDFNs or HAoSMCs at 80% confluency were sequentially transfected with siRNA followed by plasmids using Lipofectamine 2000. Cells were then cocultured with an equal number of HDFNs or HAoSMCs (as a control) or HUVECs for an additional 48 h. Cells were collected, and promoter activity was measured by luciferase assays using SteadyGlo reagent (Promega). To normalize the transfection efficiency, Hsp-β-galactosidase (LacZ) was cotransfected, and luciferase activities were normalized based on an equivalent amount of LacZ activity. Luciferase and LacZ activities were measured as described (27) and quantified using a Molecular Devices SpectraMax luminometer. All experiments were performed in duplicate and repeated a minimum of three times.

Immunoprecipitations—Approximately 2 × 10^6 cells in a 10-cm plate were washed with ice-cold Krebs-Ringer-Hepes buffer (125 mM NaCl, 4.8 mM KCl, 2.6 mM CaCl_2, 1.2 mM MgSO_4, 25 mM Hepes, and 5.6 mM glucose (pH 7.4)) and then cross-linked by 1 mM dithiobis(succinimidyl) propionate (Thermo Scientific) for 15 min at room temperature. After washing once, dithiobis(succinimidyl) propionate was quenched by 100 μl of 103 mM glycine (pH 7.5) in 1% SDS. 1 mg of protein from cell lysates was subjected to immunoprecipitation using magnetic beads preincubated with anti-HA antibody, anti-NOTCH3 antibody, mouse IgG, or rabbit IgG (Santa Cruz Biotechnology). After precipitation at 4°C for 3 h, immunoprecipitates were eluted with elution buffer (10 mM Tris (pH 7.5), 0.2 M dithiothreitol, and 5 mM EDTA) and subjected to immunoblotting.

Statistical Analysis—Data analyses were performed using GraphPad Prism, and comparisons between data sets were made using Student’s t test. Differences were considered significant if p < 0.05, and data are presented as means ± S.E. Data shown are representative of at least three independent experiments.

RESULTS

Syndecan-2 Is Regulated by Endothelial Cells through Notch Signaling—Using a screen to find genes regulated by endothelial-mural cell interaction, we identified an array of genes whose
expression was altered when cells of the vasculature were cocultured (28). One of these genes was the heparan sulfate proteoglycan syndecan-2 (SDC2) (15). Coculture of HUVECs with HDFNs, HAoSMCs, or human coronary artery smooth muscle cells led to a significant induction of syndecan-2 mRNA expression in mural cells as assessed by qPCR (Fig. 1A). The induction in the different mural cell subtypes ranged from 10-fold in fibroblasts to 4-fold in coronary artery smooth muscle cells, which exhibited a higher level of basal syndecan-2 expression. In contrast, two non-mural cell types, HepG2 and HeLa cells, showed no significant induction of syndecan-2 when cultured with HUVECs (Fig. 1B). To determine whether the induction of syndecan-2 requires cell-cell contact between neighboring endothelial and mural cells, we performed coculture experiments using Transwell inserts to physically separate the cells. Compared with control wells that had endothelial and mural cells cultured together, when endothelial cells were separated by a 0.4-μm pore membrane, the level of syndecan-2 expression remained low in the mural cells, similar to that seen in non-cocultures (Fig. 1C). These data indicate that endothelial cells induce syndecan-2 expression in mural cells and that this is dependent upon cell-cell contact.

Our laboratory previously showed the importance of Notch signaling for the communication of endothelial and mural cells (12, 28). Given that syndecan-2 induction requires cell-cell contact and Notch receptor activation relies on contact from an adjacent ligand-presenting cell, we tested the involvement of the Notch pathway. To do so, we used the γ-secretase inhibitor DAPT, which prevents Notch receptor cleavage. In the presence of endothelial cells, 3 μM DAPT blocked the up-regulation of syndecan-2 in both fibroblasts and smooth muscle cells (Fig. 2, A and B). We further confirmed the role of Notch signaling by using the dominant-negative MAML construct, which blocks Notch transcriptional activity (29). Similar to DAPT inhibition, syndecan-2 transcript induction by endothelial cells was inhibited in the presence of dominant-negative MAML (Fig. 2, C and D).

