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ACTIVATION OF NOTCH SIGNALING IN ENDOTHELIUM CAUSE UPREGULATION OF N-TERMINAL ACETYLATED HISTONE 1

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Highlights

• Notch signaling is known to be important regulator of endothelium homeostasis and cardiovascular disease. Particularly, Notch seems to be associated with pathological changes in endothelium epigenome although no such Notch effects have been found. We have discovered that activation of Notch signaling alters histone 1 repertoire in the human endothelial cells and this is the first example of epigenomic Notch targets.

Aim	The disturbance of blood flow and alteration of physiological shear stress is one of the main reasons for endothelial dysfunction. Mechanosensitive and dose-dependent Notch pathway is assumed to be an important player of endothelial dysfunction progression, but the molecular mechanisms of the influence of Notch dysregulation on endothelium are still not understood. In particular, there is no data about possible targets of Notch in the endothelial epigenome.
Methods	Here we focused on the analysis of changes in histone code of human umbilical vein endothelial cells (HUVEC) after activation of Notch. For this purpose, we transduced cells by lentiviruses with construction for Notch 1 intracellular domain (N1ICD) overexpression or by empty vector (control). Then we isolated histone enriched fraction and secretome proteins and performed their shotgun proteomics analysis on timsToF Pro instrument. Proteomics data are available via ProteomeXchange with identifier PXD032978.
Results	We found the shift in proteomics profile of HUVEC caused by Notch activation and, particularly, the increase in the levels of N-terminal acetylated forms of histone 1: H1-0, H1-3, H1-4, H1-5, H1-10. We also found changes in the cell secretome profile which are associated with the decrease in proangiogenic effect of HUVEC secretome.
Conclusion	Our data identified epigenomic Notch targets and we assume that changes in H1 repertoire might be associated with cardiovascular disease progression *in vivo*.

Keywords	Notch • Histone 1 • Endothelium • HUVEC • Histone code • Epigenome • Secretome • Shear stress

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Список сокращений

HUVEC	– human umbilical vein endothelial cells	PTMs	– post-translational modifications
NICD	– Notch intracellular domain	LC-MS/MS	– liquid chromatography coupled with tandem mass spectrometry

Introduction

Vasculature permeates our entire body and is involved in homeostasis and the progression of various diseases. Endothelial cells line the vessels and play a substantial role in the development and maintenance of the cardiovascular system. Due to its localization, the endothelium maintains a barrier between blood and peripheral tissues. It directly controls the transport of nutrients (such as sugars and amino acids) and transfers systematic signals from various hormones and cytokines.

Endothelial function is controlled by various signal cascades and stimuli and Notch signaling and mechanical shear stress are among the main ones [1, 2].

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Physiologically high shear stress is necessary for normal endothelium function and production of NO, endothelium-derived relaxing factors (EDRFs), endothelin-1 (ET-1) etc. [1, 2]. Endothelial dysfunction is associated with various forms of cardiovascular disease from atherosclerosis to chronic heart failure [2, 3]. Decrease in shear stress, particularly due to the disturbance of the blood flow by bends or vascular obstruction, leads to atherosclerosis and endothelial dysfunction [2]. In clinical studies, low shear stress correlated with plaque burden in patients with coronary artery disease [2].

Notch signaling may act as a mechanosensor [4, 5] and therefore is assumed to be one of the central signal pathways associated with endothelial dysfunction [1]. For Notch-signal transduction, receptors on the cell-membrane surface (Notch1-4 in mammals) should interact with their ligands on the surface of another cell (Delta-like ligand 1, 3, 4, and Jagged 1, 2 in humans). This interaction leads to the cleavage of Notch-receptor by gamma-secretase and release of Notch intracellular domain (NICD) which then migrates to the nucleus where it forms transcription activation complex with CSL and MAML proteins [4]. Unlike most signaling pathways, Notch-pathway has no signal amplification stage (has dose-dependent effects [6]) and is sensitive to mechanical stimuli [4].

