Evaluation of *Notch3* Deficiency in Diabetes-Induced Pericyte Loss in the Retina

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Keywords

Notch3 · Pericytes · Notch signaling · Diabetic retinopathy · *Ins2Akita*

Abstract

Loss of vascular pericytes has long been associated with the onset of diabetic retinopathy; however, mechanisms contributing to pericyte dropout are not understood. *Notch3* has been implicated in pericyte stability and survival, and linked to vascular integrity. *Notch3* mutant mice exhibit progressive loss of retinal pericytes. Given that diabetic retinopathy is associated with pericyte loss, we sought to determine whether perturbation of *Notch3* signaling contributes to diabetes-induced pericyte dropout and capillary degeneration. We utilized a pericyte-expressed LacZ transgene (*XlacZ4*) to examine pericyte loss in retinas of a type I diabetic mouse model (*Ins2Akita*) and *Notch3*-deficient mice. *Notch3* null animals showed a dramatic loss of the LacZ marker by 8 weeks of age, while *Ins2Akita* diabetic and *Notch3* heterozygous mice exhibited a much slower and subtler loss of LacZ. Although combined *Notch3* heterozy-

gosity in *Ins2Akita* diabetic animals did not show further deficits, the trypsin digest method revealed that *Notch3* haploinsufficiency increased the formation of acellular capillaries in diabetic mice. Our data further indicate that Notch signaling is blunted in diabetic retinas and in cells exposed to hyperglycemia. These results are the first to demonstrate an association between *Notch3* signaling, pericyte loss, and diabetic retinopathy.

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Introduction

Diabetic retinopathy is a major health concern for people with all types of diabetes [1, 2] and affects 5.4% of the US population aged 40 years and older. If untreated the disease leads to blindness [3]. One third of patients diagnosed with diabetes have some stage of diabetic retinopathy [4]. The disease is characterized by morphologic changes to the microvessels that include aberrant tight junctions, thickening of the basement membrane, loss of pericytes and formation of acellular capillaries that leads

to increased vascular permeability, retinal ischemia, and microaneurysms [1, 5]. One of the earliest events in diabetic retinopathy is pericyte loss or dropout, which contributes to vascular instability and disease progression [5–7]. Understanding what promotes this critical step and identifying ways to prevent pericyte dropout is an important component in controlling disease progression. However, the mechanistic link between diabetes, hyperglycemia and pericyte loss is not well defined.

Retinal pericytes are vascular support cells that surround the endothelial cell-lined vessels and provide stability and tone to promote proper blood flow [6, 8, 9]. They also serve an important role in maintenance of the blood-retina barrier and are a critical part of the neurovascular unit by regulating permeability, clearance of toxic metabolites, and neuroinflammation [10, 11]. While pericyte loss or disruption has been linked to an array of diseases, including fibrosis and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) [9, 12], it is unequivocally tied to diabetic retinopathy and is one of the earliest morphological indicators of the disease [5–7]. Some evidence has linked hyperglycemia to the cause of pericyte loss, with some indication that it is a result of increased reactive oxygen species [1, 8, 13]. Despite these important studies, the direct mechanisms contributing to pericyte dropout remain elusive.

Genetic and molecular approaches have identified certain pathways that contribute to pericyte homeostasis and function. Ligand/receptor combinations from the platelet-derived growth factor- β , transforming growth factor- β , and angiopoietin-1 signaling families are known to contribute to pericyte recruitment, migration, and stability [9, 11, 13]. Additionally, the Notch signaling pathway has also been linked to pericyte differentiation and survival [14–17]. The Notch3 receptor is enriched in vascular smooth muscle cells and pericytes, and is the causative gene of CADASIL, which is an autosomal dominant stroke disorder resulting in vascular dementia [18, 19]. Genetic deletion studies have revealed its importance in smooth muscle maturation and homeostasis and blood-brain barrier function [16–18, 20]. In the retina, *Notch3* is expressed in pericytes [21–23], and genetic deficiency results in progressive loss of retinal pericytes, which is linked to its role in cell survival [23, 24].

