Notch Signaling Functions in Retinal Pericyte Survival

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PURPOSE. Pericytes, the vascular cells that constitute the outer layer of capillaries, have been shown to have a crucial role in vascular development and stability. Loss of pericytes precedes endothelial cell dysfunction and vascular degeneration in small-vessel diseases, including diabetic retinopathy. Despite their clinical relevance, the cellular pathways controlling survival of retinal pericytes remain largely uncharacterized. Therefore, we investigated the role of Notch signaling, a master regulator of cell fate decisions, in retinal pericyte survival.

METHODS. A coculture system of ligand-dependent Notch signaling was developed using primary cultured retinal pericytes and a mesenchymal cell line derived from an inducible mouse model expressing the Delta-like 1 Notch ligand. This model was used to examine the effect of Notch activity on pericyte survival using quantitative PCR (qPCR) and a light-induced cell death assay. The effect of Notch gain- and loss-of-function was analyzed in monocultures of retinal pericytes using antibody arrays to interrogate the expression of apoptosis-related proteins.

RESULTS. Primary cultured retinal pericytes differentially expressed key molecules of the Notch pathway and displayed strong expression of canonical Notch/RBPJK (recombination signal-binding protein 1 for J-kappa) downstream targets. A gene expression screen using gain- and loss-of-function approaches identified genes relevant to cell survival as downstream targets of Notch activity in retinal pericytes. Ligand-mediated Notch activity protected retinal pericytes from light-induced cell death.

CONCLUSIONS. Our results have identified signature genes downstream of Notch activity in retinal pericytes and suggest that tight regulation of Notch signaling is crucial for pericyte survival.

Keywords: diabetic retinopathy, survival, notch signaling, coculture, pericyte, small-vessel

Clinical and experimental observations indicate that interactions between endothelial cells (ECs) and pericytes, the two cell types that comprise capillaries, are central to the integrity and function of the retinal vasculature.¹ Loss of pericytes in diabetic retinopathy is temporally associated with endothelial dysfunction, including the development of a procoagulant surface, elevated levels of leukocyte adhesion molecules, endothelial apoptosis, and vessel growth, leading to the suggestion that the absence of pericytes results in the destabilization of the vasculature.^{2–5} The association of pericytes with the newly-formed vessel and the subsequent deposition of a basement membrane⁶ morphologically mark vessel maturation during development; however, the molecular basis of vessel stability and pericyte survival in adult tissues are largely unknown.

Coculture studies have shown that proliferating ECs produce platelet-derived growth factor B (PDGF-B), which acts as a chemoattractant to recruit undifferentiated mesenchymal cells to the new vessels, and as a mitogen to increase the number of mural cells including pericytes and smooth muscle cells (SMC) precursors.^{7–9} Additionally, there is evidence demonstrating that contact between pericytes and ECs leads

to activation of latent transforming growth factor- β 1 (TGF- β 1), which suppresses endothelial migration and proliferation, and induces the differentiation of the mesenchymal cells to a pericyte lineage, all hallmarks of capillary maturation.^{8,10,11}

Recent studies of human populations with cardiovascular diseases, along with analysis of animal models, have demonstrated that Notch signaling, a master regulator of cell fate decisions, is essential for vascular development.^{12–14} Notch appears to participate in vascular remodeling as well as in the process of arterial/venous specification.^{15–19} A role for Notch signaling in the adult vasculature is suggested by the overlapping and often complementary patterns of expression of Notch signaling components in vascular cells that appear to be modulated upon injury.²⁰ A function for Notch in the adult vasculature is indicated further by the involvement of *Notch 3* mutations in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), a human degenerative condition characterized by vascular SMC pathology and ischemic stroke.^{21–23} A key pathological feature of CADASIL pathology is the progressive loss of mural cells,²³ a characteristic shared with diabetic retinopathy.