While the association of shear stress, Notch and endothelial dysfunction is well known, molecular mechanisms connecting these factors are still not understood. For example, it has been demonstrated that mutations in *Notch1* in valve leaflet endothelium are associated with general dysregulation of gene networks leading to calcific aortic valve disease progression. In turn, the recovery of normal gene networks in endothelium by XCT790 prevents calcific aortic valve disease progression [7]. One might assume that long-term dysregulation of Notch in endothelium causes changes in the epigenetic profile of the cells. While epigenetic changes in shear stressed environments are known to be involved in endothelial dysfunction [8], the possible connection of Notch and epigenetic changes in endothelium has not yet been tested.

Therefore, here we analyzed how activation of Notch signaling influences histone code of endothelial cells *in vitro*. We found that activation of Notch increased the level of N-acetylated forms of Histone 1: H1-0, H1-3, H1-4, H1-5, H1-10. We also found downregulation of telomere-complex components (Hsp90 and p23), and changes in chromatin remodeling proteins: upregulation of SMARCD2 and downregulation of SMARCA5. Those changes were also associated with changes in the secretome profile of endothelium and might have broad biomedical significance in case of endothelial dysfunction.

Methods

Cell culturing

Human umbilical vein endothelial cells (HUVEC) were bought in Pokrovsky Stem Cell Bank. HUVEC were seeded on 6-well plates (Eppendorf, Germany) gelatinized by 0.2% gelatin (Sigma Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS; Rusmedbio, St. Petersburg, Russia) in the 20×10^5 density and cultured with endothelial cell culture medium with endothelial cell growth supplement (ScienCell, Carlsbad, CA, USA) with addition of 1% of L-glutamine (Invitrogen, Carlsbad, CA, USA) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA). The incubation was performed at 37°C in humid air containing 5% CO_2.

Lentiviral transduction

For the Notch pathway activation we used lentiviral transduction with construction for Notch1 intracellular domain (N1ICD) overexpression or with empty constructions (control). This method of Notch activation was described in details before [6].

Shortly, we used a three-plasmid system for lentiviral production in HEK 293-T cell line with polyethylenimine (PolySciences, Warrington, PA, USA) transfection [9]. Lentiviral packaging plasmids (pMD2.G and pCMV-dR8.74psPAX2) were a generous gift from Prof. Didier Trono (École Polytechnique Fédérale de Lausanne, Switzerland). Modified pLVTHM plasmid was used for NICD overexpression. Open reading frame for murine N1ICD was amplified from reversely transcribed mouse ES cells mRNA and inserted to pLVTHM vector [10]. 15 ug pLVTHM with NICD or empty vector (for control transfection), 9.73 ug of pCMV-dR8.74psPAX2 and 5.27 ug of pMD2.G were mixed with 60 UL of polyethylenimine and added to HEK293-T cells in 70-80% confluence. One the second morning (16 h. after transfection) the medium was changed to the fresh medium. After 24 h. conditioned medium with produced lentivirus were collected and concentrated from the supernatant by ultracentrifugation, resuspended in 1% BSA/PBS and frozen in aliquots at −80 °C. The virus titer was defined by GFP-expressing virus; the efficiency of cell transduction was 85–90% by GFP.

qPCR

Notch pathway activation was verified by qPCR quantification of HEY1 expression (one of the main Notch targets). RNA from cultured cells was isolated using ExtractRNA (Eurogen, Moscow, Russia) and was used for reverse transcription (MMLV RT kit, Eurogen, Moscow, Russia). Real-time PCR was performed in the LightCycler 96 system (Roche, Basel, Switzerland) with SYBR Green visualization (qPCRmix-HS SYBR,

Eurogen, Moscow, Russia). We used primers for HEY1 (forward: 5' TGAGCTGAGAAGGCTGGTAC 3', reverse: 5' ATCCCAAACTCCGATAGTCC 3') level of which was calculated relatively to HPRT (forward: 5' TGACACTGGCAAAACAATGCA 3', reverse: 5' GGTCCTTTTCACCAGCAAGCT 3'). The fold change in gene expression (compared to control samples) was calculated using the ΔΔCt method.