In this study we examined the relationship of pericyte loss in *Notch3* deficiency and diabetes. Using a pericyte-expressed LacZ transgene (*XlacZ4*) [25], we show that *Notch3* null mice have substantially reduced expression of the transgene at 8 weeks of age, while heterozygous

Fig. 1. Smooth muscle cell/pericyte-specific reporter *XlacZ4* highlights the vasculature in the retina. Retinas were isolated from adult mice harboring the *XlacZ4* transgene, subjected to staining for β -galactosidase (LacZ) and flat mounted for imaging. **a–c** Transgene expression at increasing magnification.

Fig. 2. *XlacZ4* transgene expression is decreased in *Notch3*-deficient mice. Retinas from 8-week old wild-type (WT), *Notch3*^{+/-} (N3/+), and *Notch3*^{-/-} (N3/N3) mice harboring the *XlacZ4* transgene were isolated and stained for LacZ activity. **a** Graph of relative LacZ expression from indicated genotypes with wild-type control reference sample set to 1. **b–d** Representative images of flat-mounted retinas from WT, N3/+, and N3/N3 mice stained for LacZ activity. Scale bar = 500 μ m. * $p < 0.05$ versus relative controls; $n = 4$.

mice exhibit a much slower and progressive loss of the LacZ expression. Evaluation of LacZ expression in the type I diabetic *Ins2*^{Akita} mice [26–28] showed reduced expression that parallels that of *Notch3* haploinsufficiency; however, examination of *Notch3* haploinsufficiency in *Ins2*^{Akita} diabetic mice revealed no association. Utilization of trypsin digest to evaluate acellular capillaries demonstrated that *Notch3* heterozygosity did not significantly increase acellular capillaries, while the *Ins2*^{Akita} diabetic

mice had significant increases in acellular capillaries at 24 weeks of age. Importantly, *Notch3* haploinsufficiency further increased acellular capillaries in the *Ins2*^{Akita} diabetic mice. Evaluation of Notch signaling components in diabetic mice showed alterations in select transcripts, and in vitro experiments to evaluate Notch activity in cocultured vascular cells indicated that Notch signaling is blunted by hyperglycemia.

Fig. 3. Pericyte coverage is decreased in aged diabetic *Ins2^{Akita}* mice. Detection of pericytes as monitored by the *XLacZ4* transgene in aged diabetic *Ins2^{Akita}* mice. LacZ was quantified in pericytes of wild-type (WT) and diabetic *Ins2^{Akita}* (AK) mice at 12, 16, and 24 weeks of age. **a** Graph of relative LacZ expression at indicated time points. **b** Representative images of WT and AK retinas at 24 weeks of age. Scale bar = 100 μ m. * $p < 0.05$ versus relative controls; ns, not significant; $n = 6$.

Results

Notch3 Deficiency Causes a Dose-Dependent Decrease in *XLacZ4* Transgene Expression

Previously, others and we demonstrated that *Notch3* mutant mice have progressive loss of retinal pericytes and smooth muscle cells [16, 17, 23, 24]. As a simple method to track pericyte loss, we utilized an *XLacZ4* transgenic mouse line (B6.FVB-Tg(Fabp4-lacZ)4Mosh/J, Stock No.: 018625) [25], which expresses the *LacZ* reporter gene under the control of a smooth muscle- and pericyte-specific promoter, to demarcate retinal pericytes (Fig. 1) [29, 30]. The *XLacZ4* transgenic mice were crossed with mice harboring the *Notch3* null mutation (*Notch3^{-/-}*), and LacZ expression was evaluated in retinas of mice at 8 weeks of age. LacZ staining in flat-mounted retinas revealed that

in the absence of functional *Notch3* there was a dramatic loss of the *XLacZ4* transgene expression, compared to the wild-type control (Fig. 2) (WT 1.02 ± 0.152 , N3/N3 0.09 ± 0.052). *Notch3^{+/-}* retinas showed a less severe loss of *LacZ* transgene expression (N3/+ 0.73 ± 0.136), but staining was still significantly reduced and distinguishable from wild-type and *Notch3^{-/-}* mice (Fig. 2). Thus, the *XLacZ4* transgene exhibited *Notch3*-reliant expression that was dose-dependent and consistent with the loss of pericytes.