In distinction to PDGF- β and TGF- β 1, which are soluble factors, the Notch ligands are transmembrane proteins and require direct cell-cell interactions to activate the membrane-tethered Notch receptors.²⁴ In mammals, there are four paralogs of the Notch receptor (Notch 1–4) and five ligands (Jagged 1 and 2, and Delta-like 1, 3, and 4).²⁵ Mural cells are predominant sites of *Notch 3* expression in adult tissues, and in vitro studies demonstrate that *Jagged 1* expression in ECs promotes *Notch 3* expression in mural cells.^{26,27} Evidence from animal models also indicates that endothelial *Jagged 1* expression is necessary for mural cell development.²⁸

In the canonical Notch signaling pathway, interactions between the Notch receptor and its ligands Delta or Serrate/Jagged trigger proteolytic cleavage of the receptor ectodomain by ADAM (ADAM metalloproteinase domain). This is followed by a presenilin-dependent cleavage of Notch transmembrane region that releases the intracellular domain (NICD). The NICD then translocates to the nucleus where it forms a complex with recombination signal-binding protein 1 for J-kappa (S[H]/RBPJK) and mastermind (MAM) to control the expression of specific genes relevant to the control of cell fate decisions.²⁴ Notch signals are known to be highly pleiotropic, dictating cellular fates in ways that depend on cellular context.^{29,30} Thus, depending their integration with other signaling pathways, Notch signaling can influence differentiation, proliferation, or apoptotic events in a broad spectrum of tissues, including the vasculature.²⁵ In this work, we investigated the role of members of the Notch signaling pathway in pericyte survival.

MATERIALS AND METHODS

Isolation and Culture of Bovine Retinal Pericytes and Endothelial Cells

Bovine retinal pericytes and endothelial cells were isolated using previously published protocols.^{31,32} Large retinal vessels were removed during the isolation procedure; thus, our cultures were composed primarily of cells deriving from the retinal microvasculature, including pericytes and endothelial cells. Bovine retinal pericytes (BRPs) were cultured in Dulbecco's modified Eagle's medium (DMEM; Lonza, Walkersville, MD, USA), supplemented with 10% HyClone bovine calf serum (BCS; Thermo Fisher Scientific, Waltham, MA, USA), 100 U/mL of penicillin/100 mg/mL of streptomycin (Lonza), and 2 mM L-glutamine (Lonza). Bovine retinal endothelial cells (BREC) were cultured in EBM-2 Basal Medium (Lonza) supplemented with EGM-2 BulletKit (Lonza), 100 U/mL of penicillin, 100 mg/mL of streptomycin (Lonza), 2 mM L-glutamine (Lonza), and 10% FBS (Atlanta Biologicals, Flowery Branch, GA, USA) on 0.2% gelatin-coated plates. Staining with antibodies against α -smooth muscle actin (C6198; Sigma-Aldrich Corp., St. Louis, MO, USA) and DiI-acetylated low-density lipoprotein (L-3484, 10 μ g/mL; Life Technologies, Carlsbad, CA, USA) was used to assess the purity of BRP and BREC cultures, respectively (>99%, Supplementary Fig. S1).

Generation of DLL1-Expressing Cells

Primary mouse embryonic fibroblasts (MEFs) were derived from 12.5 d.p.c. *ROSA26*^{LSL-DLL1/CRE-ERT2} embryos, following standard procedures.³³ The *ROSA26*^{LSL-DLL1/CRE-ERT2} MEFs were immortalized by successive passaging following a 3T3 protocol.³⁴ Exogenous Delta-like 1 (DLL1) expression was induced at the *ROSA26* locus by CRE-mediated excision of the *loxP*-Stop-*loxP* cassette after in vitro addition of 4-hydroxytamoxifen (4-OHT) at a final concentration of 1 μ M. Expression

of DLL1 was assessed by Western blot 48 hours following the addition of 4-OHT.

