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Notch, BMP and WNT/β-catenin network is impaired in endothelial cells of the patients with thoracic aortic aneurysm☆

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A B S T R A C T

Cellular and molecular mechanisms of thoracic aortic aneurysm are still not clear and therapeutic approaches are mostly absent. The role of endothelial cells in aortic wall integrity is emerging from recent studies. Although Notch pathway ensures endothelial development and integrity, and *NOTCH1* mutations have been associated with thoracic aortic aneurysms, the role of this pathway in aneurysm remains elusive. The purpose of the present work was to study functions of Notch genes in endothelial cells of patients with sporadic thoracic aortic aneurysm.

Aortic endothelial cells were isolated from aortic tissue of patients with thoracic aortic aneurysm and healthy donors. Gene expression of Notch and related BMP and WNT/β-catenin pathways was estimated by qPCR; WNT/β-catenin signaling was studied by TCF-luciferase reporter. To study the stress-response the cells were subjected to laminar shear stress and the expression of corresponding genes was estimated by qPCR.

Analyses of mRNA expression of Notch genes, Notch target genes and Notch related pathways showed that endothelial cells of aneurysm patients have dysregulated Notch/BMP/WNT pathways compared to donor cells. Activity of Wnt pathway was significantly elevated in endothelial cells of the patients. Cells from patients had attenuated activation of *DLL4*, *SNAIL1, DKK1* and *BMP2* in response to shear stress.

In conclusion endothelial cells of the patients with thoracic aortic aneurysm have dysregulated Notch, BMP and WNT/β-catenin related signaling. Shear stress-response and cross-talk between Notch and Wnt pathways that normally ensures aortic integrity and resistance of endothelial cells to stress is impaired in aneurysmal patients.

1. Introduction

Thoracic aortic aneurysm (TAA) is a life threatening condition, which is manifested by progressive enlargement of the thoracic aorta due to destructive changes in the aortic wall. Therapeutic agents that may influence the process are absent to date and the only therapeutic decision is elective surgical intervention [1]. The

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etiologies underlying TAA are diverse and range from degenerative or hypertensive associated aortic enlargement to less common genetic disorders, such as Marfan syndrome, Ehlers-Danlos, and other syndromic connective tissue diseases [2].

Non-syndromic TAA may occur in the presence of a tricuspid (TAV) or a bicuspid aortic valve (BAV), and several lines of evidence suggest that the mechanism behind aneurysm development is distinct between the two patient groups [3] [4]; [5]. So far, a few genes such as *NOTCH1* [6] [7] [8]; [9]; and *GATA5* [10] have been associated with non-syndromic forms of BAV/TAA. It is widely accepted that *NOTCH1* mutations are associated with BAV and calcific aortic valve disease [6] [7]; [9].

Vascular smooth muscle cells (VSMC) have been considered as the main target of degeneration in the aortic wall, however, endothelial cells (ECs) have been implicated in maintaining the differentiation state of VSMC of the vessel wall [11] [12]; [5]. We have previously shown that primary endothelial cells derived from aortas of BAV/TAA patients have attenuated Notch signaling, irrespective of *NOTCH1* mutation [12], and this may reflect an impaired common stress-response mechanism in the diseased cells. Recently we and others reported *NOTCH1* gene variants and mutations in patients with aortic stenosis with TAV [13]; [14]. However, no evidence has so far been presented for the defective function of Notch pathway in the ECs of aneurismal patients with a normal tricuspid valve.

In the present study we have analyzed the expression of the Notch pathway in primary ECs from TAA patients with TAV. We show that Notch signaling is attenuated also in TAV/TAA cells. We observed over activation of Wnt and BMP signaling in the aortic ECs of TAA patients and show that the proper Notch, BMP2 and Wnt/β-catenin cooperation that is required for appropriate response to shear stress is impaired in aortic ECs of TAA patients.

2. Materials and methods

2.1. Patients

The clinical research protocol was approved by the local Ethics Committee of the Almazov Federal Medical Research Centre and was in accordance with the principle of the Declaration of Helsinki. All patients gave informed consent.

Samples of the aneurysmal wall of the thoracic aorta were harvested during aortic surgery because of thoracic aortic aneurysm with aortic diameter more than 5 cm at the Almazov Federal Medical Research Centre. Nine specimens were sampled from patients with thoracic aortic aneurysm with tricuspid aortic valve (n = 9) (Table 1). Patients with connective tissue disorders were excluded. Control aortic specimens were obtained from organ transplant donors (n = 5) and all had TAV. All tissues were sampled from the outer curvature of the thoracic aorta.

Table 1
Clinical characteristics in the study groups values are means ± S.E.M. CSA/h, ascending aortic cross-sectional area to patient height ratio TAV ($n = 9$).

