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Role of Notch signaling pathway in pathologies of aorta and aortic valve

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Role of Notch signaling pathway in pathologies of aorta and aortic valve – Aleksandra Kostina

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To my mother, who sincerely believes in me

«The most incomprehensible thing about the world
is that it is at all comprehensible».

Albert Einstein

Abstract

Notch is highly conserved signaling pathway that regulates the development and differentiation of many types of tissues and influences major cellular processes such as cell proliferation, differentiation and apoptosis. Mutations in several Notch signaling components have been associated with a number of congenital heart defects, demonstrating an essential role for Notch both in cardiovascular system development and its maintenance during postnatal life. In particular, mutations in *NOTCH1* have been linked to congenital abnormalities of aortic valve and aorta, such as bicuspid aortic valve, increasing risk of aortic dilatation and valve calcification. Therapeutic agents that may influence these disorders are absent to date and the only therapeutic decision is elective surgical intervention. Thus, understanding the mechanisms underlying left ventricular outflow tract malformations become especially important.

This study aimed to investigate the role of Notch-dependent cellular and molecular mechanisms in development of aortic and aortic valve pathologies. Using primary cells, first, we sought to compare the cellular functions of endothelial and smooth muscle cells in patients with thoracic aortic aneurysm and healthy donors. Second, since Notch signaling pathway is key regulator of endothelial-to-mesenchymal transition, the process underlying valve formation, we explored whether Notch-dependent endothelial-to-mesenchymal transition is affected in aortic endothelial cells from patients with thoracic aortic aneurysm, associated with bicuspid aortic valve – common congenital heart malformation. Third, although vascular smooth muscle cells have been considered as the main target of degeneration in the aortic wall, endothelial dysfunction might also be responsible for thoracic aortic aneurysm formation. Therefore, we addressed the role of Notch and Notch-related signaling pathways in shear stress response in endothelial cells from aortic aneurysm and healthy controls. Forth, the involvement of dysregulated Notch pathway in calcification is evident. In this work we sought to reveal early Notch-dependent mechanisms of valve calcification in patients with bicuspid- or tricuspid aortic valve associated calcified stenosis.

Our data demonstrate downregulation of smooth muscle as well as endothelial cell specific markers in the patient cells. Cellular proliferation, migration, and synthesis of extracellular matrix proteins are attenuated in the cells of the patients with thoracic aortic aneurysm compared to healthy controls. We show that endothelial cells from persons with aortic aneurysm and bicuspid aortic valve have downregulated Notch signaling and fail to activate Notch-dependent endothelial-to-mesenchymal transition in response to its stimulation by different Notch components. Activity of Wnt and BMP pathways was significantly elevated in endothelial cells from aneurysms. Furthermore, activation of *DLL4*, *SNAIL1*, *DKK1*, *TCF4* and *BMP2* was attenuated in cells of patients in response to shear stress, implying dysregulated Notch/BMP/WNT cross-talk.

We report that the expression pattern of Notch genes is altered in the aortic valve interstitial cells of patients with calcific aortic stenosis compared to those of healthy persons. Interstitial cells from bicuspid calcified valves demonstrated significantly higher sensitivity to stimuli at early stages of induced proosteogenic differentiation and were significantly more sensitive to the activation of proosteogenic *OPN*, *ALP* and *POSTIN* expression by Notch activation. Notch-dependent endothelial-to-mesenchymal transition was also more prominent in bicuspid valve derived endothelial cells compared to the cells from calcified tricuspid and healthy valves.

This study provides the first direct functional evidence that primary aortic and valvular cells from patients with left ventricle outflow tract pathologies have impaired Notch signaling pathway comparing to healthy donors. In conclusion: 1 - both endothelial and smooth muscle cells of aneurysmal aortic wall have downregulated specific cellular markers and altered functional properties, such as growth rate, apoptosis induction, and extracellular matrix synthesis; 2 – Notch-dependent endothelial-to-mesenhymal transition is attenuated in endothelial cells of patients with thoracic aortic aneurysm and bicuspid aortic valve; 3 - shear-stress response is impaired in endothelial cells of the patients with thoracic aortic aneurysm due to altered Notch/BMP/WNT/β-catenin network; 4 - early events of aortic valve calcification are Notch-dependent and differ in bicuspid and tricuspid aortic valves.

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Non-standard Abbreviations and Acronyms

TAA	thoracic aortic aneurysm
TAV	tricuspid aortic valve
BAV	bicuspid aortic valve
SMC	smooth muscle cells
ECs	endothelial cells
HAEC	human aortic endothelial cells
HUVEC	human umbilical vein endothelial cells
VEC	valve endothelial cells
VIC	valve interstitial cells
ECM	extracellular matrix
OFT	outflow tract
AVC	atrioventricular canal
EMT	endothelial-to-mesenchymal transition
NICD	Notch Intracellular Domain

1. Introduction

Notch signaling is an evolutionary conserved pathway regulating a vast range of crucial developmental process. Notch pathway is a key signaling in cell-cell communications, determining cell fate and differentiation of many types of tissues. Deceptively simple molecular design of the core pathway is in apparent contrast to multiple Notch effects. The canonical Notch signaling is at its core a straightforward signaling mechanism, which is extremely dose-sensitive due to lack of signal amplification step or utilization of secondary messengers to transmit the signal from the cell surface to the nucleus.

In accordance with a variety of cellular events that Notch pathway controls, many data are accumulated on pathogenic role of the Notch signaling cascade in a wide spectrum of human diseases including cardiovascular disorders. In the last decade it became clear that this signaling pathway plays an important role in postnatal maintenance of tissues homeostasis as well as in response to stress. Apparently, damage in intercellular Notch signaling could lead to abnormalities in tissues homeostasis, in particular in cardiovascular system and thus lead to the pathologies associated with a shift of cell differentiation.

1.1 The core of Notch pathway.

In mammals Notch family constitutes of four receptors (Notch 1, -2, -3, -4) and five ligands (Delta-like 1, -3, -4, Jagged (Serrate) 1, -2). The Notch receptors are type I single-pass transmembrane proteins with large extracellular ligand-binding region (NECD), membrane-spanning part and intercellular domain (NICD), containing among other motifs, a transcriptional activation domain **(Figure 1)**. The Notch receptors are synthesized as 300-350 kDa single precursor proteins that during maturation in the Golgi proteolitically processed by furin-like convertase at the site called S1, giving two non-covalently associated subunits (Logeat et al., 1998). The resulting two associated subunits compose the mature heterodimeric form of the protein present at the cell surface (Kopan and Ilagan, 2009; Kopan et al., 1996). The extracellular domain composed of 29-36 N-terminal-epidermal growth factor (EGF)-like repeats that are available to interact with ligands. O-linked glycosylation of these EGF repeats, including modification

by O-fucose, Fringe and Rumi glycosyltransferases, also modulate the activity of Notch receptors in response to different ligands (Kovall and Blacklow, 2010). For instance, modifications of EGF6 and 36 by Manic Fringe specifically inhibited Notch1 activation by Jagged 1 and enhances activation from Delta-like 1 (Kakuda and Haltiwanger, 2017). Many EGF repeats bind calcium ions that also can affect signaling efficiency (Raya et al., 2004). Within the extracellular domain structure, the EGF repeats are followed by unique negative regulatory region (NRR), which is composed of three cysteine-rich Lin12/Notch repeats (LNR) and heterodimerization domain. The NRR participates in preventing receptor activation in the absence of ligands (Rand et al., 2000; Sanchez-Irizarry et al., 2004).

The intercellular domain of Notch receptors consists of a recombination signal binding prtotein-1 for Jk (RBPjk)-associated molecule (RAM) domain, seven ankyrin repeats (ANK), edged by two nuclear localization signals (NLS), a transactivation domain (TAD), present in Notch 1, -2, -3, and a PEST region that participates in protein degradation (Rechsteiner and Rogers, 1996).

Notch ligands are divided into two general classes, depending on their homology to *Drosophila* prototypes Delta and Serrate, and collectively referred to as DSL family (Delta/Serrate/LAG-2). They also are type I transmembrane proteins with domain organization. The extracellular domain comprises 7-16 EGF-like repeats and DSL domain, which is unique to Notch ligands. Serrate family ligands differ from Delta-like ligands by larger number of EGF repeats and by the presence of additional cysteine-rich domain and a von Willebrand factor type C domain in the extracellular region (Fleming, 1998).

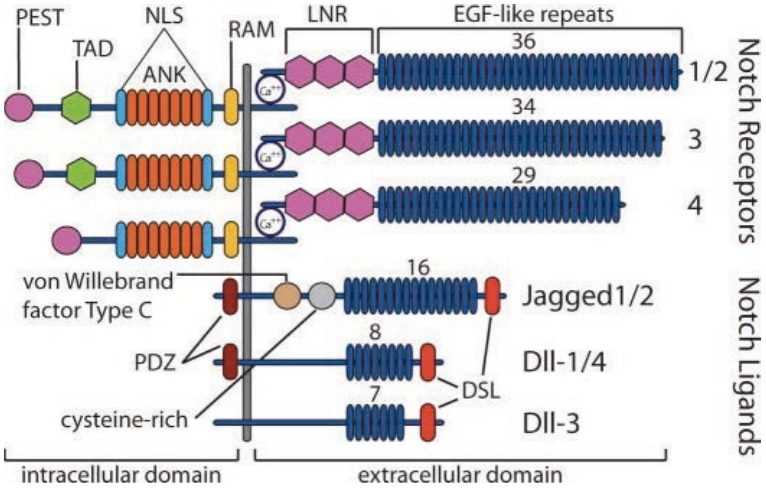


Figure 1. Notch receptors and ligands organization (Niessen and Karsan, 2007).

Transduction of Notch signal mediated by a sequence of proteolytic events and requires three key steps: 1 – ligand recognition; 2 – conformational changes and exposure of the cleavage site; 3 – assembly of nuclear transcriptional activation complexes (Bray, 2006; Kovall and Blacklow, 2010). Because both receptors and ligands are transmembrane proteins, Notch activation depends on direct cell-cell contact. Interaction between the Notch receptor of one cell and the Notch ligand of another cell (*trans*-interaction) results in conformational changes of the extracellular domain exposing a motif that is recognized and cleaved by ADAM metalloproteases at the S2 cleavage site (Brou et al., 2000). Binding of receptor and ligand in the same cell in *cis* generally leads to the inhibition of signaling (Bray, 1997; Sprinzak et al., 2010). Efficiency of ligand-receptor interaction depends on ubiquitylation and endocytosis of ligand in signaling cell promoted by E3 ubiquitin ligases such as Mindbomb-1 (Mib1). This event causes mechanical force on the receptor pulling protective NRR away to promote exposure of S2 site (Itoh et al., 2003; Kopan and Ilagan, 2009; Meloty-Kapella et al., 2012). S2 cleavage creates membrane-tethered intermediate called Notch extracellular truncation (NEXT) that is substrate for γ-secretase protease complex, containing presenilin1, presenilin2, Pen-2, Aph-1 and nicastrin (Fischer et al., 2007; Selkoe and Wolfe, 2007; Struhl and Greenwald, 2001). γ-Secretase complex cleaves the Notch receptor at the two distinct sites S3/S4 and releases

intercellular domain of Notch, which translocates to the nucleus to regulate gene transcription.

All Notch signals cause changes in gene transcription mediated by nuclear effector CSL (CBF1 in humans, Suppressor of Hairless in *Drosophila*, RBPjk in mice). In the absence of NICD CSL is bound by corepressor proteins, such as SMRT (NcoR) and SHRP (MINT/SPEN), and inhibits transcription of target genes, recruiting histone deacetylases to its promoters (Morel et al., 2001). NICD-CSL binding displaces corepressor complexes from CSL and allows recruitment of the transcriptional coactivator Mastermind-Like-1 (MAML) and histone acetyltransferases such as p300 (Wallberg et al., 2002). Formation of CSL/NICD/MAML complex results in direct transcriptional activation of target genes **(Figure 2)**.

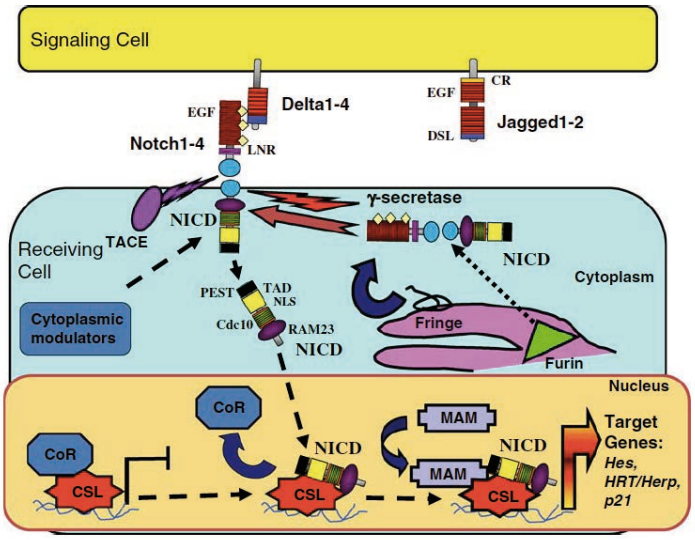


Figure 2. Schematic representation of Notch signaling pathway (de la Pompa, 2009).

The best characterized Notch target genes are members of the basic helix-loop-helix (bHLH) hairy/enchancer of split (*HESR*) family and the *Hes*-related repressor protein (*HRT/Hey/Herp* with YRPW motif) transcription factor family (Iso et al., 2003; Kokubo et al., 2005). Nevertheless, the spectrum of Notch targets is larger and numerous genes may be activated in parallel with *HESR*, including *SNAIL1*, *p21*, *MYC*, *Ephrin B2*, *Cyclin D1*, platelet-derived growth factor receptor

β (*PDGFR β*) and smooth muscle α-actin (*ACTA2*) (Andersson et al., 2011; Cohen et al., 2010; Morimoto et al., 2010; Sahlgren et al., 2008; Timmerman, 2004). The nuclear kinase CycC:CDK8 negatively regulates NICD activity, recruited by MAML it hyperphosphorylates the TAD and PEST domains of NICD resulting in ubiquitin-mediated proteasome degradation (Fryer et al., 2004). Altogether, it has been suggested that versatility of Notch signaling functions is ensured by numerous posttranslational modifications of both receptors and ligands, amount of receptors and ligands expressed in distinct cell context, and via coactivators and inhibitors that operate at every level of the signaling cascade.

1.2 Notch pathway in cardiac development.

The heart development initiates from formation of the cardiac crescent by bilateral precardiac mesoderm cells. The crescent contains two populations of precardiac cells - the first and second heart fields (FHF and SHF) that include progenitors of the first cardiac tissues, the myocardium and endocardium (Buckingham et al., 2005; Kelly et al., 2014). At this stage Notch ligand Jag1 is expressed in endocardium, labeling the presumptive valve area of the atrioventricular channel (AVC) and throughout the myocardium, while NICD and Dll4 are expressed in the endocardium (Luxán et al., 2016). These observations suggest early Notch signaling activity in heart development. The crescent fuses at the embryonic midline to form a single primary heart tube consisting of an inner endocardial layer and an outer myocardial layer separated by an extracellular matrix named cardiac jelly. At this stage Jag1 expression is maintained in the endocardium and is activated in myocardium, while restricted endocardial expression is exhibited by Dll4, Notch2, NICD and Notch4 (Del Monte et al., 2007; Luxán et al., 2016; Timmerman, 2004). The heart tube grows by addition of SHF progenitors into its anterior (arterial) and posterior (venous) poles and undergoes rightward looping morphogenesis. The initial anterior-posterior polarity changes into right-left patterning. At this stage NICD expression is relatively uniform in presumptive valve endocardium (the endocardium lining the AVC and the outflow tract (OFT)), but restricted to the endocardium at the base of the developing trabeculae in the ventricules. Jag1 is expressed in AVC

endocardium and myocardium, Notch2 is detected in chamber endocardium (Loomes et al., 2002). The FHF will give rise to the left ventricle (LV) and other parts of the heart, except the OFT, whereas the SHF will give rise to OFT and other parts except the LV.

A third cardiac tissue covering the myocardium, epicardium, develops from proepicardium, which arises from cells of splanchnic mesoderm, located at the venous pole of the embryonic heart. These cells, which express Dll4, Jag1, Notch1, NICD, Notch3 and *HEY*, migrate to cover myocardium (Grieskamp et al., 2011; del Monte et al., 2011). Epicardial cells then undergo epithelial-to-mesenchymal transition and generate a population of epicardium-derived cells, expressing Dll4, Jag1 and NICD , that contribute coronary vessel formation (Cai et al., 2009).

In late gestation Jag1 is expressed in smooth muscle cells around the valve and in chamber myocardium, Dll4 in developing coronary vessels and Notch1 in valve and chamber endocardium (Luxán et al., 2016). Epicardium, myocardium and endocardium are tightly associated and their interactions during cardiogenesis ensure highly regulated cardiac pattern, proper cell differentiation and tissue morphogenesis, resulting in formation of fully functional heart at birth.

1.2.1 Role of Notch pathway in ventricular trabeculation.

During ventricular chamber development the myocardium differentiates into two distinct layers: an outer compact zone and an inner trabecular zone. Trabeculae are sheets of cardiomyocytes forming muscular ridges lined by endocardial cells (Moorman and Christoffels, 2003). Trabecular cardiomyocytes and endocardium are in close interactions regulating cardiomyocyte proliferation and differentiation (Sedmera et al., 2000). Several signaling pathways are required for trabeculation, such as Bmp10, Nrg1/ErbB and EphrinB2/EphB4 (Chen, 2004). Notch1 activity is observed in ventricular endocardium at the base of the forming trabeculae. Dll4 and Jag1 are expressed in opposite manner: Dll4 is observed in endocardium, at the base of the developing trabeculae, whereas Jag1 is expressed in chamber myocardium with stronger expression in the cardiomyocytes forming trabeculae (D'Amato et al., 2015, 2016; Luxán et al., 2016).

Notch pathway is required for regulation of key pathways of trabeculae formation. Murine embryos lacking, systematically or in the endocardium, *NOTCH1* or *RBPjk* show impaired trabeculation (Grego-Bessa et al., 2007). In the endocardium, *EFNB2* is direct target of NICD and its expression is abrogated in Notch mutants. Neureguline1-ErbB signaling is essential for initiation of trabeculation (Meyer and Birchmeier, 1995). Neuregulin1 (*NRG1*) is expressed in endocardial cells and acts as paracrine factor for ErbB2-ErbB4 receptors expressed in cardiomyocytes (D'Amato et al., 2016; Meyer and Birchmeier, 1995). Expression of *NRG1* in endocardium is regulated by direct target of Notch – *HAND2* (VanDusen et al., 2014).

Impaired Notch pathway also affects expression of the cytokine Bmp10, a chamber specific bone morphogenetic protein (BMP) family member. Expression of Bmp10 is restricted to the trabecular myocardium. *BMP10* knockout murine embryos dead before birth and show dramatically reduction in cardiomyocyte proliferative activity (Chen, 2004).

Recent data show that heart chamber development is coordinated by sequential Notch1 receptor activation, regulated by glycosyltransferase Manic Fringe (MFng) (D'Amato et al., 2015). MFng-favoured endocardial Dll4-Notch1 signaling promotes cardiomyocytes proliferation and differentiation resulting in trabeculation. Myocardial Jag1/Jag2 – Notch1 signaling, occurring after MFng (and Dll4) downregulation in the endocardium, supports myocardial patterning, maturation and compaction (D'Amato et al., 2015, 2016).

1.2.2 Role of Notch pathway in atrioventricular canal development.

The boundary between the prospective atrial and ventricular chamber regions of the heart tube is known as the atrioventricular canal (AVC). Formation of AVC is dependent on BMP2 regulated expression of T-transcription factor 2 (*TBX2*), which inhibits chamber-specific gene expression within the myocardium of AVC (Harrelson, 2004). Cardiac-specific deletion of *BMP2* results in a failure of AVC specification, whereas mice lacking *TBX2* show defective AVC patterning and ectopic expression of chamber specific genes (Aanhaanen et al., 2009; Ma, 2005). Notch signaling pathway has been shown to restrict BMP2

expression to AVC myocardium. Expression of Notch targets *HEY1* and *HEY2* is restricted to the atria and the ventricles, respectively, but is excluded from the AVC. Ectopic expression of *NICD* in the myocardium of mouse hearts activated *HEY1* and *HEY2* expression throughout this tissue, which in turn resulted in downregulation of both *BMP2* and *TBX2* expression and loss of AVC boundaries (de la Pompa and Epstein, 2012; Luna-Zurita et al., 2010; Rutenberg et al., 2006; Watanabe, 2006). Simultaneously, inactivation of Notch signaling in *RBPjk*-targeted mutants does not affect myocardial *HEY1*, *HEY1* and *BMP2* expression (Luna-Zurita et al., 2010). Although these studies show apparent role of *HEY1* and *HEY2* in AVC formation, precise mechanisms remain to be elucidated.

1.2.3 Early valve development.

Early valve development initiates with the formation of the endocardial cushions by a process of endothelial-to-mesenchymal transition (EMT) that takes place initially in the AVC and later in the OFT endocardium. Before the beginning of cushion formation, the endocardium and the myocardium lie adjacent to each other, separated by thick hyaluronan-rich gelatinous extracellular matrix named a cardiac jelly. Myocardial signals from AVC and OFT regions instruct adjacent endocardial cells to undergo EMT. During this process endocardial cells undergo phenotypic and morphological alterations, resulting in loss of apical-basolateral polarity and disruption of intercellular junctions, and acquire the ability to degrade the basement membrane and migrate away from endothelial sheet to invade the underlying cardiac jelly (Person et al., 2005). Endocardial cells exhibit abundant expression of intercellular adhesion complexes such as E-Cadherin and integrins. In order to acquire a mesenchymal phenotype, cells must lose cell adhesion by E-Cadherin downregulation and degradation. Completing the transition, cells activate the expression of additional mesenchymal genes and proteins, such as α-smooth muscle actin (αSMA, *ACTA2*), smooth muscle protein 22α (SM22α, *TAGLN*), collagen I and III, vimentin and fibroblast-specific protein 1 (*FSFS100A4*) (Kovacic et al., 2012).

This EMT generates mesenchymal progenitor cells that contribute to valvuloseptal structure and adult valve interstitial cells (VIC) (Butcher and

Markwald, 2007). Growth and proliferation of this newly formed mesenchymal cells results in fusion of endocardial cushions within the lumen of the heart tube and formation of the initial septa. Further remodeling of the endocardial cushions by condensation and elongation results in the formation of atrioventricular and arterial valves that ensure unidirectional blood flow in the mature heart (Hinton and Yutzey, 2011; Niessen and Karsan, 2007).

Notch pathway is indispensable for early specification and initiation of EMT in the endocardial cells. Notch pathway genes are expressed in prospective valve endocardium at the beginning of EMT (Del Monte et al., 2007; Timmerman, 2004). Dll4 seems to be more relevant ligand at this stage, whereas Notch1 is a receptor with strongest endocardial expression, and the three receptors Notch1-3 are expressed in transforming mesenchymal cells of the AVC and the OFT (Timmerman, 2004). Recent data suggest sequential activation of Notch1 in the endocardium during valve morphogenesis and heart septation: first by Dll4 to induce EMT and subsequently by Jag1 to ensure proper cushion fusion and restrain mesenchymal cell proliferation before differentiation (MacGrogan et al., 2016). Co-culture of HMEC-1 microvascular endothelial cell line-1 with cells bearing ligand Jag1 results in EMT induction (Noseda, 2004). Similar phenotypes between *Notch1* and *RPBjk* mutant embryos indicates that Notch1 might be the most relevant Notch receptor in the endocardium (Timmerman, 2004). In murine model targeted inactivation of *Notch1* (or *RBPjk*) results in severely hypoplastic endocardial cushions due to impaired EMT. Histological analysis of mutants reveals that endocardial cells remain in close association, abnormally maintain adherens junctions, and do not invade the cardiac jelly, despite exhibiting features of activated premigratory endocardial cells (Timmerman, 2004). EMT involves the upregulation of the Snail family of transcription factors, which are responsible for downregulation of the cell-adhesion molecule vascular endothelial cadherin (VE-cadherin, encoded by *CDH5*) (Nieto, 2002). The proposed mechanism is that NICD-RBPjk directly activates the EMT drivers *SNAIL1/2,* whose expression is severely reduced in AVC and OFT endocardium of *Notch1* (or *RBPjk*) mutant mice (Timmerman, 2004). Concomitantly, *CDH5* expression remains abnormally stabilized in the endocardium of the mutants, suggesting that loss of *SNAIL1/2*

expression prevents downregulation of cadherin-mediated endocardial cell adhesion, that blocks EMT (Sahlgren et al., 2008; Timmerman, 2004). These *in vivo* observations were confirmed by the defective EMT in AVC and OFT explants from either Notch1-deficient mice or wild-type explants treated with Notch signaling inhibitor and cultured *in vitro* onto 3D-collagen gel (Timmerman, 2004). Numerous functional studies in mouse embryos indicate that myocardial paracrine and endocardial autocrine production of transforming growth factor beta 2 (TGFβ2) and TGFβ3 are required for endocardial cushion formation (Camenisch et al., 2002). Moreover, *Notch1* (or *RBPjk*)-deficient mice show specific reduction of transcription of TGFβ2 in AVC and OFT myocardium, suggesting that endocardial Notch function is needed for production of the myocardial TGFβ2 signal (Timmerman, 2004). Mice lacking *HRT1* (*HEY1*) and *HRT2* (*HEY2*) die during embryogenesis due to severe cardiovascular malformations, including impaired development of endocardial cushions (Fischer et al., 2007; de la Pompa, 2009).

