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Perivascular Adipose Tissue

NOTCH Signaling Networks in Perivascular Adipose Tissue

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ABSTRACT: Over a hundred years ago, mutants were detected in *Drosophila melanogaster* that led to a NOTCH in the wing tip. This original phenotype was reflected in the nomenclature of the gene family that was later cloned and characterized in the 1980s and found to be conserved across metazoans. NOTCH signaling relies on transmembrane ligands and receptors that require cellular contact for receptor activation, reflecting its role in multicellular organisms as an intercellular signaling strategy. In humans, mutations in genes encoding *NOTCH* and their ligands have been shown to promote human disease; these aspects have been extensively reviewed. Notch signaling plays important roles in vascular development (vasculogenesis and angiogenesis) and homeostasis. NOTCH signaling is also active in adipose tissue and contributes to adipocyte differentiation. In addition, NOTCH activity regulates functions of other metabolic organs. This review focuses on NOTCH activity in perivascular adipose tissue within the vascular microenvironment as defined by mouse studies and summarizes expression and potential signaling of the NOTCH signaling network in human perivascular adipose tissue. Due to the strong activity of NOTCH in regulation of metabolic function, activation of the NOTCH network in specific cell types in perivascular adipose tissue has implications for signaling to the underlying blood vessel and control of vascular health and disease.

GRAPHIC ABSTRACT: A graphic abstract is available for this article.

Key Words: adipocytes ■ angiogenesis ■ gene expression ■ homeostasis ■ sequence analysis, RNA ■ signal transduction

NOTCH signaling is an essential communication pathway found in many species,[1] guiding vital processes that help cells grow, specialize, and determine cell fate during embryogenesis.[2,3] It is conserved functionally between *Drosophila* and mammalian systems. In many species, NOTCH networks regulate adipose tissue function and therefore metabolism. For example, NOTCH signaling regulates lipid metabolism in *Drosophila*, as constitutive activation of Notch in wings or eyes of *Drosophila* larvae increased the fat body with altered expression of lipolysis and lipogenesis genes, leading to impaired lipid metabolism.[4] While the core NOTCH signaling components remain conserved between *Drosophila* and mammals, species-specific adaptations exist. There are 4 mammalian NOTCH proteins (NOTCH1−4) compared with 1 in *Drosophila*, *Notch*. The expansion of NOTCH pathway components in mammalian species shows its complex and specialized function in multiple organs. This review will focus on NOTCH signaling in mice and humans.

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In addition to adipose tissue, NOTCH plays unique roles in other tissues related to metabolism. In the liver, biliary cell differentiation is influenced by NOTCH,[5] which also controls glucose production, glycogen breakdown, and fat storage.[6–10] These functions affect insulin sensitivity and fat buildup. In the pancreas, NOTCH supports the growth of progenitor cells,[11] impacts β-cells, and modulates insulin release.[12,13] NOTCH signaling supports blood vessel health by managing lipid transport and promoting the growth of new vessels[14,15] but conversely has been found to promote

Nonstandard Abbreviations and Acronyms

ADAM	a disintegrin and metalloprotease
Ang II	angiotensin II
BAT	brown adipose tissue
CCL2	chemokine (C-C motif) ligand 2
DLL	delta-like ligand
GPX4	glutathione peroxidase 4
HES	hairy/enhancer of split
HEY	hairy/enhancer of split related with YRPW motif protein
HFD	high-fat diet
IFN-γ	interferon-γ
IL	interleukin
JAG	Jagged
MAML	mastermind-like protein
Mib	mind bomb
N1ICD	Notch1 intracellular domain
NECD	Notch extracellular domain
Neur	neuralized
NICD	Notch intracellular domain
NOX	NADPH oxidase
PDGFRB	platelet-derived growth factor receptor-β
PVAT	perivascular adipose tissue
RBP-J	recombination signal binding protein-J
snRNA-seq	single-nucleus RNA sequencing
TNFα	tumor necrosis factor-α
UCP1	uncoupling protein 1
WAT	white adipose tissue

Highlights

- Notch signaling network components are expressed in adipose tissues in mouse and human, including perivascular adipose tissue.
- Mouse studies show that Notch regulates cell differentiation and tissue metabolism in multiple tissues, including adipose, liver, pancreas, and the vasculature.
- In mouse perivascular adipose tissue, Notch signaling is associated with activated Notch signaling, which is the opposite with calorie restriction; Notch activation in perivascular adipose tissue drives adipocyte whitening.
- In human adipose tissue, Notch signaling components are expressed in vascular cells, adipocyte progenitors, and immune cell populations.

inflammation that promotes atherosclerosis.[16] NOTCH signaling also impacts oxidative phosphorylation, glycolysis, and mitochondrial dynamics.[17] For example, in mouse primary hepatic macrophages and a murine macrophage cell line, NOTCH activation boosts mitochondrial oxidative phosphorylation and reactive oxygen species production, fueling inflammation.[18] Further, in cancer cells, NOTCH induces energy production shifts toward pathways that fuel cell growth and survival by reshaping the mitochondria.[19,20] These connections make NOTCH relevant to conditions of metabolic dysfunction, including type 2 diabetes,[21] nonalcoholic fatty liver disease,[22] and cardiovascular disease.[23]

NOTCH SIGNALING NETWORK COMPONENTS

NOTCH signaling activation is initiated via contact between neighboring cells expressing NOTCH proteins or their ligands. NOTCH receptors (NOTCH1−4 in mammals) are single-pass transmembrane proteins containing an NECD (notch extracellular domain), a transmembrane domain, and an NICD (notch intracellular domain).[24]