Analysis of Pericytes and Acellular Capillaries in Diabetic Mice with Notch3 Deficiency

Ins2^{Akita} mice are an established model of type I diabetes and have been shown to have pericyte dropout [26–28, 31, 32]. *Ins2^{Akita}* male mice become hyperglycemic at 4

Fig. 4. Characterization of combined *Ins2^{Akita}* and *Notch3* deficiency. Blood glucose measurements (**a**) and body weights (**b**) of wild-type (WT), *Notch3*^{+/-} (N3/+), *Ins2^{Akita}* (AK), and *Ins2^{Akita};Notch3*^{+/-} (AK;N3/+) mice. **c** LacZ activity measured in indicated genotypes. * $p < 0.05$ versus relative controls; ns, not significant; $n \geq 7$.

weeks of age and begin to develop retinal complications at 12 weeks [26–28]. Using the *XlacZ4* transgene we evaluated pericytes in *Ins2^{Akita}* diabetic mice at 12, 16, and 24 weeks of age. Quantification of LacZ staining revealed no significant difference in pericyte numbers between wild-type and diabetic mice at 12 and 16 weeks (Fig. 3a); how-

ever, there was a significant decrease in LacZ staining at 24 weeks (Fig. 3a, b) (WT24 1.0 ± 0.131 , AK24 0.78 ± 0.142). At 24 weeks the vessels of the diabetic mice appeared thinner, with less LacZ-positive pericytes at the periphery of the blood vessels.

Given that the *XlacZ4* transgene could detect pericyte loss in both *Notch3*-deficient and *Ins2^{Akita}* diabetic mice, we next tested whether the combination of *Notch3* deficiency and diabetes would show an exacerbated loss of pericytes at 24 weeks. Because *Notch3* homozygous null mice have severe pericyte loss prior to the onset of a diabetic phenotype in *Ins2^{Akita}* animals, we utilized *Notch3* heterozygous mice to cross with *Ins2^{Akita}* mice for these analyses. Comparing blood glucose and body weights of control and *Ins2^{Akita}* mice showed the expected increase and decrease in these parameters. Importantly, the combined *Notch3*^{+/-};*Ins2^{Akita}* mice were not statistically different from *Ins2^{Akita}* animals (Fig. 4a, b). Evaluation of the pericyte population with the *XlacZ4* transgene revealed that compared to *Notch3*^{+/-} and *Ins2^{Akita}*, the combined *Notch3*^{+/-};*Ins2^{Akita}* mice were not significantly different. Compared to the wild-type control, all 3 genotypes (*Notch3*^{+/-}, *Ins2^{Akita}*, and *Notch3*^{+/-};*Ins2^{Akita}*) were different from control, but not from one another (Fig. 4c) (WT 1.00 ± 0.159 , N3/+ 0.59 ± 0.124 , AK 0.68 ± 0.147 , AK;N3 0.63 ± 0.212). The reduced *XlacZ4* transgene expression may reflect pericyte injury rather than pericyte loss based on evidence that this marker is downregulated in response to mechanical vascular injury [11, 25].

In diabetic retinopathy, pericyte loss is accompanied by acellular capillary formation [26, 33]. Acellular capillaries are basement membrane tubes without cell nuclei that have at least one fourth of the normal capillary diameter [26, 33]. To further examine the consequence of *Notch3* deficiency in diabetic mice, we utilized trypsin digestion to quantify acellular capillaries of the retina. Interestingly, at 24 weeks *Notch3*^{+/-} retinas were indistinguishable from wild-type controls (Fig. 5a, b). Conversely, *Ins2^{Akita}* mice exhibited an increase in acellular capillaries, which was exacerbated in the *Notch3*^{+/-};*Ins2^{Akita}* mice (Fig. 5a, b) (WT 49.35 ± 1.46 , N3/+ 51.55 ± 4.62 , AK 69.4 ± 4.33 , AK;N3 89.75 ± 3.12).