Gain-of-function studies support the importance on Notch signaling for EMT. Constitutive Notch1 activity in the endocardium enables ectopic noninvasive EMT of ventricular endocardial cells, conferring valvular features to nonvalvular, "EMT-resistant" ventricular endocardium (Luna-Zurita et al., 2010). In zebrafish model overexpression of *NICD* in heart leads to formation of hypertrophic atrioventricular valves (Timmerman, 2004).

Moreover, the integration of endocardial Notch and myocardial BMP2 has been revealed to promote EMT. Notch1 is required for *SNAIL1* expression and BMP2 signaling regulates *SNAIL1* activation through Alk3/6 receptors in endocardium and inhibition of glycogen synthase kinase-3β (Gsk3β) activity (Luna-Zurita et al., 2010; Ma, 2005). Endocardial Notch1 induces the expression of *WNT4,* which acts as a paracrine factor to upregulate *BMP2* expression in the adjacent AVC myocardium to signal EMT (Luxán et al., 2016; Wang et al., 2013). The role of BMPs in initiation of EMT is supported by *in vivo* analysis of mice lacking myocardial *BMP2* expression, which show no AVC endocardial cushion mesenchymal cell formation (Ma, 2005; Rivera-Feliciano and Tabin,

2006). All these findings show that Notch activity is required for endocardial EMT.

1.2.4 Notch activity in outflow tract development and remodeling.

The OFT is a structure at the arterial pole of the heart connecting the right and left ventricles with the pulmonary trunk and aorta, respectively. OFT development depends on coordinated interactions between multiply cell types originating from within and outside the heart: the endocardium, the endocardium-derived mesenchyme, cardiac and smooth muscle precursors from the SHF, and cardiac neural crest (CNC) (Hutson and Kirby, 2007). During cardiogenesis the single vessel arised from the looped heart, truncus arteriosus, is remodeled under the influence of invading CNC progenitors and septates to form two vessels, the aorta and the pulmonary trunk. Simultaneously, the OFT rotates and aligns with the ventricles.

The CNC cells are a multipotent population of progenitor cells arised from the dorsal neural tube that among other cell types give rise to melanocytes, nerve tissue, muscle, cartilage and bone. Deficient CNC contribution into the OFT results in persistent truncus arteriosus because of lack of mesenchymal contribution to the aortic-pulmonary septum (Neeb et al., 2013). In addition, CNC play crucial role in positioning the cushions and patterning the valve cusps within the developing OFT cushions (Luxán et al., 2016; Phillips et al., 2013). Cells of SHF and CNC are not randomly intermixed in the OFT and ascending aorta. SHF mesenchymal cells are dominantly located in the aortic root, while CNC cells populate the intimal edge of ascending aorta (Harmon and Nakano, 2013; Pfaltzgraff et al., 2014; Waldo et al., 2005). In the developing aortic valve CNC cells populate the aortic surface, whereas SHF cells populate the ventricular surface of the valve (Yassine et al., 2017).

Notch elements are broadly expressed in developing OFT (High et al., 2007; Loomes et al., 2002). Deletion of the Notch ligand Jag1, or inhibition of Notch signaling within the SHF resulted in impaired development of neighboring tissue because of defective EMT and CNC migration (High et al., 2009). Deficient SHF contribution to the OFT causes a spectrum of malformations such as double outlet

right ventricle and over-riding aorta, which represents a set of incorrect alignment defects (Neeb et al., 2013). These malformations are closely to those seen in human with Allagile syndrome, caused by mutations in *JAG1* or *NOTCH2,* and among others characterized by right-sided OFT defects (Li et al., 1997; McDaniell et al., 2006; Oda et al., 1997). Double heterozygous for the *JAG1* null allele and *NOTCH1* hypomorphic allele mutant mice exhibit characteristic Allagile syndrome abnormalities including OFT developmental defects (McCright et al., 2002). Conditional inhibition of Notch signaling in CNC derivatives resulted in a spectrum of outflow tract and aortic arch malformations, associated with decreased differentiation and expression of smooth muscle cell (SMC) markers (High et al., 2007). Thus, Notch signaling seems to be an important mediator of interactions between SHF, CNC and OFT endocardium/endothelium.

1.2.5 Notch signaling in the vasculature.

Vasculogenesis is the de novo development of endothelial cells (ECs) from a dispersed population of mesodermally derived endothelial cell progenitors - angioblasts, whereas angiogenesis is the formation of new vessels from preexisting vessels. Angioblasts first differentiate and form the primary vascular plexus, which then is remodeled during angiogenesis, involving sprouting, bridging, and intussusception (vessel splitting), to generate the mature vascular system. Angiogenesis requires ECs to adopt specialized phenotypes and functions. Leading ECs, named the tip cells, are migratory and invasive, and are thought to guide new sprouts. Tip cells are followed by stalk cells, which elongate, proliferate and stabilize the nascent vessel and establish a lumen (Betz et al., 2016; Potente et al., 2011).

Tissue hypoxia is key trigger factor of the new vessel branches formation. It induces secretion of pro-angiogenic growth factors and cytokines, such as vascular endothelial growth factor (VEGF), which binds VEGF receptors (VEGFRs) on surface of ECs and promotes proliferation, migration and tip cell formation (Simons et al., 2016). ECs with high activation of VEGFR2 and VEGFR3 became tip cells and upregulate expression of *DLL4* (Blanco and Gerhardt, 2013; Potente and Mäkinen, 2017). DLL4 induces stalk cell phenotype

in adjacent ECs by Notch1 signaling activation, resulting in downregulation of VEGFR2 and VEGFR3 and activation VEGFR1 expression, that sequesters VEGF. Thus, Dll4-Notch1 signaling establishes differential VEGFR expression and VEGF sensitivity, that in turn ensure tip-stalk cell specification (Blanco and Gerhardt, 2013; Zarkada et al., 2015). During spouting, the levels of VEGFRs are permanently changed depending on ECs environment, allowing asymmetric cell division and reaction on new cellular neighbours (Blanco and Gerhardt, 2013; Costa et al., 2016). Thus, stalk cells can become tip cells and conversely, ensuring dynamic position changes in the growing vessels (Jakobsson et al., 2010).

Dll4-deficient embryos as well as *RBPjk* mutant and *HEY1/HEY2* double mutant embryos exhibited severe vascular defects and did not express arterial markers (Duarte, 2004; Fischer et al., 2004; Gale et al., 2004; Krebs, 2004). Notch ligand Jag1 has been shown to play antagonistic role during sprouting angiogenesis. *JAG1* loss-of-function mutant mice have reduced sprouting angiogenesis in retina, while *JAG1* overexpression enhanced angiogenesis and resulted in increased number of tip cells. Thus, Jag1 seems to act as a proangiogenic signal during postnatal retinal angiogenesis (Benedito et al., 2009; Pedrosa et al., 2015).

Moreover, Notch signaling is among key pathways regulating arterial venous specification (Domenga et al., 2004). Both, Notch deficient and Notch gain-of-function mouse embryos develop arteriovenous malformations (Duarte, 2004; Gale et al., 2004; Krebs, 2004). Ectopic *NOTCH4* and *NOTCH1* expression in endothelial cells resulted in development of arteriovenous malformations and embryonic vascular remodeling defects (Kim et al., 2008; Krebs et al., 2010). *EphrinB2*, one of key arterial markers, is direct target of Notch signaling (Grego-Bessa et al., 2007). *EphrinB2* loss-of-function mutants displayed the arteriovenous malformations their presented in embryos with Notch1 conditional activation in endothelial cells. Inducible expression of an activated *NOTCH4* in adult mice resulted in vessel arterialization (Carlson et al., 2006; Gridley, 2010).

To support the growing network of endothelial cell tubes, mural cells are recruited and differentiate into smooth muscle cells (SMC) and pericytes. This

recruitment is largely driven by a gradient of PDGF-β secreted by endothelial cells (Hellstrom et al., 1999). Notch ligands expressed by endothelial cells can activate Notch signaling in these mural cells, which in turn ensure integrin adhesion to the endothelial basement membrane and induces maturation and differentiation (Scheppke et al., 2012). Among all Notch receptors Notch2 and Notch3 appear to be the most important for SMC. These two receptors affect the phenotype and functions of SMC (Baeten and Lilly, 2015; Liu et al., 2015). Notch signaling in the endothelium of the vessel is positioned to mediate differentiation of underlying SMC, ensuring integrity of the vessel wall (Pedrosa et al., 2015). Mice with an endothelial deletion of *JAG1* displayed decreased SMC differentiation and expression of SMC markers (High et al., 2008). Moreover, activation of Notch signaling in SMC by endothelial-expressed Jag1 leads to increased expression of *NOTCH3* and *JAG1* (Baeten and Lilly, 2017). This finding suggests the lateral induction of Notch signaling within multiple SMC layers, ensuring the promotion of differentiation initially induced by endothelium signal (Hoglund and Majesky, 2012; Liu et al., 2009; Manderfield et al., 2012). Notch pathway has been shown to increase expression of PDGF-β receptor. Thus, active Notch signaling in SMC may increase migration in response to PDGF-β (Jin et al., 2008). Notch1 and Notch3 were shown to facilitate SMC migration in vitro (Sweeney et al., 2004). Notch target gene *HEY2* was also demonstrated to promote proliferation, migration and formation of neointimal layer in a mouse injury model (Sakata, 2004). All these observations evidence that Notch signaling pathway is indispensable for recruitment of mural cells.

SMC are not “terminally differentiated”, possessing to exist in multiple phenotypes allowing a wide range of functions, such as contraction, proliferation or synthesis and secretion of extracellular matrix (ECM). A “differentiated” SMC phenotype is generally determined as a quiescent cell, expressing genes encoding contractile proteins, such as αSMA (*ACTA2*), smooth muscle myosin heavy chain (*MYH11*), transgelin known as SM22α (*TAGLN*) and calponin (*CNN1*). This is required cell state for mature vessels to maintain physiological homeostasis. The effect of Notch signaling on SMC phenotype is controversial. Notch signaling has been linked to SMC differentiation both *in vitro* and *in vivo* (Boucher et al., 2012;

Doi et al., 2006; Noseda, 2006). At the same time, some data suggest Notch signaling to repress SMC differentiation and maintenance of the contractile SMC phenotype (Morrow, 2005; Proweller et al., 2005; Sweeney et al., 2004).

The proliferation of SMC is negatively related to their differentiation status. Mitotically active cells are considered as "dedifferentiated" or "synthetic" (Owens, 2004). In several papers Notch pathway has been shown to promote SMC proliferation (Campos, 2002; Sweeney et al., 2004). At the same time, the Notch2 receptor has been shown to localize in non-proliferating regions of injured vessels and it inhibited proliferation through cell-cycle arrest (Baeten and Lilly, 2017; Boucher et al., 2013). Interestingly, while both Notch2 and Notch3 promote contractile differentiation, they oppositely affect SMC proliferation, thereby underlining abundant Notch-dependent regulation of SMC phenotype. Moreover, Notch signaling has been involved in suppression of apoptosis and promotion of cell survival, apparently, with receptor specific effect and integration with other signal pathways (Baeten and Lilly, 2015, 2017; Liu et al., 2010; Sweeney et al., 2004).

SMC and their progenitors are key contributors to the ECM production. Notch activity has been demonstrated to promote ECM synthesis, whereas inhibition of Notch activity resulted in decreased expression of collagen IV and laminin, but increased expression of *MMP2* and *MMP9* (Lilly and Kennard, 2008; Yao et al., 2015).

Thus, it is obvious that proper vasculature development and its further maintenance, including sprouting angiogenesis, endothelial cell specification, recruitment and differentiation of mural cells, ECM production and arteriovenous specification, is tightly regulated by Notch signaling pathway.

1.3 Notch pathway in left ventricular outflow tract pathologies.

Mutations in components of Notch pathway result in a spectrum of left ventricular outflow tract malformations. The most prevalent abnormalities are bicuspid aortic valve disease and calcification of the aortic valve. Bicuspid aortic valve describes an aortic valve with two rather three leaflets, where adjacent cusps fuse to form a single aberrant cusp **(Figure 3)**.

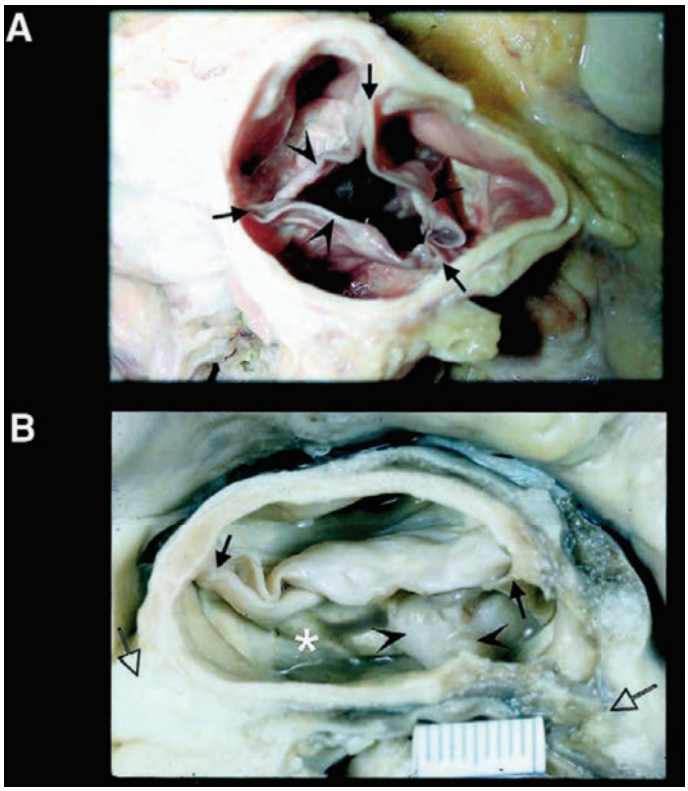


Figure 3. (A) Normal tricuspid aortic valve. (B) Congenitally bicuspid aortic valve, one cusp (asterisk) larger than the other (Fedak et al., 2002).

Bicuspid aortic valve (BAV) is the most common congenital heart malformation, occurring in 1% to 2% of the population (Prakash et al., 2014). The most frequent complication of BAV is aortic stenosis, attended by the premature fibrosis, stiffening and calcium deposition (Fedak et al., 2002). Although only <2% of the population has BAV, but they represent about 50% of the patients undergoing aortic valve replacement (Roberts, 2005). Calcification also occurs at an earlier age in BAV compared to individuals with the normal tricuspid anatomy (tricuspid aortic valve, TAV) (Beppu et al., 1993; Fedak et al., 2002).

NOTCH1 mutations have been associated with BAV and have been found in individuals with various left ventricular outflow tract (LVOT) abnormalities, such as hypoplastic left heart syndrome, coarctation of the aorta and aortic valve stenosis (Garg et al., 2005; Koenig et al., 2017; McKellar et al., 2007). Recently it has been shown that *NOTCH1* haploinsufficiency promotes proosteogenic and inflammatory gene expression and dramatically influences the capacity of human iPS-derived endothelial cells to resist shear stress, this may explain the aortic

pathology in the case of *NOTCH1* mutations (Theodoris et al., 2015). It has been suggested that the valve defect may arise during development of the aortic valvular cusps and aortic media from CNC cells (Bonderman et al., 1999). Combined loss of *NOTCH1* downstream targets, *HEY1* and *HEYL* causes impaired EMT in mice (Fischer et al., 2007). Functional studies on the missense *NOTCH1* mutations associated with LVOT malformations have shown reduced receptor signaling associated with defective EMT in HMEC-1 microvascular endothelial cell line used as a model (Riley et al., 2011).

The mechanisms underlying heart valve calcification are not fully elucidated. The progressive valve fibrosis and mineralization were earlier believed to be a passive degenerative process, but it is now recognized as an active disease process driven by the cells originating to the aortic valve (Goldbarg et al., 2007; Pomerance, 1967; Rutkovskiy et al., 2017). Normal aortic valve leaflets have two cell types: valve interstitial cells (VIC) and valve endothelial cells (VEC) (Rutkovskiy et al., 2017). It has been suggested that VIC are the main functional units of the valve that undergo mineralization (Rabkin-Aikawa et al., 2004). However, the VEC may also participate in the mineralization process (Cheng et al., 2013; Hjortnaes et al., 2015; Shapero et al., 2015; Yao et al., 2013). Ability of VEC to EMT may also contribute valve calcification. Human pulmonary VEC were capable of responding to TGFβ to produce VIC phenotype (Paruchuri et al., 2006). In addition, mechanical strain was shown to enhance EMT of VEC through TGFβ/Smad and Wnt/βcatenin signaling cascade (Balachandran et al., 2011). The data regarding the role of Notch in aortic valve calcification are controversial. Notch1 can prevent osteogenic calcification, however, opposite data suggest that Notch1 promote osteogenic calcification in human VIC (Acharya et al., 2011; Nigam and Srivastava, 2009; Zeng et al., 2013). However, haploinsufficiency for a *RBPjk* predisposes to diet-induced calcific aortic valve disease in mice (Nus et al., 2011). Notch pathway seems to play complex role in aortic valve calcification, which should be interpreted with respect to the process timeline, the species and the end point employed in the study (Rutkovskiy et al., 2017).

BAV patients are at increased risk for aortic dilation, aneurysm, and dissection (Fedak et al., 2003, 2002; Gleason, 2005). Thoracic aortic aneurysm

(TAA) is a life threatening condition, which is manifested by progressive enlargement of the thoracic aorta due to altered cellular composition and degeneration of the ECM in the aortic wall. The pathogenesis of aneurysm formation in the monogenic syndromes has been extensively studied (Lindsay and Dietz, 2011). However, the molecular and cellular mechanisms of the other forms, which constitute the majority of TAA, remain largely unknown and conservative therapy is currently absent for this disease (Atkins, 2014). Non-syndromic TAA may occur in the presence of TAV or BAV, and several lines of evidence suggest that the mechanism behind aneurysm development is distinct between the two patient groups (Balistreri et al., 2013; Folkersen et al., 2011; Ignatieva et al., 2017; Kjellqvist et al., 2013). SMC have been considered as the main target of degeneration in the aortic wall, however, as endothelial cells have a substantial influence on smooth muscle cell differentiation via Notch pathway, recent studies suggest that TAA may be associated with endothelial dysfunction (Ali et al., 2014; Baeten and Lilly, 2015; Liu et al., 2015). In addition to mutations in *NOTCH1* (Dong et al., 2015; Freylikhman et al., 2014; Kerstjens-Frederikse et al., 2016; McBride et al., 2008; Mohamed et al., 2006), gene *GATA5* also has been associated with non-syndromic forms BAV/TAA (Padang et al., 2012, 2015). Recently, variants and mutations of *NOTCH1* gene have been reported in patients with TAV-associated LVOT abnormalities (Ducharme et al., 2013; Irtyuga et al., 2017).

Altogether, it is obvious, that mutations in genes encoding components of the Notch pathway as well as impaired Notch signaling during cardiogenesis lead to a broad spectrum of LVOT disorders.

2. Materials and Methods

2.1 Patients.

The clinical research protocols were approved by the local Ethics Committee of the Almazov National Medical Research Center and were in accordance with the principle of the Declaration of Helsinki. All patients gave informed consent.

Samples of the aneurysmal wall of the ascending aorta were harvested during surgery for aneurysm corrections at the Almazov National Medical Research Center. All samples were from aneurysms with a diameter of >5 cm measured by preoperative transthoracic echocardiography. Patients with connective tissue disorders were excluded. The patients are described in **Table 1**. Control aortic specimens were obtained during organ harvesting from organ transplant donors (n = 16) with the authorization of the law of Russian Federation and in accordance with the Declaration of Helsinki. Donors used as controls had no evidence of aneurysmal disease and all had TAV.

Valve interstitial cells (VIC) and valve endothelial cells (VEC) were isolated from aortic valves explanted during aortic valve replacement at the Almazov National Medical Research Centre. Patients with known infective endocarditis and rheumatic disease as well as patients with left ventricular systolic dysfunction were excluded from the study. VIC and VEC from normal aortic valves were isolated from healthy valves obtained from explanted hearts from recipients of heart transplantation (n = 11). Due to the low incidence of BAV in the population all healthy valves were TAV. Clinical data regarding patients with aortic stenosis in BAV and TAV are shown in **Table 2**.

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords after birth at the Almazov Medical Research Centre.

Table 1. Clinical characteristics of TAA patients.

Parameters	TAV (n = 13)	BAV (n = 22)
Male gender (%)	46	59
Age (years)	71.3 ± 2.53 (range 55–84)	62.1 ± 1.87 (range 42–79)

Aortic diameter (cm)	5.6 ± 0.18*	5.9 ± 0.16*†
Aortic CSA/h (cm2/m)	6.6 ± 0.6*	7.6 ± 0.6*
Peak valve gradient (mmHg)	83 ± 9	86 ± 11
Mean valve gradient (mmHg)	55 ± 7	59 ± 9
Aortic valve area index (cm2/m2)	0.39 ± 0.02	0.38 ± 0.02
Hypertension (%)	84*	81*
Medication		
Angiotensin receptor blockers (%)	38*	18
Statins (%)	0	41*†
Aspirin (%)	31	14

Values are means ± SEM. *$P < 0.05$ compared with donors; †P<0.05 compared with TAV; CSA/h, ascending aortic cross-sectional area to patient height ratio.

Table 2. Clinical characteristics of patients with calcified aortic valve stenosis.

Parameters	Patients with BAV Me (25th; 75th percentile) (n=13)	Patients with TAV Me (25th; 75th percentile) (n=17)	p
Age, years	59.0 (53.0;66.0)	63.0 (58.0;67.0)	0.133
Gender	F -14 (36.8%); M – 24 (63.2%)	F -15 (46.8%); M – 17 (53.2%)	0.472
AV, Vmax, m/s	4.70 (4.20;5.00)	4.40 (3.95;4.90)	**0.007**
AV dpmean, mm Hg	57.00 (48.00;66.00)	47.00 (35.00;57.00)	**0.006**

Aortic diameter at the proximal ascending aorta, mm	41 (35;44)	36 (32;40)	**0.038**
Body mass index (BMI), kg/m^2	27.40 (25.51;30.34)	32.62 (26.79;34.94)	**0.022**
Total cholesterol, mmol/L	5.59 (4.42;6.25)	4.91 (4.23;6.11)	0.307
High-density lipoproteins (HDL-C), mmol/L	1.27 (1.08;1.41)	1.16 (1.04;1.46)	0.533
Low-density lipoproteins (LDL-C), mmol/L	3.50 (2.57;4.45)	3.54 (2.77;4.17)	0.758
C-reactive protein (CRP), mg/L	1.56 (1.10;3.86)	3.01 (1.06;4.70)	0.451

BMI - body mass index; AS-aortic stenosis; HDL-C – high-density lipoprotein cholesterol; LDL-C – low-density lipoprotein cholesterol.

2.2 Cell cultures.

2.2.1 Primary cell cultures.

Smooth muscle cells and aortic endothelial cells were isolated from tissue fragments of aortic wall of BAV- and TAV-associated thoracic aortic aneurysm patients and of healthy donors. All tissues were sampled from the outer curvature of the ascending aorta and the tissue was immediately dissected to separate medial layers followed by either freezing or enzymatic digestion.

Valve endothelial cells and valve interstitial cells were isolated from aortic valve leaflets of patients with BAV- and TAV-associated calcified aortic valve stenosis as well as from tricuspid aortic valve of healthy donors.

All cell cultures were maintained at 37°C in a humid 5% CO_2 atmosphere.

2.2.2 Isolation of human aortic endothelial cells (HAEC).

Under sterile conditions tissue fragments of aortic wall were dissected away from the adventitia. After washing in Phosphate-buffered Saline (PBS), the tissue fragments were first incubated for 30 minutes at 37°C with 0.1% collagenase solution (Collagenase, Type III, ≥100 units/mg, Worthington Biochemical Corporation, USA). Then endothelial layer was removed mechanically by scalpel blade, collagenase solution with endothelial cells was collected and centrifuged for 5 minutes at 300 g. The pellet containing endothelial cells was washed twice with Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, USA) and plated onto 3 cm^2 culture dish covered with 0.2% gelatin (Sigma-Aldrich, USA) in Endothelial Cell Medium (ECM) (ScienCell, USA) and incubated at 37°C. On the next day endothelial cells were washed with PBS and culture medium was changed. The cells were used in experiments at passages 2-5.

2.2.3 Isolation of human smooth muscle cells.

To obtain smooth muscle cells (SMC) cultures the cells were isolated as previously described (Kirschenlohr et al., 1996). Briefly, the remaining after HAEC isolation aortic tissue was incubated for 1 hour at 37°C in 0.1% collagenase solution (Collagenase, Type III, ≥100 units/mg, Worthington Biochemical Corporation, USA). Then aortic tissue fragments were washed with fresh DMEM supplemented with 20% of Fetal Calf Serum and gentamycin. Using scissors enzymatic digested tissue was cut into small pieces (cubes 1-2 mm on a side) and transferred on T-25 flask. The cells were maintained in DMEM with 20% of Fetal Bovine Serum (FBS), 2mM L-glutamine and 100units/ml penicillin/streptomycin and were used in experiments between passages 2–5.