We found a 20-fold increase in HEY1 level which correlated with our previous data in these experimental conditions and confirmed activation of Notch pathway.

Protein isolation

For secretome collection, in 48 h. of incubation after lentiviral transduction conditioned medium was collected (n = 6), centrifuged on 500g and then on 1500g for 15 min to remove cell contaminations. Then protein was precipitated by 4 volumes of iced cold acetone (LC-MS Grade; LiChrosolv) and washed with methanol (LC-MS Grade; LiChrosolv). Protein pellet was air-dried and resuspended in 8M Urea (Sigma Aldrich, St. Louis, MO, USA) in 50 mM ammonium bicarbonate (Sigma Aldrich, St. Louis, MO, USA).

Histone isolation was performed in the same cells by Histone Extraction Kit (n = 2; ab113476; Abcam, Cambridge, UK) according to the manufacturer recommendations.

Sample preparation for shotgun proteomics

Protein concentrations were measured by Qubit fluorimeter (Thermo Fisher Sci, Waltham, MA, USA) with QuDye Protein Quantification Kit (Lumiprobe, Moscow, Russia). We used 20 ug of each secretome and 2.5 ug of each histone enriched fraction were used for sample preparation. Samples were incubated for 1 h at 37°C with 5 mM DTT (Sigma Aldrich, St. Louis, MO, USA) with subsequent incubation in 15 mM iodoacetamide for 30 min in the dark at room temperature (Sigma Aldrich, St. Louis, MO, USA), followed by quenching with 5 mM DTT. the samples were diluted with seven volumes of 50 mM ammonium bicarbonate and incubated for 16 h at 37°C with Trypsin Gold in ratio 1:50 (Promega, Madison, WI, USA).

Tryptic peptides were desalted by solid-phase extraction using stage tips. Stage-tips were prepared by filling of polypropylene Vertex pipette tips (200 µL; SSIbio, USA) with six layers of C18 reversed-phase excised from Empore 3M C18 extraction disks (3M-Corporation, Maplewood, MN, USA). The desalted peptides were evaporated in a Labconco Centrivap Centrifugal Concentrator (Labconco, USA) and dissolved in water/0.1% formic acid (LC-MS Grade; LiChrosolv) to approximate final concentration of 250 ng/ul for further LC-MS/MS analysis.

LC-MS/MS analysis

Approximate 500 ng of peptides were used for shotgun proteomics analysis by LC-MS/MS with ion mobility in TimsToF Pro mass spectrometer (Bruker Daltonics, Bremen, Germany) with nanoElute UHPLC system (Bruker Daltonics, Bremen, Germany). UHPLC was performed in two-column separation mode with Acclaim™ Pep-Map™ 5 mm Trap Cartridge (Thermo Fisher Scientific, Waltham, MA, USA) and Bruker Ten separation column (C18 ReproSil AQ, 100 mm × 0.75 mm, 1.9 µm, 120 A; Bruker Daltonics, Bremen, Germany) in gradient mode with 400 nL/min flow rate with column temperature at 50°C. Phase A was water/0.1% formic acid, phase B was acetonitrile/0.1% formic acid (LC-MS Grade; LiChrosolv). The gradient was from 2% to 35% phase B for 35 min, then to 95% of phase B with subsequent wash with 95% phase B for 10 min. The column was equilibrated with 4 column volumes before each sample. CaptiveSpray ion source was used for electrospray ionization with 1600 V of capillary voltage, 3 l/min N2 flow, and 180 °C source temperature. The mass spectrometry acquisition was performed in automatic DDA PASEF mode with 1.1 s cycle in positive polarity with the fragmentation of ions with at least two charges in m/z range from 100 to 1700 and ion mobility range from 0.60 to 1.60 1/K0. The analysis of samples of histone enriched fraction was performed in analytical triplicates.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD032978 and 10.6019/PXD032978.