**NOTCH2** and **NOTCH3** are prominently expressed in vascular smooth muscle cells (30–32). To determine whether one or both of these Notch receptors is responsible for the transactivation of syndecan-2, we performed knockdown experiments with **NOTCH2** and **NOTCH3** utilizing siRNA (Fig. 3 and supplemental Fig. 1). Under coculture conditions, knockdown of **NOTCH2** significantly blocked syndecan-2 induction by endothelial cells, whereas knockdown of **NOTCH3** had little effect (Fig. 3, A and B). siRNA inhibition of both the **NOTCH2** and **NOTCH3** receptors resulted in a complete loss of the inductive effects of endothelial cells on syndecan-2 expression. To determine whether the Notch receptors are sufficient for syndecan-2 gene expression, we overexpressed the intracellular domains of **NOTCH2** (NICD2) and **NOTCH3** (NICD3) by lentivirus transduction in smooth muscle cells and measured syndecan-2 expression (Fig. 3, C and D). The data show that both **NOTCH2** and **NOTCH3** can promote syndecan-2 expression in smooth muscle cells. Together, these results show that Notch signaling...
is both sufficient and necessary for the endothelial cell-dependent expression of syndecan-2.

To determine whether syndecan-2 expression might be regulated by Notch2 and Notch3 in vivo, we examined the highly vascularized yolk sacs of mouse embryos with mutations in the Notch2 and/or Notch3 gene at embryonic day 10.5. qPCR was performed to determine expression of syndecan-2 in mice lacking one (+/−) or both (−/−) wild-type copies of Notch2 and Notch3. **, p < 0.01; *, p < 0.05.

Syndecan-2 Regulates Notch Signaling—Previously, we showed that endothelial cells activate Notch signaling in neighboring mural cells to promote an autoregulatory loop resulting in NOTCH3 induction, followed by NOTCH3-dependent differentiation of smooth muscle cells (12). Because syndecan-2 is a target of Notch signaling, we asked if the up-regulation of syndecan-2 might affect Notch signaling in smooth muscle cells. To address this, we first examined endothelial cell-activated Notch signaling using a CBF1-luciferase reporter construct, which serves as a general Notch signaling sensor. HAoSMCs were cotransfected with the CBF1-luciferase or control luciferase construct along with siRNA to knock down syndecan-2 expression (Fig. 5, A and B). Following coculture with endothelial cells, Notch transcriptional activity was measured by luciferase assays. As published previously (12), endothelial cells promoted robust activity of the CBF1-luciferase reporter construct, which serves as a general Notch signaling sensor. HAoSMCs were cotransfected with the CBF1-luciferase or control luciferase construct along with siRNA to knock down syndecan-2 expression (Fig. 5, A and B). Following coculture with endothelial cells, Notch transcriptional activity was measured by luciferase assays. As published previously (12), endothelial cells promoted robust activity of the CBF1-luciferase reporter construct, which serves as a general Notch signaling sensor. However, when syndecan-2 was knocked down in smooth muscle cells, Notch signaling was greatly attenuated (Fig. 5A), and furthermore, NOTCH3 expression was decreased, as a likely consequence of its inability to autoactivate its own expression (Fig. 5, C and D).
To more precisely examine the downstream effect that the loss of syndecan-2 has on Notch signaling, we measured the expression of known targets of \textit{NOTCH3} in smooth muscle cells cocultured with endothelial cells. Consistent with our previous findings, expression of \textit{HEYL}/\textit{HRT3}, PDGF receptor-\textit{β}, smooth muscle α-actin, SM22α, and \textit{CNN1} (calponin-h1) was up-regulated in smooth muscle cells by endothelial cell coculture (Fig. 6, A–F). In the absence of syndecan-2, however, the expression of most of these genes was abrogated. \textit{CNN1} showed a slight but not significant decrease, suggesting that it is regulated differently. These data indicate that syndecan-2 facilitates Notch signaling in smooth muscle cells and is an important mediator of smooth muscle differentiation by regulating the expression of some smooth muscle genes.