Notch Signaling Is Perturbed by Hyperglycemia

To determine whether diabetic conditions affect Notch signaling, we evaluated expression by quantitative (q) PCR in whole retinas of wild-type and *Ins2^{Akita}* mice at 24 weeks of age. *Notch3* and *jagged1* (*Jag1*) have been characterized as the major receptor/ligand pair that facilitates endothelial cell-pericyte interactions [34–36]. Expression

Fig. 5. Acellular capillaries are reduced in *Notch3*-deficient and diabetic *Ins2^{Akita}* mice. Retinas were collected from 24-week-old wild-type (WT), *Notch3*^{+/-} (N3/+), *Ins2^{Akita}* (AK), and *Ins2^{Akita};Notch3*^{+/-} (AK;N3/+) mice. Retinal trypsin digestion was performed to detect the change of acellular capillaries. Pictures were taken under a light microscope at 200 $\times$ magnification, and acellular capillaries were quantified in 8 midretinal fields and averaged. **a** Number of acellular capillaries per field in the midretinas of indicated genotypes. **b** Representative images of trypsin digests from indicated genotypes. Arrows indicate acellular capillaries. Scale bar = 25 μ m. * p < 0.05 versus relative controls; ns, not significant; n = 5.

analysis of *Notch3* showed no significant difference between control and diabetic mice, while the *Jag1* ligand exhibited increased expression in retinas of *Ins2^{Akita}* mice (Fig. 6a). Downstream targets, *Hes1* and smooth muscle α -actin (*Acta2*), showed a significant decrease in the dia-

betic retinas, whereas *HeyL* was not changed (Fig. 6b). These data indicate that Notch signaling is altered in diabetes, and that certain Notch components may be selectively targeted. Previously our lab demonstrated that endothelial cells via the *Jag1* ligand increase *Notch3* expres-

Fig. 6. Select Notch signaling components are perturbed in diabetic mice. qPCR to detect expression of Notch signaling genes from retinas of wild-type (WT) and diabetic *Ins2^{Akita}* (AK) mice relative to *Rpl13A* as reference gene. **a** Receptor, *Notch3* and ligand, *Jag1*. **b** Notch-dependent downstream genes, *Hes1*, *HeyL*, and smooth muscle α -actin (*Acta2*). * $p < 0.05$ versus relative controls; ns, not significant; $n = 12$.

sion and activate Notch signaling in cocultured smooth muscle cells and pericytes [36]. Using a Notch-sensitive luciferase reporter transfected into human retinal pericytes, we can measure endothelial cell-dependent Notch activation in a cell-specific manner (Fig. 7a). To evaluate whether Notch activation is influenced by hyperglycemia, we measured Notch signaling in transfected retinal pericytes cultured alone or with human retinal endothelial cells in normal and high glucose conditions. The data show that Notch activation by cocultured endothelial cells is blunted by hyperglycemia (Fig. 7b) (cocultured low glucose 5.6 ± 1.81 , cocultured high glucose 4.3 ± 2.13). We also examined the expression of *Notch3* or *Jag1* in hyperglycemic conditions but did not observe signifi-

Fig. 7. Endothelial cell-pericyte Notch signaling is blunted in hyperglycemia. **a** Diagram of Notch sensor (5 × CBF) luciferase assay used to detect Notch activity in cocultured human retinal pericytes. Pericytes were transfected with the Notch sensor prior to culturing with or without human retinal endothelial cells in low (5 mM) or high (25 mM) glucose for 48 h. **b** Robust induction of Notch activity in pericytes by cocultured endothelial cells, which is blunted in the presence of hyperglycemia. * $p < 0.05$ versus relative controls; $n = 4$.

cant differences (not shown), suggesting that expression does not reflect Notch activity. These data, together with the decrease in Notch target genes in diabetic mice suggests that Notch signaling is perturbed by diabetes.