NECDs inhibit NOTCH receptor activation in the absence of ligand.[25] NICDs regulate the binding of transcription factors or the degradation of the ICD.[26] There are 5 NOTCH ligands: JAG (Jagged) 1, JAG2, DLL (delta-like ligand) 1, DLL3, and DLL4. The interaction of ligands with NOTCH is controlled by EGF (epidermal growth factor)-like repeats in the extracellular domain of the ligands.[27] After binding, NOTCH ligands are ubiquitylated by E3 ubiquitin ligase Neur (neuralized) and Mib (mind bomb) and are activated for Epsin-mediated endocytosis.[28] This leads to conformational changes of NOTCH and S2 cleavage by ADAM (a disintegrin and metalloproteinase) domain–containing proteins.[29] The residual NECD can undergo endocytosis or be cleaved further by γ-secretase (S3 cleavage) to release the NICD.[30] The NICD is translocated into the nucleus where it participates in multiprotein transcriptional regulation complexes. NICD binds with RBP-J (recombination signal binding protein-J) and MAMLs (mastermind-like proteins), leading to the release of corepressor proteins and the recruitment of coactivators.[31] This NICD-containing complex then binds to NOTCH target genes via the DNA-binding motif of RBP-J to regulate transcription. Target genes include *HEY* (hairy/enhancer of split related with YRPW motif protein) family and *HES* (hairy/enhancer of split) family.[32] These targets genes play important roles in cardiovascular or adipose tissue function (Table). Aside from this canonical signaling, the NOTCH pathway can be activated independent of ligand binding through endosomal trafficking.[37] In addition, noncanonical NOTCH pathways can bypass the RBP-J transcriptional complex to regulate processes such as oncogenesis.[38]

NOTCH REGULATES ADIPOSE TISSUE METABOLISM

Through nonshivering thermogenesis, brown adipose tissue (BAT) contributes significantly to energy dissipation

Table. NOTCH Signaling Impact in Adipose and Cardiovascular Tissue

NOTCH-related genes	Functions in cardiometabolic processes
Hes1	Transcription of pro-adipogenic genes such as peroxisome proliferator-activated receptor gamma and C/EBPα was markedly suppressed by Hes1 overexpression in mesenchymal stem cells during early adipogenesis.[33]
Hes5	Notch3-Hes5 in vascular smooth muscle cells promoted oxidative and endoplasmic reticulum stress and activated redox signaling. This led to pulmonary vascular dysfunction and pulmonary hypertension.[34]
Hey1/2	Hey1/2 regulated embryonic vascular development and medial arterial cell fate decision.[35]
Pdgfrb	Pdgfrb regulated the development of adipose tissue neovascularization. Pdgfrb-null mice showed reduced vascularity and were protected from diet-induced obesity.[36]
Ccl2	The DLL4 (delta-like ligand)-Notch signaling axis increased Ccl2 expression and promoted a proinflammatory macrophage phenotype, leading to insulin resistance and cardiometabolic diseases.[16]

and body temperature maintenance during both acute and chronic cold exposure.[39] UCP1 (uncoupling protein 1) expression and function during cold stress are the main molecular determinants. UCP1 is an inner mitochondrial transporter that releases heat as a byproduct of the electron transport chain's energy production.[40] Converting adipose tissue into a more brown-like and high UCP1 expression phenotype has garnered attention due to its potential as a treatment for obesity because of its ability to burn calories. Mice have established brown adipose depots into adulthood, making it the primary model organism used to investigate BAT. Additionally, there are tiny BAT or BAT-like depots around important organs such as arteries.[41] Mouse studies have shown that NOTCH plays a part in the shift from white to beige (or brown-like) adipocytes. Deletion of Notch1 or RBPJ in adipocytes with aP2-Cre dramatically converted white adipocytes into brown or beige adipocytes.[42] More intriguingly, enhanced systemic glucose metabolism and insulin sensitivity coincided with phenotypic alterations in white adipose tissue (WAT). Conversely, constitutive NOTCH signaling in adipose tissue suppressed transformed BAT into a WAT-like tissue. Mechanistically, phenotypic alterations were accomplished by blocking the NOTCH downstream target Hes1, which binds to the promoter region of beige adipocyte genes including Prdm16 and Ppargc1α, encoding PGC1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha). Additionally, this modulation resulted in decreased UCP1.[42] In obesity, insulin resistance was further exacerbated by NOTCH1 activation, which also promoted proinflammatory macrophage polarization via the DLL4-NOTCH axis[16] and stimulated the release of proinflammatory cytokines such as TNFα (tumor necrosis factor-α).[43] Recent findings also showed that NOTCH

signaling suppressed brown adipogenesis during development through its downstream target PDGFRβ, and inhibition of RBP-J promoted brown adipogenesis. Conditional deletion of NOTCH signaling in PDGFRβ+ populations, including pericytes, improved glucose metabolism in postnatal mice that were fed a high-fat and high-sugar diet. Moreover, loss of NOTCH signaling in the PDGFRβ+ pericytes during development prevented diet-induced impairment of glucose metabolism in juveniles.[44]

From an evolutionary perspective, during times of food scarcity, the ability of NOTCH signaling to regulate adipogenesis and lipid accumulation could have been 1 mechanism to promote efficient energy storage. This type of regulation would maintain essential developmental and homeostatic functions. Consistent with this idea, the NOTCH pathway is regulated by nutritional cues. Calorie restriction treatment in mouse models greatly decreased the amount of NOTCH signaling in perivascular adipose tissue (PVAT).[45] However, in the age of calorie abundance, overactive Notch signaling impairs adipose tissue metabolism, obesity, and obesity-related metabolic disorders.