2.2.4 Isolation of human valve endothelial cells.

Valve endothelial cells (VEC) were isolated form human aortic valve leaflets. Valve leaflets were washed with PBS and incubated for 10 minutes at 37°C with 0.2% collagenase solution (Collagenase type IV, ≥160 units/mg, Worthington Biochemical Corporation, USA). Then the valve tissue was vortexed for 1 min to remove endothelial cells, supernatant was collected and centrifuged

for 5 minutes at 300 g. The pellet of cells was washed with DMEM and plated onto 3 cm^2 culture dish covered with 0.2% gelatin (Sigma-Aldrich, USA) in Endothelial Cell Medium (ECM) (ScienCell, USA) and incubated at 37°C. On the next day endothelial cells were washed with PBS and culture medium was changed. The endothelial cells between passages 2-5 were used for experiments.

2.2.5 Isolation of human valve interstitial cells.

To isolate valve interstitial cells (VIC) the remaining after VEC isolation valve tissue was incubated with 0.2% collagenase solution (Collagenase type IV, ≥160 units/mg, Worthington Biochemical Corporation, USA) for 24 hours at 37°C. Then tissue was pipetted repeatedly to break up the tissue mass and centrifuged for 5 minutes at 300 g. The pellet containing VIC was resuspended in DMEM supplemented with 15% FBS, 2mM L-glutamine and 100units/ml penicillin/streptomycin, and plated onto T-75 flask. The interstitial cells were used in experiments at passages 2-5.

2.2.6 Isolation of human umbilical vein endothelial cells.

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical vein by enzymatic dissociation as previously described (Baudin et al., 2007). In brief, the vein was washed with PBS, filled by 0.1% collagenase solution (Collagenase, Type II, Worthington Biochemical Corporation, USA) and incubated in PBS at 37°C for 10 minutes. The collagenase solution containing cell suspension was centrifuged for 5 minutes at 300 g. The pellet of cells was twice washed with DMEM, resuspended and seeded onto 3 cm^2 culture dish covered with 0.2% gelatin (Sigma-Aldrich, USA) in Endothelial Cell Medium (ECM) (ScienCell, USA) and incubated at 37°C. Primary cells between passages 2-5 were used for experiments.

2.2.7 Continuous cell line.

Cell line of human embryonic kidney 293T (HEK293T) was used for lentiviral production. HEK293T cells were maintained in DMEM supplemented

with 10% FBS, 2mM L-glutamine and 100 units/ml penicillin/streptomycin on 10 cm^2 culture dish and subcultured using a 1:20 splitting ratio twice on week.

2.3 Magnetic cell separation.

CD31 also known as PECAM-1 (platelet endothelial cell adhesion molecule-1) is transmembrane glycoprotein constitutively expressed on the surface of endothelial cells. Endothelial cells (HAEC and VEC) were purified from interstitial cells using magnetic cell separation (MACS) with anti $CD31^+$-conjugated microbeads (Miltenyi Biotec, Germany) according to the manufacturer's directions. Endothelial cells were treated with Trypsin/EDTA (Gibco, USA), collected and centrifuged for 5 minutes at 300 g. Then cells were resuspended in 60 µl of medium, mixed with 20 µl of FcR Blocking Reagent and 20 µl of CD31 MicroBeads. The suspension was incubated for 15 minutes at 4°C. Then endothelial cells were loaded onto column placed in the magnetic field. The labeled CD31+ cells were retained on the column while unlabeled cells ran through. After removal of the column from the magnetic field, the magnetically retained CD31+ cells were eluted as the positively selected cell fraction and directly taken into culture. Purity of the endothelial cells was confirmed by immunocytochemistry staining with anti CD31 and anti vWF antibodies.

2.4 Cell proliferation and migration assay.

The cell proliferation rate was assayed with the cultured cell growth curves. The cells were plated at equal density and counted each two days. In these experiments 80×10^3 of SMC were seeded into 6-well plates; 20×10^3 of HAEC were seeded into 12-well plates.

Cell migration was examined using “scratch” wound assay as described previously (Mathew et al., 1996). Cells were grown to confluence on 6-well plates; after the cells formed a monolayer the medium was exchanged for serum free medium containing 10 mM hydroxyurea and 10 ng/mL PDGF_BB growth factor to inhibit proliferation and to stimulate migration and the cell monolayer was scraped with a 200P pipette tip to create a cell-free zone. The number of cells migrated into the wounded area was counted after 6 and 24 hours.

Experiments were performed in duplicates and then repeated three times.

2.5 Cell apoptosis assay.

Hydrogene peroxide (H_2O_2) is the most widely used apoptosis inducer (Whittemore et al., 1994). Exposure to H_2O_2 triggers apoptosis via the mitochondrial pathway leading to release of cytochrome *c* from mitochondria and activation of caspases-9 and -3 (Singh et al., 2007; Xiang et al., 2016). In the viable cells phosphatidylserin is located on the cytoplasmic surface of the cell membrane. In the apoptosis, phosphatidylserin is translocated from the cytoplasmic face to the outer leaflet of the membrane, exposing phosphatidylserin to the external cellular environment where it can be detected. Annexin V is a cellular protein used to detect apoptotic cells by its ability to bind to phosphatidylserine.

For estimation of apoptosis SMC were plated at a density 10×10^3 cells/cm^2. After 48 hours 10×10^{-3} M hydrogen peroxide (H_2O_2) was added to the culture medium. Two hours later the cells were removed and labeled with FITC-conjugated annexin V (Sigma-Aldrich, USA). The number of annexin V labeled cells was estimated by flow cytometry using Calibur II (BD, USA).

2.6 Induction of endothelial-to-mesenchymal transition.

Endothelial-to-mesenchymal transition (EMT) was induced in "direct" and "co-culture" experimental systems. For direct induction 50×10^3 either of HAEC or VEC were seeded onto 6-well plates covered with 0.2% gelatin and transduced overnight with saturating concentration of the lentiviral concentrate encoding *DLL1*, *DLL4*, *JAG1*, *JAG2* (only for HAEC) and *NICD* relatively. "Direct" EMT was also induced by treatment with 5ng/ml of TGFβ1 in the culture media.

For co-culture stimulation 25×10^3 of HUVEC were plated onto 6-well plates covered with 0.2% gelatin and transduced overnight with Dll1-, Dll4-, Jag1- and Jag2-bearing lentiviruses respectively. After 24 hours 25×10^3 of HAEC were seeded onto HUVEC with fresh ECM.

Responsive markers of EMT such as *SNAIL1*, *SLUG*, *FSPS100A4*, *HES1* and *HEY1* were estimated by qPCR after 72 hours and 14 days of EMT

stimulation. αSMA encoding by *ACTA2* was late responsive and reliable marker of EMT measured by qPCR as well as by immunocytochemistry staining only after 14 days of EMT induction. Primer sequences are represented in **Table 3**.

2.7 In vitro Flow model.

Prior to flow, 75×10^4 of HAEC were plated on teflon-bordered cell culture slides (75x25x10 mm, Flexcell International Corp., Hillsborough, NC, USA) coated with 0.2% gelatin and cultured for 40 hours at 37°C. The culture slides were then inserted into a parallel-plate Streamer device (Flexcell International Corp., Hillsborough, NC, USA) and exposed either to laminar or oscillatory flow of 12 dynes/cm2 for 6 hours 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.). The Osci-Flow regulates flow direction by closing and opening pinch valves that constrict or release tubing in the valves. For laminar flow exposure the steady flow during experimental period was applied to the cells. For oscillatory flow exposure, flow was regulated in oscillatory manner. Cells cultured the same experimental period under static conditions were used as controls. After flow experiments total RNA was isolated and expression of shear stress responsive genes was estimated by qPCR. **Table 4** presents the list of analyzed genes. Primer sequences are represented in **Table 3**.

2.8 Co-culture of valve endothelial and interstitial cells.

Within the tissue cells are closely associated and can regulate each other's phenotype and activities such as proliferation, migration and apoptosis. The co-culture experiments model the situation in vivo and provide detailed information about the molecular basis of cell-cell interactions, and knowledge of cellular events governing the differentiation.

One day before co-culture 120×10^3 of VIC were plated on 12-well plates coated with 0.2% gelatin. At the same time HUVEC were seeded on a culture dish covered with 0.2% gelatin and transduced with saturating concentration of NICD-bearing lentivirus. After 24 hours HUVEC were reseeded and 120×10^3 of HUVEC were added to VIC with fresh DMEM supplemented with 15% FBS, 2mM L-

glutamine and 100 units/ml penicillin/streptomycin. After adhesion of HUVEC, the cell culture medium was replaced by osteogenic medium. The osteogenic medium was changed every other day.

2.9 Induction of osteogenic differentiation.

The osteogenic potential of cells was tested via treatment with osteogenic medium (DMEM supplemented with 15% FBS (HyClone), 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, 50 μg/ml ascorbic acid, 0.1 μM dexamethasone and 10 mM b–glycerophosphate) for 21 days. The osteogenic medium was changed every other day. Early assay was performed after 96 hours. The expression of the following proosteogenic genes was measured by qPCR: *HEY1, BMP2*, *RUNX2*, *SPRY1*, *OPN, OPG, POSTIN*, *CTNNB1*, *SOX9, ALP*. Primer sequences are represented in **Table 3**. After 10 days of osteogenic medium treatment alkaline phosphatase activity was assayed. After 21 days osteogenic differentiation was demonstrated by Alizarin Red.

2.10 Alkaline phosphatase activity assay and Alizarin Red staining.

Alkaline phosphatase staining was performed using Sigma BCIP®/NBT kit (Sigma-Aldrich, USA) according to the manufacturer's directions after 10 days of osteogenic induction. Cells were washed with PBS and incubated with alkaline phosphatase working solution for 10-15 minutes at room temperature. Alkaline phosphatase activity appeared as blue deposition and plates were photographed with digital camera.

Alizarin Red staining is widely used as a reagent for calcium staining. Sites of calcium are covered and surrounded by an orange-red precipitate due to free ionic calcium forms precipitate with alizarin. After 21 days of osteogenic differentiation the calcium deposits were detected by Alizarin Red staining. Cells were washed with PBS, fixed in 70% ethanol for 1 hour, washed twice with distillated water and stained using Alizarin Red solution (Sigma-Aldrich, USA). Matrix calcification was showed with red calcium phosphate deposition. The images were analyzed for the ratio of differentiated and undifferentiated cell areas with MosaiX software (Carl Zeiss microsystems, Germany).

2.11 RNA isolation.

Total RNA was extracted from the cells using Trizol reagent (Invitrogen, USA) according to the instructions of the manufacturer. Cells were washed with PBS and 1 ml of Trizol reagent was added to the culture plate to lyse the cells. The lysate was homogenized by pipet and incubated for 5 minutes at room temperature to allow complete dissociation of nucleoproteins complex. To separate phases 200 µl of chlorophorm were added, the sample was incubated for 2-3 minutes at room temperature and centrifuged for 15 minutes at 12000 g at 4°C. The mixture separated into a lower red phenol-chloroform layer (containing the DNA and proteins), interphase, and a colorless upper aqueous phase containing RNA. The clear aqueous phase was transferred to a new tube avoiding transferring any of interphase or organic layer. To precipitate the RNA 500 µl of isopropanol was added, mixed and overnight incubated at -20°C. Then the sample was centrifuged for 15 minutes at 12000 g at 4°C. Total RNA precipitate forms as white gel-like pellet at the bottom of the tube. The pellet was resuspended in 1 ml of 70% ethanol, briefly vortexed and centrifuged for 5 minutes at 7500 g at 4°C. Pellet of RNA was dried and dissolved in 20 µl of RNase-free water. The RNA was stored at -80°C. The concentration and quality of RNA were estimated by spectrophotometr Nanodrop 3300 (ThermoScientific, USA) and 1.5% agarose gel electrophoresis.

2.12 Reverse transcription and qPCR assay.

Total RNA was reverse transcribed to cDNA with MMLV RT kit (Eurogen, Russia). 1 µg of RNA, 20 µM of Random$(dN)_{10}$ primer and RNase-free water were incubated for 2 minutes at 70°C. The solution of 5x first strand buffer, 10 mM dNTP, 20 mM DTT and RNase-free water was added to previous reaction and mixed with 1 µl (100 units/µL) of MMLV Reverse Transcriptase. The synthesis reaction lasted 10 minutes at 25°C, 1 hour at 37°C, 10 minutes at 70°C respectively.

Real-time PCR was performed with 1 µl of complementary DNA and 5x SYBRGreen PCR Mastermix in the Light Cycler system ABI 7500 (Applied

Biosystems, USA) using 100 nM specific forward and reverse primers for target genes. The thermocycling conditions were as follows: 95 °C for 5 minutes, followed by 45 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. A final heating step of 65 °C to 95 °C was performed to obtain melting curves of the final PCR products.

Corresponding gene expression level was normalized to housekeeping genes *HPRT (Hypoxanthine-guanine phosphoribosyltransferase)* or *GAPDH (glyceraldehyde-3-phosphate dehydrogenase)* from the same samples. Changes in target genes expression levels were calculated as fold differences using the comparative ΔΔCT method. Primer-BLAST tool was used to design target-specific primers. All primer sequences are presented in **Table 3**.

Table 3. Primer sequences.

Gene		**Primer 5' – 3'**
NOTCH1	Forward	GTCAACGCCGTAGATGACC
	Reverse	TTGTTAGCCCCGTTCTTCAG
NOTCH2	Forward	ATGGTGGCAGAACTGATCAAC
	Reverse	TTGGCAAAATGGTCTAACAGG
NOTCH3	Forward	GGAGCCAATAAGGACATGCAGGAT
	Reverse	GGCAAAGTGGTCCAACAGCAGC
NOTCH4	Forward	GTTGTGACAGGGTTGGGACT
	Reverse	CAGCCCAGTGGGTATCTCTG
DLL1	Forward	CTACTACGGAGAGGGCTGCT
	Reverse	CCAGGGTTGCACACTTTCTC
DLL4	Forward	AGGCCTGTTTTGTGACCAAG
	Reverse	CTCCAGCTCACAGTCCACAC
JAG1	Forward	TGCCAAGTGCCAGGAAGT
	Reverse	GCCCCATCTGGTATCACACT
HEY1	Forward	TGGATCACCTGAAAATGCTG
	Reverse	CGAAATCCCAAACTCCGATA
HES1	Forward	AGCACAGAAAGTCATCAAAG

	Reverse	AGGTGCTTCACTGTCATTTC
SNAIL1	Forward	CTCTTTCCTCGTCAGGAAGC
	Reverse	GGCTGCTGGAAGGTAAACTC
SLUG	Forward	ATGAGGAATCTGGCTGCTGT
	Reverse	CAGGAGAAAATGCCTTTGGA
ACTA2	Forward	GTTACTACTGCTGAGCGTGAG
	Reverse	CAGGCAACTCGTAACTCTTC
VIMENTIN	Forward	ACACCCTGCAATCTTTCAGACA
	Reverse	GATTCCACTTTGCGTTCAAGGT
CALPONIN	Forward	CAGCATGGCGAAGACGAAA
	Reverse	GCTCCTGCTTCTCTGCGTACTT
SM22α	Forward	AACAGCCTGTACCCTGATGG
	Reverse	ATGACATGCTTTCCCTCCTG
CDH5	Forward	TGGACAAGGACACTGGCGA
	Reverse	ACAACCGATGCGTGAACACA
FSPS100A4	Forward	AACTAAAGGAGCTGCTGACCC
	Reverse	AAGTCCACCTCGTTGTCCC
HPRT	Forward	TGACACTGGCAAAACAATGCA
	Reverse	GGTCCTTTTCACCAGCAAGCT
AXIN2	Forward	AGTGTGAGGTCCACGGAAAC
	Reverse	CTGGTGCAAAGACATAGCCA
BMP2	Forward	GCCAAGCCGAGCCAACAC
	Reverse	CCCACTCGTTTCTGGTAGTTCTTC
CDC20	Forward	CTCAGGCCATGGCTTTGCA
	Reverse	GCTGCTGCGGATGCCACT
CDCA2	Forward	TTGCCGTTCTCAGTTCTCCTAA
	Reverse	CAGAAGAAATCAATTGGTCAGTGTT
COL15A1	Forward	TGGCCACGGAGGTCAGTTCA
	Reverse	CACAAGGCGGACGCCATG

CXCL12	Forward	AAGGTCGTGGTCGTGCTGGT
	Reverse	CTCAGGCTGACGGGCTTCC
CYP1B1	Forward	CACTATCACTGACATCTTCGGCG
	Reverse	ACGACCTGATCCAATTCTGCCT
DKK1	Forward	CGTGCAAATCTGTCTCGCCTG
	Reverse	TGGTTTCCTCAATTTCTCCTCGG
EFNB2	Forward	GCCAGACAAGAGCCATGAAGA
	Reverse	TCTGGACGTCTTGTTGGATCTTT
GREM1	Forward	GGACCCGCCGCACTGACA
	Reverse	CTTCAGCAGCCGGCAGCAG
MGP	Forward	AGAGAGGATCCGAGAACGCT
	Reverse	GCGTTCGCAAAGTCTGTAGTC
MMP10	Forward	GTGAAACAGTTTAGAAGAAAGGACA
	Reverse	GGCAGAATCAACAGCATCTCT
MMP19	Forward	GAGGCAGCCTCGTTGTGG
	Reverse	GGCAGGTTCAAGATGCGG
MMP24	Forward	GATTGGAGGAGACACCCACTTTGA
	Reverse	CATGCACAGCCACCAGGAAGA
PDE2A	Forward	GCAACAGGCCGATGGAGATG
	Reverse	CGCTTTGGGGAACAGGTCCT
POSTIN	Forward	CCCAGCAGTTTTGCCCATT
	Reverse	TGTGGTGGCTCCCACGAT
RASSF4	Forward	GCCATGTGAGAAGATCGCCA
	Reverse	GTCCAGCACCGGCATTTCA
SMAD1	Forward	GCACAGTCTGTGAACCATGGATTT
	Reverse	GTAACATCCTGGCGGTGGTATTC

SOX7	Forward	CAAGGACGAGAGGAAACGGCT
	Reverse	TCAGCGCCTTCCACGACTTT
SOX9	Forward	GACGCTGGGCAAGCTCT
	Reverse	GTAATCCGGGTGGTCCTTCT
OPN	Forward	TCACCTGTGCCATACCAGTTAAA
	Reverse	TGGGTATTTGTTGTAAAGCTGCTT
TCF4	Forward	GGTCTGGGCTCAGGGTATGGA
	Reverse	TGGTTTGGCAGAAGAGAATGGC
THSD1	Forward	GGAGGAGTGCATGCTAATTCAGAG
	Reverse	CACACTCGGCGACGCTCTC
TXNRD1	Forward	CGATTCCGTCAAGAGATAACAACA
	Reverse	GCCTTGTGTAACTTCTCCAGCATT
vWF	Forward	GGCAGCTGTTCTTATGTCCT
	Reverse	TGGAACTCATTGTTTTGTGG
PECAM	Forward	AGACGTGCAGTACACGGAAG
	Reverse	GATGTCCTTCCAGGGATGTG
GAPDH	Forward	CAAGGTCATCCATGACAACTTTG
	Reverse	GTCCACCACCCTGTTGCTGTAG
RUNX2	Forward	TGGATCACCTGAAAATGCTG
	Reverse	CGAAATCCCAAACTCCGATA
CTNNB1	Forward	ACCACAAGCAGAGTGCTGAA
	Reverse	GCTTGCATTCCACCAGCTTC
OPG	Forward	AAACGGCAACACAGCTCACAAGAA
	Reverse	GCACGCTGTTTTCACAGAGGTCAA
ALP	TaqMan	Hs00740632_gH

SMAD6	TaqMan	Hs00178579_m1
MMP2	TaqMan	Hs01548727_m1
TIMP1	TaqMan	Hs00171558_m1
TIMP3	TaqMan	Hs00165949_m1
STAT1	TaqMan	Hs01013996_m1
STAT6	TaqMan	Hs00598625_m1
TGFBR2	TaqMan	Hs00234253_m1

2.13 Promotor activity assay.

Firefly luciferase is widely used in cell biology as a reporter to study gene expression at transcriptional level and other cellular events coupled to gene expression, such as receptor activity, intracellular signal transduction, mRNA processing, etc. The enzyme firefly luciferase catalyzes the chemical reaction in which luciferin is converted to oxiluciferin. Some of the energy released by this reaction is in the form of light.

To estimate canonical Wnt activity lentiviral TOP flash reporter construct based on 7TFC (Fuerer and Nusse, 2010) with following measurement of TCF activity were used (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 (Korinek et al., 1997) 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, USA) according to the manufacturer recommendations. The cells were washed with PBS and incubated with 5x lysis buffer for 15 minutes at room temperature. In opaque multiwell plate 100 µl per sample of the Luciferase Assay Reagent were dispensed. 20 µl of cell lysate were added to the Luciferase Assay Reagent and plate was placed in the luminometer. Luciferase activity was measured with Synergy2 (BioTek, USA). Samples were normalized by protein content using

Pierce BCA Protein Assay Kit (ThermoScientific, USA).

2.14 Genetic constructs and Lentiviruses.

Lentiviral packaging plasmids pLVTHM, psPAX2 и pMD2.G were a generous gift of D.Trono (École Polytechnique Fédérale de Lausanne, Switzerland; http://http://tcf.epfl.ch); pLVTHM was modified by the addition of the T7 tag and chloramphenicol resistance gene (cm), resulting in the pLVTHM-T7-cm vector. Open reading frame for murine Notch intracellular domain (NICD) was amplified from reversely transcribed mouse ES cells mRNA, using the 5'-GGCGCGCCTCTGGATCCAGTGCTGCTGTCCCGCAAG-3' and 5'-CCACTAGTGCGGCCGCTTATTTAAATGCCTCTGGAATGTG-3' primers. cDNA of murine *DLL1*, *DLL4*, *JAGGED1* and *JAGGED2* were kind gift from Prof. Shigeru Chiba (University of Tsukuba Japan) and Dr. Katsuto Hozumi (Tokai University School of Medicine, Japan) (Abe et al., 2010). The *NICD* PCR fragment was cleaved with AscI and SpeI, then cloned in frame of the T7 tag, replacing the cm gene within pLVTHM-T7-cm. Similarly, the *DLL1* was cloned at the AscI and SpeI restriction sites of pLVTHM-T7-cm, the *DLL4* and *JAG2* at AscI and EcoRI restriction sites of pLVTHM-T7-cm, and the *JAG1* into BamHI and EcoRI restriction sites of the LeGO-G/BSD (Addgene). Lentiviral production was performed as described previously (Malashicheva et al., 2007). The day before the transfection 2.5×10^6 of HEK293T cells were plated onto 10 cm^2 culture dishes. After 24 hours 10 cm^2 culture dishes of nonconfluent HEK293T cells were cotransfected with 15 μg plasmid with gene of interest, 5.27 μg pMD2.G, and 9.73 μg packaging pCMV-dR8.74psPAX2 by calcium-phosphate method. The transfection mix was as follow: plasmids, 440 μl of 0.1x TE buffer pH8.0, 75.3 μl of 2.5 M $CaCl_2$, sterile water up to 760 μl and 760 μl of 2x HeBS added dropwise under agitation. The following day the medium was changed to fresh, and the cells were incubated for 24 hours to obtain high-titer virus production. Produced lentivirus was concentrated from supernatant by ultracentrifugation for 2 hours at 70000 g at 4°C. The pellet containing lentivirus was resuspended in 400 μl of 1% BSA/PBS, incubated for 1 hour on ice and frozen in aliquots at -80°C.

The virus titer was defined with GFP-expressing virus. For analysis, 10×10^4 of HEK293T cells were plated onto 6-well plate and transduced by 1 μl, 10 μl and 100 μl of GFP virus respectively. Cells without infection were used as control. After 72 hours the cells were treated with Trypsin/EDTA and resuspended in 1% BSA/PBS. The number of GFP-positive cells was determined by flow cytomentry using Calibur II (BD, USA). The efficiency of primary cells transduction was 90–95% by GFP.

2.15 Immunocytochemistry.

After growing on cover slides, cells were fixed for 20 minutes in 1% paraformaldehyde and then for 5 minutes in methanol at -20°C. Fixed cells were permeabilized in 1% BSA/0.1% Triton X-100/PBS for 5 minutes and washed with PBS followed by blocking in 1% BSA/PBS for 1 hour. Then cells were incubated for 1 hour at room temperature with primary antibodies. Following primary antibodies were used: SMA (sc-32251, Santa Cruz, USA), SM22α (ab14106, Abcam, UK), vimentin (sc-6260, Santa Cruz), VE-cadherin (MAB938, RandD, USA), von Willebrand factor (ab20435, Abcam), CD31 (ab24590, Abcam). Then cells were washed with PBS three times for 5 minutes and incubated for 40 minutes in dark at room temperature with secondary antibodies, conjugated with Alexa488 or Alexa546 (Invitrogen, USA). Then cells were washed with PBS three times for 5 minutes and DAPI was used to visualize nuclei. Microphotographs were taken using AxioObserver Microscope (Zeiss) at x20 magnification with AxioVision software.

2.16 Immunoblotting.

Proteins were extracted from medial tissues or cells. Specimens were homogenized in a lysis buffer (50 mM Tris (pH 8), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 5 mM EDTA), containing protease inhibitors (Roche, Germany). Protein concentration was estimated by Pierce BCA Protein Assay Kit (ThermoScientific, USA). Extracts were separated by 10% sodium dodecyl sulfate–polyacrilamide gel electrophoresis (SDS–PAGE); 20 μg of prepared protein sample were loaded. Primary antibodies used: SMA (Abcam),

vimentin (M072529, DAKO), beta-Actin (ab 6276, Abcam), calponin (Sigma-Aldrich), SM22α (ab14106, Abcam), collagen I, fibrillin and elastin. Secondary antibodies were conjugated with peroxidase (BioRad, USA). Positive bands were quantified by densitometry using a gel documentation system Fusion Fix (Vilber Lourmat) and Fusion-Capt software. Bands were normalized using β-actin or α-tubuline staining.