Materials and Methods

Mice

All strains were obtained from Jackson Laboratory and maintained in C57BL/6 background: *Notch3* (B6;129S1-*Notch3*^{tm1Grid/J}, Stock No.: 010547) [37], Akita (C57BL/6-*Ins2^{Akita}*/J, stock No.: 003548) [26], XLacZ4 (B6.FVB-Tg(Fabp4-lacZ)4Mosh/J, Stock No.: 018625) [25]. Genotyping of mice was carried out by PCR following Jackson Laboratory protocols. All mouse studies were per-

formed in accordance with protocols approved by the Institutional Animal Care and Use Committee at the Research Institute at Nationwide Children's Hospital. For experiments with diabetic mice, only heterozygous *Ins2^{Akita}* male mice were used, with wild-type male mice used as controls. Age-matched mice were grouped and evaluated based on relevant genotypes. Blood glucose was measured following an 8-hour fasting period using tail vein blood with AlphaTrak 2 Blood Glucose Strips (Abbott) and Glucometer. Experimental groups of mice were weighed every week after weaning on a consistent schedule.

LacZ Staining on Whole-Mount Retinas

For staining of β -galactosidase activity, eyes were isolated from mice at indicated time points and fixed in 2% formaldehyde (Fisher) plus 0.2% glutaraldehyde (Sigma) for 30 min. The cornea and lens were removed, and retinas were placed in staining solution overnight at room temperature. The staining solution to detect LacZ activity consisted of 5 mM $K_4Fe(CN)_6$, 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.02% NP-40, and 0.1% X-gal in PBS buffer (all chemicals from Sigma). After staining, retinas were postfixed with 4% formaldehyde overnight. The remaining sclera and vitreous were removed to isolate retinas, and radial incisions were made at equal intervals along the retinal edge. Retinas were flat mounted with Vectashield (Vector Laboratories). LacZ images were captured using a Zeiss AXIO microscope with ZEN pro software and equivalent settings. LacZ was quantified with NIH ImageJ software using a fixed threshold to measure colored pixels. Relative LacZ expression is graphed with a wild-type control reference sample set to 1.

Trypsin Digestion of Mouse Retina and Staining

Eyes were fixed in 2% paraformaldehyde at room temperature overnight. On the second day, retinas were isolated and digested in 3% Difco Trypsin 250 (BD Biosciences) for 6 h at 37°C. After careful removal of internal limiting membranes, the neuroretinal tissue was gently brushed away. Next, retinas were allowed to stand in 0.5% Triton X-100 solution until the complete removal of neurons. Preparations of retinal vascular networks were set onto glass microscope slides in distilled water, air-dried and stained with periodic acid-Schiff and hematoxylin for histologic evaluation. Acellular capillaries were counted at 200 $\times$ magnification in 8 fields per retina in multiple midretinal areas and averaged.

RNA Isolation and qPCR

Total RNA was extracted from mouse retinas using TissueLyser (Qiagen) homogenation in TRIzol (Invitrogen) according to the manufacturer's instructions. RNA was reverse transcribed with M-MLV reverse transcriptase (Promega) to generate cDNA. qPCR was performed using a StepOne PCR system (Applied Biosystems) with Power SYBR Green. The relative difference in various transcripts was calculated by the $\Delta\Delta C_T$ method using ribosomal protein L13a (*Rpl13a*) as the internal control/reference gene. Primer sequences for mouse transcripts were as follows: *Notch3* forward 5'-TTG TCT GGA TGG AAG CCC ATG T-3'; *Notch3* reverse 5'-ACT GAA CTC TGG CAA ACG CCT-3'; *Jag1* forward 5'-GGC TTC TCA CTC AGG CAT GAT A-3'; *Jag1* reverse 5'-GTG GGC AAT CCC TGT GTT TT-3'; *Hes1* forward 5'-CCC CAG CCA GTG TCA ACA C-3'; *Hes1* reverse 5'-TGT GCT CAG AGG CCG TCT T-3'; *HeyL* forward 5'-CGC AGA GGG ATC ATA GAG AAA CG-3'; *HeyL* reverse 5'-GCC AGG GCT CGG GCA TCA AAG AA-3'; *Acta2* forward 5'-TCC TGA CGC TGA AGT ATC