To assess whether syndecan-2 is sufficient to activate Notch signaling and smooth muscle gene expression, we overexpressed syndecan-2 cDNA by lentivirus transduction in smooth muscle cells and measured gene expression by qPCR and Western blotting. Syndecan-2 was overexpressed by \textasciitilde50-fold (Fig. 7A). In smooth muscle cells cultured alone, the overexpression of syndecan-2 could not induce \textit{NOTCH3} expression or the expression of Notch signaling targets \textit{HRT3}, PDGF receptor-\textit{β}, and smooth muscle α-actin (Fig. 7). Moreover, in cells cocultured with HUVECs, in which Notch signaling is activated, overexpression of syndecan-2 could not further induce any of the tested Notch targets (Fig. 7).

\textbf{Syndecan-2 and NOTCH3 Physically Interact}—Our data indicated that syndecan-2 modulates Notch signaling, and because both proteins are localized within the cell membrane, we speculated that syndecan-2 was facilitating Notch signaling through direct binding to Notch receptors. Because our attempts to use commercial antibodies to detect human syndecan-2 were unsuccessful, we performed co-immunoprecipitation experiments with a HA-tagged full-length syndecan-2 (HA-SDC2) protein, followed by immunoblotting to detect endogenous \textit{NOTCH3}. HA-SDC2 was lentivirally transduced into cells, followed by immunoprecipitations with IgG or anti-HA antibodies. \textit{NOTCH3}-specific immunoblots demonstrated that \textit{NOTCH3} protein was pulled down with syndecan-2 (Fig. 8). The reverse experiment of immunoprecipitating endogenous \textit{NOTCH3}, followed by probing for HA-tagged syndecan-2 by Western blotting, showed a similar result (Fig. 8). Thus, our data show that these two proteins physically associate in cultured cells, providing a mechanism by which syndecan-2 modulates Notch signaling.

\textbf{DISCUSSION}

The signaling events that govern the interaction of vascular cells are critical for proper formation and function of blood vessels. The data presented here provide mechanistic insight into how cells within the vasculature communicate to control the function of each other. Previously, we demonstrated a role for \textit{NOTCH3} in endothelial cell/smooth muscle cell communication (12). Our data showed that Notch signaling is important for endothelial cell-induced differentiation of smooth muscle cells, but the mechanisms down-
stream of NOTCH3 were not defined. Here, we have shown that syndecan-2 is also induced by endothelial cells and is dependent upon NOTCH2 and NOTCH3 for this up-regulation. Moreover, in the absence of syndecan-2, the expression of downstream targets of Notch signaling is attenuated, indicating that syndecan-2 acts as a facilitator of Notch receptor activity. Consistent with this, in the absence of activated Notch signaling, syndecan-2 is not sufficient to induce expression of Notch target genes.

Notch signaling, particularly Notch3, has been shown to be important for the regulation of smooth muscle differentiation (12, 30, 33). Our data suggest that syndecan-2 acts to promote smooth muscle differentiation via modulation of Notch signaling. A precise role for syndecan-2 in smooth muscle has not been reported; however, studies with syndecan family members suggest a potential function in controlling the switch between proliferation and differentiation. Deletion of syndecan-1 causes increased intimal hyperplasia and smooth muscle proliferation particularly in response to PDGF-B (34). Loss of syndecan-4 limits neointimal hyperplasia and reduces smooth muscle proliferation (35). An earlier report showed a requirement for syndecan-4 in thrombin-induced proliferation (36). Unpublished in vitro results from our laboratory indicate that syndecan-2 inhibits smooth muscle proliferation, consistent with a role in governing differentiation in collaboration with Notch signaling. Whether syndecan-2 has functions independent of the Notch pathway in smooth muscle cells remains to be determined.