2.17 Transformation of bacteria and plasmid isolation.

Competent cells *E. coli* of strain Top10 were used for transformation (genotype: F– mcrA Δ(mrr-hsdRMS-mcrBC) Φ80lacZΔM15 ΔlacX74 recA1 araD139 Δ(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG). 1 μg of plasmid was added to 50 μl of competent cells; the competent cell/DNA mixture was incubated on ice for 30 minutes followed by heat shock for 20 seconds at 42°C and 2 minutes incubation on ice. Then cells were incubated for 1 hour in 37°C shaking incubator with 950 μl of LB media (10 g/L NaCl, 5 g/L yeast extract, 10 g/L tryptone) without antibiotic. After the transformation was plated onto 10 cm^2 LB/agar dish containing 100 μg/ml ampicillin and let grow overnight at 37°C.

Plasmid DNA isolation was performed using the HiPure Plasmid Filter Maxiprep Kit (Invitrogen, Germany) according to the manufacturer's directions. The concentration and quality of DNA were estimated by spectrophotometr Nanodrop 3300 and 0.8% agarose gel electrophoresis.

2.18 Statistics.

qPCR data on gene expression were analyzed using GraphPad Prism and R software (version 2.12.0; R Foundation for Statistical Computing, Vienna, Austria). 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.

3. Results

3.1 Proliferation and migration of smooth muscle cells from patients with thoracic aortic aneurysm and healthy donors.

Specific degenerative processes associated with reduced cell number in the vessel tissue are observed in the thoracic aortic aneurysm (TAA) aortic wall (Milewicz et al., 2008). To evaluate the possible smooth muscle cell (SMC) contribution to these changes, we compared the rate of SMC proliferation and migration in healthy donors and patients. TAA is regarded as a group of etiologically different diseases rather than a particular illness. Recent genetic studies showed that TAA accompanied and unaccompanied by bicuspid aortic valve (BAV) differed (Folkersen et al., 2011; Kjellqvist et al., 2013). Basing on these observations, we formed three experimental groups: patients (n = 9) with TAA without aortic valve defects (TAV-TAA), patients (n = 7) with TAA accompanied with aortic valve defect (BAV-TAA), and healthy donors (n = 7). Smooth muscle cells were isolated from tissue fragments of TAA and from the aortic wall of healthy donors.

The SMC proliferation rate was assayed with the cultured cell growth curves. The cells were seeded at equal density and counted each two days for proliferation assay. The results are presented in **Figure 4A**. It is seen that SMC proliferation rate in TAA patients is lower than in healthy donors regardless of valve defect.

The SMC migration rate was estimated by scratch assay. Migrated cells were counted after 6 hours and 24 hours **(Figure 4B)**. It was found that the SMC migration rate was higher in TAV-TAA compared to healthy donors. No significant difference was observed in the SMC migration rate between BAV-TAA and healthy donors. These results agree with genetic data showing that BAV- and TAV-associated TAA are different pathologies. Thus, SMC decreased proliferation may be a reason for diminished cell number in TAA aortic wall. The increased SMC migration rate observed in TAV-TAA patients does not affect the decrease in the cell number in the patient aortic tissue and may be produced by pro-inflammation process common for TAV-TAA (Milewicz et al., 2008).

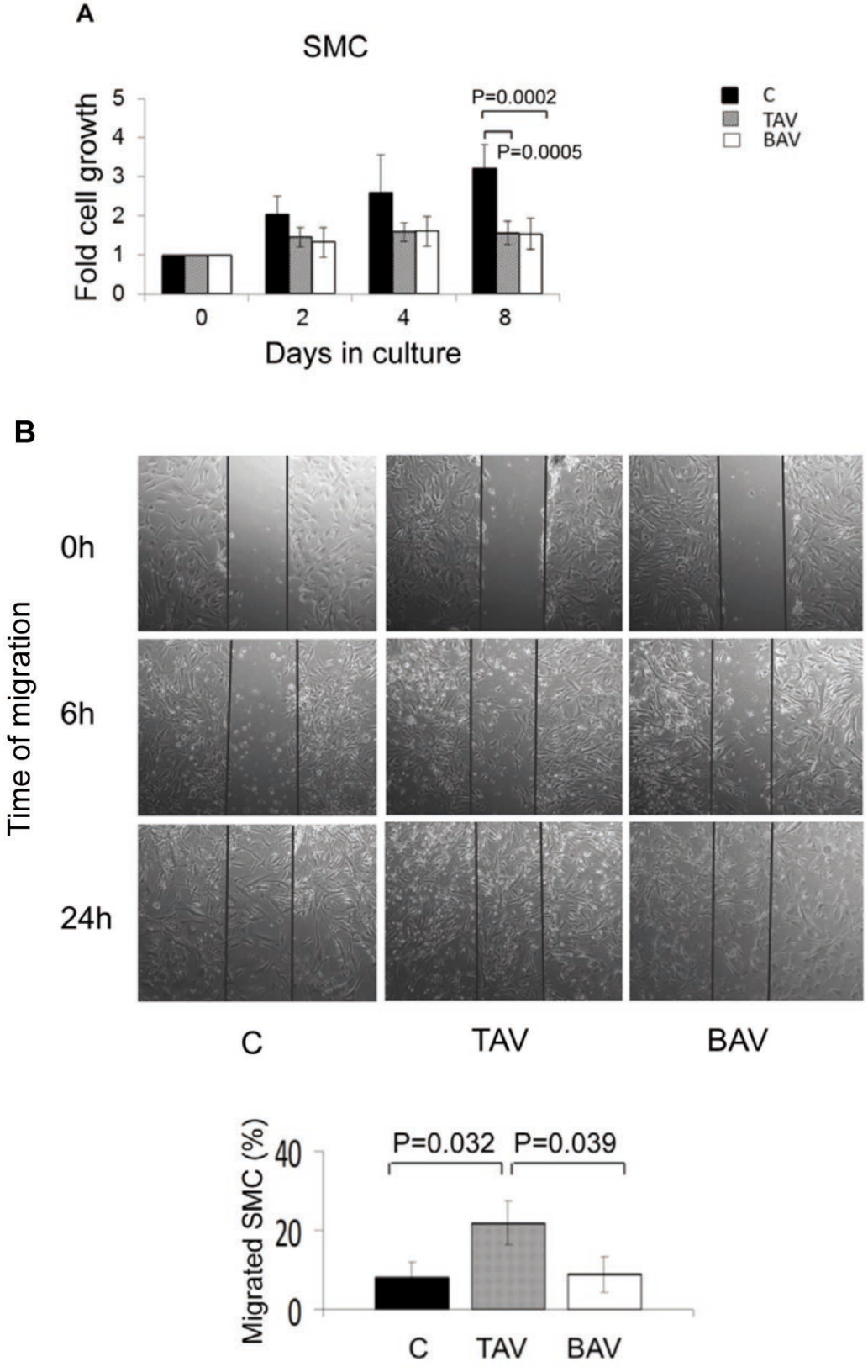


Figure 4. Proliferation and migration characteristics of SMC from patients

with aortic aneurysm with either tricuspid aortic valve (TAV, n= 5) or bicuspid aortic valve (BAV, n= 5) and controls (C, n= 5). (A) SMC proliferation; (B) SMC migration. Groups are compared using Mann Whitney non-parametric test; *p<0.05.

3.2 Expression of smooth muscle cell markers in smooth muscle cells from thoracic aortic aneurysm patients and healthy donors.

Smooth muscle cells from aneurysms of the thoracic aorta and from control aortas were analyzed regarding the expression of SMC markers like α-smooth muscle actin (SMA), vimentin, and SM22α. **Figure 5** shows typical immunofluorescent staining of SMC from control aortas and from aneurysms in patients with BAV and TAV. Both BAV- and TAV-derived SMC appeared to have decreased level of SMA, vimentin, and SM22α. However, there were no visible differences between SMC from patients with BAV and TAV-associated aneurysm.

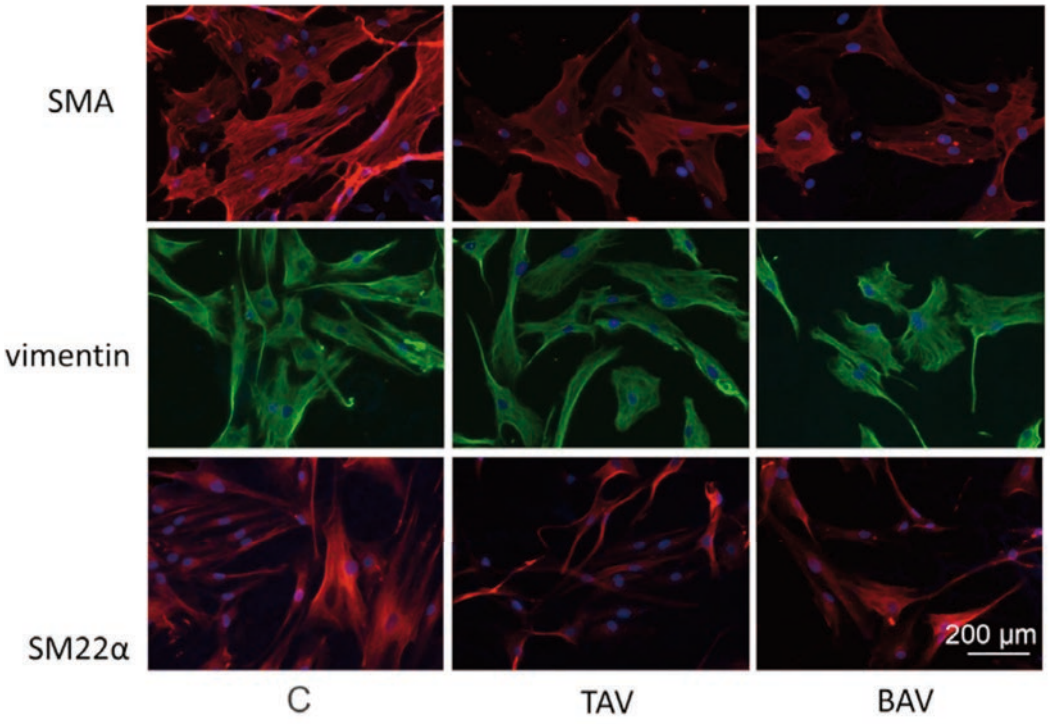


Figure 5. Expression of smooth muscle cell markers in SMC from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid

aortic valve (BAV) and controls (C) determined by immunocytochemical staining of α-smooth muscle actin (SMA), vimentin and SM22α. Scale bar corresponds to 200 μm.

At protein level expression of SMA was reduced both in the BAV- and TAV-derived SMC and in the aortic media **(Figure 6)**. However, although SMA was lower in aortic media of aneurysms from patients with BAV than in controls, it was still higher than in patients with TAV. mRNA level of SMA was significantly lower only in BAV-derived SMC. At both mRNA and protein level expression of vimentin was reduced in the BAV-derived SMC, however the protein content of vimentin in aortic media from TAV-derived SMC also was reduced. SM22α expression was decreased only in SMC and aortic media from patients with TAV. Relative mRNA content of calponin was significantly increased in TAV-derived SMC.

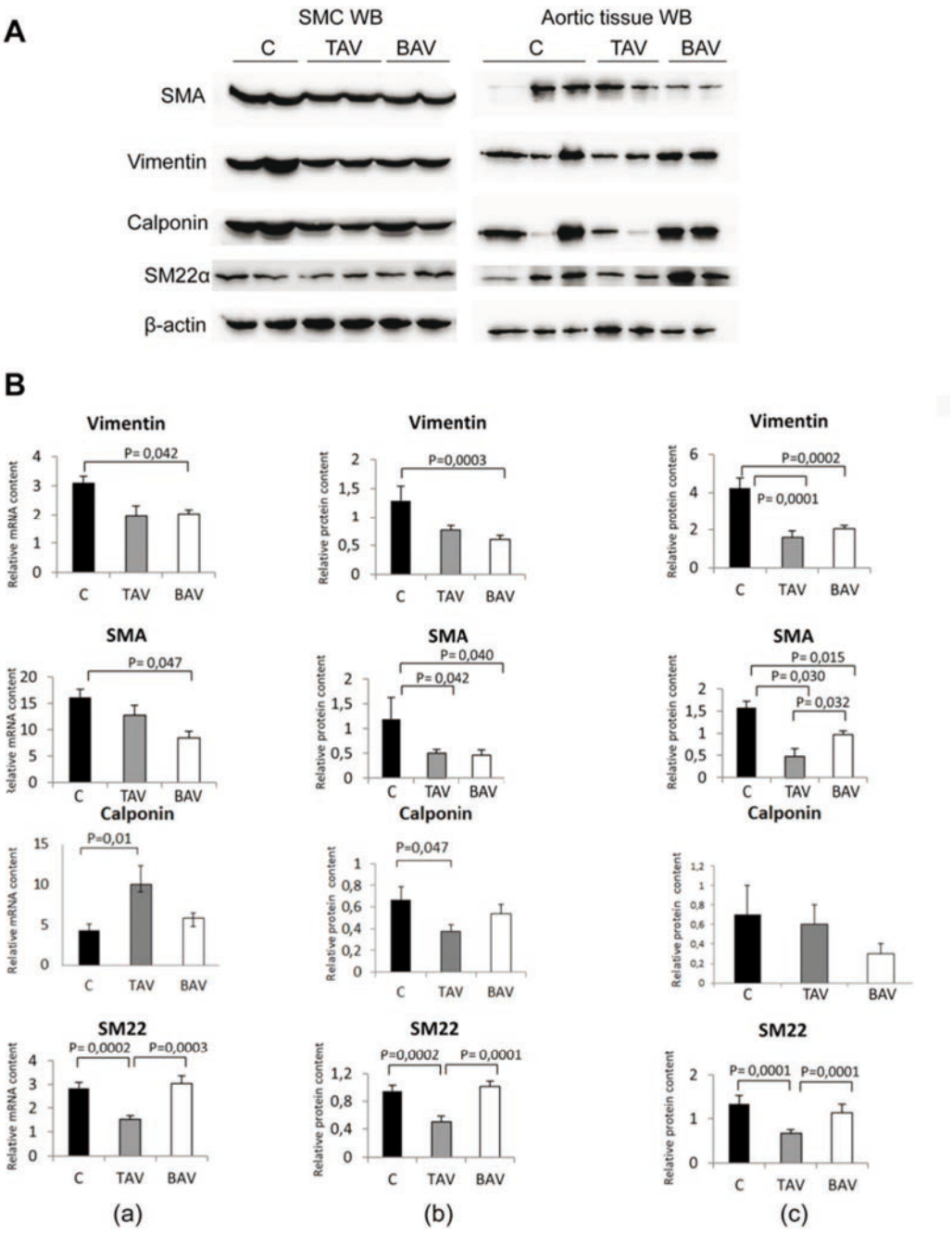


Figure 6. Expression of smooth muscle cell markers in SMC from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and controls (C). mRNA level was determined by qPCR; protein level was determined by Western blot. The bands were normalized by β-actin. (A) Representative Western blot picture for protein level in SMC; (B) (a) mRNA level in SMC: (C, n= 10; TAV, n= 13; BAV, n= 11). (b) Protein level in SMC: (C, n= 10; TAV, n= 13; BAV, n= 11). (c) Protein level in aortic

media: (C, n= 11; TAV, n= 13; BAV, n= 17). Groups are compared using Mann-Whitney non-parametric test; *p<0.05.

3.3 Apoptosis in smooth muscle cells from patients with thoracic aortic aneurysm and healthy donors.

Reduced cell number has been shown in aortic tissue of thoracic aortic aneurysm patients and may reflect increased apoptotic level in the vessel wall cells. Indeed, the cell number positive for DNA double strand breaks (an apoptotic marker) is increased in the media of the wall of thoracic aneurysms (Schmid et al., 2003). It has also been suggested that oxidative stress is responsible for abdominal aortic aneurysm (AAA) (McCormick et al., 2007) and TAA (Liao et al., 2008). We have compared the cell number labeled with annexin V in SMC cultures derived from TAA patients and healthy donors **(Figure 7A).** The number of annexin V-positive cells was significantly higher in SMC cultures from patients with both BAV and TAV and did not depend on whether TAA is accompanied with valve defects or not. Oxidative injury might be a cause of increased wall weakness in both AAA and TAA (McCormick et al., 2007). The ability of oxidative stress to cause apoptosis might be altered in SMC from aneurysms. To test this hypothesis H_2O_2 was added to SMC. We measured apoptosis as a residual between the percentage of annexin V-positive cells after H_2O_2 treatment and the amount of annexin V-positive cells in normal cultures ("baseline"). **Figure 7B** shows that apoptotic induction was higher in cells derived from donors than from TAA patients. Reduced apoptotic induction caused by H_2O_2 was observed in cultures established from BAV- and TAV-TAA patients. These results show that SMC ability to enter apoptosis produced by H_2O_2 is diminished in TAA patients.

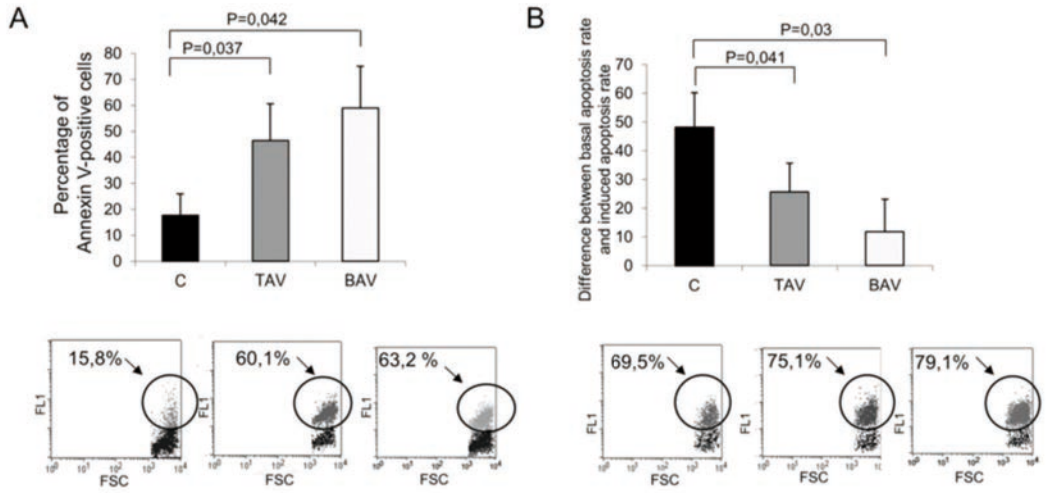


Figure 7. Apoptosis level in cultured SMC from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and controls (C). (A) The level of "baseline" SMC apoptosis in culture. The diagram shows the percentage of annexin V-positive SMC in vitro estimated by flow cytometry. The lower panel shows representative plots from the analysis of live SMC in culture: (C, n= 5; TAV, n= 5; BAV, n= 5); (B) apoptosis induction by H_2O_2. The diagram shows the residual between the percentage of annexin V-positive cells after H_2O_2 treatment and the level of annexin V-positive cells in untreated cultures. The lower panel shows representative plots from the analysis of SMC treated with H_2O_2: (C, n= 5; TAV, n= 5; BAV, n= 5). Arrows mark annexin V-positive cells. Groups are compared using Mann-Whitney non-parametric test; *p<0.05.

3.4 Matrix protein content in aortas of patients with thoracic aortic aneurysm and healthy donors.

Degenerative processes observed in TAA tissues are accompanied by a modified structure and level of a number of extracellular matrix (ECM) proteins. To evaluate SMC contribution to ECM protein synthesis (elastin, fibrillin, and collagen I) in the aortic wall we estimated the protein content in aortic media specimens and in SMC protein extracts by Western blot. The elastin and fibrillin content was significantly reduced in aortic media from aneurysms in both BAV and TAV patients (**Figure 8)**. The elastin content in SMC was increased in both

BAV- and TAV-TAA while the fibrillin level was significantly lower only in TAV-TAA.

Collagen I content was higher in aortic media from both types of aneurysm patients but was not significantly changed in SMC from aneurysm patients. However, the amount of collagen I was higher in SMC of aneurysms from patients with TAV only **(Figure 8)**.

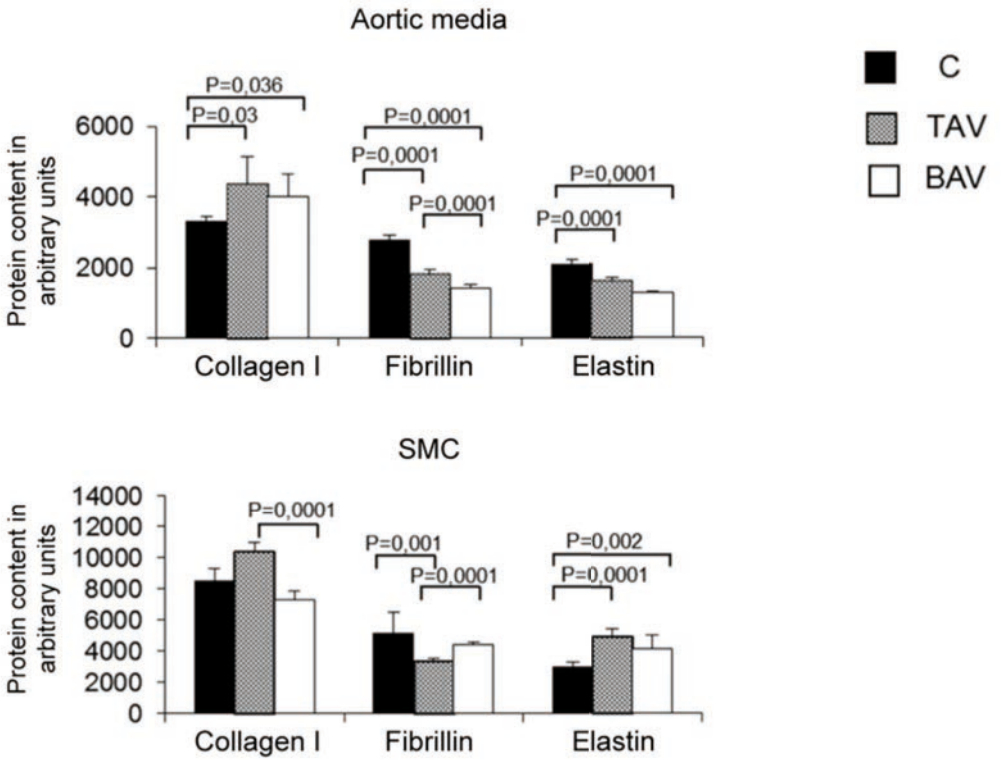


Figure 8. Matrix protein level in SMC from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and controls (C) determined by Western blot. The diagrams represent the results of densitometry. The bands were normalized by β-actin. (C, n= 10; TAV, n= 3; BAV, n= 17). Groups are compared using Mann-Whitney non-parametric test; *p<0.05.

3.5 Proliferation rate, expression of endothelial markers and matrix protein content in aortic endothelial cells from patients with thoracic aortic aneurysm and healthy donors.

Although SMC are considered to be the main functional unit in the aortic wall, endothelial cells (EC) are also involved in the function of aortic wall and therefore their changed function could contribute to the aortic pathology. A role of the endothelial cells in the developing of aneurysms is largely unknown, although in recent study endothelial dysfunction has been shown to affect BAV-TAA (Ali et al., 2014). To test if EC are also functionally changed in the aorta of TAA patients we compared EC cultures from TAA patients and donors.

Since we observed significant decline of proliferation rate in diseased SMC we compared also proliferation of EC from BAV- and TAV-TAA patients and healthy donors. The cells were seeded at equal density and counted each two days for proliferation assay. Endothelial cells proliferation rate in both BAV and TAV aneurysm was lower compared to healthy donors, but endothelium from patients with BAV had lower proliferation rate than EC from aneurysms of patients with TAV **(Figure 9)**.

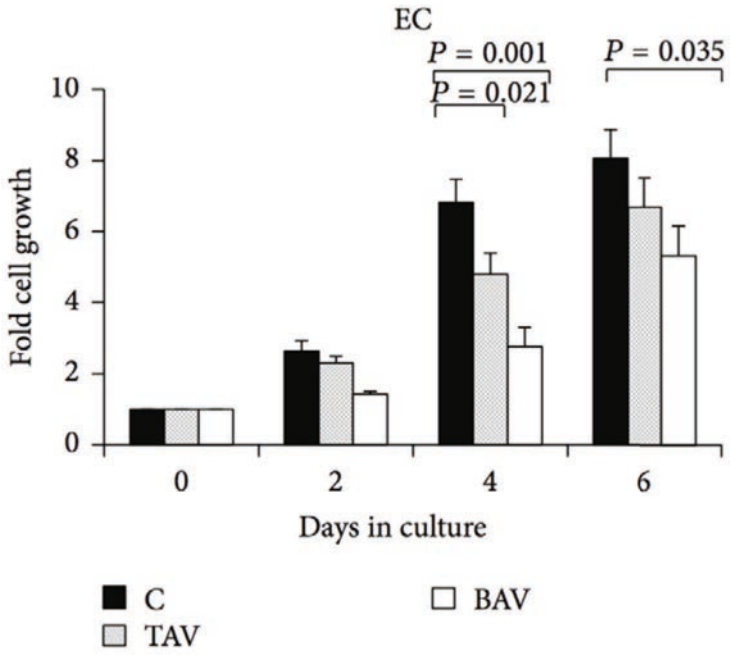


Figure 9. Proliferation characteristic of endothelial cells from patients with aortic aneurysm with either tricuspid aortic valve (TAV, n= 5) or bicuspid

aortic valve (BAV, n= 5) and controls (C, n= 5). Groups are compared using Mann-Whitney non-parametric test; *p<0.05.