Targeting the NOTCH pathway may enhance adipose tissue and overall metabolism. Several studies conducted in the last decades have shown vital roles of NOTCH in controlling metabolism in adipose tissue. Studies in defined and primary cell lines showed that NOTCH can regulate adipocyte metabolism, including differentiation and dedifferentiation processes. Adipogenesis in 3T3-L1 preadipocytes was regulated by NOTCH in 2 opposing ways depending on the context: either by suppression of DLK1 (delta-like noncannonical Nocth ligand 1), an inhibitor of differentiation, or by downstream HES1 activation, which then suppressed expression of adipogenesis genes such as C/ebpα and Ppar-γ.[46] Experiments in primary white adipocytes derived from transgenic mice with constitutively activated NOTCH in the adipose tissue showed that NOTCH signaling could drive dedifferentiation of white adipocytes and tumorigenic transformation. This was caused by inhibition of fatty acid metabolism, which further led to deficiency in the PPARG (peroxisome proliferator-activated receptor gamma) pathway and reduced expression of mature adipocyte genes, including Adipoq and Fabp4. The dedifferentiated adipocytes could be rescued by supplementation with the PPARG ligand rosiglitazone.[47] The vasculature in fat tissue modulates adipose tissue phenotypes through paracrine communication between endothelial cells and adipocytes. Under homeostatic conditions, adipose tissue endothelial cells regulate lipolysis and fatty acid uptake in the adipose tissue. Under pathological conditions, impaired adipose tissue blood flow and endothelial dysfunction caused hypoxia, inflammation, and fibrosis in the adipose tissue.[48] Inducible expression of N1ICD (Notch1 intracellular domain) in endothelial cells led to

constitutive NOTCH1 signaling, decreased WAT and adipocyte size, decreased vessel area/cell, and increased adipose fibrosis in male mice.[49] This indicates the close association of the vasculature with adipose tissue phenotype and the multiple cellular targets of Notch signaling within adipose tissue stroma.

NOTCH IN PVAT

In the mouse, PVAT surrounding the thoracic aorta shares characteristics with BAT, including an abundance of mitochondria and multilocular lipid droplets. Because genes like *Prdm16* and *Ucp1* are highly expressed, mouse thoracic PVAT also exhibits thermogenic activity.[50] Mesenteric and abdominal PVATs are more similar to WAT.[51] PVAT not only provides structural support to vasculature but also secretes various bioactive molecules to the underlying vessels.[52] In doing so, it regulates vascular activity and inflammation, impacting vascular health and disease. Through the release of chemokines, PVAT controls endothelial function. During Ang II (angiotensin II)–induced hypertension, PVAT has been shown to produce the chemokine RANTES (regulated upon activation normal T-cell expressed and secreted) and increase the recruitment of inflammatory cells, which further compromises endothelial function.[53] Furthermore, inflammatory cytokines such as IL (interleukin)-6, IL-17, and IFN-γ (interferon-γ) produced by PVAT may control vascular smooth muscle cell migration, proliferation, and constriction.[54] Last, PVAT releases adipokines to the nearby vessel. These chemicals have been implicated in the modulation of the redox state of arteries through the NOX (NADPH oxidase) pathway.[55] Healthy PVAT protects against vascular pathology by releasing vasoprotective adipokines such as adiponectin, which prevents atherosclerosis by inhibiting proliferation of smooth muscle cells, vascular remodeling, and lipid accumulation in macrophages.[56] On the contrary, obesity led to a change in the secretory profile compared with healthy PVAT. The expression of anti-inflammatory adipokines, such as adiponectin, was markedly decreased,[57] while proinflammatory cytokines such as IL-6, IL-8, MCP-1 (monocyte chemoattractant protein 1), IFN-γ, and IL-17 were greatly increased in PVAT during obesity, corresponding to a strong inflammatory response.[58,59]

Our group recently showed that mouse thoracic PVAT is also regulated by NOTCH signaling.[45] We showed that a 12-week high-fat diet (HFD) treatment induced pathological changes, including expansion of lipid content, adipocyte hypertrophy, and whitening of PVAT, and a decreased thermogenic molecular profile; thermogenic markers such as PGC1α and PAT2 (proton-coupled amino acid transporter 2) were reduced, while the inflammatory marker *Itgam* was elevated. Further, sequential window acquisition of all theoretical mass spectra mass spectrometry, a data-independent acquisition technique

that enables comprehensive and reproducible quantification of proteins across samples, determined that NOTCH proteins were dramatically upregulated in PVAT from mice fed an HFD compared with mice fed a control diet. This was also confirmed by separate experiments at the transcript and protein levels. *NOTCH1* and *NOTCH2*, and downstream genes *Hes1* and *Hey1*, were upregulated compared with PVAT from mice fed a control diet. Interestingly, activation of NOTCH after HFD feeding was exclusive to PVAT, as NOTCH levels in WAT were not different between mice fed with HFD or control diets, and there was reduced NOTCH signaling in BAT from mice fed an HFD. This corroborates the proteomics data, which showed that PVAT has a distinct molecular profile compared with BAT despite their shared similarities. Interestingly, we also found that mice under 30% calorie restriction for 5 weeks showed dramatic reduced levels of NOTCH pathway components (NOTCH2, JAG1, HES1, and HES5). To determine whether NOTCH activation could phenotypically mimic the effects of HFD on PVAT, we expressed a constitutively active *N1ICD* in mature adipocytes by crossing *N1ICD* mice with *Adipoq-Cre* mice.[45] We showed that NOTCH activation in mice fed a control diet led to conversion of PVAT into white-like adipocytes as characterized by the expansion of PVAT adipocytes. This was independent of changes in global metabolism, as body weight, free cholesterol, and circulating triglycerides were unchanged with constitutive NOTCH signaling in adipocytes. Inhibition of NOTCH with γ-secretase inhibitor reduced lipid accumulation independent of adipocyte dedifferentiation.