Very little is known about the regulation of syndecan-2 gene expression. Expression levels have been reported in many cell types and linked to certain cancers (13–15). One report showed that syndecan-2 levels increase upon treatment with tumor necrosis factor-α (37). In vascular smooth muscle cells, FGF2

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was shown to induce the expression of syndecan-4, but not syndecan-2 (38). Our results show that Notch signaling regulates syndecan-2 gene expression in vitro and in vivo. We have shown that both NOTCH2 and NOTCH3 are necessary and sufficient for syndecan-2 induction in smooth muscle cells. Currently, we do not know if the NICD and cofactor CBF (RPB-Jx) bind directly to the syndecan-2 gene to activate its transcription. Like syndecan-2, Notch receptors are widely expressed and are known to be important for development and tumor progression (8). Our results examined Notch-dependent activation of syndecan-2 only in dermal fibroblasts and coronary artery and aortic smooth muscle cells. Given the potential overlap of Notch receptors and syndecan-2 expression in other cell types, it is interesting to speculate that Notch receptors regulate syndecan-2 expression in other tissues, and in turn, syndecan-2 acts to govern Notch signaling.

One of the most interesting findings from our study is that syndecan-2 acts to reinforce Notch signaling in smooth muscle cells. Syndecan-2 appears to do this through direct protein-protein interaction. The syndecan family has been reported to interact with growth factors and their receptors (13, 14), and specifically syndecan-2 has been shown to directly bind to the TGFβ type III receptor, betaglycan (24). More interestingly, Pisconti et al. (39) demonstrated a direct link between syndecan-3 and Notch1 in skeletal muscle satellite cells. These authors showed that Notch1 and syndecan-3 directly interact and that syndecan-3 regulates Notch1 cleavage by ADAM17/tumor necrosis factor-α-converting enzyme. Thus, like growth factor receptors, Notch family members may be common targets for syndecan regulation. Our results show for the first time that syndecan-2 is a target of Notch signaling in smooth muscle cells, and syndecan-2 acts in a feedforward loop to enhance the
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Dev. Cell 21, 193–215

2. Gaengel, K., Genové, G., Armulik, A., and Betsholtz, C. (2009) Endothelial-mural cell signaling in vascular development and angiogenesis. Arterioscler. Thromb. Vasc. Biol. 29, 630–638

3. Brouillard, P., and Vikkula, M. (2003) Vascular malformations: localized defects in vascular morphogenesis. Clin. Genet. 63, 340–351

4. Mombouli, J. V., and Vanhoutte, P. M. (1999) Endothelial dysfunction: from physiology to therapy. J. Mol. Cell. Cardiol. 31, 61–74

5. Gridley, T. (2007) Notch signaling in vascular development and physiology. Development 134, 2709–2718

6. Morrow, D., Guha, S., Sweeney, C., Birney, Y., Walsh, T., O’Brien, C., Walls, D., Redmond, E. M., and Cahill, P. A. (2008) Notch and vascular smooth muscle cell phenotype. Circ. Res. 103, 1370–1382

7. Baron, M. (2003) An overview of the Notch signaling pathway. Semin. Cell Dev. Biol. 14, 113–119

8. Bray, S. J. (2006) Notch signaling: a simple pathway becomes complex. Nat. Rev. Mol. Cell. Biol. 7, 678–689

9. Fischer, A., and Gessler, M. (2007) Delta–Notch—and then? Protein interactions and proposed modes of repression by Hes and Hey bHLH factors. Nucleic Acids Res. 35, 4583–4596