Endothelial cells from aneurysms of thoracic aorta and from control aortas were analyzed regarding the expression of endothelial markers like vWF (von Willebrand factor), CD31/PECAM (platelet endothelial cell adhesion molecule), VE-cadherin as well as smooth muscle cell marker SMA to confirm that the cultures were not contaminated with SMC. **Figure 10** demonstrates primary cultures and immunofluorescence staining of the EC. Endothelial markers appeared to be reduced in endothelium in aneurysms from patients with both TAV and BAV (**Figure 10)**.

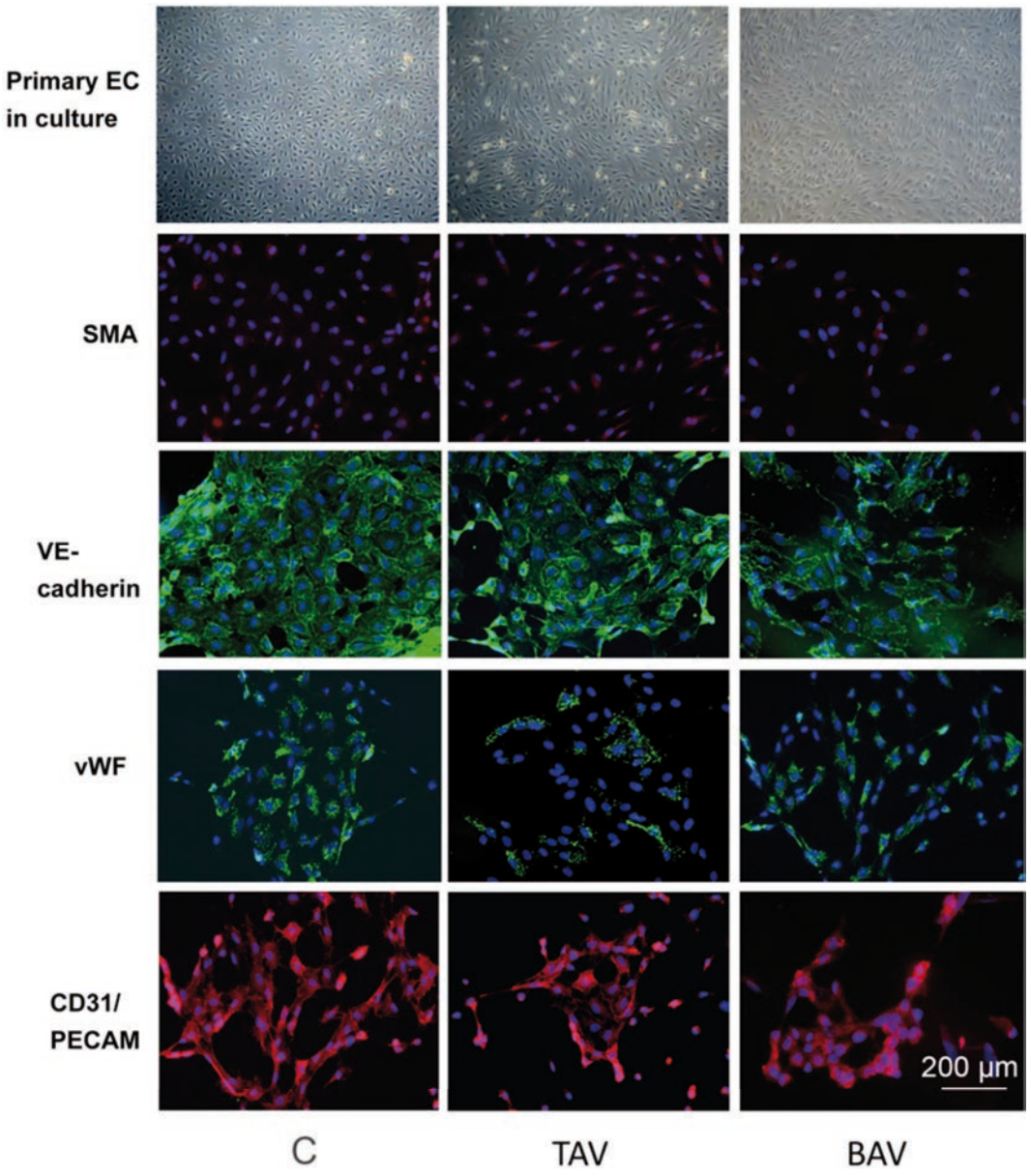
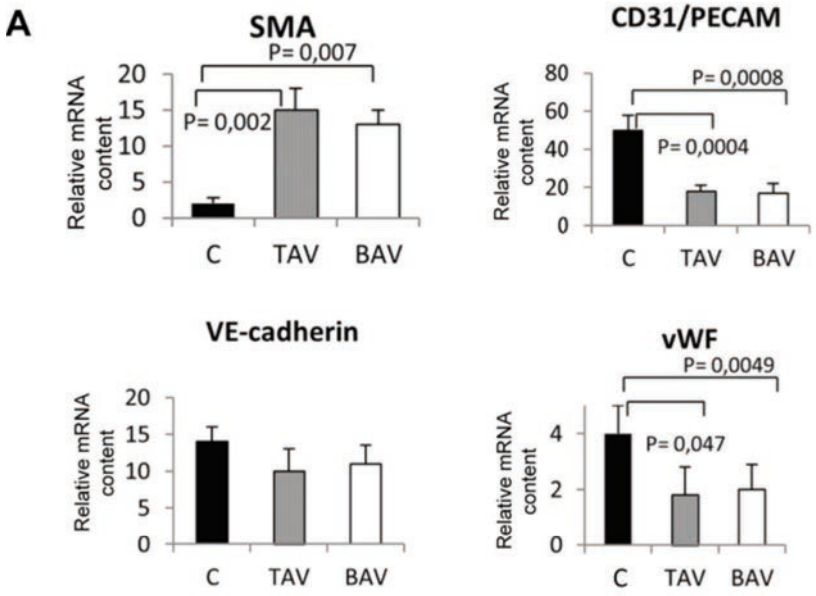


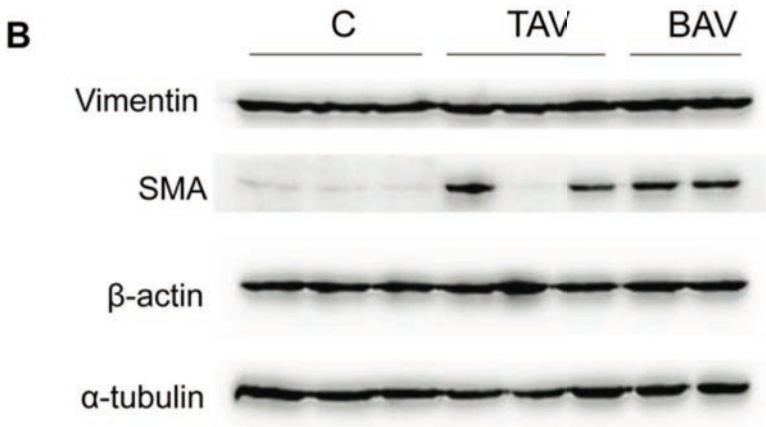
Figure 10. Characterization of the aortic endothelial cells from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and controls (C). Upper panel represents typical aortic endothelial cell cultures from control and aneurysmal aortas. The SMA staining confirms the lack of medial SMC contamination in the endothelial cell cultures. VE-cadherin, vWF, and CD31/PECAM staining confirms endothelial nature of the isolated cells. Scale bar corresponds to 200 µm.

We compared also the mRNA level of SMA, PECAM/CD31, VE-cadherin and vWF in EC derived from control aortas and from aneurysms. The expression

of SMA was elevated in both BAV- and TAV-derived EC **(Figure 11A)**. Typical SMA microfilament staining was not observed in our endothelial cultures; thus, the elevation of SMA mRNA level was not due to contamination with SMC. Additionally, the protein level of SMA determined by Western blot was increased in aneurysmal EC **(Figure 11B)**. Expression of the endothelial markers vWF and PECAM/CD31 was substantially decreased in EC from patients and confirms changes observed with immunocytochemical staining. The level of VE-cadherin mRNA was not changed **(Figure 11A)**.

The culture media from EC was analyzed for elastin, collagen I, and fibrillin content. Our data show that aortic EC as well as SMC are capable of synthesizing these proteins. Moreover, EC from TAA patients synthesize fragmented collagen I which suggest that EC contribute to the impaired matrix biosynthesis **(Figure 11C)**.


A
SMA
P= 0,007
P= 0,002
Relative mRNA content
20
15
10
5
0
C
TAV
BAV
CD31/PECAM
P= 0,0008
P= 0,0004
80
60
40
20
0
VE-cadherin
vWF
P= 0,0049
P= 0,047
4
2


B
C
TAV
BAV
Vimentin
SMA
β-actin
α-tubulin

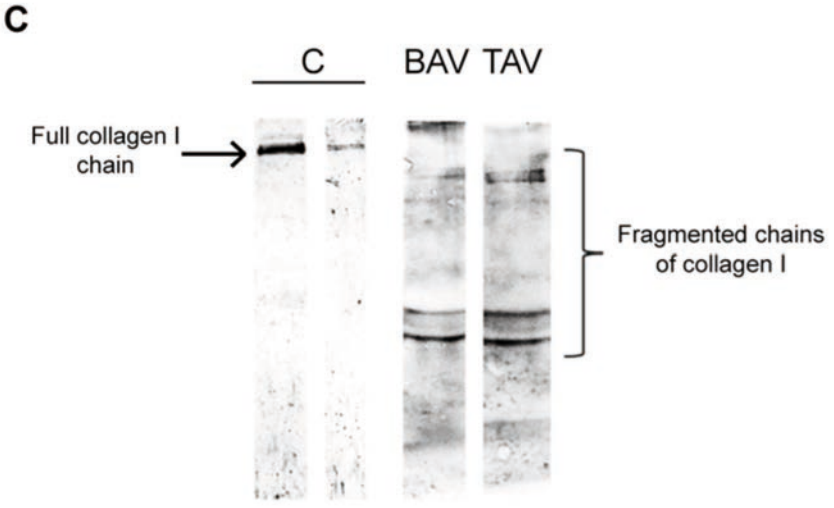

C
C
BAV
TAV
Full collagen I chain
Fragmented chains of collagen I

Figure 11. Expression of smooth muscle cell markers and endothelial cell markers in endothelial cells from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and controls (C). mRNA level was determined by qPCR; protein level was determined by Western blot. The bands were normalized by β-actin. (A) mRNA in endothelial cells: (C, n= 5; TAV, n= 5; BAV, n= 5); (B) representative Western blot picture for SMA and vimentin protein level in endothelial cells; (C) collagen I protein level in endothelial cell culture medium. Groups are compared using Mann-Whitney non-parametric test; *p<0.05.

3.6 Baseline level of Notch signaling pathway in aortic endothelial cells from patients with thoracic aortic aneurysm and healthy donors.

Our findings demonstrated substantial phenotypic changes in endothelial cells derived from aortic wall of patients with TAA. Notch signaling pathway plays a critical role in cardiac outflow tract (OFT) development and loss of Notch pathway results in spectrum of OFT defects including aortic abnormalities (High et al., 2009). Notch1 has been shown to be required within endothelial cell lineage for proper OFT development (Koenig et al., 2016). Notch pathway has been reported to be decreased in the ascending aorta wall specimens of patients with BAV (Sciacca et al., 2013).

We address the role of Notch signaling pathway in human aortic endothelial cells (HAEC) from patients with TAA and healthy donors. Initially, we assessed the levels of expression for key genes belonging to Notch pathway – *NOTCH1*, *NOTCH2*, *NOTCH3*, *NOTCH4*, *DLL1*, *DLL4*, *JAG1* in endothelial cells of BAV- and TAV-TAA patients and healthy controls **(Figure 12)**. Endothelial cells from both BAV- and TAV-TAA patients had significantly lower mRNA levels of *NOTCH1*, *NOTCH3, NOTCH4* and *DLL4* compared to controls. The level of *NOTCH2* expression was significantly lower only in HAEC of TAV-TAA patients, whereas the expression levels of *DLL1* and *JAG1* were significantly higher in endothelium from TAV-TAA patients. The mRNA level of direct Notch target *HEY1* was significantly elevated in both BAV- and TAV-TAA. Expression

of other direct Notch target gene *HES1* was changed neither in BAV- nor TAV-derived endothelial cells.

SNAIL1 one of the master regulator of EMT and Notch1 induces elevation in its expression during endothelial-to-mesenchymal transition (EMT) (Nieto, 2002; Timmerman, 2004). Its mRNA level was significantly higher in HAEC of BAV patients compared to TAV-TAA and control cells. However, the expression level of other main regulator of EMT *SLUG* (*SNAIL2*) was not different between BAV-TAA patients and controls. Our data suggest alterations of baseline Notch signaling in aortic endothelial cells of BAV- and TAV-TAA patients.

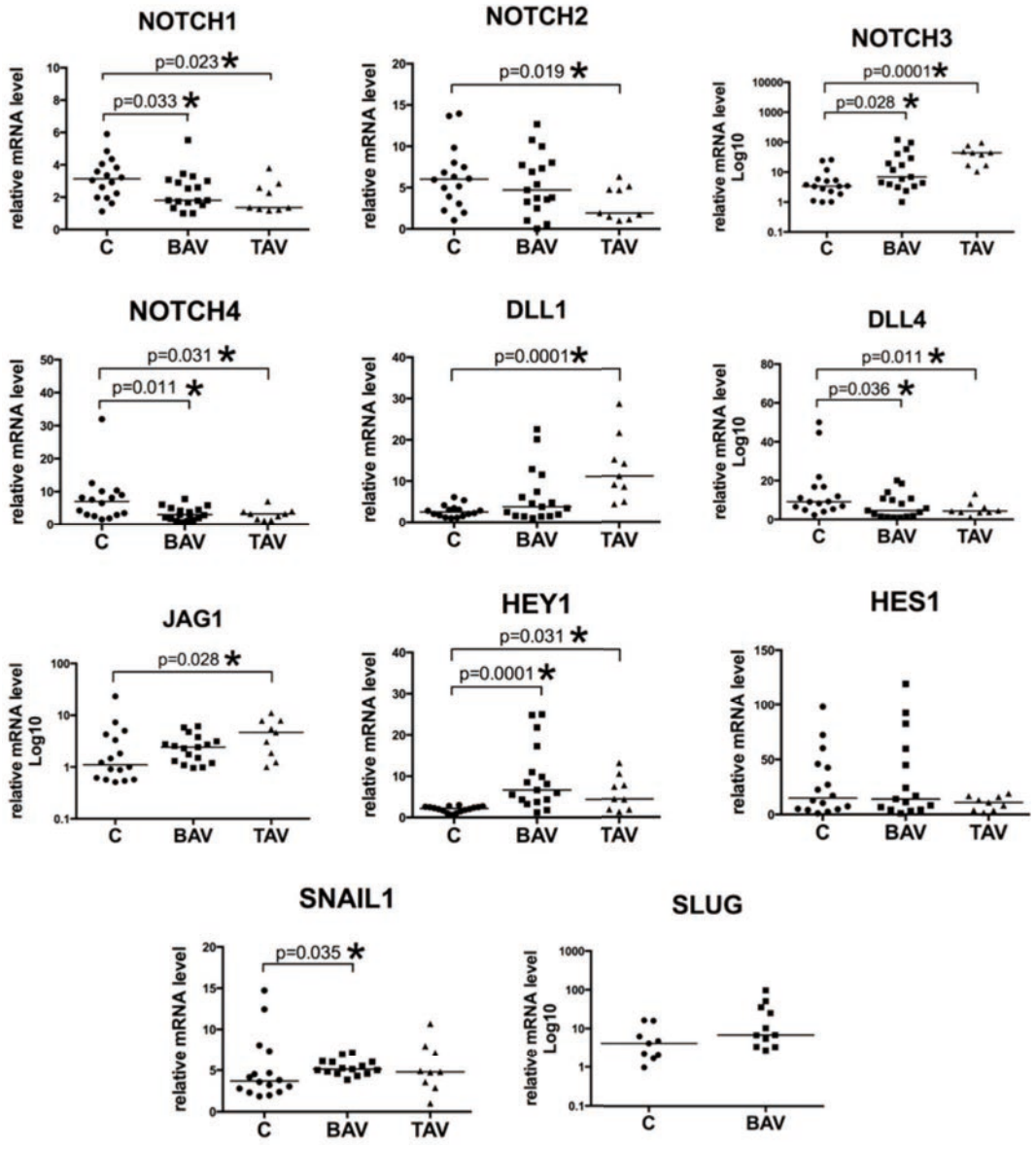


Figure 12. Expression level of Notch receptors, ligands and transcriptional targets in the aortic endothelial cells from the patients with thoracic aortic aneurysm associated with either tricuspid aortic valve (TAV, n= 9), or bicuspid aortic valve (BAV, n= 15), and controls (C, n= 15). mRNA level was determined by qPCR. Groups are compared using Mann-Whitney non-parametric test; line represents the median; *p<0.05.

3.7 Experimental models for induction of endothelial-to-mesenchymal transition.

Patients with BAV are at increased risk for the most of aortic pathologies, including ascending aortic aneurysm (Fedak et al., 2003, 2002; Gleason, 2005). Bicuspid aortic valve is a result of impaired valve formation during the endothelial-to-mesenchymal transition (EMT) (Kovacic et al., 2012), but the proper mechanisms of development defective valve remain unclear. We hypothesized that the altered baseline level of Notch pathway in BAV-TAA patients affects EMT.

To establish an experimental model for estimating EMT efficiency in HAEC of BAV-TAA patients we induced EMT in human umbilical vein endothelial cells (HUVEC) and control HAEC either by introduction of Notch intracellular domain (NICD) with lentivirus or by the addition of TGF-β as it was described earlier (Niessen et al., 2008; Yang et al., 2008). Stimulated cells lost cobblestone morphology after addition of NICD-bearing virus or TGF-β suggesting loss of endothelial phenotype and transformation to mesenchymal cells.

During cardiac EMT, endocardial cells undergo significant changes in gene expression including Notch-dependent induction of *ACTA2 (*αSMA), *SNAIL1*, and *SLUG* (Noseda, 2004). Previously in our laboratory additional screening of 11 genes described as markers of EMT was made for their up or down regulation at EMT for HAEC and HUVEC after TGF-β or NICD-induction (Zeisberg and Neilson, 2009). Among responsive early EMT markers by qPCR were *HEY1*, *HES1*, *FSPS100A4*, *SLUG* which were up regulated after EMT induction. These genes were used in further study **(Figure 13A)**. SMA was late responsive and

reliable marker of EMT in HAEC and HUVEC by qPCR as well as by immunofluorescent staining after 14 days of EMT induction **(Figure 13B)**. *HEY1* and *HES1* are among main Notch transcriptional targets (Andersson et al., 2011) and were up regulated at mRNA level in endothelial cells in NICD-induced cultures. Thus, both HAEC and HUVEC activate EMT program in response to either Notch1 intracellular domain activation or to TGF-β stimulation at approximately equal extent although activating slightly different level of responsive genes. This may reflect differences in the origin of the two endothelial lineages.

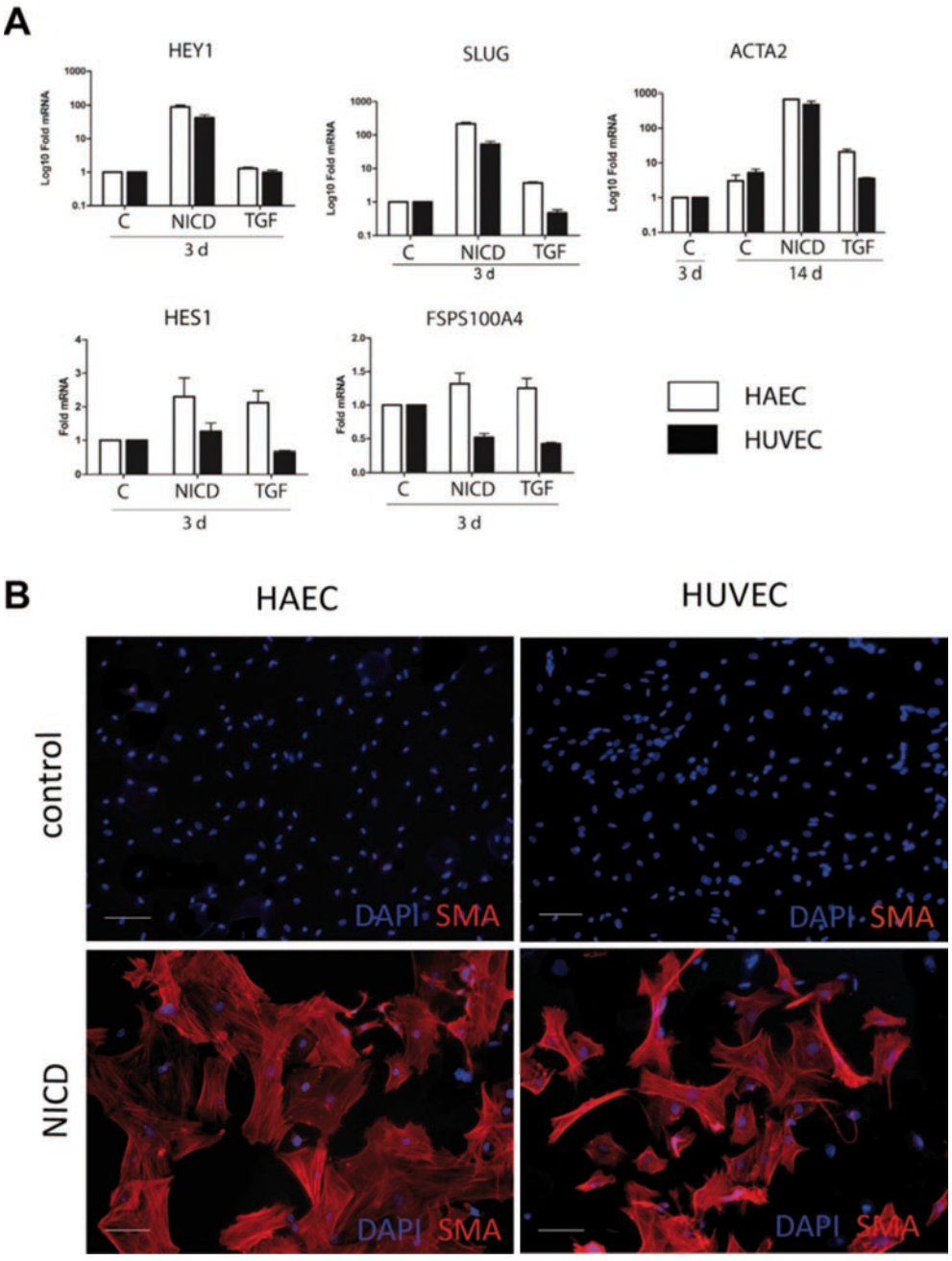


Figure 13. Induction of endothelial-to-mesenchymal transition (EMT) in endothelial cells derived from human aorta (HAEC) and human umbilical vein (HUVEC) by either introduction of Notch-intracellular domain (NICD) or by TGF-β. (A) Expression of responsive genes 3 and 14 days after EMT induction estimated by qPCR; (B) SMA induction in HAEC and HUVEC 14 days after EMT induction by immunocytochemical staining. Scale bar corresponds to 200 µm.

Notch is known as a complex and lineage-specific signaling with several receptors and ligands (Andersson et al., 2011), therefore we explored the efficiency of different Notch ligands to induce EMT in HAEC using "direct" and "co-culture" systems of EMT induction. In the direct system we modified HAEC with lentiviruses bearing one of the Notch ligands (Dll1, Dll4, Jag1, Jag2) and analyzed the expression of key early EMT markers **(Figure 14)**. For induction of EMT in co-cultures HUVEC were transduced with a lentivirus bearing one of the ligands (Dll1, Dll4, Jag1, Jag2) and then co-cultured with HAEC. **Figure 14** shows that both types of induction activate genes responsible for EMT in HAEC but with different extent and with the activation of different sets of genes specific for each ligand. Dll1 and Jag1 had similar modes of EMT induction: direct introduction of Dll1 or Jag1 into HAEC caused up regulation of *SLUG* and *FSPS100A4*, whereas the same inducers used in co-culture caused only moderate up regulation of the same target genes. On the opposite, Dll4 and Jag2 induced significant up regulation of EMT markers only in co-culture but not at direct introduction into HAEC. Notch targets *HEY1* and *HES1* were significantly up regulated only in co-culture experiments for all four ligands studied, but were not up regulated when the same genes were directly introduced into HAEC. All together these our results reflect versatile function of different Notch components in EMT. Described experimental settings were used in further experiments with the cells from BAV patients.