Male gender (%)	46
Age (years)	71.3 ± 2.53 (range 55–84)
Aortic diameter (cm)	5.6 ± 0.18
Aortic CSA/h (cm^2/m)	6.6 ± 0.6
Peak valve gradient (mmHg)	83 ± 9
Mean valve gradient (mmHg)	55 ± 7
Aortic valve area index (cm^2/m^2)	0.39 ± 0.02
Hypertension (%)	84
Medication	
Angiotensin receptor blockers (%)	38
Statins (%)	0
Aspirin (%)	31

2.2. Primary cultures

Human aortic endothelial cells (HAEC) were isolated from tissue fragments of patients after surgery for aneurysm corrections as described [12]. The cells were used in experiments at passages 2–5.

2.3. In vitro flow model

HAECs were plated on gelatin-coated teflon-bordered cell culture slides (75 × 25 × 10 mm, Flexcell International Corp.) and cultured for 40 h with 5% CO2 at 37 °C. The culture slides were then inserted into a parallel plate Streamer device (Flexcell International Corp., Hillsborough, NC, US) and exposed to laminar flow of 12 dyn/cm^2 for 6 h with 5% CO2 at 37 °C. A Masterflex L/S peristaltic pump was used to generate flow, and the frequency was determined by the Osci-Flow flow controller (Flexcell Internationl Corp.). Cells cultured under static conditions were used as controls. Total RNA was isolated using Qiagen miRNeasy Mini Kit (Qiagen, Hilden, Germany), according to manufacturer's instructions and quantified using NanoDrop ND-1000 (NanoDrop Technologies). In total, cells from n = 8 TAA patients and n = 5 donors were used.

2.4. qPCR analysis

Total RNA (1 μg) was reverse transcribed with MMLV RT kit (Eurogen, Russia). Real-time PCR was performed with 1 μL cDNA and SYBRGreen PCR Mastermix (Eurogen, Russia) in the Light Cycler system using specific forward and reverse primers for target genes. Primer sequences are available upon request. Changes in target genes expression levels were calculated as fold differences using the comparative ΔΔCT method. The mRNA levels were normalized to *HPRT* mRNA.

2.5. Genetic constructs and lentiviruses

Lentiviral packaging plasmids were a generous gift of Didier Trono (École Polytechnique Fédérale de Lausanne, Switzerland); pLVTHM was modified by the addition of the T7 tag and chloramphenicol resistance gene (cm), resulting in the pLVTHM-T7-cm vector. The pCS2 vector containing stabilised β–catenin resistant to proteolysis due to S33A mutation was kindly provided by Ralf Kemler, MPI Freiburg. S33A β-catenin gene was amplified by PCR using the following primers:

Asc-Bcat GGCGCGCCCATGGCTACTCAAGCTG,

Nde-Bcat GGAATTCCATATGTTACAGGTCAGTATCAAACC

The PCR product was cleaved with AscI and NdeI, then cloned in frame of the T7 tag replacing the cm gene within pLVTHM-T7-cm resulting in the pLVTHM-b-catenin-S33A plasmid for lentiviral system. Lentiviral production was performed as described previously [12]. The virus titer was defined by GFP-expressing virus; the efficiency of primary endothelial cell transduction was 90–95% by GFP. The efficiency of transgene expression with S33A β-catenin bearing virus verified by ICH staining with the antibodies to β-catenin and it was 90–95%.

2.6. Promoter activity assay

To estimate canonical Wnt activity we used lentiviral TOP flash reporter construct and measured TCF activity (Addgene 24307). In the construct the expression of the firefly *luciferase* gene is regulated by seven tandem TCF binding sites upstream of a minimal TK promoter [15] and the level of TCF/LEF promoter activity indicates

the transcriptional activation of WNT/β-catenin pathway. Cell lysis was performed using Luciferase Assay System (Promega) according to the manufacturer recommendations. Luciferase activity was measured with Synergy2 (BioTek, USA). Samples were normalized by protein content using Pierce BCA Protein Assay Kit (Thermo Scientific).

2.7. Statistics

Values are expressed as means ± SEM. Groups were compared using the Mann–Whitney non-parametric test. A value of $P \leq 0.05$ was considered significant. Statistical analysis was performed by using R software (version 2.12.0; R Foundation for Statistical Computing, Vienna, Austria). Principal component analysis (PCA) was performed to determine, which continuous variables discriminate between groups of TAA and donors. Continuous variables were qPCR gene expression data from 2^-ΔΔCT (RQ) estimation. PCA and the analysis of differential gene expression were performed using Phantasus tool (https://genome.ifmo.ru/phantasus/) with integrated limma instrument [16].