We further detected molecular changes in PVAT following *N1ICD* expression in adipocytes. These included increased expression of inflammatory markers such as MAC-1 (macrophage 1 antigen) and decreased thermogenesis markers such as UCP1 and PGC1α. Detailed examination of changes in protein signatures using sequential window acquisition of all theoretical mass spectra mass spectrometry revealed that proteins involved in mitochondrial dysfunction (eg, acyl-coenzyme A dehydrogenase long chain) and oxidative phosphorylation pathways (eg, succinate dehydrogenase complex flavoprotein subunit A) were differentially expressed in PVAT with constitutively activated NOTCH signaling.[60] Seahorse assays using differentiated PVAT adipocytes from *N1ICD;Adipoq-Cre* and control mice showed that mitochondrial respiration and ATP production were significantly downregulated in PVAT-derived adipocytes with constitutive NOTCH activation, which confirmed that NOTCH activation impaired mitochondrial function. Further in vivo and in vitro analyses of transcript and protein level changes in PVAT revealed that the serine/threonine protein kinase 1-parkin mitophagy pathway was regulated by NOTCH activation. This was accompanied by changes in mitochondrial dynamics, as we observed significantly reduced expression of mitochondrial fusion regulators

(eg, dynamin-like GTPase [OPA1] long isoforms) and increased expression of mitochondrial fission regulators (eg, dynamin-related protein 1). Previous studies have linked changes in mitochondrial dynamics to altered adipose tissue physiology. Shifting of mitochondrial dynamics from fission toward fusion promotes differentiation of mesenchymal stem cells to adipocytes,[61] and mitochondrial biogenesis is also activated during adipogenesis. Mitochondrial dynamics also regulate the physiology of thermogenic adipocytes.[62] The Parkin-dependent mitophagy pathway modulates the beige-to-white adipocyte transition. In addition, mitophagy modulates adaptive thermogenesis in BAT, and Parkin-null mice were protected from diet-induced insulin resistance through over-activation of thermogenesis.[63] Therefore, the whitening phenotype of PVAT adipocytes in the Notch transgenic mice could be caused by elevated mitophagy in the PVAT of *N1ICD;Adipoq-Cre* mice.

In addition to mitochondrial dysfunction, we also detected ferroptosis in PVAT after *N1ICD* expression in adipocytes. We found that lipid peroxidation was significantly increased, and expression of ferroptosis regulator GPX4 (glutathione peroxidase 4) was significantly downregulated in adipocytes within PVAT. These data suggest that the NOTCH pathway promotes oxidative stress in PVAT, which may impair the function of the underlying vessel. This was confirmed by wire myography experiments showing that vasoreactivity of the blood vessels from *N1ICD;Adipoq-Cre* compared with the control mice was significantly altered. Aortae from *N1ICD;Adipoq-Cre* mice had increased vasoconstriction and decreased vasorelaxation in a PVAT-dependent and age-dependent manner. Taken together, these data show that NOTCH signaling regulates mitochondrial metabolism in PVAT, leading to altered phenotypes including reduced thermogenesis capacity and vasoprotective function, affecting vascular physiology.

IMPLICATIONS OF NOTCH SIGNALING IN HUMAN METABOLIC AND CARDIOVASCULAR HEALTH

There are conserved features of cell identities in PVAT between mouse and human species.[64] However, there are also distinctions, and it is important to consider whether patterns of expression of NOTCH components in human PVAT support the functional roles identified in model organisms. With relation to cardiovascular health, endothelial cell NOTCH activation contributed to atherogenesis.[65] In the context of adipose tissue, NOTCH activation in samples from individuals with obesity has been associated with insulin resistance and inflammation.[42] NOTCH activity in other metabolic organs has also been described. NOTCH signaling was shown in the human liver, where increased NOTCH signaling correlated with a greater severity of nonalcoholic fatty liver disease and insulin resistance.[66] In pancreatic β-cells from type 2 diabetic donors, inhibition of NOTCH signaling improved insulin secretion.[12]

While there are not direct studies on NOTCH signaling in human PVAT, examining existing transcriptomic data for NOTCH-related molecules from human samples offers a valuable exploration. This approach allows us to infer the potential role of NOTCH signaling in human PVAT and draw comparisons with findings from animal models. We analyzed 2 key studies that provide single-cell transcriptomic data from human PVAT samples.[64,67] These data sets offer a unique opportunity to explore the expression patterns of NOTCH pathway components in various cell populations within human PVAT. Angueira et al[64] performed single-nucleus RNA sequencing (snRNA-seq) on human adult PVAT samples from the ascending aorta from donors with coronary artery disease undergoing coronary artery bypass grafting. This analysis revealed a complex cellular landscape including presumptive fibroblastic and smooth muscle–like adipocyte progenitor cells and provided valuable insights into the developmental origins and cellular composition of human aortic PVAT under conditions of cardiovascular disease. In addition, Fu et al[67] utilized single-cell RNA sequencing to characterize macrophages in human PVAT. A single-cell transcriptomic library was prepared from the stromal vascular fraction from PVAT located on the left anterior descending artery of the excised heart from patients undergoing end-stage heart transplants. This study broadened the transcriptomic library of immune cell populations in PVAT, resulting in exciting discoveries surrounding the role subpopulations of immune cells play in alleviating fibrosis. We refer to the former data set as PVAT from coronary artery disease and the latter as PVAT from heart failure. snRNA-seq was used to profile adipocytes in the Thoracic PVAT study, which are too large and fragile for conventional single-cell RNA sequencing, by isolating nuclei instead of whole cells. In contrast, the single-cell RNA sequencing study focused on immune cells. Together, these approaches provide complementary insights into PVAT biology.