CGA TA-3'; *Acta2* reverse 5'-GGT GCC AGA TCT TTT CCA TGT C-3'; *Rpl13a* forward 5'-TCC CTG CTG CTC TCA AGG-3'; *Rpl13a* reverse 5'-GCC CCA GGT AAG CAA ACT T-3'.

Cell Culture

Primary human retinal pericytes (Cell-Systems) were grown in Dulbecco's modified Eagle's medium (Mediatech Inc.) supplemented with 5% fetal bovine serum (Hyclone), 2 mM glutamine, 1 mM sodium pyruvate, and 100 U/mL penicillin-streptomycin. Primary human retinal microvascular endothelial cells (Cell-Systems) were maintained in EBM-2 media (Lonza) supplemented with the bullet kit as recommended. Cells between passages 6 and 9 were used for all experiments. All cultures were maintained in humidified 5% CO₂ at 37°C.

Plasmid Transfection and Reporter Assays

A Notch sensor containing 5 $\times$ CBF1 binding sites upstream of the luciferase reporter was generated as described [36, 38]. For normalizing transfection efficiency, CMV promoter-driven secreted embryonic alkaline phosphatase (SEAP) (Addgene: #24595) reporter plasmid was cotransfected with the luciferase reporter [38]. To measure Notch transcriptional activity, retinal pericytes at 80% confluency were transfected with the reporter plasmids using Lipofectamine 3000 (Invitrogen). Following transfection, pericytes were cultured in media with high (25 mM) or low (5 mM) glucose, and retinal endothelial cells were added to select wells for coculture and activation of Notch signaling for 48 h. All coculture and control alone experiments were performed in media consisting of EBM-2 supplemented with the bullet kit and indicated glucose concentration. Luciferase activity was measured with the BrightGlo assay kit (Promega), and SEAP activity was measured with a Phospha-Light System (Applied Biosystems). Activity was quantified using a Molecular Devices SpectroMax luminometer [36, 38]. All experiments were performed in duplicate and repeated a minimum of 3 times.

Statistical Analysis

Data analyses were performed using GraphPad Prism. Comparisons between data sets were made using Student's *t* test or ANOVA, with differences considered significant if $p < 0.05$. Data are presented as means $\pm$ SD, unless otherwise indicated. Data shown are representative of at least 3 independent experiments, and/or the *n* value is indicated with data.

Discussion

Diabetic retinopathy is one microvascular complication associated with diabetes and a leading cause of blindness [1, 2]. One of the first indicators of diabetic retinopathy is a loss of pericytes surrounding the blood vessels. This leads to progression of the disease that includes increased vascular permeability, capillary degeneration, retinal microaneurysms and hemorrhage, and devolves into the proliferative stage, which ultimately leads to blindness [5–7]. While pericyte dropout is a defining feature of the early stages of the disease, the mechanisms that

contribute to this important step are not known. Data from several labs have demonstrated that Notch signaling, in particular the Notch3 receptor, is essential for pericyte stability and survival, and therefore seemed a likely candidate as a contributor to diabetes-induced pericyte loss [14–17, 23]. To examine this possible link, we examined pericyte loss in *Notch3* mutant and diabetic mouse models using a pericyte-expressed LacZ reporter transgene. The data indicate that loss of the pericyte marker is profoundly affected by *Notch3* deficiency. Previous studies with the *XlacZ4* transgene revealed this marker is downregulated in response to vascular crush injury [11, 25], and thus loss of *Notch3* may be a perceived insult that promotes this event. Others and we have previously shown loss of *Notch3* promotes apoptosis in the retina [16, 23, 39]. *Notch3* haploinsufficiency and the *Ins2^{Akita}* diabetic mice exhibited similar temporal profiles of LacZ-positive pericytes and did not show an additive or cooperative effect in combination, which is surprising given they both contribute to pericyte loss. These data suggest that the *XlacZ4* transgene may not be a suitable tool for monitoring pericyte loss due to its downregulation in response to various stressors.