Mono- and Coculture Experiments

For reporter assays, primary cultures of BRPs were transfected using Amaxa Basic Nucleofector Kit Primary Smooth Muscle Cells (VPI-1004, D-033 program; Lonza) with 4 μ g of a Notch signaling-sensitive TP1-luciferase³⁵ and 0.5 μ g Renilla pRL-TK vector (E2241; Promega, Madison, WI, USA) before plating in 12-well culture plates (1.30 $\times$ 10⁵ cells per well, 62406-165; VWR International Products, Atlanta, GA, USA). In experiments testing the effect of lunatic fringe (LFng), 2 μ g TP1, 0.25 μ g Renilla, and 2 μ g LFng or control vector were cotransfected (LFng-V5 construct was described previously³⁶). At 24 hours after plating, BRPs were cocultured with an equal number of cells expressing the Notch ligand, DLL1, or control cells in culture medium supplemented with 1% BCS for 48 hours before harvest. Luciferase activity was detected with a dual-luciferase reporter assay (E1910; Promega) using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Notch activity was normalized to the signal obtained from BRPs cocultured with control cells. Unpaired *t*-test was used to identify statistical significance. *P* values < 0.05 were considered significant.

For Notch loss of function (LOF) studies, BRPs monocultures or cocultures were incubated with either dimethyl sulfoxide (control) or Compound E (1 nM or 100 nM; Enzo Life Sciences, Farmingdale, NY, USA) for 48 hours. To isolate BRPs from cocultures, DLL1 cells were labeled with PKH67 live dye (PKH67GL; Sigma-Aldrich Corp.) before coculture and excluded using fluorescence-activated cell sorting (FACS) using a BD LSRII (BD Biosciences, San Jose, CA, USA). For Notch gain of function (GOF) studies, BRPs (monoculture) were transfected with a vector containing the intracellular domain (ICD) of *Notch 3* fused to green fluorescent protein (GFP, the N3ICD-GFP construct was described previously³⁶).

Microarray and PCA Analysis

Available datasets from published *Notch 3* knockout microarray studies were analyzed using Partek Genomics Suite Software (caudal vessels-tail arteries, downloaded from the Gene Expression Omnibus database [accession code GSE36437³⁷], and aortas and brain-derived SMC/pericytes [accession code GSE58368³⁸]). A 2-way ANOVA analysis was conducted to investigate changes in gene expression according to genotype and tissue origin.

Gene Expression Screen and Quantitative PCR (qPCR)

Primer3 software was used to design PCR primers across exons using sequences from the *Bos Taurus* Ensembl database.³⁹ cDNA was prepared from 900 ng of RNA using the iScript cDNA synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and probed for qPCR using Faststart Universal SYBR Green Master (Hoffmann-La Roche, Basel, Switzerland). Fold-change was calculated as the ratio of 2^{− $\Delta\Delta$ Ct} and normalized to a reference gene following the MIQE guidelines.⁴⁰ The gene screen included 77 targets selected from the microarray datasets mentioned above.^{37,38} These candidate genes were selected for further analysis based upon statistical significance, fold-change, and gene ontology classification. In addition, 25 genes with known functions related to the Notch pathway, 26 genes associated with apoptosis, one gene of special interest (*NG2*), and four reference genes (*beta-actin*, *18-S*, *GAPDH*, *HPRT*) were included in the analysis. All genes were tested in

the LOF (monoculture and coculture) and further validated in the GOF (monoculture) experiments described above.

Western Blot

Protein extracts (in RIPA buffer, 20 µg per lane) were loaded onto a 4% to 12% bis-TRIS Novex gel (Life Technologies) in denaturing conditions. Antibodies against mouse DLL1 (ab10554, 1:800; Abcam, Cambridge, MA, USA) were used to demonstrate expression of the transgene. As loading control, mouse α -tubulin was detected using DM1A antibody (T6199, 1:20,000; Sigma-Aldrich Corp.). All primary incubations were performed overnight at 4°C in TBST + 5% milk. Detection of immunoblot was performed by ECL Prime (Amersham-GE Healthcare, Pittsburgh, PA) after secondary incubation with horseradish peroxidase (HRP)-coupled antibodies against rabbit or mouse IgGs (1:10,000; Amersham-GE Healthcare), two hours at room temperature in tris-buffered saline with Tween (TBST) + 5% milk.