We categorized the NOTCH network into the following targets of interest: ligands and receptors (*JAG1*, *JAG2*, *DLL1*, *DLL3*, *DLL4*, *NOTCH1*, *NOTCH2*, *NOTCH3*, and *NOTCH4*), receptor posttranslational modification (*FRINGE* family), receptor cleavage proteins (γ-secretase complex), regulators (*NUMB* and *NUMBL* [NUMB-like]), transcriptional partners (*RBPJ*, *MAML1*, *MAML2*, and *MAML3*), and downstream effectors/targets (*HES* family, *HEY* family, *PDGFRB* [platelet-derived growth factor receptor-β], and *CCL2* [chemokine (C-C motif) ligand 2]). Our goal in reporting these data is to identify the cell types in human PVAT that express NOTCH signaling network components to infer potential NOTCH signaling activities in human PVAT. This analysis revealed
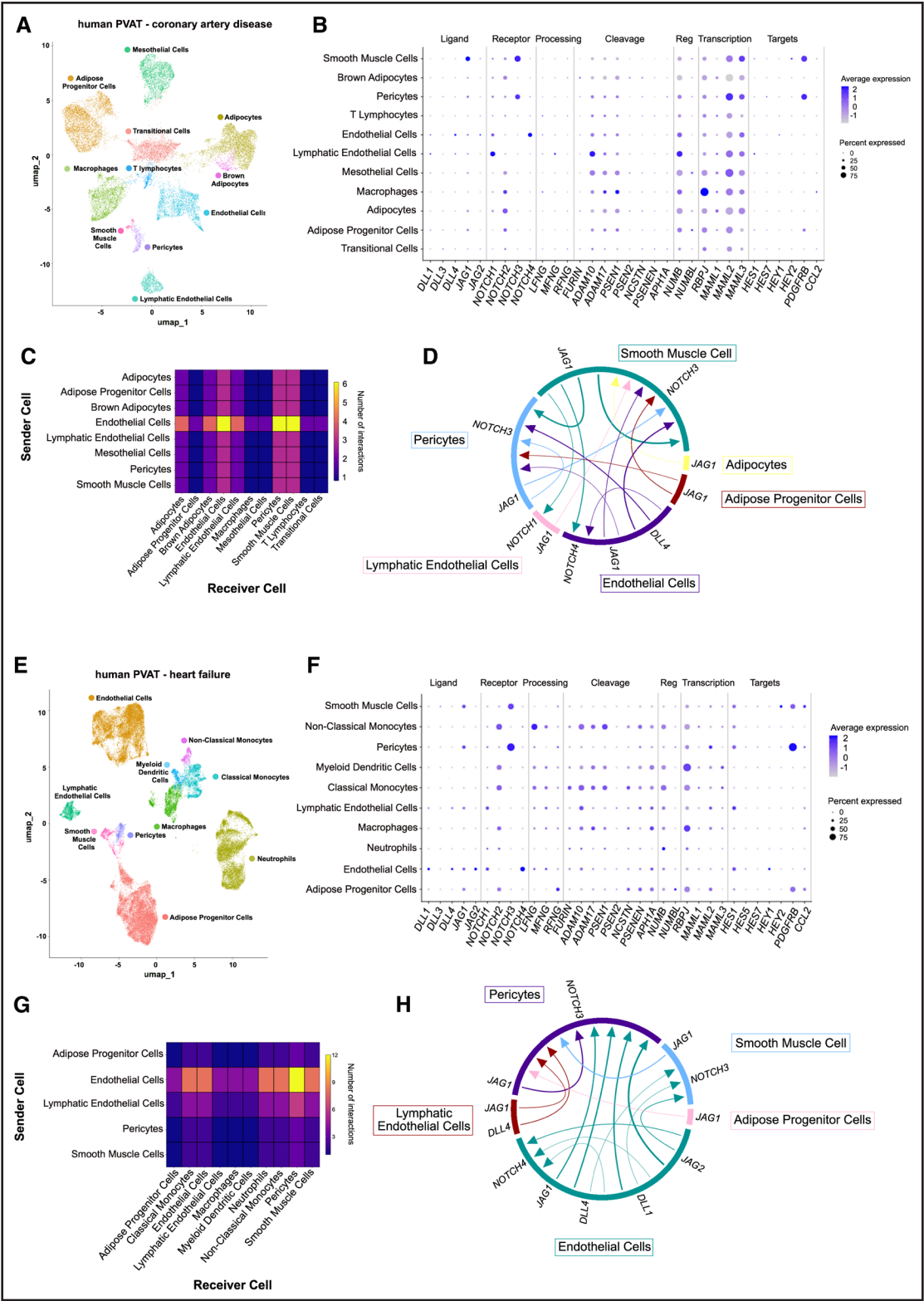
Figure 1. Analysis of NOTCH signaling components in human perivascular adipose tissue (PVAT).

Data processing was performed primarily using Seurat v5.1.0.[68] Barcoded droplets with RNA counts >400 and below the 98th percentile, RNA features >200, and mitochondrial percentage (as a proportion of features) ≤15% were retained. Doublets were removed using Solo,[69] provided via scVI[70] v1.2.2, using objects built within the scanpy[71] v1.10.4 framework. SeuratDisk aided in bridging between Seurat and scanpy. Batch-corrective integration of samples was performed using scVI, and mitochondrial percentage was utilized as a continuous covariate, from which the latent embeddings were extracted and imported into Seurat for downstream analyses. The ScType[72] databases of white adipose tissue and immune cell population markers were utilized to educate population identification, as the publicly available data did not contain annotation labels. Raw counts were log normalized (scale factor, 10 000), all genes were linearly scaled and centered with no (*Continued*)

interesting patterns and potential cell type–specific roles of NOTCH signaling in human PVAT (Figure 1).