Protein identification

Protein identification was performed in Peaks Xpro software (Bioinformatics Solutions Inc., Waterloo, ON, Canada) with parent mass error tolerance 10 ppm and fragment mass error tolerance 0.05 ppm, protein and peptide FDR less than 1%, two possible missed cleavage sites, proteins with at least two unique peptides were included for further analysis. Cysteine carbamidomethylation was set as fixed modification. Methionine oxidation, acetylation of protein N-term, asparagine, and glutamine deamidation were set as variable modifications. For histone data analysis we used human protein SwissProt database (uploaded on 2 March 2021; 20,394 sequences) while for secretome analysis we mixed human and bovine databases to exclude proteins from bovine serum. CRAP contaminants database was used for both searches (version of 4 March 2019).

2.8 Statistical analysis

Peak area was used for further statistical analysis in R (version 4.1.2) [11]. For secretome study we used proteins identified in no less than 75% of samples while for proteomics analysis of histone enriched fraction we used proteins identified in no less than 80% of samples. Then we performed imputation of missed values

by k-nearest neighbors by the "impute" package for both datasets. Secretomes and proteomes of histone enriched fraction from control HUVEC and cells with activated Notch signaling were compared by principal component analysis (package MixOmis) and differential expression analysis (Limma package).

We revealed potential differences in N-terminal acetylated forms of histone 1, thus, for analysis of differentially expressed N-acetylated peptides we extracted such peptides from the original dataset. We used peptides identified in no less than 75% of samples. Missed values were replaced by zero. Differential expression analysis of N-acetylated peptides was performed by the "limma" package.

Reproducible code for data analysis is available from github.com (https://github.com/ArseniyLobov/Analysis-of-changes-in-HUVEC-histone-code-under-Notch-activation).

Functional annotation was performed by the Database for Annotation, Visualization and Integrated Discovery v6.8 (https://david.ncifcrf.gov/, accessed on 28 March 2022) GO "biological processes" database.

Results

Changes of HUVEC proteomics profile during activation of Notch signaling

We obtained protein extract enriched in histones, but it also included some other proteins. Our dataset included 1013 proteins after exclusion of proteins identified by less than two unique peptides and missed values in more than two samples.

We performed enrichment analysis of this dataset against GO "cellular compartment" database and found that most of the proteins had nuclear (589 proteins, p-Value = 2,5E-85) or cytosol (551 proteins, p-Value = 4,3E-79) localization. Other various compartments were also presented but much less enriched in our dataset such as extracellular exosome (362 proteins, p-Value = 3,6E-101), membrane (353 proteins, p-Value = 3,6E-79), extracellular region (121 proteins, p-Value = 9,6E-2) and mitochondrion (103 proteins, p-Value = 1,8E-4). Thus, obtained data is also informative for analysis of overall changes in HUVEC's physiology caused by activation of Notch signaling.

HUVECs before and after activation of Notch formed separate clusters in the principal component analysis (figure 1*A*). We found statistically significant 83 upregulated and 79 downregulated proteins presented in the supplementary materials 1 (adjusted P-value <0.05, LogFC > |1|; supplementary table S1 available via https://github.com/ArseniyLobov/Analysis-of-changes-in-HUVEC-histone-code-under-Notch-activation). Pathway enrichment analysis against GO "biological processes" database revealed that upregulated processes were associated with mRNA synthesis and processing and mitochondrial electron transport; while down regulated proteins were associated with translation (chaperone complex assembly and ribosome) and telomerase complex (figure 1*B*).

Among specific upregulated proteins we would like to emphasize several forms of histone 1: H1-0 (LogFC 2.15, adj. P-value 0.01), H1-3 (LogFC 1.98, adj. P-value 0.017), H1-1 (LogFC 1.68, adj. P-value 0.005), H1-5 (LogFC 1.5, adj. P-value 0.006), H1-2 (LogFC 1.42, adj. P-value 0.002), H1-10 (LogFC 1.36, adj. P-value 0.015); and SMARCD2 – SWI/SNF-related matrix-associated actin-dependent regulator of chromatin (LogFC 2, adj. P-value 0.034; supplementary materials 1).