3. Results

3.1. Alteration in Notch signaling in ECs of TAV patients

We measured the expression levels of key genes belonging to Notch pathway, namely– *NOTCH1-4*, *JAG1*, *DLL1*, *DLL4* in human aortic endothelial cells (HAEC) of TAA patients and healthy controls (donors) (Fig. 1). This revealed a significantly lower level of mRNAs for *NOTCH1*, *NOTCH2, NOTCH4* and *DLL4*, but significantly higher levels of *NOTCH3* and *DLL1* expression in TAA patients as compared to donor controls. Our data suggest alterations of baseline Notch signaling in aortic ECs of TAA patients.

Next we estimated the expression of key genes of several major pathways including antiosteogenic, antioxidant, antiatherogenic and proinflammatory pathways (Fig. 1, Supplementary Fig. 1). The mRNA levels of direct Notch target *HEY1* and TGF-β/BMP effector, *BMP2,* were significantly up regulated while *GREM1* was down regulated in the cells of the patients (Fig. 1A). WNT/β-catenin effectors, *TCF4*, *DKK1* and *STAT6* were also upregulated in the patients. PCA analysis shows that HAEC from the patients are different by gene expression profiles from the cells derived from aortic tissues of healthy persons. Our data suggest that HAEC of TAA patients have dysregulated Notch/BMP/WNT pathways comparing to donor cells.

3.2. Cross-talk between Notch and Wnt/β-catenin pathways

BMP2 has been shown to activate WNT/β-catenin signaling cascade, driving osteogenic mineralization of vascular progenitors [17]. Since we observed differential expression of effectors of WNT/β-catenin, *BMP2, DKK1*, *STAT6* and *TCF4,* we estimated the level of the WNT/βcatenin signaling in the diseased and healthy cells using TOP flash using TOPflash reporter construct (Fig. 3).

To verify how activated WNT/β-catenin operated in the HAEC of the TAA patients in comparison to healthy donors we overexpressed S33A mutated stabilised β-catenin in the cells via lentiviral transduction or added a specific inhibitor of Gsk3 activity, CHIR99021, to the culture medium (Fig. 3). Firstly, TCF activity was significantly elevated in the HAEC of TAA patients even at a basal level indicating possible differences in WNT/β-catenin signaling between the HAECs of the two groups. Secondly, the diseased cells also demonstrated a significant increase of the TCF-dependent luciferase activity in response to inhibition of Gsk3 by CHIR99021 (Fig. 3a), but not by the S33A β-catenin alone. However, the fold activation was significantly lower in diseased cells comparing to control (Fig. 3b), possibly due to the high initial level of the signaling. The level of *AXIN2* expression, a direct WNT transcriptional target (Fig. 3c), reflects the same tendency, showing the failure of activation in response to WNT, either by S33A β-catenin or CHIR99021. Thus, the WNT/β-catenin pathway activity is substantially elevated in the HAECs of TAA patients.

WNT/β-catenin pathway has been reported to modulate endothelial Notch/Dll4 signaling in mouse development [18]. We assessed how activation of WNT/β-catenin influences *DLL4* expression in adult HAEC (Fig. 4). We activated WNT/β-catenin in HAEC either by transduction of S33A β-catenin-bearing lentivirus or by the addition of CHIR99021. Correspondingly, we observed increase in *AXIN2* expression; inhibition of Gsk3 activity had more prominent effect on *AXIN2* expression than S33A β-catenin alone.