Light Box Incubator

A Forma Scientific (model 3159; Thermo Fisher Scientific) incubator was modified to fit a light box producing a broad-spectrum light source (1200 lux, 0.15 mM/cm²). For light exposure, the BRP/DLL1 coculture plates were placed on top of a stack of two empty culture plates (353043, approximately 4.5 cm, directly on top of the light box; VWR International Products). For dark conditions, cells were kept on the top shelf of the same incubator covered from the light with aluminum foil. To prevent heat damage, temperature of the culture medium and surrounding space was measured over time to ensure a constant 37°C in the light and dark conditions.

Antibody Staining and FACS Analysis

Cocultures were carried out as detailed above by plating equal numbers of untransfected BRPs with DLL1 or control cells (6.5×10^4 cells of each type). After 24 hours, cells were collected by trypsinization, washed and fixed, and permeabilized using Cytofix/Cytoperm reagents, following the provider's instructions (554714; BD Biosciences). Cells were stained using cy3-labeled anti-smooth muscle actin (C6198, 1:500 dilution; Sigma-Aldrich Corp.) and cleaved caspase-3 FITC (9579S, 1:500 dilution; Cell Signaling Technology, Beverly, MA, USA) before FACS. Unpaired *t*-tests were used to compare percentages of apoptotic pericytes between conditions.

Antibody Arrays

The BRP monocultures were plated at a density of 4.4×10^5 cells/cm² (~3.3 million BRPs per T75 flask) in four T75 flasks for 24 hours before transfection with N3ICD-GFP, or with control, EGFP, using Lipofectamine 2000, following the provider's instruction (two flasks per construct, 11668019; Life Technologies). At 24 hours after transfection, cells were washed, lysed, and harvested using the protocol and reagents supplied in the Proteome Profiler Human Apoptosis Array kit following the provider's protocols (ARY009; R&D Systems, Minneapolis, MN, USA). A total of 300 µg of protein per condition was applied to each array. The mean intensity of the pixels comprising each dot in the image was quantified via an algorithmic-based computer system developed by Wimasys Image Analysis (Munich, Germany). A variance analysis was conducted on each array to identify candidate proteins responsive to Notch misregulation. As previously described, proteins were considered differentially expressed if the

absolute value of the difference between control and experimental conditions exceeded three times the variation.²³

RESULTS

Expression of Notch Pathway Genes in Cultured Microvascular Retinal Cells

Notch signaling was examined in primary cultures of BRPs and BREC. Comparative gene expression analyses of early passage (passages 1–3) cells in monoculture identified *Notch 3* as a gene preferentially expressed in BRPs, whereas *Notch 4* and *Delta-like 4* were more abundant in BREC (Supplementary Table S1). *Jagged 2*, *DLL1*, and *manic fringe*, a glycosyltransferase known to modulate Notch activity,⁴¹ were identified as genes specific to BREC. In contrast, *Notch 2* and *RunX1* (runt-related transcription factor 1), a known downstream target of Notch activity,⁴² were more highly expressed in BRPs compared to BREC. Upon coculture, the expression of the Notch downstream target *HeyL* was increased in BRPs compared to BREC, indicating that BREC function as the Notch signal-sending cells, whereas BRPs operate as the Notch signal-receiving cells (1.6-fold, $P < 0.05$).

Ligand-Dependent Notch Activity in Retinal Pericytes

The ECs and pericytes are known to interact during vascular development and in the adult through multiple signaling mechanisms, including TGF- β -TGF- β R, angiopoietin-Tie2, PDGF- β -PDGFR- β , S1P-Edg, and Notch.⁴³ Relevant to this work, multiple Notch receptors, ligands, and regulators are expressed in cultured retinal cells (Supplementary Table S1). To begin to elucidate the role of Notch signaling using a system that could be controlled easily, we developed a coculture system using BRPs and a mesenchymal cell line generated from an inducible mouse model expressing DLL1,²³ a Notch ligand prominently expressed in ECs (Supplementary Table S1; Fig. 1A). In this coculture, Notch signaling was on average 10-fold higher in BRPs cocultured with DLL1 cells compared to BRPs cocultured with control cells ($P < 0.001$, Fig. 1B).