Among downregulated proteins we singled out MYB Binding Protein 1a (MYBBP1A; LogFC –1, adj. P-value 0.001), SWI/SNF Related, Matrix Associated, Actin Dependent Regulator of Chromatin, Subfamily A, Member 5 (SMARCA5; LogFC –2.3, adj. P-value 0.006), Heat Shock Protein 90 Alpha Family Class A Member 1 (HSP90AA1; LogFC –2.9, adj. P-value 0.01),

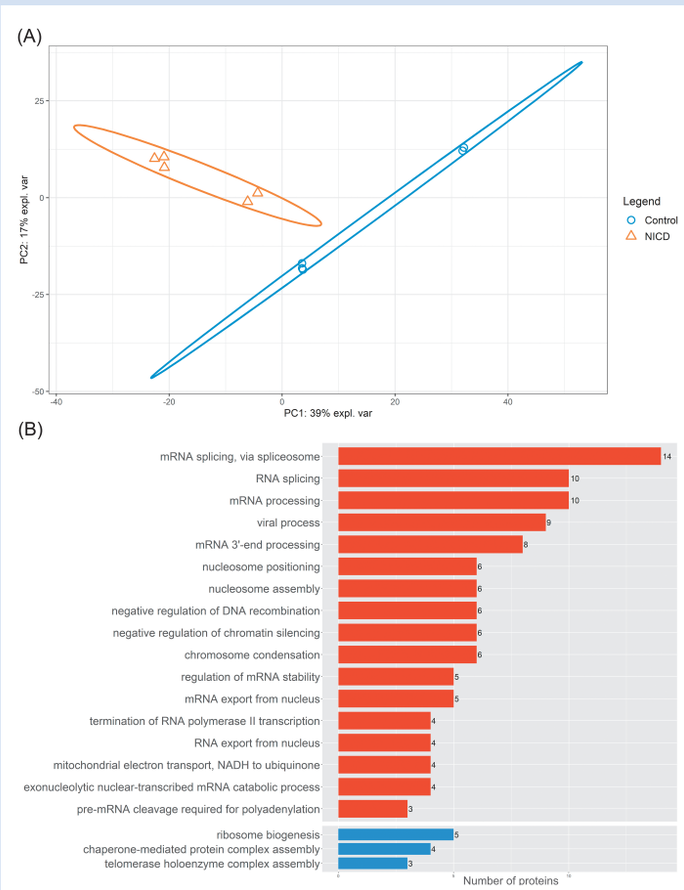

Figure 1. Comparison of proteomic profiles of human umbilical vein endothelial cells (HUVEC) with or without activation of Notch pathway. (*A*) Principal component analysis of HUVEC transduced by lentivirus with construction for Notch1 intracellular domain overexpression (N1ICD) or by empty lentivirus (Control). (*B*) Results of pathway enrichment analysis of proteins upregulated (red, upper pathways) or downregulated (blue, bottom pathways) after NICD overexpression against GO "biological processes" database

Profilin 1 (PFN1; LogFC –1.5, adj. P-value 0.04), Zyxin (ZYX; LogFC –2.3, adj. P-value 0.02).

Activation of Notch caused downregulation of secretome proteins, necessary for normal endothelium functioning

One of the central roles of endothelium *in vivo* is to regulate biological processes in blood and in the vessel wall. Thus, we also performed the analysis of HUVEC secretome in control and under activation of Notch-signaling. Due to the sensitivity of endothelium to depleted medium we analyzed standard condition medium with highly abundant serum proteins. Thus, we were able to include to statistical analysis only 159 proteins and to identify three endothelium secretome proteins downregulted by activation of Notch signaling: Pentraxin-related protein (PTX3; LogFC –2.35, adj. P-value 5.6E-6), Plasminogen activator inhibitor 1 (SERPINE1; LogFC –1.68, adj. P-value 0.00014), Thrombospondin-1 (THBS1; LogFC –2.47, adj. P-value 0.041).