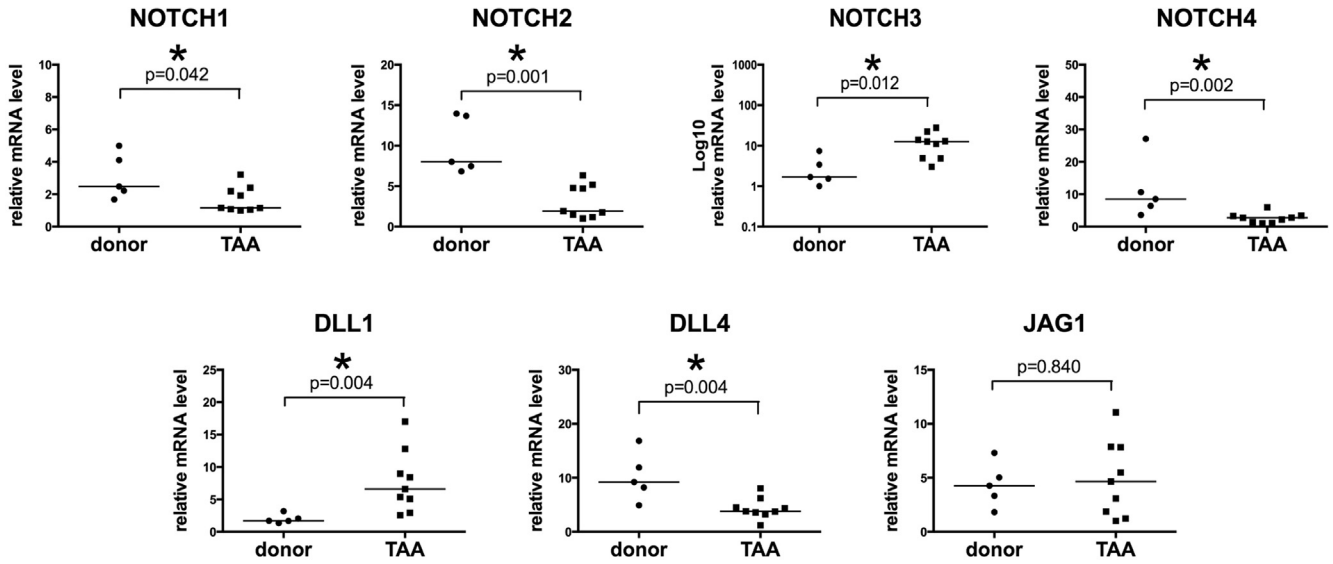

Fig. 1. Expression levels of Notch receptors and ligands in the aortic endothelial cells from the patients with thoracic aortic aneurysm (TAA), n = 9 or control cells (donor), n = 5. Groups are compared using Mann-Whitney nonparametric test; line represents the median; *p < 0.05.