The functionality of the assay was tested using a γ -secretase inhibitor, Compound E, and the glycosyltransferase LFng, a fringe orthologue expressed in BRPs (Fig. 1C). Fringe has been shown to modulate the sensitivity of Notch receptors to specific ligands depending upon cellular context,^{41,44} but its role in regulation of Notch activity in pericytes has not been explored to our knowledge. Treatment with Compound E led to a nearly 85% decrease in Notch signaling ($P < 0.001$), whereas overexpression of LFng enhanced DLL1-mediated Notch signaling in BRPs by more than 55% ($P < 0.005$, Fig. 1C). These results confirmed that Notch signaling is induced effectively in the coculture system, demonstrates a sensitive dynamic range, and illustrates the functionality of fringe regulation of Notch activity in retinal pericytes.

Charting Downstream Targets of Notch Activity in Retinal Pericytes

To identify candidate genes downstream of Notch signaling in pericytes, we analyzed previously published datasets reporting expression profiling of *Notch 3*-deficient mouse models, a Notch receptor paralogue highly expressed in mural cells.^{37,38} Principal component (PC) analysis⁴⁵ was used to evaluate similarities or differences in gene expression between samples from *Notch 3* knockout versus controls across tissues, including aorta, brain-derived SMC/pericytes, and caudal

FIGURE 1. Delta-like 1-mediated activation of Notch signaling in retinal pericytes. (A) Western blot of lysates from ROSA26LSL-Dll1/CRE-ERT2 cells treated with 4-OHT or DMSO. (B) Bovine retinal pericytes were transfected with a TP1-luciferase Notch reporter before coculture with mesenchymal cells induced to express DLL1 or control cells for 48 hours in the presence of γ -secretase inhibitor Compound E or DMSO. (C) Bovine retinal pericytes were transfected with LFng or a control plasmid and TP1-Luciferase before coculture with DLL1 or control cells. Overexpression of LFng led to a significant enhancement of DLL1-mediated Notch signaling in BRPs. *** $P < 0.005$.

FIGURE 2. Principal component analysis was used to reduce the dimensionality of the data and to allow visualization and clustering of microarray gene expression from three datasets corresponding to *Notch 3* LOF in cells/arteries from different tissues. (A, B) Percentage in each PC indicates the proportion of total variation represented by that component. The PCs are uncorrelated and ordered by descending magnitude. Note that in (B) most of the *large ovals* representing *Notch 3* LOF are located in the coordinates corresponding to negative values. (C) *Dot plot* of *Notch 3* expression across tissues.

FIGURE 3. Genes regulated by Notch LOF in retinal pericytes. **(A)** Bovine retinal pericytes were cocultured with PKH-labeled cells expressing DLL1 or control cells in the presence of Compound E (100 nM) or DMSO for 48 hours before separation by FACS. The *Array* column indicates if a gene has known function in the Notch pathway, apoptosis, or if it was selected for analysis in BRPs because it was misregulated in N3KO mice. Fold-change indicates gene expression differences in BRPs exposed to Compound E compared to controls as measured by qPCR. **(B)** Heat map represents changes in gene expression in BRP monocultures in the presence of increasing concentrations of Compound E (1 or 100 nM) for 48 hours before RNA harvest. *P* values of the differences between control and Compound E 100 nM are <0.001 in every case, except when listed as not significant (NS).