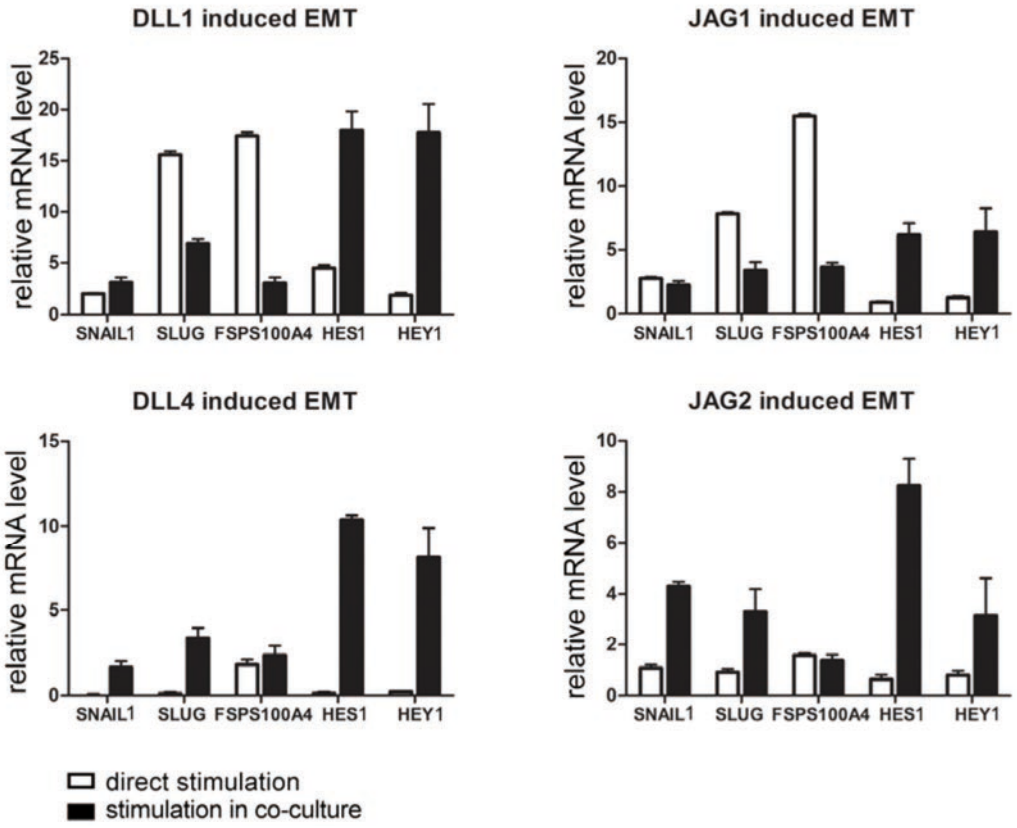


Figure 14. Comparison between direct endothelial-to-mesenchymal transition (EMT) induction by Notch ligands and induction of EMT in co-culture. The expression of corresponding EMT markers was verified 3 days after the induction of EMT by qPCR.

3.8 Efficiency of endothelial-to-mesenchymal transition in HAEC of BAV patients and healthy donors.

To test the hypothesis that Notch signaling is impaired in aortic endothelial cells of BAV-TAA patients we analyzed EMT induction in models described above and compared effectiveness of EMT in HAEC from the patients with control cells by gain of mesenchymal markers and loss of endothelial markers.

First we induced EMT in BAV-TAA cells and in controls either by introduction of NICD or addition of TGF-β **(Figure 15)**. We observed significantly impaired NICD-induced activation of *HEY1* and *SLUG* after 3 days of EMT induction by NICD in HAEC of BAV-TAA; *HES1*, *FSPS100A4* and

ACTA2 also were not activated after 14 days in response to EMT induction by NICD in the cells of patients. Similarly, TGF-β-induced activation of the key EMT markers was significantly reduced in diseased HAEC **(Figure 15A, B)**. The loss of endothelial markers was observed in both HAEC of patients and controls but was not different between 2 groups **(Figure 15C)**.

Up regulation of *ACTA2* expression induced either by NICD or TGF was also impaired in the cells from patients. **Figure 15D** demonstrates failure to activate NICD and TGF-β –induced EMT program in the cells of BAV patients by SMA staining of the cells. Endothelial cells of patients failed to properly form actin fibers during EMT compared to controls.

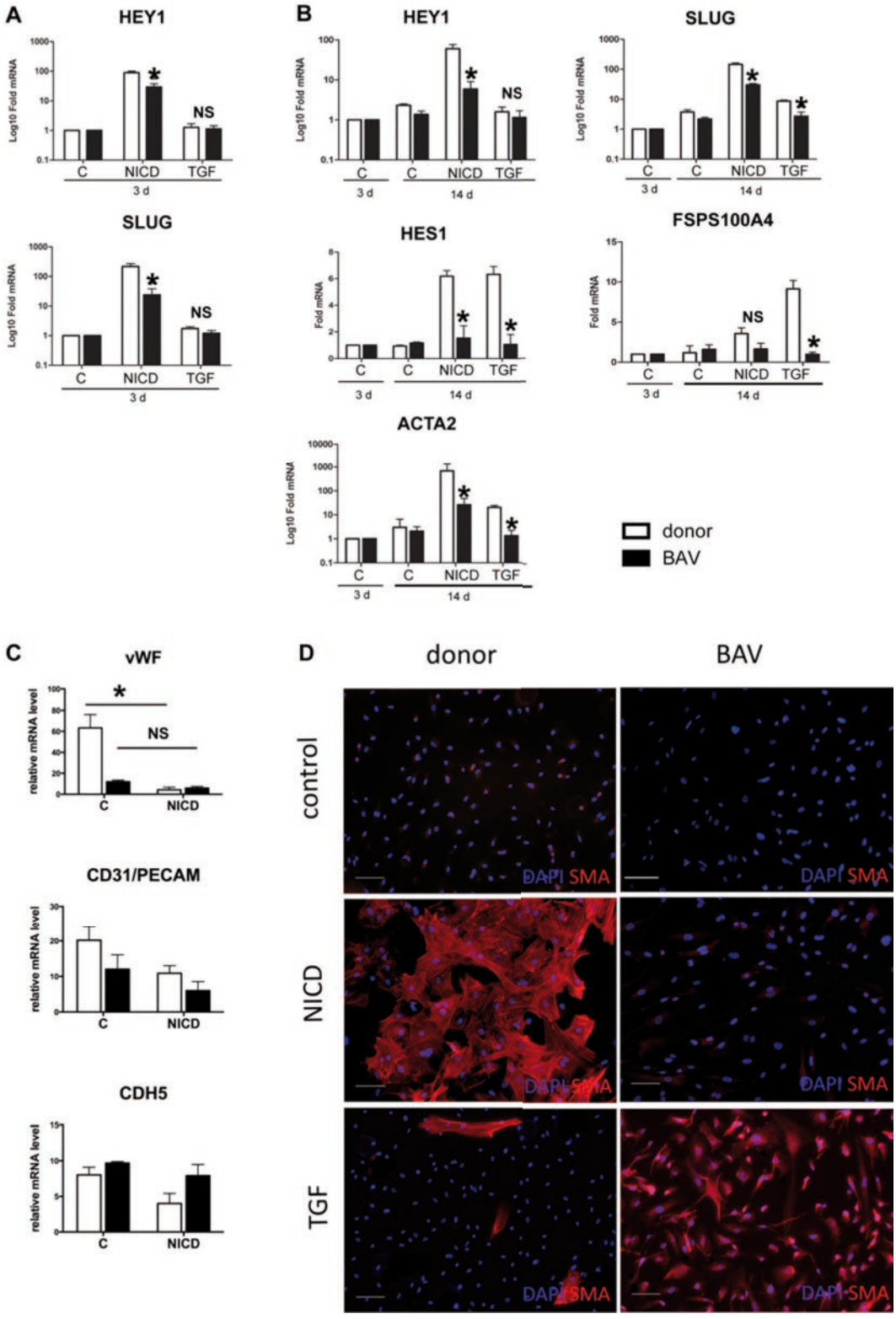


Figure 15. Comparison of endothelial-to-mesenchymal transition (EMT) induction effectiveness between HAEC from the patients with thoracic aortic aneurysm associated with bicuspid aortic valve (BAV) and control HAEC (donor) by either NICD or TGFβ. (A) Expression of mesenchymal markers 3

days after the induction; (B) expression of mesenchymal markers 14 days after the induction; (C) expression of endothelial markers 7 days after the induction; (D) expression of SMA by immunocytochemical staining 14 days after the induction. Scale bar corresponds to 200 μm. mRNA level was determined by qPCR. The initial level of a given gene was normalized between different samples and the fold change was estimated. Groups are compared using Mann-Whitney non-parametric test; asterisks (*p<0.05) indicate differences between control donor and BAV groups.

To be activated Notch signaling pathway requires cell-to-cell contacts when transmembrane receptor of one cell binds transmembrane ligand of adjacent, but not the same cell, to induce cleavage of NICD. Whereas ligands activate Notch on the surface of neighboring cells, several studies show that ligands block Notch activity within the same cell (Li and Baker, 2004). Therefore "co-culture" model of EMT induction, when not all cells express ligands, seems to be more correctly model the situation in vivo than "direct" model.

We analyzed EMT induction in HAEC from donors and patients in co-culture with the ligand-expressing cells using the set of four Notch ligands **(Figure 16)**. We observed impaired ligand-induced upregulation of *HEY1* and *HES1* as well as *SNAIL1* and *SLUG* in diseased cells after 3 days of EMT induction **(Figure 16A)**. Expression of *HEY1*, *FSPS100A4*, *SLUG* and *ACTA2* also was not activated in HAEC of patients after 14 days of EMT induction **(Figure 16B)**. Staining for SMA confirmed observations revealed in gene expression assay **(Figure 16C)**. Our data confirm the findings obtained in direct EMT stimulation that the cells of BAV patients fail to activate EMT program in response to activation of Notch ligands.

Previously in our laboratory DNA sequencing of blood samples from BAV-TAA patients was performed to verify if any of the BAV-TAA patients had mutations in *NOTCH1* gene. It has been defined that none of the patients included in current study had mutations in exons of *NOTCH1*, previously implicated in BAV formation (Freylikhman et al., 2014).

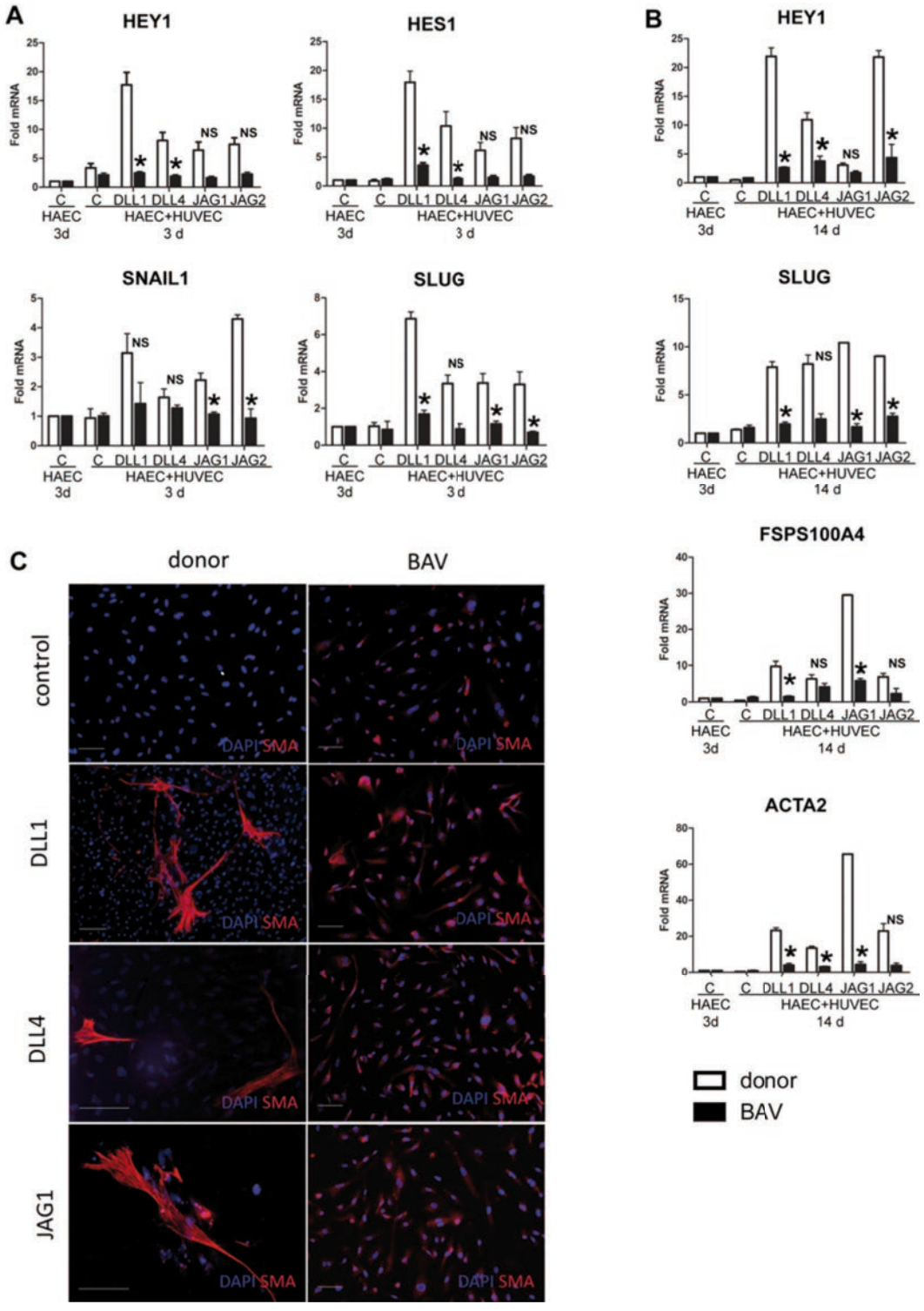


Figure 16. Comparison of endothelial-to-mesenchymal transition (EMT) induction effectiveness between HAEC from the patients with thoracic aortic aneurysm associated with bicuspid aortic valve (BAV) and control HAEC (donor) by co-culture with HUVEC expressing one of the ligands Dll1, Dll4,

Jag1 or Jag2. (A) Expression of EMT-responsive markers 3 days after the induction; (B) Expression of EMT-responsive markers 14 days after the induction; (C) expression of SMA by immunocytochemical staining. mRNA level was determined by qPCR. Groups are compared using Mann-Whitney non-parametric test; asterisks (*p<0.05) indicate differences between donor and BAV groups. Scale bar corresponds to 200 µm.

3.9 Notch1-Jagged1 feedback loop in HAEC of BAV patients and healthy donors.

Notch functions as a part of positive feedback loop in which Notch receptor activation promotes Notch ligand expression in surrounding cells thus relaying a signal, a process known as lateral induction (Ross and Kadesch, 2004). *JAG1* has been shown to be a direct transcriptional Notch target (Manderfield et al., 2012). Therefore we tested if this feedback loop is active in the cells of BAV patients. We transduced HAEC with NICD-bearing lentivirus and verified up regulation of Notch genes in control donor cells and in the cells from the patients **(Figure 17)**. Our data show that *JAG1* expression induced by NICD was substantially reduced in the cells of BAV patients.

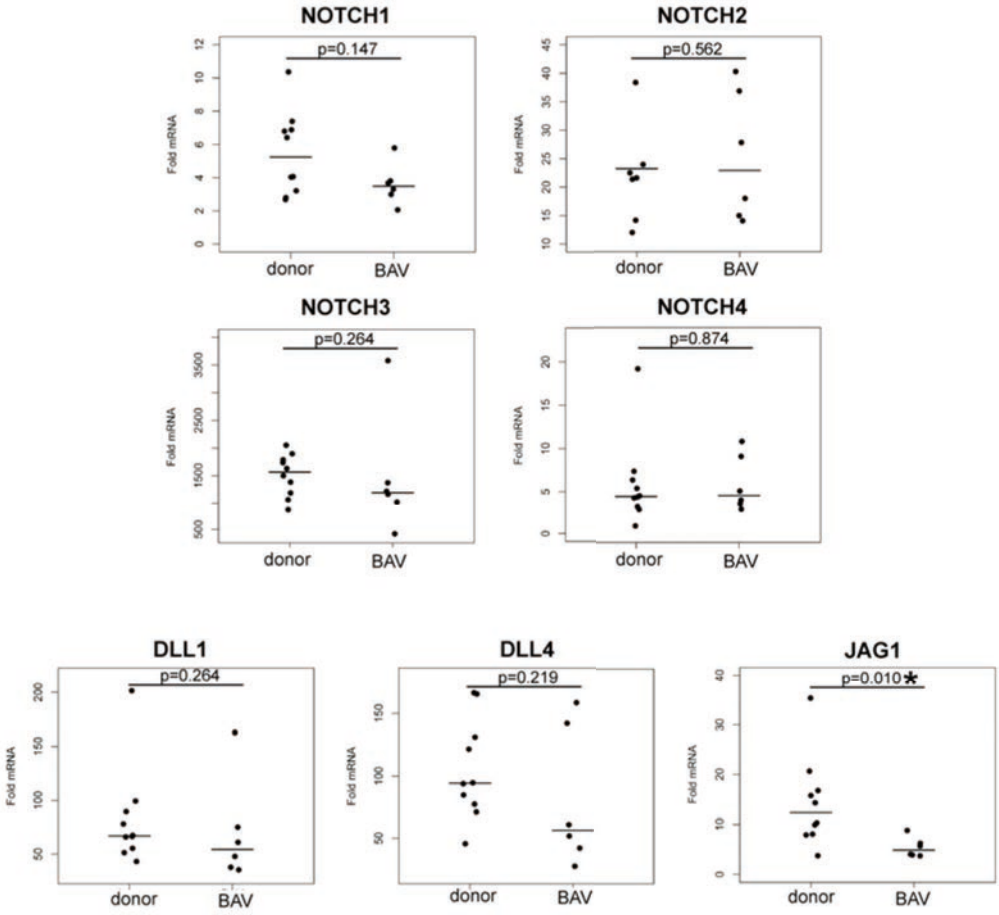


Figure 17. Lateral induction of Notch genes by NICD. HAEC from the patients with thoracic aortic aneurysm associated with bicuspid aortic valve (BAV, n=6) and control HAEC (donor, n=9) were transduced with NICD-bearing lentivirus and the expression of Notch family genes was verified after 72 h by qPCR. Groups are compared using the Mann–Whitney non-parametric test; asterisks (*p<0.05) indicate differences between donor and BAV groups.

3.10 The baseline level of several major signaling pathways in HAEC of TAA patients and healthy donors.

NOTCH1 haploinsufficiency as well as *NOTCH1* inactivation by siRNA in endothelial cells cause dysregulation in several major pathways, associated with a proper shear stress response, including antiosteogenic, antioxidant,

antiatherogenic and proinflammatory pathways (Theodoris et al., 2015; White et al., 2015). We observed obvious changes in expression of Notch related genes in HAEC of the patients with TAA, therefore we looked at the expression of key genes of the pathways highlighted in (Theodoris et al., 2015; White et al., 2015) and belonging to pathways that cross talk to Notch in order to better characterize HAEC from TAA patients **(Table 4)**.

Table 4. Genes of different pathways analyzed in the HAEC of the patients with TAA in comparison to the cells of healthy donors.

Gene	Pathway
NOTCH1	Notch ligands and receptors
NOTCH2	
NOTCH3	
NOTCH4	
DLL1	
DLL4	
JAG1	
HEY1	Notch targets
HES1	
SNAIL1	
CDC20	Cell cycle
CDCA2	
PDE2A	Phosphodiesterase 2
TXNRD1	Antioxidant
RASSF4	Ras effector
THSD1	Endothelial cell-cell interaction
EFNB2	Arterial specificity
GREM1	Anti-osteogenic
DKK1	
CYP1B1	Anti-atherogenic
COL15A1	Extracellular matrix
TCF4	Wnt effector
CXCL12	Pro-inflammatory
BMP2	Pro-osteogenic
MGP	Inhibitor of vascular mineralization
AXIN2	Wnt traget
SOX7	Pro-osteogenic Wnt related
POSTIN	Pro-osteogenic
TGFBR2	TGF-beta
STAT1	Jak/STAT
STAT6	
SMAD1	Wnt effector

SMAD6	
MMP2	Metalloproteases and inhibitors
MMP10	
MMP19	
MMP24	
TIMP1	
TIMP3	

We observed a decrease in expression of cell cycle genes *CDC20* and *CDCA2* in HAEC of TAV-TAA patients. mRNA level of TGFβ pathway related gene *TGFBR2* was elevated only in endothelium from BAV-TAA patients **(Figure 18)**. Surprisingly, expression of arterial marker and downstream Notch1 target *EFNB2* was not altered in the cells of the patients **(Supplementary Figure 1)**. Among about 30 genes of several pathways responsible for stress response in endothelium (Fernández Esmerats et al., 2016; Theodoris et al., 2015) we observed significant upregulation of proosteogenic BMP signaling effectors *DKK1* and *BMP2* in the cells of the patients; antagonizing BMP secreted factor *GREM1* was downregulated in HAEC from TAV patients. Genes of Wnt, such as effector *TCF4* and protein of anti-inflammatory IL-4 mediator *STAT6* were significantly upregulated in the cells of the TAA patients. Expression of antiatherogenic *CYP1B1* was in contrary decreased in both groups of the patients; expression of *TIMP1* and *TIMP3* was elevated in the HAEC of the patients. Expression of other genes represented in **Table 4** is shown in **Supplementary Figure 1**.

Our data suggest that HAEC of BAV- and TAV-TAA patients have dysregulated pathways responsible for a proper shear stress response.

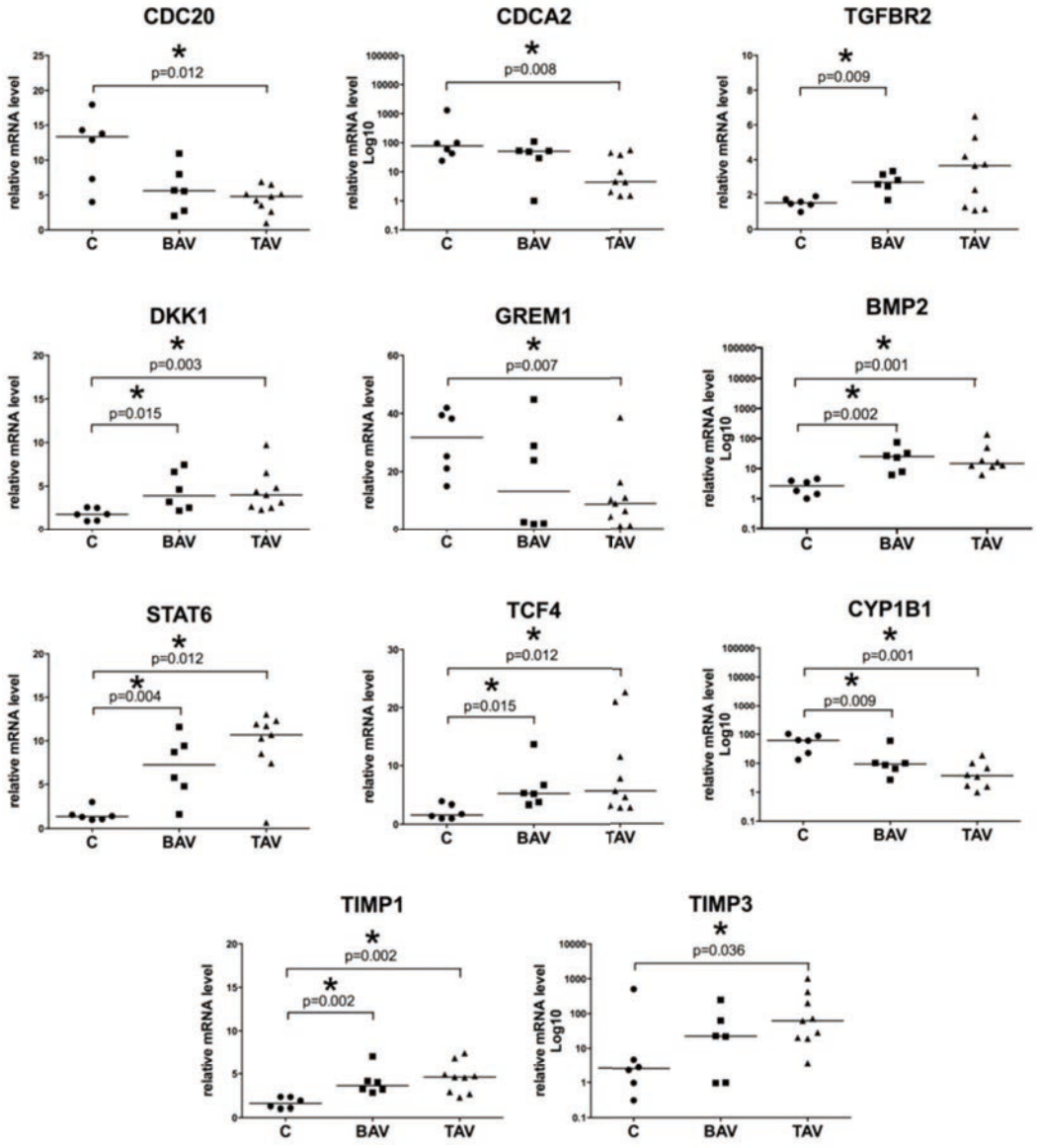


Figure 18. Genes dysregulated in the endothelial cells from patients with thoracic aortic aneurysm with either tricuspid aortic valve (TAV, n= 9) or bicuspid aortic valve (BAV, n= 6) and control HAEC (C, n= 6). Groups are compared using Mann-Whitney non-parametric test; line represents the median; asterisks (*p<0.05) indicate differences between donor and patient groups.