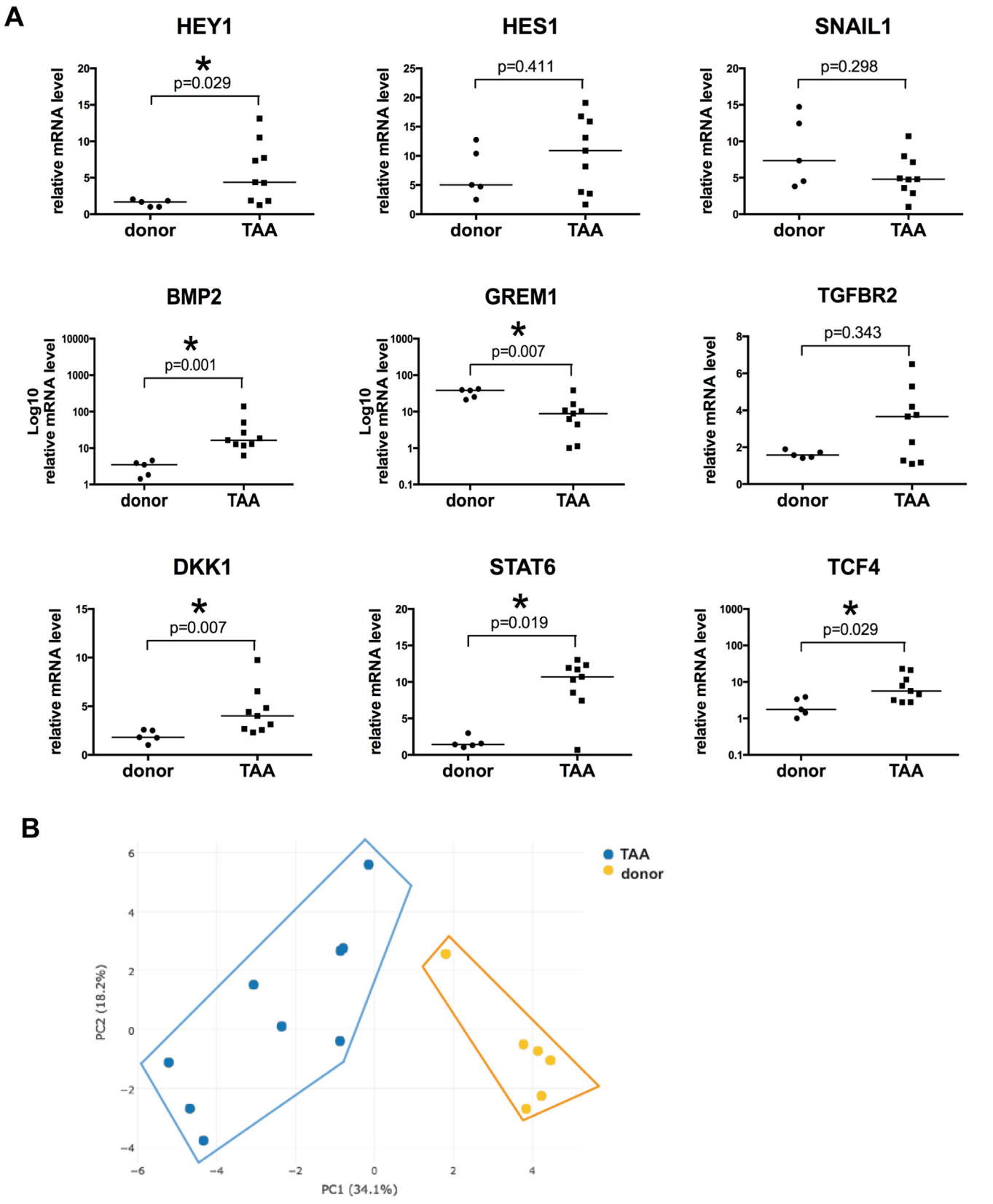

Fig. 2. Genes dysregulated in the aortic ECs from the patients with thoracic aortic aneurysm (TAA), n = 9 or control cells (donor), n = 5.A. Notch target genes (*HEY1*, *HES1*, *SNAIL*) as well as the expression of genes belonging to pathways that cross talk to Notch such as TGF-β/BMP (*BMP2*, *GREM1*, *TGFRB2*) and WNT/β-catenin (*TCF4*, *DKK1*, *STAT6*) pathways is shown. Groups are compared using Mann-Whitney non-parametric test; line represents the median; *p < 0.05. B. PCA analysis showing differences in gene expression between HAEC from healthy and diseased aortas. See also suppl. Fig. 1.

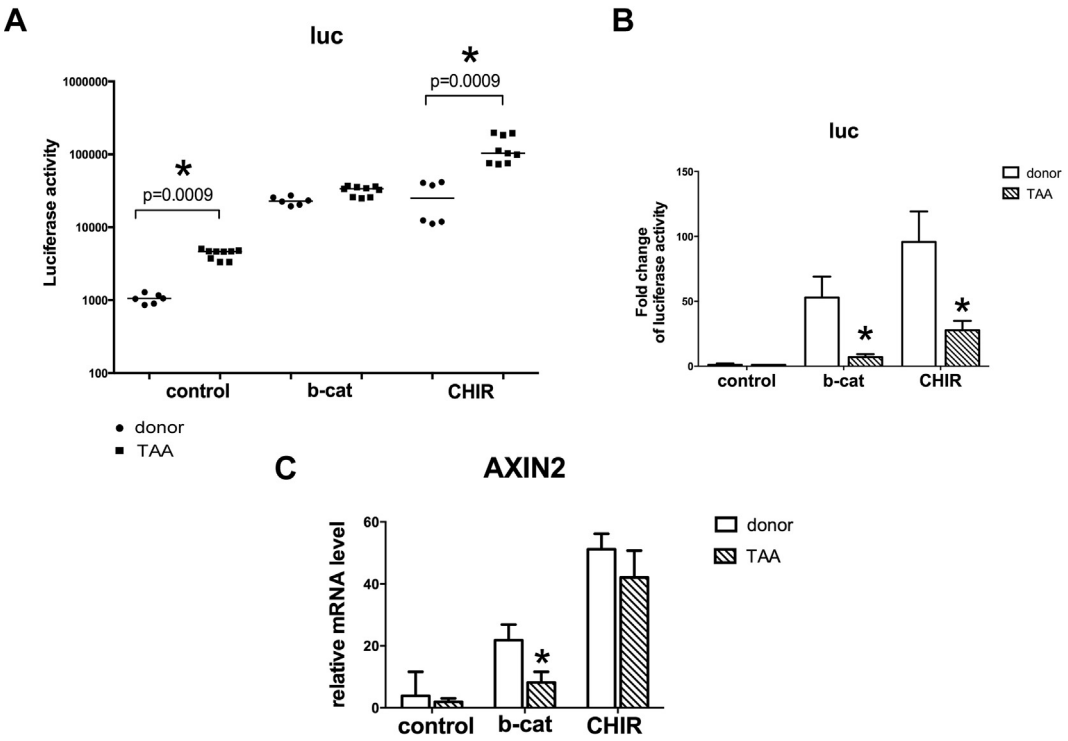

Fig. 3. Wnt activity in the HAEC of patients with TAA. Wnt was activated by a lentiviral transduction of proteolysis resistant S33A mutant of β-catenin into the cells or by addition of specific Gsk3 inhibitor CHIR99021. Wnt-activity was estimated with TOPFlash reporter construct as well as by the expression of *AXIN2*. (A) Dotted graph represents the basal level of luciferase activity in the HAEC; (B) bar graphs represent fold change of luciferase activity (mRNA level) in non-stimulated cells relative to the stimulated cells. (C) The mRNA level of direct WNT/β-catenin target *AXIN2* after the introduction of mutant β-catenin into the cells or by addition of specific Gsk3 inhibitor CHIR99021. HAEC from the patients with thoracic aortic aneurysm (TAA), n = 9; control HAEC (donor), n = 6; line represents the median. Groups are compared using Mann-Whitney non-parametric test. *$p < 0.05$.