vessels (tail arteries). More than 70% of the total variability in gene expression was included in three PCs displayed in Figure 2. The PC1, encompassing approximately 50% of the variation, clearly clustered each of the gene arrays according to their tissue source. Interestingly, PC2, corresponding to approximately 18% of the variation, separated the caudal and brain-derived SMC/pericytes from the aorta (Fig. 2A). Lastly, PC3, which visually corresponded to genotype in each of the clusters, represented approximately 5% of the total variation (Fig. 2B). Despite the effect of tissue origin, 2-way ANOVA analysis revealed a list of 28 genes that were differentially expressed between *Notch 3* knockout and controls including, as expected, *Notch 3* itself (Supplementary Fig. S2). The analysis identified novel candidate targets of *Notch 3* activity (Supplementary Fig. S2) and revealed that *Notch 3* expression in aorta is much lower than that in caudal arteries or brain-derived SMC/pericytes, a finding consistent with the notion of a prominent role for *Notch 3* in small vessels (Fig. 2C, Supplementary Fig. S2).³⁷

We further analyzed the effects of Notch-LOF and GOF in BRPs on the expression of 132 genes using qPCR (see Supplementary Table S2). This list of genes included 77 candidate genes selected from the microarray analysis of *Notch 3* knockout mice^{37,38} as well as genes with known functions related to the Notch and apoptosis pathways (as described in the Methods). For Notch LOF, we used Compound E, a γ -secretase inhibitor that has been used effectively in multiple systems for discovery of Notch targets, and was effective at abrogating Notch activity in retinal pericytes (Fig. 1C).⁴⁶ The Notch LOF analysis using coculture identified 17 genes using an arbitrary cutoff ($P \leq 0.05$, >50% change, Fig. 3A). *HeyL* was the most down-regulated gene (0.22) in cells exposed to Compound E, followed by *Wnt5B* (0.28-Wingless-Type MMTV Integration Site Family, Member 5B), whereas *PCSK6* (Protein Convertase Subtilisin/Kexin Type 6) scored as the most upregulated gene (1.83). *Hey1* and *Hey2*, which, like *HeyL*, have been described previously as canonical downstream

targets of Notch activity, also were downregulated in Notch LOF by 0.36 and 0.47, respectively, validating the approach.

Consistent with previous reports of an autoregulatory loop between *Notch 3* and *Jagged 1* expression in vascular mural cells, Notch LOF led to a significant decrease in the expression of *Notch 3* (0.42) and *Jagged 1* (0.49) in BRPs.²⁶ In addition, Notch inhibition led to increased expression of *Notch 2* (1.76) receptor and *ADAM10* (1.53), a metalloprotease involved in the S2 ligand-dependent cleavage of Notch.⁴⁷ *Radical* and *lunatic Fng* also were upregulated in Notch LOF, although not to a level that reached the pre-established threshold (1.21 and 1.32, respectively, $P = 0.04$ for both). *HMOX1* (heme oxygenase 1) and *catalase*, which have crucial roles in clearance of reactive oxygen species, and *TNFRSF10A* (tumor necrosis factor receptor superfamily member 10A) also were upregulated in BRPs under Notch LOF conditions (1.53, 1.54, and 1.68, respectively). Of the 17 genes modulated by Notch LOF in coculture, 15 also were modulated consistently by Notch in monoculture conditions, although the differences were less robust (Fig. 3B).

Notch GOF was achieved through overexpression of a constitutively active *Notch 3* construct in retinal pericyte monocultures, an approach that is used widely in the field to uncover transcriptional targets of Notch activity bypassing the requirement for ligand-mediated activation.⁴⁸ *Notch 3* was selected for this analysis because its expression is restricted to mural cells in adult tissues, and *Notch 3* mutations have been linked to mural cell pathology in animal models and humans with CADASIL.^{21–23} The Notch GOF analysis in monoculture identified 56 genes downstream of Notch activity in pericytes using the same cutoff defined for LOF (Supplementary Table S3). *Hes1*, a canonical downstream target of Notch activity, was the most upregulated gene (4.05). *HeyL*, *Hey1*, and *Hey2*, which were downregulated in Notch LOF, conversely were upregulated in Notch GOF, further validating the approach (2.46, 2.57, 1.89, respectively). *Jagged 2*, a ligand that showed significantly lower levels of expression in BRPs compared to

FIGURE 4. Apoptosis-related proteins regulated by Notch GOF in retinal pericytes. (A) Bovine retinal pericyte monocultures were transfected with N3ICD-GFP or GFP constructs and cultured for 24 hours before cell harvest. Duplicate *dot blots* indicate signal for each specific protein. Prominent *dots* at the edges of the arrays represent alignment controls. Coordinates for all the proteins are provided in Supplementary Table S4 (B) Shorter exposure of image show in (A).