NOTCH LIGAND AND RECEPTOR EXPRESSION IN HUMAN PVAT

NOTCH1 was predominantly expressed in lymphatic endothelial cells and endothelial cells. *NOTCH2* was observed at the highest levels in adipocytes and immune cells from the myeloid lineage, with some expression in adipose progenitors. *NOTCH3* was highly expressed in pericytes and smooth muscle cells, and *NOTCH4* was only expressed in endothelial cells, consistent with known patterns. This distribution suggests that different cell types within PVAT may be poised to respond to NOTCH signaling in unique ways, potentially influencing their function and interactions with neighboring cells. The expression of NOTCH ligands in human PVAT appeared to be more limited and cell type specific. *JAG1* was predominantly expressed in smooth muscle cells, endothelial cells, and pericytes. Utilization of Liana, a cell signaling interaction resource, which infers relationships based on transcriptomic data, identified the most Notch signaling interactions in coronary artery disease PVAT were between endothelial cells and pericytes or smooth muscle cells (Figure 1C). The strongest relationships were seen between smooth muscle cells and pericytes, and the most abundant signaling was *JAG1* originating from smooth muscle cells and *DLL4* originating from endothelial cells (Figure 1D). Notch signaling in heart failure PVAT had more ligand diversity but was more 1-dimensional, with the majority of interactions taking place between endothelial cells and pericytes (Figure 1G), executed through *JAG1*, *JAG2*, *DLL1*, and *DLL4* ligand dissemination (Figure 1H). These patterns suggest that vascular-associated cells are the major contributors of NOTCH signaling in human PVAT.

NOTCH POSTTRANSLATIONAL MODIFICATIONS

The family of O-fucosylpeptide-3-beta-N-acetylglucosaminyltransferases (*LFNG*, *MFNG*, and *RFNG*) is involved in posttranslational glycosylation of NOTCH that regulates ligand-receptor interactions. Impairment of these processes leads to human disease.[75] In human coronary artery disease PVAT, expression was generally low for all *FNG* transcripts, with *MNFG* expressed in a small proportion of lymphatic endothelial cells. From heart failure PVAT, *FNG* was expressed in populations of immune cells including monocytes and macrophages and smaller populations of lymphatic endothelial cells. *RFNG* was uniquely expressed in adipocyte progenitor cells.

PROTEOLYTIC CLEAVAGE OF NOTCH

FURIN generates the initial cleavage of NOTCH on its way to maturity in the plasma membrane, and components of the γ-secretase complex mediate processing post-ligand stimulation. Coronary artery disease PVAT expressed primarily *ADAM10*, *ADAM17*, and *PSEN1* in many cells, with particularly high levels of *ADAM10* in lymphatic endothelial cells. Expression of other components, including *NCSTN*, *PSENEN*, and *APH1A*, was only detectable in the PVAT from heart failure. Of interest, monocytes and macrophages in this PVAT data set had the most robust proportion of cells expressing the γ-secretase complex.

NUMB GENES

NUMB proteins regulate a variety of pathways, including NOTCH signaling.[76] NUMB and its homolog NUMBL are NOTCH inhibitors. Mechanistically, they promote NOTCH degradation and reduce their activation. This maintains a balance between differentiation and proliferation, as dysregulation of NUMB/NUMBL can lead to unchecked NOTCH activity, contributing to cancer and cardiovascular disease.[77,78] In our human PVAT data sets, *NUMB* was expressed in a broad pattern, with little *NUMBL* detected.

TRANSCRIPTIONAL PARTNERS

Within the nucleus, NICD participates in regulatory complexes to mediate gene expression. The DNA-binding

Figure 1 Continued. regressed variables, and highly variable genes (2000) were identified using the vst method. **A**, Uniform manifold approximation and projection (UMAP) embeddings calculated from 40 latent dimensions and a resolution of 2.4, of 3 previously published human aortic PVAT samples diagnosed with coronary artery disease.[64] UMAP_1 and UMAP_2 represent the 2 primary dimensions of a UMAP embedding, where cells with similar transcriptional profiles cluster together, allowing visualization of cellular relationships and heterogeneity within the tissue. **B**, Expression levels of NOTCH signaling components in human coronary artery disease PVAT displayed in a DotPlot. **C**, Heatmap representing the number of Notch signaling interactions occurring between different cell populations in thoracic PVAT. Rows correspond to sending cell populations (ligand-expressing cells), while columns represent receiving cell populations (receptor-expressing cells). Interactions were calculated using the R package Liana[73] and ranked by the logfc method. **D**, Circos plot visualizing the top 15 intercellular signaling interactions inferred from Notch ligand-receptor pairs in single-nucleus RNA sequencing data of coronary artery disease PVAT. Each color represents a cell type, and the arcs between them indicate ligand-receptor interactions facilitating Notch signaling. Cell signaling visualizations were generated using the R package CCPlotR.[74] **E**, UMAP (dim, 55; res, 1.2) of 10 previously published human heart failure PVAT samples.[67] **F**, Dotplot displaying expression levels of NOTCH signaling components in cardiac human aortic PVAT. **G**, Heatmap of cell signaling interactions found within coronary PVAT. **H**, Circos plot displaying the 15 strongest interactions between cell types found within coronary PVAT. Reg indicates regulation.

protein RBP-J had low expression across many cells, with the exception of immune cells, including myeloid dendritic cells and macrophages. The MAML family of transcriptional coactivators, essential for NOTCH-mediated gene expression, showed a consistent presence across most cell populations in the PVAT from coronary artery disease, particularly *MAML2* and *MAML3*. Similar expression, albeit fewer proportion of cells, expressed these genes in the populations from heart failure PVAT.