10. Gridley, T. (2010) Notch signaling in the vasculature. Curr. Top. Dev. Biol. 92, 277–309

11. High, F. A., Lu, M. M., Pear, W. S., Loomes, K. M., Kaestner, K. H., and Epstein, J. A. (2008) Endothelial expression of the Notch ligand Jagged1 is required for vascular smooth muscle development. Proc. Natl. Acad. Sci. U.S.A. 105, 1955–1959

12. Liu, H., Kennard, S., and Lilly, B. (2009) NOTCH3 expression is induced in mural cells through an autoregulatory loop that requires endothelial expressed JAGGED1. Circ. Res. 104, 466–475

13. Alexopoulou, A. N., Multhaupt, H. A., and Couchman, J. R. (2007) Syndecans in wound healing, inflammation, and vascular biology. Int. J. Biochem. Cell Biol. 39, 505–528

14. Tkachenko, E., Rhodes, J. M., and Simons, M. (2005) Syndecans: new kids on the signaling block. Circ. Res. 96, 488–500

15. Essner, J. J., Chen, E., and Ekker, S. C. (2006) Syndecan-2. Int. J. Biochem. Cell Biol. 38, 152–156

16. Chen, E., Hermanson, S., and Ekker, S. C. (2004) Syndecan-2 is essential for angiogenic sprouting during zebrafish development. Blood 103, 1710–1719

17. Fears, C. Y., Gladson, C. L., and Woods, A. (2006) Syndecan-2 is expressed in the microvasculature of gliomas and regulates angiogenic processes in microvascular endothelial cells. J. Biol. Chem. 281, 14533–14536

18. Noguer, O., Villena, J., Lorita, J., Vilaró, S., and Reina, M. (2009) Syndecan-2 down-regulation impairs angiogenesis in human microvascular endothelial cells. Exp. Cell Res. 315, 795–808

19. Kramer, K. L., and Yost, H. J. (2002) Ectodermal syndecan-2 mediates left-right axis formation in migrating mesoderm as a cell-nonautonomous Vg1 cofactor. Dev. Cell 2, 115–124

20. Granés, F., García, R., Casaroli-Marano, R. P., Castel, S., Rocamora, N., Reina, M., Ureña, J. M., and Vilaró, S. (1999) Syndecan-2 induces filipodia by active cdc42Hs. Exp. Cell Res. 248, 439–456

21. Arrington, C. B., and Yost, H. J. (2009) Extra-embryonic syndecan-2 regulates organ primordial migration and fibrillogenesis throughout the zebrafish embryo. Development 136, 3143–3152

22. Choi, S., Kim, Y., Park, H., Han, I. O., Chung, E., Lee, S. Y., Kim, Y. B., Lee, J. W., Oh, E. S., and Yi, J. Y. (2009) Syndecan-2 overexpression regulates adhesion and migration through cooperation with integrin α2. Biochem. Biophys. Res. Commun. 384, 231–236

23. Couchman, J. R. (2010) Transmembrane signaling proteoglycans. Annu. Rev. Cell Dev. Biol. 26, 89–114

24. Chen, L., Klass, C., and Woods, A. (2004) Syndecan-2 regulates transforming growth factor-β signaling. J. Biol. Chem. 279, 15715–15718

25. McCright, B., Gao, X., Shen, L., Lozier, J., Lan, Y., Maguire, M., Herzlinger, D., Weinmann, G., Jiang, R., and Gridley, T. (2001) Defects in development of the kidney, heart, and eye vasculature in mice homozygous for a hypomorphic Notch2 mutation. Development 128, 491–502

26. Krebs, L. T., Xue, Y., Norton, C. R., Sundberg, J. P., Beatus, P., Lendahl, U., Joutel, A., and Gridley, T. (2003) Characterization of Notch3-deficient

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REFERENCES

1. Armulik, A., Genové, G., and Betsholtz, C. (2011) Pericytes: development, physiological, and pathological perspectives, problems, and promises.
mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. *Genesis* 37, 139–143