BRECs, was the most downregulated gene upon *Notch 3* activation in BRPs (0.22). *Notch 2*, *ADAM10*, and *LFng* were downregulated by Notch GOF, although the differences did not reach the pre-established cutoff (0.63, $P < 0.001$; 0.87, $P = 0.03$; 0.52, $P < 0.001$, respectively). Of the 26 apoptosis genes examined, 20 were misregulated by Notch GOF in BRPs (Supplementary Table S2). Of these, only *TNFRSF6* was downregulated (0.42), whereas all others were upregulated, including *TNFRSF10A* (1.9) and *HMOX1* (2.9), also increased by Notch LOF).

SMTN (1.72-smoothelin), a marker of mural cell differentiation, was upregulated by Notch GOF (Supplementary Table S3). To further investigate a possible role of Notch signaling in pericyte differentiation, the expression of *NG2*, a more specific marker (chondroitin sulfate proteoglycan 4), was examined⁴⁹; *NG2* was upregulated by Notch GOF (2.01) and downregulated by Notch LOF (0.61, $P = 0.03$).

Regulation of Pericyte Survival Through Notch Signaling

An antibody array was used to explore the effect of altering Notch signaling in BRPs in monoculture on the expression of 35 proteins relevant to apoptosis. Bovine retinal pericytes in GOF assay were transfected with N3ICD-GFP or GFP plasmids 24 hours before cell harvest and protein isolation. Consistent with the observations from the qPCR analysis, Notch GOF was associated with misregulation of proteins relevant to apoptosis. Notch GOF led to an increase of Bcl-x (2.79-fold), clusterin (1.28), and SMAC/Diablo (1.09, Fig. 4). In contrast, these same proteins were downregulated in BRPs, transfected with just GFP, in the LOF (compound E) assay: Bcl-x (0.72) clusterin (0.55), and SMAC/Diablo (0.73, Fig. 5).

To examine the effect of more physiological levels of Notch activity on pericyte survival, BRPs were cocultured with cells expressing DLL1 under light exposure conditions that have been reported previously to trigger cell death.⁵⁰ Light exposure

FIGURE 5. Apoptosis-related proteins regulated by Notch LOF in retinal pericytes. (A) Bovine retinal pericyte monocultures were transfected with GFP and incubated with DMSO or 100 nM Compound E for 48 hours before cell harvest. Duplicate *dot blots* indicate signal for each specific protein. Prominent *dots* at the edges of the arrays represent alignment controls. Coordinates for all the proteins are provided in Supplementary Table S4 (B) Shorter exposure of image show in (A).

FIGURE 6. Tight regulation of Notch signaling is crucial for pericyte survival. (A) BRPs were cocultured with DLL1-expressing cells or control cells in light or dark conditions before labeling with an anticlaved caspase-3 antibody and FACS. Bovine retinal pericytes exposed to light had a 2.3-fold increase in cleaved caspase-3 signal compared to dark ($*P < 0.01$), which was significantly reduced by coculture with DLL1-expressing cells ($**P < 0.02$). (B) Our working model for the regulation of pericyte cell survival by Notch signaling. In this study, ligand-mediated Notch signaling has been shown to be protective for pericytes (as seen in [A]), whereas LOF and GOF result in misregulation of cell survival pathways.

was associated with a significant increase in cleaved caspase-3 in BRPs cocultured with control cells as measured by FACS (Fig. 6). In contrast, Notch activation induced by coculture with DLL1-expressing cells led to a significant decrease in cleaved caspase-3 in BRPs exposed to light. Together these data provided evidence to indicate that tight regulation of Notch signaling dosage is crucial for pericyte survival.