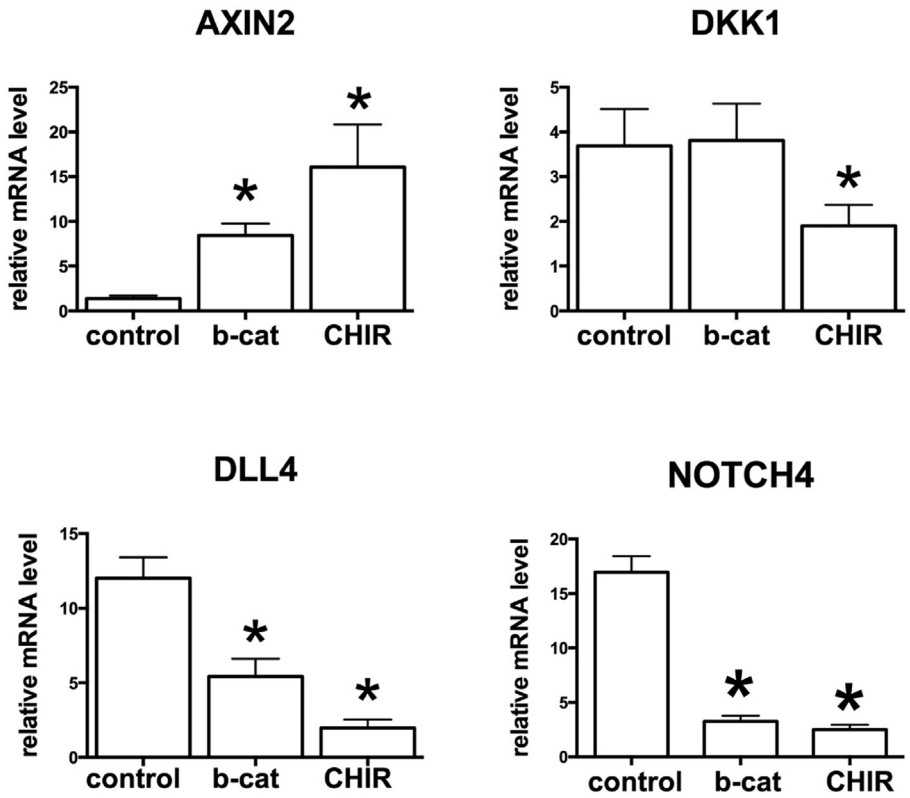

Fig. 4. Cross-talk between Wnt and Notch in adult human aortic endothelial cells (HAECs). Cells were transduced with S33A mutant β-catenin-bearing lentivirus or cultured in the presence of Gsk3 inhibitor CHIR99021. Groups are compared using Mann-Whitney non-parametric test. *$p < 0.05$ for the difference between control and stimulated cells.

Both direct S33A β-catenin introduction and inhibition of Gsk3 activity decreased expression of *DLL4* and *NOTCH4*. *DKK1* expressionwas also decreased after inhibition of Gsk3, but S33A β-catenin alone was not able to decrease *DKK1* expression. Our data suggest that activity of WNT/βcatenin itself could influence the level of Notch signaling by Dll4 and Notch4 in the adult HAECs. Correspondingly, *DLL4* and *NOTCH4* mRNA level was lower in the HAECs of the patients comparing to healthy cells (Fig. 1).

3.3. Shear stress response is impaired in the endothelial cells of the patients with thoracic aortic aneurysm

The above data suggest the dysregulation of BMP and WNT/β-catenin pathways in ECs of the TAA patients. These pathways are known to be activated in response to cellular stress including shear stress [19]. To reveal the difference in the expression of genes associated with stress response between diseased and healthy aortic ECs, we compared the shear stress response of HAECs from patient and donor cells to laminar flow that is the relevant blood flow in the aortas with a tricuspid valve. A comparison between the activation of Notch/BMP/WNT/β-catenin related genes in cells of the patients and donors (Fig. 5, Supplementary Fig. 2) showed the most striking differences in the expression of *DLL4*, *SNAIL1* and *BMP2*, *DKK1* as a result of exposure to laminar shear stress (Fig. 5A). In patient cells *DLL4* and *SNAIL1* were not up regulated to the same level as in donor cells. On the other hand, *BMP2* was up regulated in control cells, whereas the diseased cells had already elevated *BMP2*, and flow did not elevate it any further. *DKK1* level dropped in response to flow in both control and diseased cells; but the absolute level of *DKK1* was different between patient and controls in the flow-stressed cells (Fig. 5A) and remained higher in the cells of the patients.