27. Kennard, S., Liu, H., and Lilly, B. (2008) Transforming growth factor-β (TGF-β) down-regulates Notch3 in fibroblasts to promote smooth muscle gene expression. *J. Biol. Chem.* 283, 1324–1333

28. Lilly, B., and Kennard, S. (2009) Differential gene expression in a coculture model of angiogenesis reveals modulation of select pathways and a role for Notch signaling. *Physiol. Genomics* 36, 69–78

29. Weng, A. P., Nam, Y., Wolfe, M. S., Pear, W. S., Griffin, J. D., Blacklow, S. C., and Aster, J. C. (2003) Growth suppression of pre-T acute lymphoblastic leukemia cells by inhibition of notch signaling. *Mol. Cell. Biol.* 23, 655–664

30. Domenga, V., Fardoux, P., Lacombe, P., Monet, M., Maciazek, J., Krebs, L. T., Klonjowski, B., Berrou, E., Mericskay, M., Li, Z., Tournier-Lasserve, E., Gridley, T., and Joutel, A. (2004) Notch3 is required for arterial identity and maturation of vascular smooth muscle cells. *Genes Dev.* 18, 2730–2735

31. Li, Y., Takeshita, K., Liu, P. Y., Satoh, M., Oyama, N., Mukai, Y., Chin, M. T., Krebs, I., Kotliikoff, M. I., Radtke, F., Gridley, T., and Liao, J. K. (2009) Smooth muscle Notch1 mediates neointimal formation after vascular injury. *Circulation* 119, 2686–2692

32. Varadkar, P. A., Kraman, M., and McCreight, B. (2009) Generation of mice that conditionally express the activation domain of Notch2. *Genesis* 47, 573–578

33. Liu, H., Zhang, W., Kennard, S., Caldwell, R. B., and Lilly, B. (2010) Notch3 is critical for proper angiogenesis and mural cell investment. *Circ. Res.* 107, 860–870

34. Fukai, N., Kenagy, R. D., Chen, L., Gao, L., Daum, G., and Clowes, A. W. (2009) Syndecan-1: an inhibitor of arterial smooth muscle cell growth and intimal hyperplasia. *Arterioscler. Thromb. Vasc. Biol.* 29, 1356–1362

35. Ikese, M., Matsu, Y., Ohta, D., Danzaki, K., Ito, K., Kanayama, M., Kurotaki, D., Morimoto, J., Kojima, T., Tsutsui, H., and Uede, T. (2011) Syndecan-4 deficiency limits neointimal formation after vascular injury by regulating vascular smooth muscle cell proliferation and vascular progenitor cell mobilization. *Arterioscler. Thromb. Vasc. Biol.* 31, 1066–1074

36. Rauch, B. H., Millette, E., Kenagy, R. D., Daum, G., Fischer, J. W., and Clowes, A. W. (2005) Syndecan-4 is required for thrombin-induced migration and proliferation in human vascular smooth muscle cells. *J. Biol. Chem.* 280, 17507–17511

37. Halden, Y., Rek, A., Atzenhofer, W., Szilak, L., Wabnig, A., and Kungl, A. J. (2004) Interleukin-8 binds to syndecan-2 on human endothelial cells. *Biochem. J.* 377, 533–538

38. Cizmeci-Smith, G., Langan, E., Youkey, J., Showalter, L. I., and Carey, D. J. (1997) Syndecan-4 is a primary-response gene induced by basic fibroblast growth factor and arterial injury in vascular smooth muscle cells. *Arterioscler. Thromb. Vasc. Biol.* 17, 172–180

39. Pisconti, A., Cornelison, D. D., Olguín, H. C., Antwine, T. L., and Olwin, B. B. (2010) Syndecan-3 and Notch cooperate in regulating adult myogenesis. *J. Cell Biol.* 190, 427–441