DISCUSSION

Pericyte loss is a hallmark of the microangiopathy that characterizes diabetic retinopathy and other small-vessel diseases.⁴³ Although incompletely understood, multiple lines of evidence indicate that communication between ECs and pericytes is crucial for microvascular stability during development and in the adult.⁵¹ Here, we showed that Notch signaling, a cell signaling mechanism that depends upon direct cell-cell interactions, regulates transcriptional outputs crucial for survival in retinal pericytes. The notion that Notch signaling has an important role in retinal vessels is supported by recent publications that report expression of Notch molecules in retinal vessels, demonstrate a requirement for *Notch 3* in pericytes investment of newly forming capillaries, and implicate Notch signaling in laser-induced neovascularization and oxygen-induced retinopathy.^{12,52–54}

In our analyses, Notch GOF and LOF led to significant changes in the expression of genes and proteins relevant to cell survival. Bcl-x, clusterin, and SMAD/Diablo, all of which have key roles in the intrinsic apoptosis pathway, were identified in our antibody arrays as candidate mediators of cell death upon Notch signaling misregulation. Importantly, previous work showed that specific Clusterin isoforms sequester Bcl-x, leading to apoptosis through the release of Bax.⁵⁵ Furthermore, Clusterin accumulation was found previously in degenerating vessels from humans with CADASIL and in animal models carrying *Notch 3* mutations.²³

Notch activity is regulated tightly in multiple tissues and there are numerous examples of human pathologies associated with Notch hyperactivation or haploinsufficiency.⁵⁶ Several lines of evidence support the notion that, during the process of making cell fate choices, neighboring cells “compete” for particular fates that are determined by levels of Notch and its ligands. This, in combination with the fact that the Notch pathway, unlike many other cell signaling mechanisms, does

not include an enzymatic amplification step, may explain the extraordinary sensitivity of cells to the levels of Notch activity.⁵⁷

Our finding that Notch LOF and GOF led to upregulation of *TNFRSF10A* and *HMOX1* suggests that pericytes interpret abnormal levels of Notch signal as a cell stress signal; in pericyte monocultures Notch inhibition was protective, whereas Notch hyperactivation (GOF) resulted in induction of apoptotic pathways. In contrast, Notch activation induced by coculture with DLL1-expressing cells was protective. These findings indicated that specific cellular outcomes depend upon Notch signaling levels (~10-fold induction for DLL1-mediated activation versus ~50-fold induction for constitutively active *Notch 3*, Fig. 1 and data not shown), although a role for cellular context (monoculture versus coculture) cannot be ruled out. Previous reports have demonstrated that overexpression of constitutively active *Notch 3* in aortic SMCs is associated with cell proliferation and resistance to apoptosis.⁵⁸ This contrasts with our finding in pericytes, where GOF led to induction of apoptotic genes and proteins. Whether these differences represent specific molecular programs for pericytes versus SMC or are a result of different experimental approaches deserves further investigation.

Our integrated analysis of expression profiling datasets from *Notch 3* knockout mice showed that gene expression varies significantly according to tissue source and vessel caliber. Importantly, *Notch 3* expression was found to be significantly higher in mural cells from small vessels compared to those from large vessels, raising the possibility that *Notch 3* transcriptional activity may regulate context-dependent signaling circuitries in small vessels. In fact, cell-specific transcriptional programs may help to explain the phenotypic differences observed between pericytes versus aortic SMC. Further characterizing these cell-specific transcriptomes requires overcoming significant methodological obstacles, because pericytes and SMCs share many markers commonly used for cell isolation from intact tissues, and primary cultured pericytes may be affected by culture conditions.

Our results have identified the molecular circuitry downstream of Notch activity and, moreover, indicate a central role for Notch signaling in retinal pericyte survival. Manipulation of Notch, or the transcriptional targets identified in this work, may lead to novel therapeutic approaches to regulate cell fate and survival of pericytes.

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