Pericyte loss is a hallmark of diabetic retinopathy and is suggested to contribute to capillary degeneration in disease [13]. Using trypsin digest to assess acellular capillaries revealed that *Notch3* heterozygosity showed no significant difference in capillary degeneration at 24 weeks, while *Ins2^{Akita}* diabetic mice exhibited a significant increase in acellular capillaries compared to wild-type control. Interestingly, combined *Notch3* haploinsufficiency and *Ins2^{Akita}* diabetes resulted in an additional increase in capillary degeneration, suggesting that perturbation of Notch3 likely diminishes the vascular protecting function of pericytes and therefore causes retinal capillaries to be more sensitive to diabetic conditions. *Notch3* haploinsufficiency in diabetic mice does not affect pericyte loss but does affect the number of acellular capillaries, which is difficult to interpret. Possibly diabetes and haploinsufficiency both impair Notch signaling in pericytes, preventing an additive effect. However, this does not apply to the capillaries themselves, and there could be pericyte-independent functions of Notch3 that are responsible for capillary loss.

In examining Notch signaling components in retinas of diabetic mice, we evaluated *Notch3* receptor and *Jag1* ligand, which are known to play prominent roles in endothelial cell to mural cell (smooth muscle and pericytes) signaling [35, 36, 40]. At the transcript level, we observed no significant difference in *Notch3* expression between

normal and diabetic retinal tissue, while *Jag1* showed an increase in diabetic mice compared to controls (WT 0.99 ± 0.291 , AK 1.378 ± 0.769). The increase in *Jag1* is consistent with previous publications [41, 42], and indicates that diabetes may influence Notch signaling through altering the receptor/ligand ratio. Indeed, examination of known downstream targets of Notch signaling [34] showed varied differences, with *Hes1* and smooth muscle α -actin (*Acta2*) having decreased expression in diabetic mice indicative of reduced Notch activity (*Hes1* WT 0.69 ± 0.291 , AK 0.32 ± 0.335 , *Acta2* WT 0.86 ± 0.798 , AK 0.51 ± 0.666); however, *HeyL* remained unchanged. This implies that diabetic effects on Notch signaling are not straightforward and may be indirect. Given that the retina is composed of an array of different cell types, it is difficult to assess whether these changes, or lack thereof, can be directly linked to Notch signaling in vascular cells. To address the direct effect of hyperglycemia on vascular cells, we utilized a previously published coculture model [36, 43] to evaluate Notch activity between endothelial cells and pericytes. Utilization of a Notch sensor luciferase construct transfected into retinal pericytes followed by coculture with retinal endothelial cells, we demonstrate robust endothelial cell-dependent Notch activation. Comparing Notch activity in normal glucose versus high glucose culture conditions, we observed hyperglycemia blunted the Notch signaling response. Thus, these data indicate that glucose affects Notch signaling, and this ability to perturb Notch signaling between endothelial cells and pericytes may be a contributing factor to pericyte loss in diabetes. Notch signaling has been directly linked to pericyte survival and support of the microcirculation [14–17, 23]. These data are the first to demonstrate a link between Notch3 signaling and pericyte loss associated with diabetes. Our results suggest that disruption of endothelial cell-dependent Notch activity in pericytes may be a key contributor to pericyte dysfunction or loss. Further studies are needed to determine the causal relationship between hyperglycemia, Notch signaling, and pericyte dropout.

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Disclosure Statement

The authors have no disclosures.

Author Contributions

H.L. and W.Z. performed experiments, analyzed and interpreted data, helped prepare, and edited the manuscript. B.L. performed experiments, analyzed and interpreted data, wrote and edited the manuscript.

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