TRANSCRIPTIONAL TARGETS

The *HES/HEY* family of genes are typical downstream transcriptional targets of NOTCH signaling. Additionally, we assessed PDGFRB and CCL2 as targets of interest in adipogenesis. CCL2 is an important inflammatory regulator that leads to increased recruitment of adipose tissue macrophages and insulin resistance in obesity. The *CCL2* gene contains the RBP-J binding site in its promoter.[6] As mentioned, *CCL2* expression was upregulated in murine thoracic PVAT of mice with constitutively activated Notch in adipose tissue. Therefore, Notch could potentially regulate PVAT inflammation through CCL2. PDGFRβ promotes white adipogenesis of progenitors in both mouse and human adipose tissue.[79] RBP-J also binds to an intronic region of *PDGFRβ*,[42] via which NOTCH signaling modulates white adipogenesis through transcriptionally regulating PDGFRβ. In the coronary artery disease PVAT, *HES/HEY* was not detected, with the exception of *HES1* in a small proportion of lymphatic endothelial cells. In the heart failure PVAT, *HES1* was more broadly expressed at low levels, with the highest detection in lymphatic endothelial cells and endothelial cells. In addition, subpopulations of smooth muscle cells expressed *HEY2*, while a proportion of endothelial cells expressed *HEY1*. PDGFRB was confirmed as a strong marker of human pericytes and smooth muscle cells. *CCL2* expression was limited to smooth muscle cells from heart failure PVAT.

NOTCH PATHWAY EXPRESSION IN OTHER ADIPOSE DEPOTS COMPARED WITH PVAT

BAT and WAT have been characterized and compared in human transcriptomics analyses.[80] The overlap of expression profiles of PVAT compared with BAT and WAT in the human adipose tissue is unknown. We compared PVAT expression of Notch components to the expression in human WAT and BAT. We utilized human visceral and subcutaneous WAT snRNA-seq data[81] (Figure 2A through 2D), originally analyzed to reveal depot-specific differences in adipose progenitor subpopulations, as well as human deep-neck BAT snRNA-seq data[80] (Figure 2E through 2H). We specifically leveraged snRNA-seq for WAT analysis due to the unique technical challenges associated with conventional single-cell RNA sequencing

in mature adipocytes. We made no active choice with regard to which technique was best for human BAT, as it has only been described using snRNA-seq.

BAT showed generally low expression of NOTCH and their ligands, with the exception of *NOTCH3* in pericytes and smooth muscle cells, where small subsets of cells also expressed *JAG1*, consistent with *NOTCH* signaling in the adipose vasculature. Small subpopulations in BAT also expressed *NOTCH2*, including macrophages, adipocytes, and adipose progenitor cells. Cell signaling interaction analysis found the total number of Notch signaling interactions to be low (Figure 2C), with the strongest signals being *JAG1* expression from smooth muscle cells and adipose progenitor cells (Figure 2D). Cells in BAT did not express significant levels of *FNG*, *HES*, or *HEY* genes. Similar to PVAT, BAT had low levels and broader expression of *ADAM* genes, *PSEN1*, *RBPJ*, *MAML2*, and *MAML3* (Figure 2B).

The human WAT data set in general had higher cell proportions expressing NOTCH components overall compared with human BAT, although both seemed to have more restricted expression compared with cell populations in human PVAT. There were conserved patterns of expression, with subpopulations of vascular and immune cells having significant expression of *NOTCH*, *ADAM* genes, *PSEN1*, NUMB, *RBPJ*, *MAML2*, *MAML3*, and *PDGFRB* (Figure 2H). Similar to what was observed in PVAT, Notch signaling interactions were most abundant in vascular-related cell types: endothelial cells, smooth muscle cells, and pericytes (Figure 2G). The strongest interactions emphasize those populations and show the involvement of multiple ligands (*JAG1/2* and *DLL4*) and receptors (*NOTCH1–4*).

Given different expression patterns of NOTCH signaling components in distinct cell types within different human adipose depots, tailored therapeutic strategies should be considered in targeting Notch signaling. For example, endothelial cells in human PVAT are the primary target sites for *NOTCH1*, while *NOTCH2* in immune cells and adipocytes is preferentially targeted in human PVAT and WAT.