On the contrary, *DKK1* and *BMP2*, as well as Wnt effectors, *STAT6* and *TCF4,* were already elevated in non-stimulated ECs of the patients (Fig. 2). We observed elevation of *BMP2* expression by flow in donor cells while no change was observed in the flow-stimulated diseased cells. Absolute level of *DKK1* expression was significantly higher in the diseased versus healthy cells in both non-stimulated (Fig. 2) and flow-stressed cells (Fig. 5A). PCA analysis of gene expression profiles (Fig. 5B) shows that HAEC derived from the patients and healthy donors form separate clusters by gene expression in response to shear stress. We conclude that stress response was attenuated in the HAECs of TAA patients.

4. Discussion

In this study, we show that ECs from TAA patients are impaired in several important pathways such as Notch, BMP and WNT/β-catenin compared to the cells of healthy donors. We also report attenuation of shear stress response in the aortic ECs of the patients with TAA.

Notch pathway is indispensable for endothelial differentiation and maintenance during adult life [20]. We have recently shown that ECs from BAV/TAA have impaired Notch-dependent EMT irrespective of *NOTCH1* mutations [12]. Here we indeed show that the expression of different components of the Notch pathway is altered in of TAV/TAA patients.

Due to the absence of signal amplification step or utilization of secondary messengers to transmit the signal from the cell surface to the nucleus, Notch signaling is extremely dose sensitive. Strict dosage dependence of Notch during development has been reported in human and other mammals [21] and a tight regulation of both signal sending and receiving cells is crucial for optimal outcome in physiological settings. By altering the amount of available ligands and receptors, different scenarios for Notch activation could be generated [22]. Therefore, our data proposes that dysregulation of Notch pathway in the EC may play a role in the development of TAA.

Moreover, Notch signaling in the endothelium of the vessel mediates the differentiation of underlying SMC, ensuring integrity of the vessel wall [23]. This is compatible with our earlier reports in which we demonstrated a decreased expression of contractile markers in aortic SMC of TAA patients [5], but the connection between dysregulation of endothelial Notch and SMC contractile phenotype in TAA patients requires further elucidations.

We observed a strong elevation of WNT/β-catenin signaling in the diseased ECs. This pathway controls vascular stability through remodelling, junction assembly, and pericyte recruitment [24]. The sequential and parallel interactions between the BMP and WNT/β-catenin signaling controls mineralization, and intracellular/extracellular fine-tuning of signal duration and strength [25]. We show a significantly elevated expression of WNT/β-catenin antagonist, *DKK1*, in the diseased cells. Activation of Dkk1 has been associated with endothelial integrity [26]. Our data is in accordance with previously published report [5] and is suggestive of an overall attenuation of endothelial integrity and function in the cells of TAA patients.

Fluid shear stress is involved in stem cell and mesenchymal progenitor differentiation into vascular ECs and plays an important role in endothelial homeostasis [27]. The aortic wall is subjected to constant mechanical stress and the ability of the vessels to resist this stress is important for proper vascular function. In response to application of laminar shear stress, a differential expression of *DLL4*, *SNAIL*, *BMP2* and *DKK1* was observed between the patients and healthy donor ECs. This gave further support to the role of Notch, WNT/β-catenin and BMP pathways in maintaining endothelial integrity as has been reported by others [18] [28] [29]; [30]. *DLL4* was up regulated in response to shear stress in healthy cells whereas its expression remained low in the patient cells. Activation of Notch, in particular *DLL4* in response to flow, is an important factor for stress resistance [23] and this function seems to be compromised in the cells of TAA patients.

A cross talk between Wnt and Notch pathways has been shown to be important for the early endothelial patterning in vertebrate development [18] [28]; [31]. In our experiments, activation of WNT/β-catenin either by inhibition of Gsk3 kinase or by introduction of proteolysis resistant S33 β-catenin mutant, down regulated the expression of *DLL4*. This was in accordance with data obtained with patient cells where strong activation of WNT/β-catenin was accompanied by down regulation of *DLL4* and *NOTCH4* and general loss of endothelial properties. Hence, fine-tuned cross-talk between several pathways is responsible for the proper maintenance of endothelial state in the adult aorta and this cross-talk is attenuated in the diseased cells. Recent studies suggest that vascular endothelial growth factor (VEGF), ETS factors, Sox and Notch regulate *DLL4* expression in a complex cascades that may be further impacted by the canonical WNT/β-catenin pathway [18]; [31]. Despite the fact that even subtle changes in *DLL4* expression impairs vascular development [23], the regulatory mechanisms for the fine-tuning of Dll4/Notch signaling during vascular development in vivo still remain to be defined [30].

This study has two major limitations. Firstly, the number of patients used in the study was not large. Secondly, the in vitro experiments with isolated ECs in the absence of an SMC layer cannot reflect the complexity of signaling in the whole aortic wall due to cell-cell communication residing in different layers of aorta as well as cells entering these layers via systemic connection. Nevertheless, we suggest that our findings are relevant for finding potential targets to ameliorate endothelial integrity.