3.11 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 (Shao et al., 2005).

Since we observed differential expression of effectors of WNT/β-catenin, such as *BMP2, DKK1*, *STAT6* and *TCF4* in the cells of the patients we estimated the level of the whole WNT/βcatenin signaling in the diseased and healthy cells. We measured TCF/LEF promoter activity indicating transcriptional activation of Wnt/β-catenin pathway. The TOPflash reporter construct, in which expression of the firefly *luciferase* gene is regulated by six tandem TCF binding sites upstream of a minimal TK promoter (Madden et al., 1997), was used to measure TCF activity **(Figure 19A, B)**.

Canonical Wnt signaling promotes endothelial survival, junction stabilization, proliferation and is essential for vessel stability (Dejana, 2010). The canonical Wnt signaling mechanism involves suppression of Gsk3 activity and subsequent stabilization of β-catenin. To verify how activated WNT/β-catenin operated in the HAEC of the TAA patients in comparison to healthy donors we overexpressed S33A mutated stabilized β-catenin in the cells via lentiviral transduction or added a specific inhibitor of Gsk3 activity, CHIR99021, to the culture medium **(Figure 19)**. 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 HAEC of the patients with TAA and healthy donors. Secondly, the diseased cells also demonstrated a significant increase of the TCF-dependent luciferase activity in response to inhibition of Gsk3 by CHIR99021, but not by the S33A β-catenin alone **(Figure 19A)**. However, the fold activation was significantly lower in diseased cells comparing to control, possibly due to the high initial level of the signaling **(Figure 19B)**. The level of *AXIN2* expression, a direct WNT transcriptional target, reflects the same tendency, showing the failure of activation in response to WNT, either by S33A β-catenin or CHIR99021 **(Figure 19C)**. Thus, the WNT/β-catenin pathway activity is substantially elevated in the endothelial cells of TAA patients.

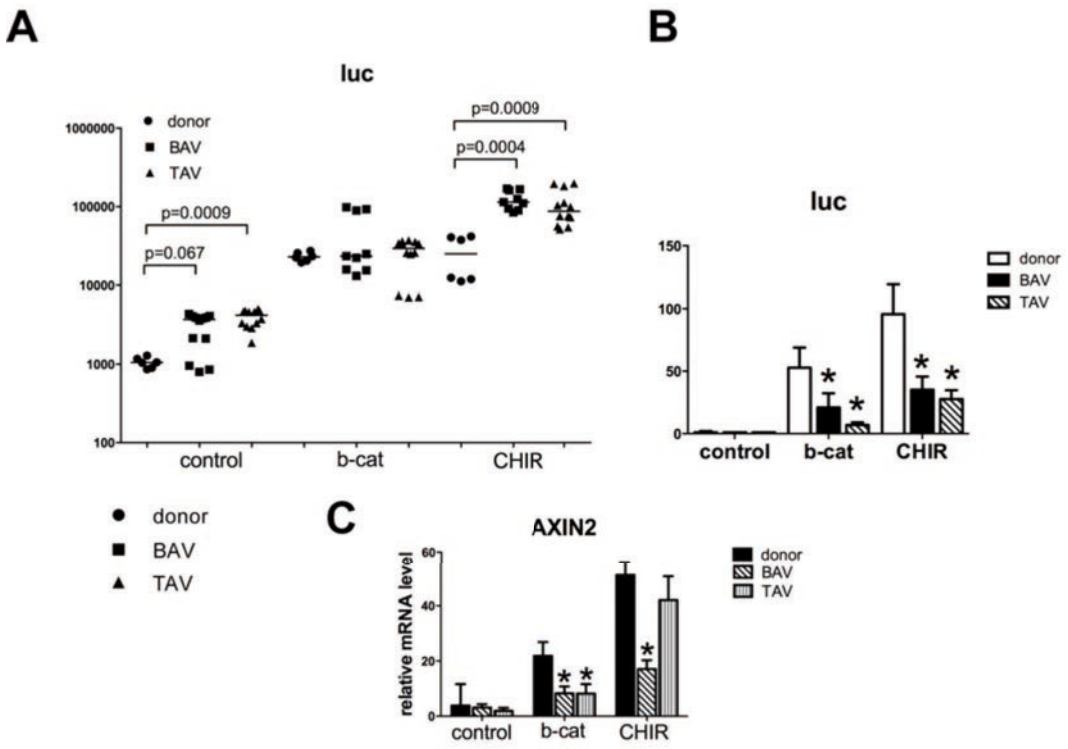


Figure 19. Wnt activity in the endothelial cells from patients with thoracic aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and control HAEC (donor). (A) Dotted graph represents the basal level of luciferase activity in the HAEC; (B) bar graphs represent fold change of luciferase activity in non-stimulated cells relative to the stimulated cells; (C) the mRNA level of direct WNT/β-catenin target *AXIN2* after Wnt activation. Groups are compared using Mann-Whitney non-parametric test; line represents the median; asterisks (*p<0.05) indicate differences between donor and patient groups.

WNT/β-catenin pathway has been reported to modulate endothelial Notch/Dll4 signaling in mouse development (Corada et al., 2010). To understand if the observed changes in Notch and Wnt activity in the endothelial cells of TAA patients were due to cross-talk between the pathways we assessed how activation of Wnt/β-catenin influences *DLL4* expression in adult HAEC **(Figure 20)**. 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. Both direct S33A β-catenin introduction and inhibition of Gsk3 activity decreased expression of *DLL4*, with more prominent effect of CHIR99021. *DKK1* expression 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 in the adult HAEC **(Figure 20)**. Thus, cross-talk and balance between Notch, Wnt and Wnt inhibitor Dkk1 ensure endothelial integrity in adult aortic endothelial cells. Correspondingly, *DLL4* mRNA level was lower in the HAEC of the patients comparing to healthy cells **(Figure 12)**.

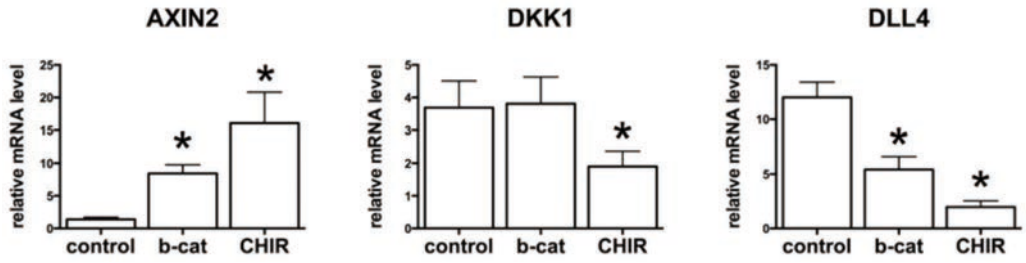


Figure 20. Cross-talk between Wnt and Notch pathways in adult human endothelial cells. Groups are compared using Mann-Whitney non-parametric test; asterisks (*p<0.05) indicate differences between non-stimulated (control) and stimulated cells.

3.12 Shear-stress response in HAEC of patients with TAA and healthy donors.

The above data suggest the dysregulation of BMP and WNT/β-catenin pathways in HAEC of patients with TAA. These pathways are known to be activated in response to cellular stress including shear stress (Theodoris et al., 2015). To reveal the difference in the expression of genes associated with stress response between diseased and healthy aortic HAEC, we compared the shear stress response of HAEC from patients and control cells to the following two types of shear stress: laminar flow imitating steady blood flow in the aorta and

oscillatory flow imitating disturbed flow also seen in the aorta especially with BAV. Endothelial cultures before and after both types of flow experiments are shown in **Figure 21.**

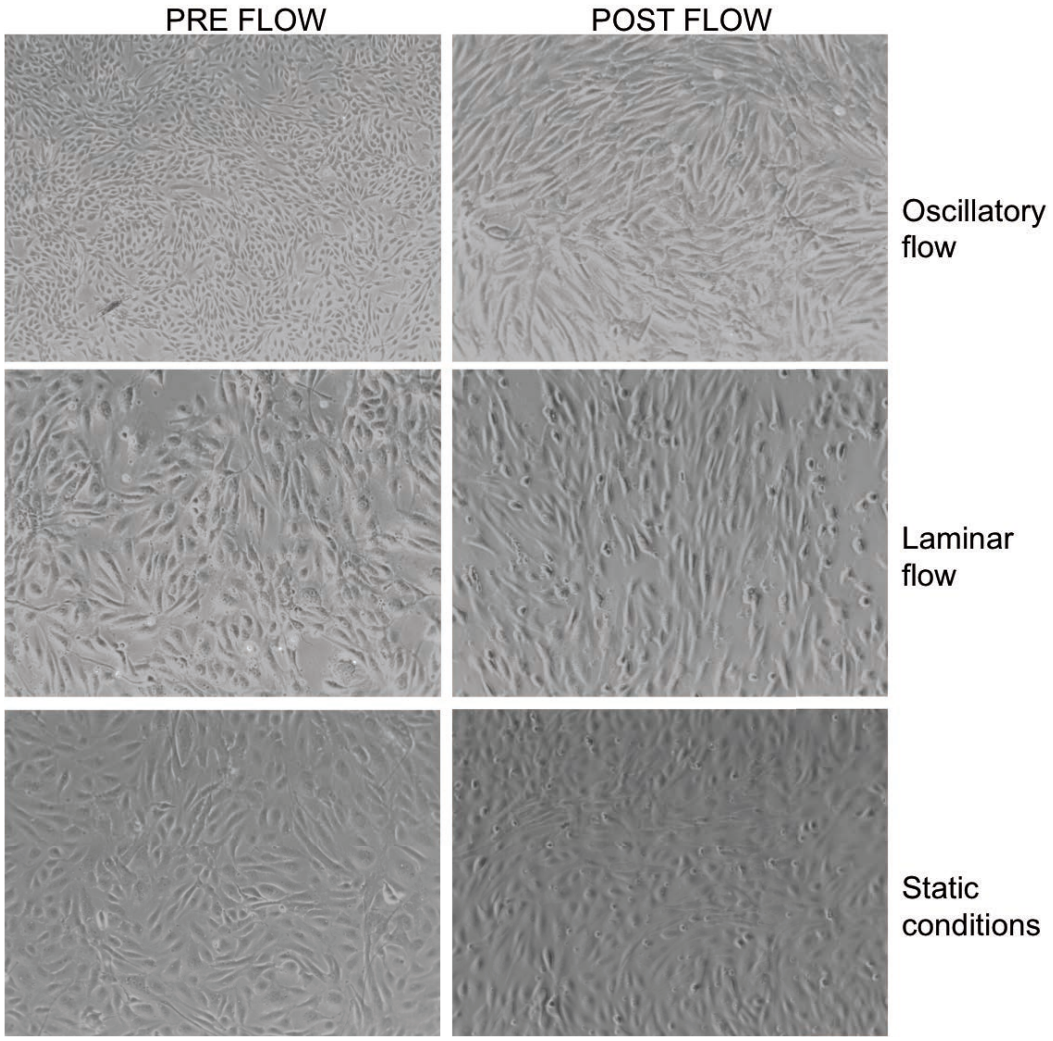


Figure 21. Control endothelial cell cultures prior and after exposure to oscillatory or laminar flow. Magnification 5x.

We analyzed expression of Notch/BMP/WNT/β-catenin related genes in diseased and control HAEC. The response of normal HAEC to both types of flow is shown in **Figure 22**. Both types of flow show similar tendency in activation shear-stress responsive genes in control HAEC with more prominent activation by oscillatory flow.

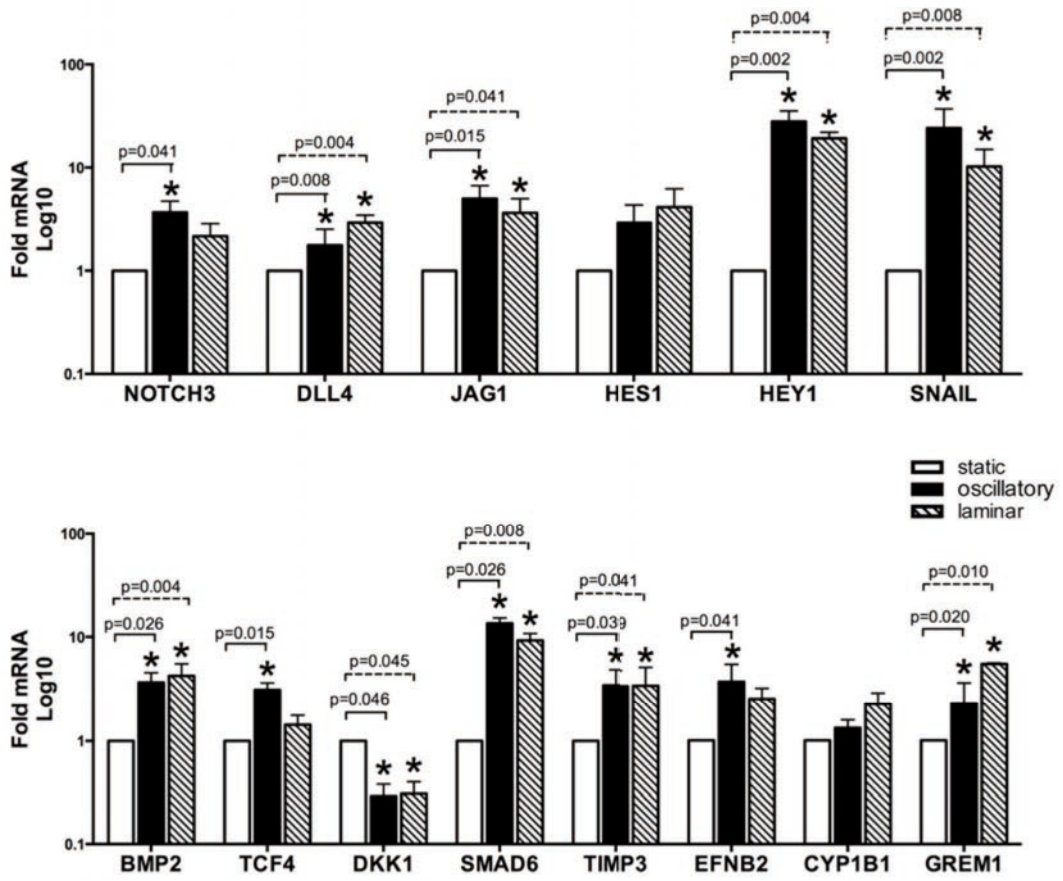


Figure 22. Genes that were up or down regulated in response to oscillatory or laminar flow in control HAEC. Groups are compared using Mann-Whitney non-parametric test; asterisks (*p<0.05) indicate differences between non-stimulated (static, n= 6) and flow-stimulated cells (oscillatory, n= 6; laminar, n= 5).

A comparison between the activation of Notch/BMP/WNT/β-catenin related genes in cells of the patients and donors showed the most striking differences in the expression of *DLL4*, *SNAIL1* and *BMP2*, *DKK1*, *TCF4* as a result of exposure to oscillatory or laminar shear stress **(Figure 23)**. *DLL4* and *SNAIL1* were not up regulated to the same level as in donor cells in both BAV and TAV HAEC in response to oscillatory flow. Whereas these genes were not activated in response to laminar flow only in TAV HAEC.

DKK1 and *BMP2*, as well as Wnt effectors *STAT6* and *TCF4*, were already elevated in non-stimulated HAEC of the patients **(Figure 18)**. *BMP2* and *TCF4*

expression was upregulated at both type of flow in control cells while we did not observe any fold change in *BMP2* and *TCF4* expression after flow in the diseased cells, possibly, due to elevated baseline level of these genes. *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 and remained higher in the cells of the patients. On the contrary, we observed elevation of *BMP2* expression by flow in donor cells while no change was observed in the flow-stimulated diseased cells **(Figure 23)**. We conclude that stress response is attenuated in the endothelial cells of TAA patients**.**

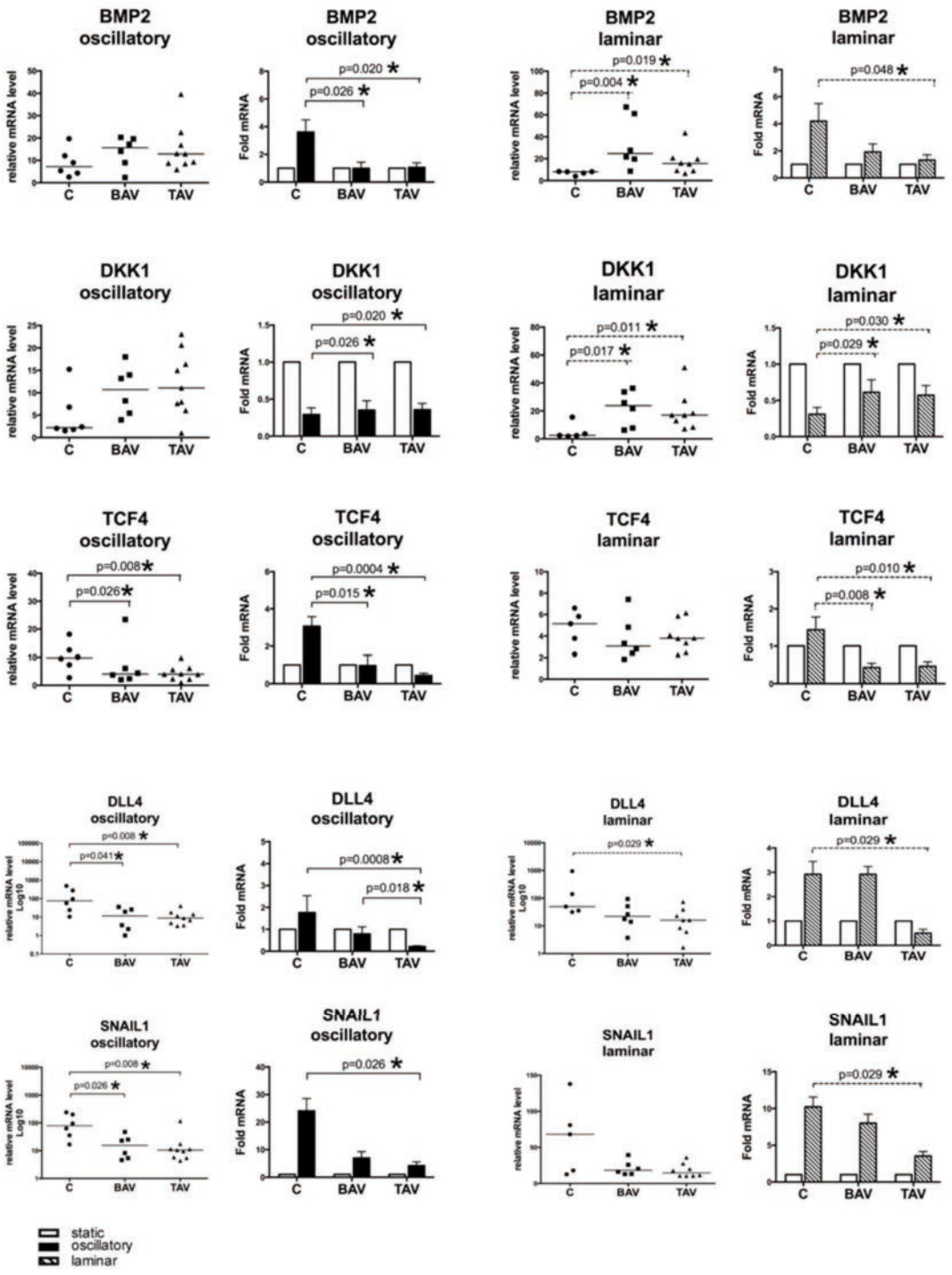
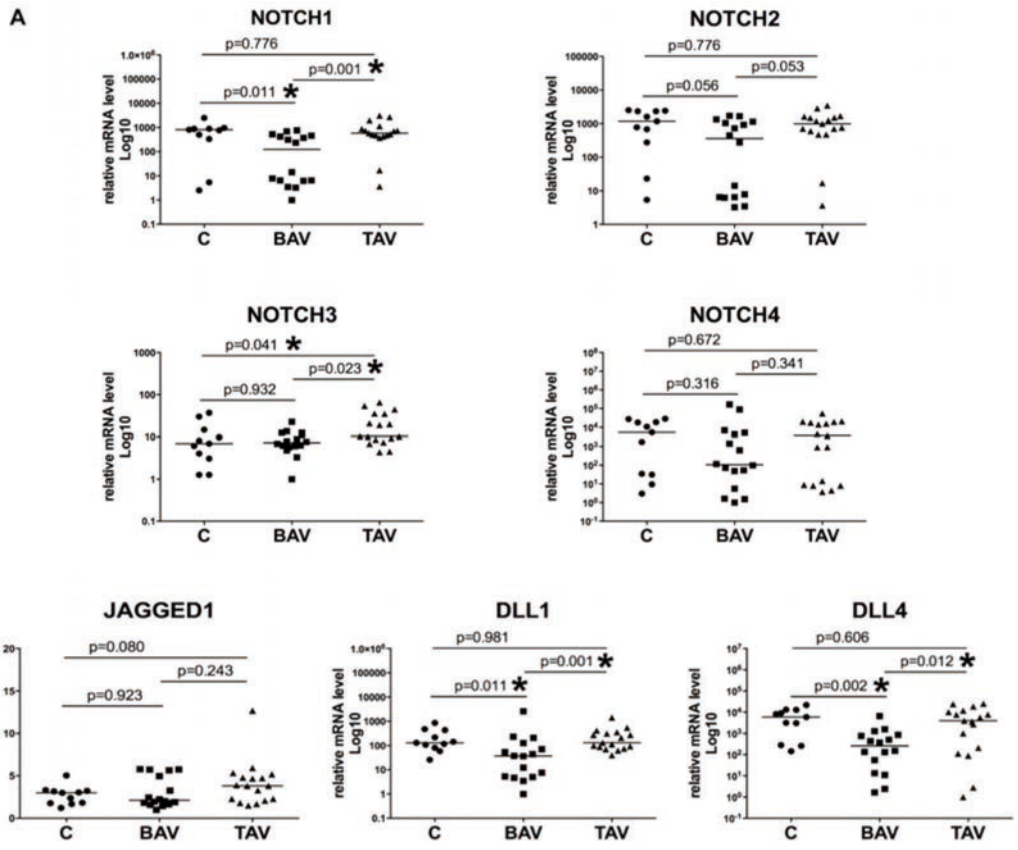


Figure 23. Shear stress response in the endothelial cells from patients with thoracic aortic aneurysm with either tricuspid aortic valve (TAV, n= 9) or bicuspid aortic valve (BAV, n= 6) and control HAEC (C, n= 6). Dotted graphs represent relative mRNA level in the cells subjected to the flow estimated by qPCR. Bar graphs represent fold change of mRNA level in non-

stimulated cells (static) to the level in the flow-stimulated (laminar/oscillatory) cells. Groups are compared using Mann-Whitney nonparametric test; line represents the median; asterisks (*p<0.05) indicate differences between control and patient groups.

3.13 Expression of Notch receptors and ligands in cells from calcified and healthy valves.

We used the cells isolated from the patients with calcific aortic stenosis with either tricuspid (TAV) or bicuspid (BAV) morphology of the aortic valve. The control samples (C) were isolated from healthy tricuspid valves because of the low occurrence of bicuspid aortic valve in the population. We measured the expression of key genes of the Notch pathway, namely *NOTCH1-4* as well as Notch ligands *JAG1*, *DLL1*, *DLL4* in human VIC and VEC **(Figure 24)**. In VIC expression of *NOTCH1*, *DLL1*, and *DLL4* were decreased in BAV compared to both TAV and controls whereas *NOTCH2* was decreased with borderline significance. *NOTCH3* expression was increased in TAV compared to both healthy valves and BAV **(Figure 24A)**. Surprisingly, none of these parameters were altered in VEC in either BAV or TAV group comparing to control group **(Figure 24B)**. Our data show dysregulation of Notch components at baseline expression level in interstitial cells from patients, mostly with BAV.