SUMMARY

In mouse models of dietary modification, metabolic dysfunction caused by diet-induced obesity elevated NOTCH signaling selectively in PVAT, whereas conversely, calorie restriction suppressed NOTCH signaling. Constitutive activation of Notch signaling in mice fed a normal chow diet phenocopied the whitening phenotype, showing that NOTCH can convert this thermogenic depot to more of a WAT phenotype. This was associated with decreased mitochondrial function and ferroptosis. The impact of activated NOTCH signaling in PVAT was seen in vasoreactivity of the underlying vessel, where the aorta contracted more to stimuli and had impaired dilation. These
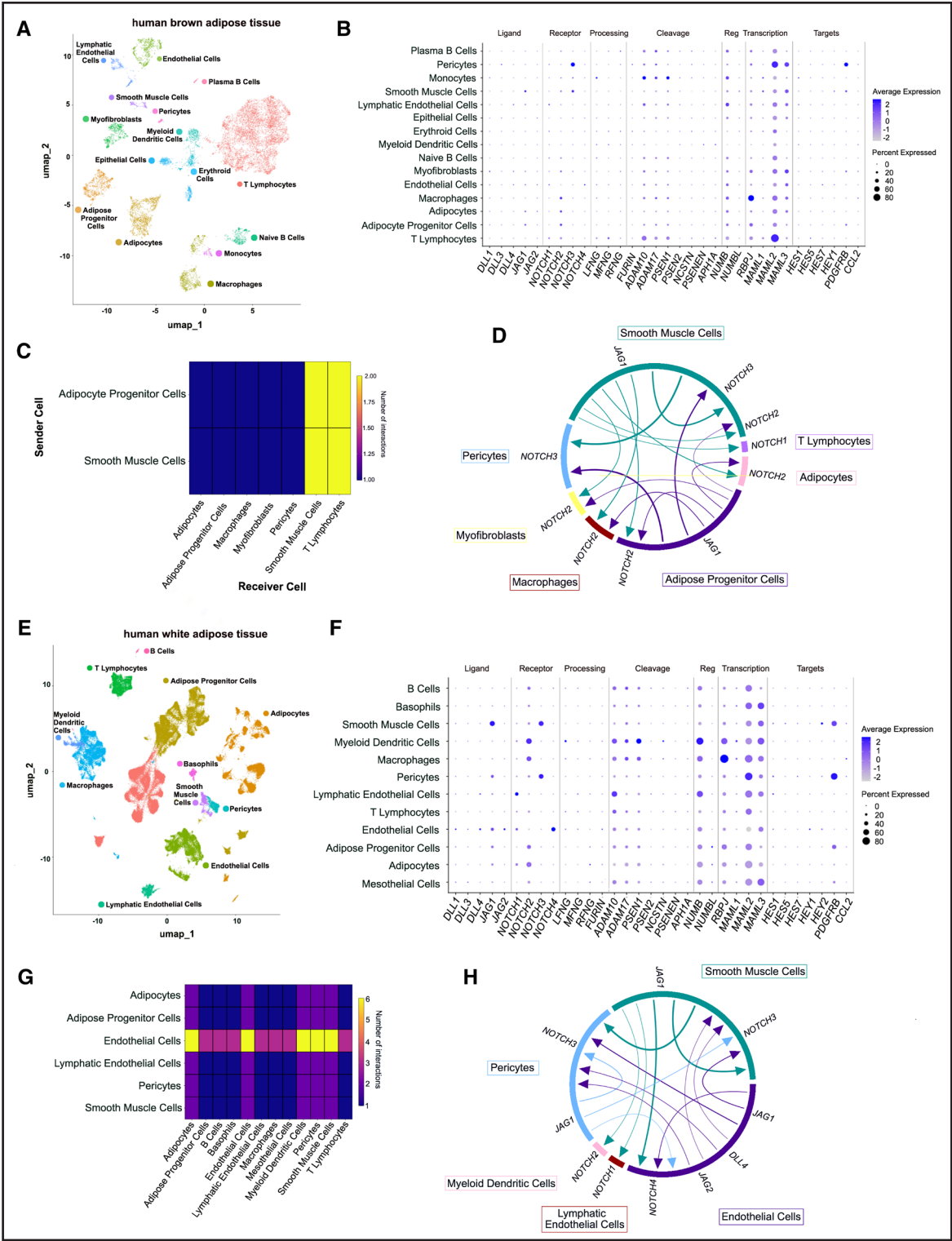
Figure 2. Analysis of NOTCH signaling components in human white adipose tissue (WAT) and brown adipose tissue (BAT).
To investigate depot-specific expression patterns of NOTCH signaling components, we analyzed single-nucleus RNA sequencing data from human BAT and WAT, including subcutaneous and visceral depots. Data processing and analysis followed the identical methodology outlined in Figure 1. **A**, Uniform manifold approximation and projection (UMAP) of the BAT data set (dim, 55; res, 0.8), visualizing cell-type clustering. **B**, Dotplot displaying expression levels of NOTCH signaling components in deep-neck BAT. **C**, Heatmap representing the number of Notch-mediated cell signaling interactions in BAT, where rows correspond to ligand-expressing (sending) cell populations and columns represent receptor-expressing (receiving) cell populations. **D**, Circos plot visualizing the 15 strongest intercellular signaling interactions inferred from Notch ligand-receptor pairs within BAT. Each arc represents a signaling event between cell populations. **E**, UMAP of the WAT data set (dim, 40; res, 2.4). **F**, Dotplot displaying expression levels of NOTCH signaling components in human WAT. **G**, Heatmap of cell signaling interactions within WAT, formatted as described in **C**. **H**, Circos plot depicting the 15 strongest NOTCH signaling interactions between cell types in WAT. Reg indicates regulation.

data show the influence of NOTCH signaling in PVAT as a mediator of vascular function. Current Notch research in mouse models shows several limitations in translating findings to human health. First, the current mouse model used to study Notch-associated diseases, such as cardiovascular diseases, cannot totally replicate the human condition due to the complexity of human pathologies.[82] Second, noncanonical Notch signaling, which also plays roles in cardiovascular function,[83] has been mostly studied in mice. There is a knowledge gap in defining the roles of noncanonical Notch in human physiology and pathologies. Last, the function of Notch signaling is cell context dependent, which makes it challenging to summarize findings from different model organism studies to benefit human health. Future studies should also focus on developing a humanized cardiovascular disease model that can improve the external validity of the findings in animal studies. In addition, context-specific studies need to be conducted to dissect the roles of different components of Notch signaling, including those in the noncanonical Notch pathway (eg, transmembrane domain), in regulating human physiology.

Single-cell transcriptomic studies of human tissues allow a snapshot of gene expression associated with particular cell types. The expression patterns observed in these data sets provide valuable insights into the potential roles of NOTCH signaling in human PVAT. The cell type–specific expression of NOTCH and ligands suggests that NOTCH signaling may be involved in regulating cell-cell interactions and maintaining the complex cellular composition of PVAT. As expected, evidence for expression of the NOTCH network was found in vascular cells (endothelial and lymphatic endothelial cells and mural cells), adipocyte progenitors, and immune populations. The low expression of some of the components of the NOTCH network is not unexpected. This overall pattern of low-to-absent expression across these critical NOTCH pathway components suggests a tightly regulated and transient NOTCH signaling activity in human PVAT under basal conditions. It is also likely that expression of the network components is only required in a temporally transient manner to induce transcriptional outputs, to allow for turning off the signal until activated again. Particularly for the *Hes/Hey* family proteins, which act as oscillators, the half-life of mRNAs can be under 1 hour.[84] Thus, low expression of transcriptional targets might be expected even with active NOTCH signaling. Translational studies querying human tissues will continue to provide verification of the expression of targets defined in experimental model organisms. The data suggest that in human PVAT, NOTCH signaling is an interesting target as a modifier of the vascular phenotype, particularly in cardiovascular disease.

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Disclosures

None.

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