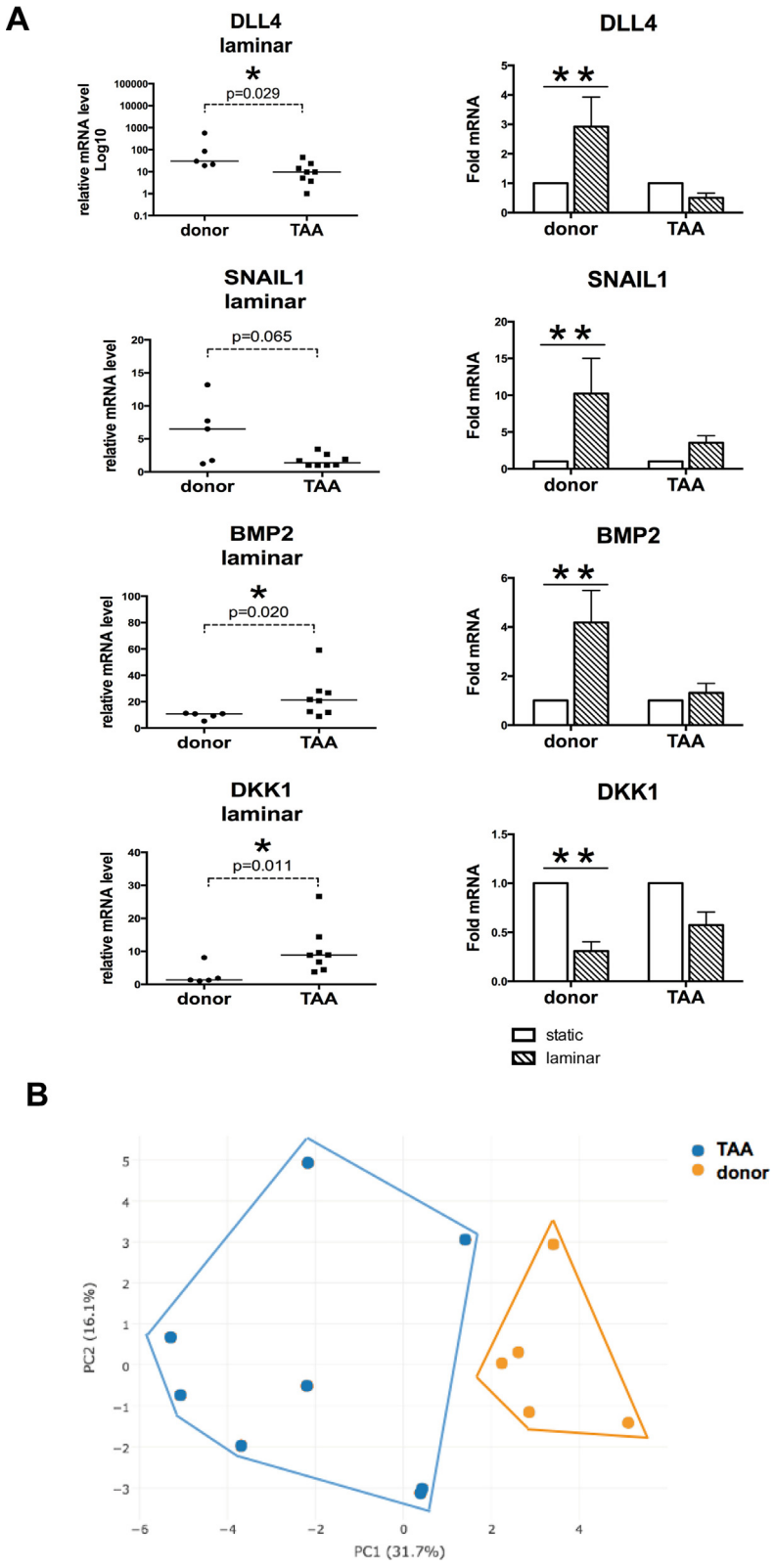

Fig. 5. Shear stress response in the HAEC of the patients with thoracic aortic aneurysm. A. Dotted graphs represent relative mRNA level in the cells subjected to the flow. Bar graphs represent fold change of mRNA level in non-stimulated level (static) to the level in the flow-stimulated (laminar) cells. Endothelial cells from the patients with thoracic aortic aneurysm (TAA), n = 9; control cells (donor), n = 5. Groups are compared using Mann-Whitney nonparametric test; line represents the median; **, *$p < 0.05$. B. PCA analysis showing differences in gene expression between HAEC from healthy and diseased aorta in response to shear stress. See also suppl. Fig. 2.

Conflicts of interest

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.atherosclerosissup.2018.08.002

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