Pathway enrichment analysis of these proteins revealed, that they are associated with atherosclerosis and positive regulation of angiogenesis.

Activation of Notch signaling increase Histone 1 N-terminal acetylation

Changes in histone 1 repertoire are related to epigenetic changes as well as their post-translational modifications (PTMs). We have not found any changes in histone 2, 3 and 4 PTMs after Notch activation, but found differences in N-acetylated forms of Histone 1. Thus, we performed qualitative analysis of N-terminal acetylated peptides identified by our data. 162 such peptides were included in statistical analysis. We found 22 differentially expressed N-acetylated peptides (adj.P.Val <0.05 and LogFC > |1|; supplementary materials 1) 9 of which were peptides of various forms of histone 1 (*table*).

Discussion

Blood flow changes causing shear stress are the main factor of endothelial dysfunction [4]. Dose-dependent Notch pathway assumed to be chemosensory effector of shear stress [3]. While epigenetic changes are known to be important in endothelial dysfunction progression, we are the first to describe possible changes in endothelial epigenome caused by Notch pathway activation. We found that Notch increase level of N-acetylated forms of histone1 (H1-0, H1-3, H1-4, H1-5, H1-10).

Notch signaling is known to be regulated by epigenetic in the level of Notch component expression or expression of Notch target genes [12, 13]. Nevertheless, we found only several examples were activation of Notch pathway caused epigenetic changes. Ting et al. (2018) demonstrated that DLL4 (Notch-ligand able to increase NICD level in recipient cell) dynamically increase H3K4 methylation in specific locus through upregulation of SMYD3 [14]. Similarly, Kim et al. (2012) found that activation of Notch1 suppressed the expression of WNT target genes in human colorectal cancer cells through histone methyltransferase SET domain bifurcated 1 (SETDB1) [15]. Thus, we assume that in the endothelial context there are some protein targets which may implement changes in level of N-acetylated H1 described by us.

N-terminal modifications of histones remain a relatively understudied area of research, especially in case of histone 1. It is known that H1 N-terminal acetylation may occur by initiator methionine or other N-terminal amino acids if initiator methionine was excised by aminopeptidases [16]. Most of N-acetylated peptides found by us were acetylated by serine (*table*), but biological relevance of it remains unknown. One of the most studied sites of H1 acetylation is acK34 of histone 1.4. Acetylation of this site assumed to activate transcription [17], but these data are not relevant to N-terminal acetylation in endothelial model which we describe here.

In some cases, H1 acetylation molecular participants have been studied: for example, acetyltransferase GCN5 acetylates H1.4 in human cells from several different lines [18]; nucleosome assembly protein 1 (NAP-1) inhibits acetylation of H1 by acetyl-transferase p300 in Drosophila cells [19]. We have not identified upregulated acetyl-transferase or aminopeptidases which might be associated with H1 acetylation, but we found downregulation of N-alpha-acetyltransferase 10 (NAA10) and methionine aminopeptidase 2 (METAP2) – proteins which technically able to acetylate N-terminal end

N-terminal acetylated peptides of Histone 1 which change their expression during Notch pathway activation.

Uniprot accession	Gene name	Peptide sequence	logFC	adj.P.Val
P16401	H1-5	M.S(+42.01)ETAPAETATPAPVEKSPAKK.K	3,00	4,38E-05
		M.S(+42.01)ETAPAETATPAPVEKSPAK.K	1,86	8,54E-07
		M.S(+42.01)ETAPAETATPAPVEK.S	1,08	0,0054
P16402	H1-3	M.S(+42.01)ETAPLAPTIPAPAEKTPVKK.K	1,49	0,0049
		M.S(+42.01)ETAPLAPTIPAPAEKTPVK.K	1,47	0,0013
P07305	H1-0	M.T(+42.01)ENSTSAPAAKPK.R	2,25	0,0112
		M(+42.01)TENSTSAPAAKPK.R	1,75	0,0085
P10412	H1-4	M.S(+42.01)ETAPAAPAAPAPAEKTPVKK.K	1,15	0,0047
Q92522	H1-10	M.S(+42.01)VELEEALPVTTAEGMAK.K	1,12	0,0436

and remove N-terminal methionine respectively. Therefore, we assume that changes in histone repertoire might be associated with some changes in acetyltransferase and aminopeptidase activity, but it needs additional confirmation.

In case of endothelial dysfunction and effect of shear stress, researchers generally focus on PTMs of core histones (e.g. H3) [8] and we found no data about PTMs of H1 in that context. Still, we found some changes in proteomics profile which might be associated with endothelial dysfunction. We found downregulation of protein components of telomerase active complex – Hsp90 (HSP90AA1 and HSP90AB1) and p23 (PTGES3; figure 1) [20]. Decrease of telomerase activity is associated with endothelial aging and senescence *in vitro* and associated with atherosclerosis *in vivo* [21]. We also found downregulation of Zyxin – mechanotransducer associated with reactions to stretch-induced stress [22].

Finally, we found that Notch induced downregulation of secreted endothelial proteins: Pentraxin-related protein (PTX3), Plasminogen activator inhibitor 1 (SERPINE1) and Thrombospondin-1 (THBS1). Similar pattern of Notch dependent decrease in expression of secreted SERPINE1 and PTX3 was already demonstrated in other models [23, 24]; while thrombospondin-1 was demonstrated to be involved in mechanotransduction [25]. Therefore, our data is in a good accordance with previous investigations. Downregulation of these factors lead to dysregulation of vascular remodeling and endothelium proliferation and thus associated with atherosclerosis and endothelial dysfunction [23–25].

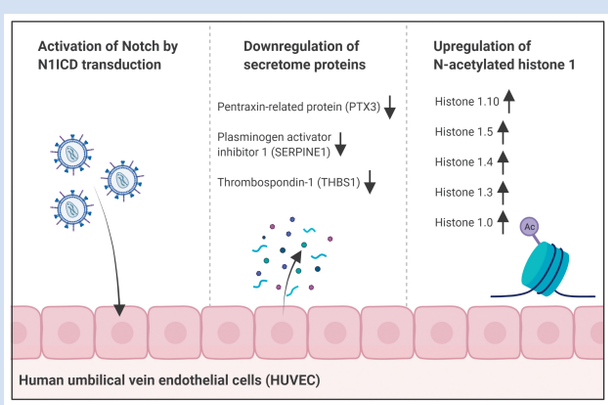

Figure 2. Graphical summary of Notch signaling activation effect on human umbilical vein endothelial cells. Created with BioRender.com

Conclusions

Notch signaling is mechanosensitive pathway associated with endothelium dysfunction and atherosclerosis. We found that its activation in human umbilical vein endothelial cells (HUVEC) cause changes in epigenetic profile by increase in level of N-acetylated histone 1 as well as in HUVEC proteomic profile and secretome. We argue that changes in histone 1 repertoire might be associated with atherosclerosis and endothelium dysfunction *in vivo* and seems fruitful to additional study in clinical samples. Summary of our study is presented in figure 2.

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Conflicts of interest

A.A. Lobov declares no conflict of interest. D.A. Perepletchikova declares no conflict of interest. E.A. Repkin declares no conflict of interest. A.B. Malashicheva declares no conflict of interest.

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Supplementary Materials

Table S1: the results of differentially expressed genes analysis and pathway enrichment analysis.

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Author Contribution Statement

LAA – contribution to the concept of the study, data collection, analysis and interpretation, manuscript writing, approval of the final version, fully responsible for the content

PDA – data interpretation, editing, approval of the final version, fully responsible for the content

REA – data interpretation, editing, approval of the final version, fully responsible for the content

MAB – data interpretation, editing, approval of the final version, fully responsible for the content

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