A
NOTCH1
p=0.776
p=0.001 *
p=0.011 *
relative mRNA level Log10
C
BAV
TAV
NOTCH2
p=0.776
p=0.053
p=0.056
NOTCH3
p=0.041 *
p=0.023 *
p=0.932
NOTCH4
p=0.672
p=0.341
p=0.316
JAGGED1
p=0.080
p=0.243
p=0.923
DLL1
p=0.981
p=0.001 *
p=0.011 *
DLL4
p=0.606
p=0.012 *
p=0.002 *

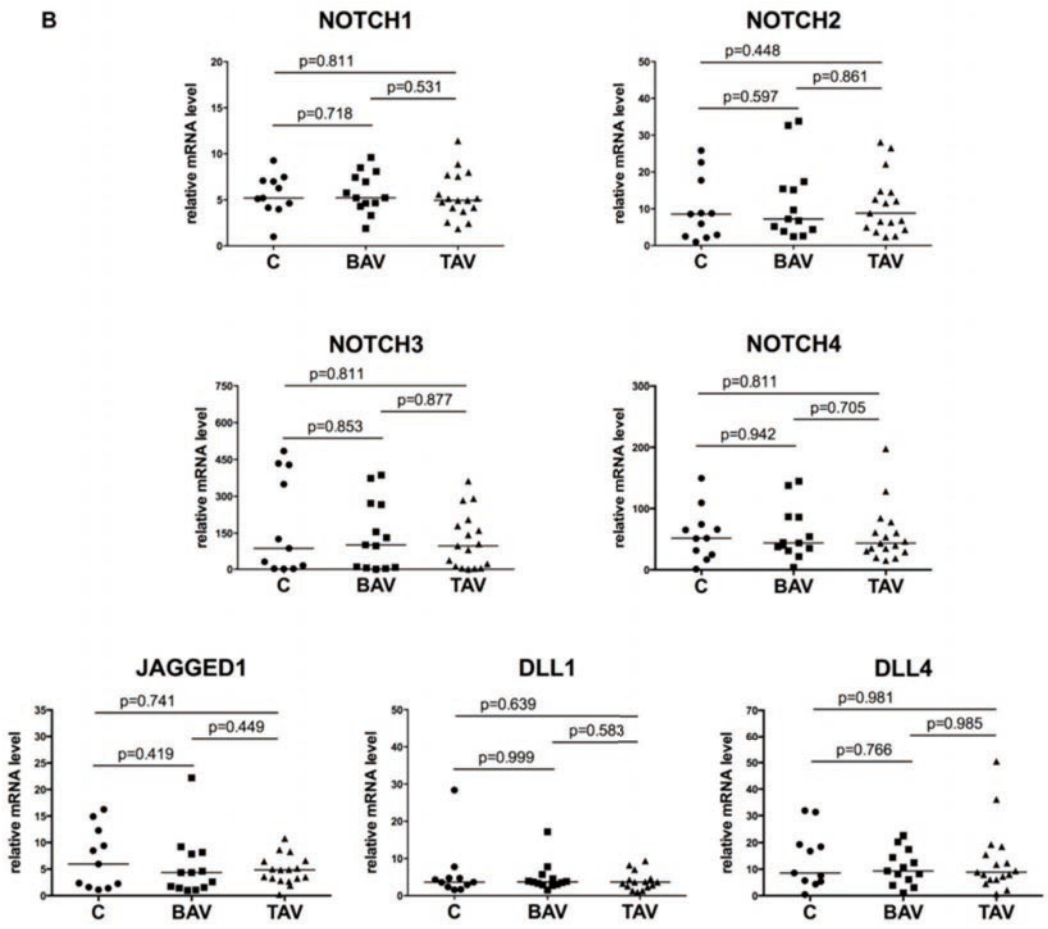


Figure 24. Expression level of Notch receptors and ligands in the valve interstitial cells VIC (A) and endothelial cells VEC (B) from the patients with calcific aortic stenosis with either tricuspid aortic valve (TAV, n= 17) or bicuspid aortic valve (BAV, n= 13) and from healthy control valves (C, n= 11). mRNA level was determined by qPCR. Groups were compared using Mann-Whitney non-parametric test; line represents the median; *p<0.05.

3.14 Induced calcification in interstitial cells from tri- and bicuspid aortic valves.

We compared the sensitivity to osteogenic stimuli of VIC from the patients and healthy controls **(Figure 25)**. Twenty-one day after stimulation with osteogenic medium calcification occurred in diseased VIC as shown by strong

Alizarin Red staining, but without any difference between VIC from BAV and TAV. No Alizarin Red staining was seen in VIC from healthy valves.

Expression of genes that might be involved in calcification was measured in VIC by qPCR after stimulation with osteogenic medium: *BMP2* (bone morphogenetic protein 2), *RUNX2* (runt related transcription factor 2), *POSTIN* (periostin), *CTNNB1* (beta-catenin), *SOX9* (sex determining region Y0-box 9), *OPN* (osteopontin), *SPRY1* (Sprouty1). *RUNX2* and *SPRY1* were lower in unstimulated VIC from BAV patients as compared to healthy controls. However, unstimulated VIC from TAV patients were not different from healthy controls. *OPN* expression was higher in non-stimulated VIC from TAV than in BAV and in controls. There were no differences between unstimulated cells derived from either BAV, TAV or controls regarding *BMP2*, *POSTIN*, *CTNNB1*, and *SOX9*.

After 21 days of osteogenic induction *RUNX2* and *SPRY1* expression increased in all three groups comparing to non-stimulated cells, but without difference between groups. *SOX9* was upregulated only in TAV after osteogenic stimulation. *BMP2* was higher in TAV than in BAV and controls after osteogenic stimulation. *POSTIN* and *CTNNB1* showed neither differences between groups nor changes after stimulation with osteogenic medium.

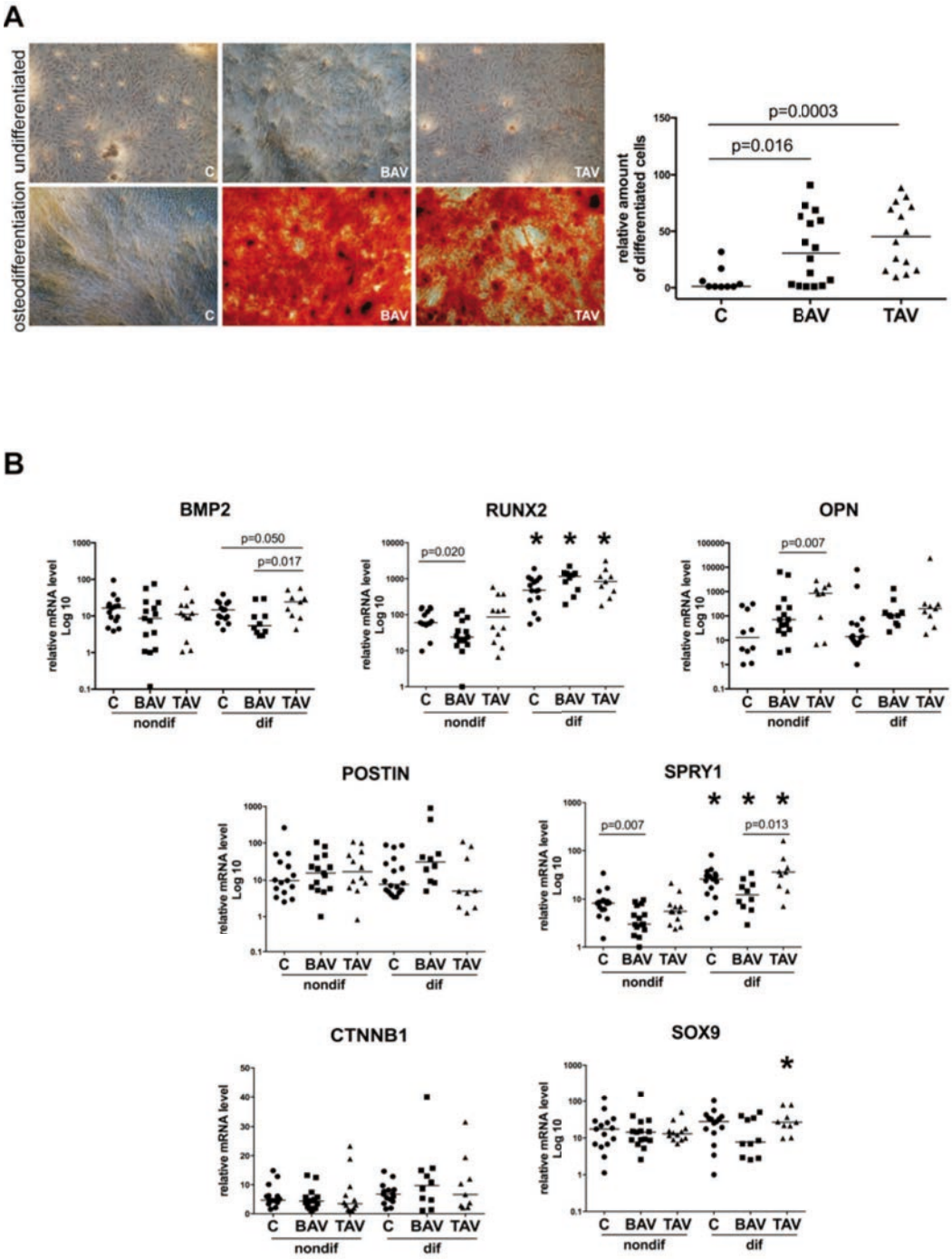


Figure 25. Sensitivity of VIC from the patients with calcific aortic stenosis with either bicuspid aortic valve (BAV, n= 13) or tricuspid aortic valve (TAV, n= 17) and from healthy control valves (C, n=11) to osteogenic stimulation. (A) Left panel shows representative image of VIC differentiation with calcification revealed by red color. Right panel represents digital quantification of calcium deposition. (B) mRNA expression by qPCR of

proosteogenic genes in the BAV, TAV and control groups of VIC. The groups were compared using Mann-Whitney non-parametric test, line represents the median. P-value for the differences between the groups is given only for the significant differences (*p<0.05). Asterisks indicate the significant differences (*p<0.05) of mRNA content between undifferentiated and differentiated cells for a given group (BAV, TAV or control, correspondingly).

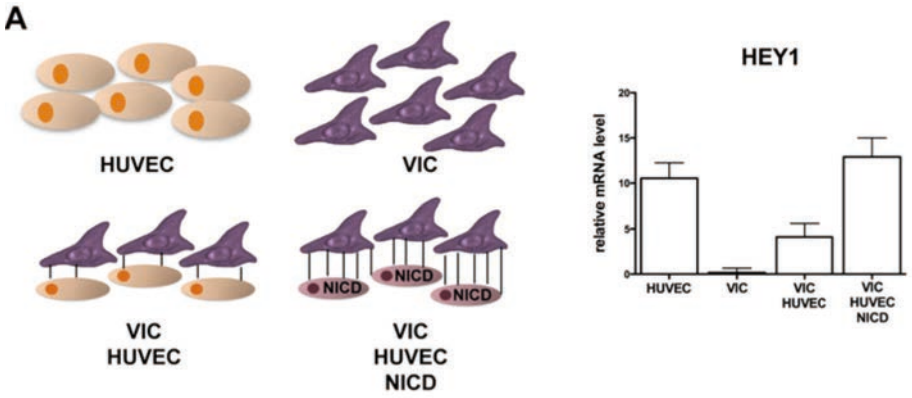
3.15 Notch-dependent initiation of osteoblast differentiation in interstitial cells from tri- and bicuspid aortic valves.

As we observed Notch genes alterations in the VIC of BAV patients with aortic stenosis, we asked whether alteration in Notch signaling activity could differently influence osteogenic signaling in BAV and TAV derived VIC.

To assess if the initial mechanisms of calcification in BAV and TAV-derived VIC are dependent on Notch signaling we used co-culture of VIC with human umbilical endothelial cells (HUVEC). These co-culture experiments model the situation *in vivo* when endothelial cells communicate with mesenchymal cells via Notch signaling (Lilly, 2014; Lin and Lilly, 2014a). HUVEC, not VEC, were used because they give more uniform endothelial cell cultures. Notch was activated in HUVEC by transduction with lentiviruses bearing Notch Intracellular Domain (NICD). The next day the HUVEC were seeded on VIC **(Figure 26A, left panel)**. *HEY1*, main Notch target, was elevated in co-cultures and this elevation was more prominent when Notch was activated in endothelial cells **(Figure 26A, right panel)**. The day after start of co-culture we induced osteogenic differentiation by addition of osteogenic medium. The early expression of Notch target *HEY1* and proosteogenic markers was measured in the co-cultures after three days of exposure to osteogenic medium in the absence (control) or presence of Notch activation **(Figure 26B)**. Expression of *HEY1* was similar in all three groups without Notch activation. However, when Notch was activated, *HEY1* was the highest in the co-cultures that contained BAV-derived VIC. Co-cultures with VIC from BAV patients demonstrated significantly higher expression of *OPN*, *ALP* and *POSTIN* after osteogenic stimulation. Activation of

Notch caused even higher elevation of *OPN*, *ALP* and *POSTIN*, which was significantly higher in the co-cultures containing BAV derived VIC. The expression of the other proosteogenic genes was not responsive to changes in Notch activity.

Increased alkaline phosphatase 10 days after the initiation of osteogenic stimulation confirmed that Notch activation induced osteogenic phenotype more readily in BAV-derived co-cultures with more intensive staining of the co-cultures containing VIC derived from BAV patients and activated Notch **(Figure 26B, lower panel)**.



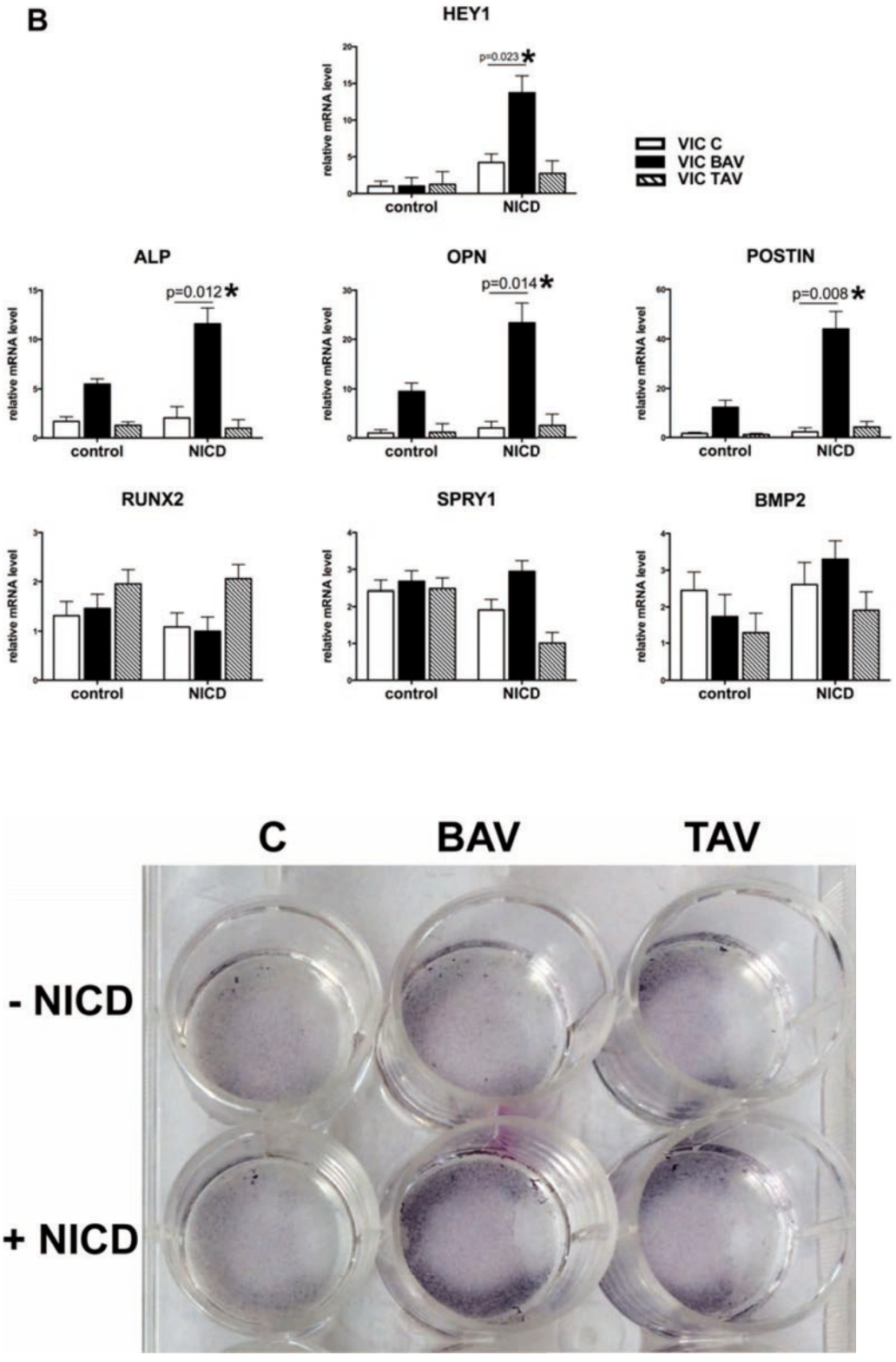


Figure 26. Notch activity distinctly influences osteogenic differentiation of VIC of patients with calcified bicuspid aortic valve (BAV, n= 5) and tricuspid aortic valve (TAV, n= 5). (A) Activation of *HEY1* expression in co-cultures of

VIC from normal valves with HUVEC with or without activation of Notch. (B) Upper panel: level of proosteogenic gene transcription (estimated by qPCR). The groups were compared using Mann-Whitney non-parametric test. P-value for the differences between the groups is given; *p<0.05. Lower panel: alkaline-phospatase staining.

3.16 Notch-dependent endothelial-to-mesenchymal transition in endothelial cells from tri- and bicuspid aortic valves.

Recently it has been shown that endothelial-to-mesenchymal transition (EMT) precedes the onset of calcification in valve cells (Hjortnaes et al., 2015). We studied Notch-dependent EMT activation in VEC derived from BAV and TAV patient and healthy valves. For Notch-induced EMT we used a previously described model with introduction of NICD into VEC by lentiviral transduction (Kostina et al., 2016). Expression of Notch-responsive genes *HEY1* and *SLUG*, which activate the initial stage of EMT, was measured after three days **(Figure 27).** In VEC from BAV expression of both *SLUG* and *HEY1* was higher than in TAV and healthy controls. After 10 days *ACTA2* expression encoding αSMA, the definitive EMT marker, was not different between BAV and TAV, but was significantly less than in control cells. Immunocytochemical staining of control cells for αSMA showed well differentiated cells that undergone EMT and terminated by appearance of true mesenchymal cells with nice actin fibers **(Figure 27).** At the same time endotelial cells from BAV and TAV patients failed to form actin fibers properly that suggested failure of EMT process.

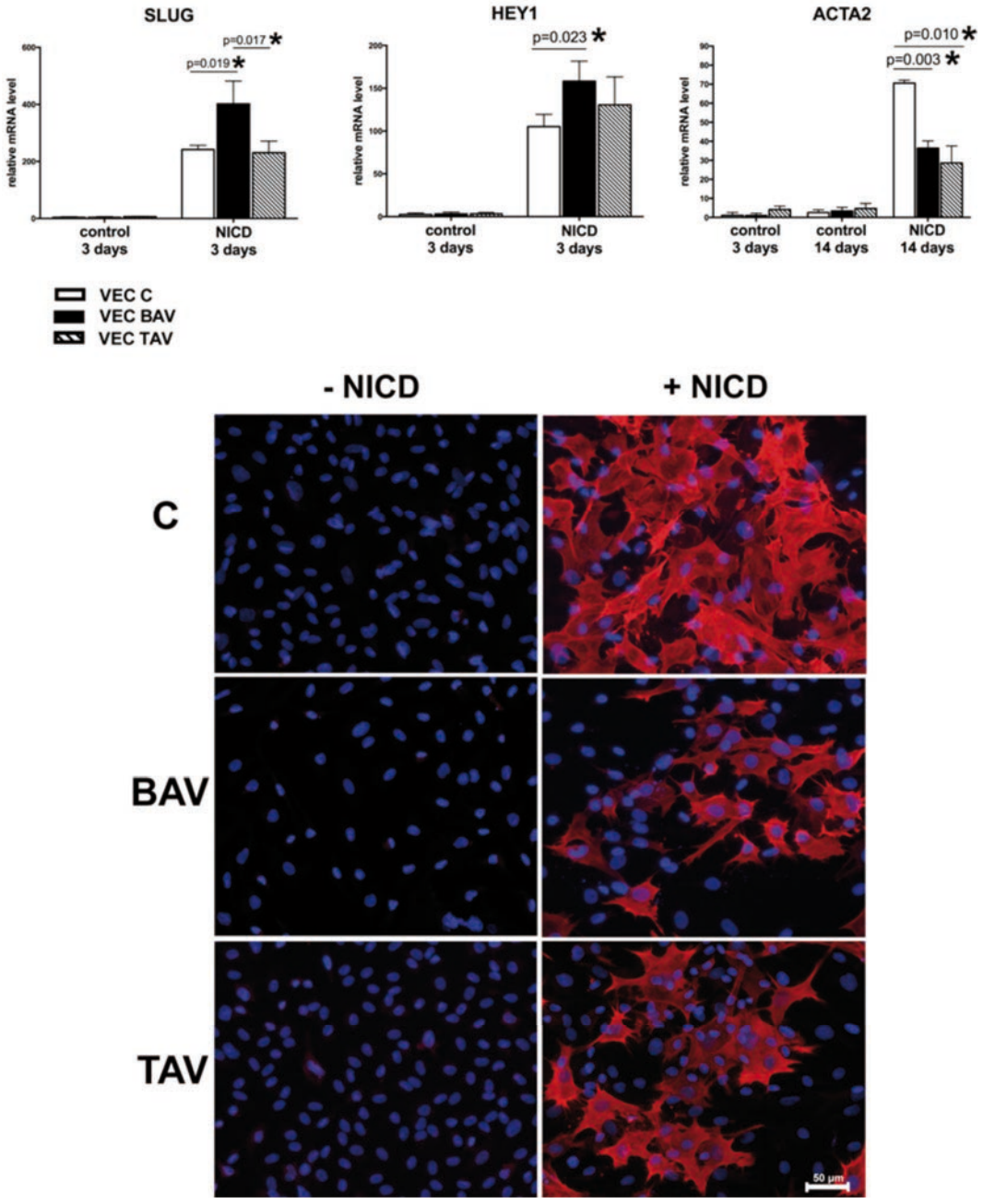


Figure 27. EMT initiation in the VEC derived from the patients with calcified BAV and TAV or control cells derived from healthy valves (C). Gene expression was analysed by qPCR (upper panel). Immunocytochemical staining of the cells with antibody against αSMA (lower panel). Scale bar corresponds to 50 µm. The groups were compared using Mann-Whitney non-parametric test. P-value for the differences between the groups is given; *p<0.05.

4. Discussion.

Notch pathway is indispensable for heart development and maintenance of cardiovascular system during postnatal life. Mutations in Notch components are associated with a number of congenital defects of the left ventricular outflow tract, particularly with bicuspid aortic valve, but the mechanisms of realization of genetic mutations remain to be defined. This study provides the first direct functional evidence that primary aortic and valvular cells from patients with left ventricle outflow tract pathologies have impaired Notch signaling pathway comparing to healthy donors.

4.1 Endothelial and smooth muscle cells in thoracic aortic aneurysm.

Thoracic aortic aneurysm (TAA) may be associated with either bicuspid or tricuspid aortic valve (BAV or TAV, respectively). Whether BAV-related aortic dilatation is a consequence of a constant stress due to valve configuration or it is a consequence of a developmental mistake is widely discussed. Moreover, no additional defects are revealed in some patients and aneurysm develops at TAV background. Aortic wall integrity depends on individual functions of endothelial (ECs) and smooth muscle cells (SMC) regulated by their mutual communication. Healthy cellular properties are changed in the pathology, apparently due to altered intercellular interactions.

We addressed phenotypic and functional properties of cellular components of the aortic wall from patients with TAA and from healthy donors (Malashicheva et al., 2016). We observed decreased SMC proliferation rate in both patient groups. Previously it has been demonstrated that the cells from BAV-TAA patients proliferate more slowly than those from healthy donors (Blunder et al., 2012). Thus, in addition to other similar data our results indicate that SMC have reduced proliferative potential in TAA patients both with BAV and TAV. These data explain the observations that the SMC number in aortic wall samples in TAA patients is less than in healthy donors (El-Hamamsy and Yacoub, 2009).

SMC from thoracic aneurysms demonstrated decreased expression of key SMC proteins such as SMA, SM22α and vimentin. These data suggest that the cells from the aneurysm aortic wall are in less differentiated state comparing to

normal aortic wall. This finding is in line with the studies that reported changes in SMC functionality during TAA formation (Della Corte et al., 2008; Forte et al., 2013; Ignatieva et al., 2017). Of most importance, we revealed the primary changes of endothelial cells from TAA patients. Our findings are in agreement with a recent publication describing endothelial dysfunction in BAV patients (Ali et al., 2014; Jia et al., 2017).

Notch pathway is an important regulator of SMC (Boucher et al., 2012; Domenga et al., 2004; Liu et al., 2010). We revealed altered baseline level of Notch signaling in endothelial cells of TAA patients in comparison to endothelial cells of healthy persons. This observation is well in line with the findings that endothelial cells influence differentiation and functions of underlying SMC through Notch signaling (High et al., 2008; Lin and Lilly, 2014b; Pedrosa et al., 2015). Endothelial-expressed Jag1 has been shown to induce differentiation and maturation of SMC via Notch3 activation during vessel wall constructing (Hoglund and Majesky, 2012; Liu et al., 2009). Expression of *JAG1* was attenuated in EC of TAV-TAA that may reflect the impaired SMC differentiation and maturation resulted in TAA formation irrespectively of BAV.

Cultured SMC from aneurysm patients demonstrated surprisingly high amounts of annexin V positive cells suggesting a high level of apoptosis in the aortic wall of aneurysm patients (Della Corte et al., 2008). Surprisingly, the SMC of TAA patients were relatively resistant to apoptosis induced by H_2O_2. Philippi and coauthors showed that SMC from aneurysms in BAV patients had the poorest resistance to oxidative stress, but they did not show a baseline level of apoptosis in their SMC cultures (Phillippi et al., 2009). Notch pathway has been reported to suppress apoptosis and promote cell survival (Baeten and Lilly, 2015; Li et al., 2009; Sweeney et al., 2004; Wang et al., 2012). Apoptosis/proliferation rate is a very important parameter of a cellular population turnover. We suggest that both decreased proliferation rate and increased apoptosis contribute to the loss of cells in the aortic wall in aneurysms.

Comparison of the content of some extracellular matrix proteins in aortic tissue samples, SMC lysates and supernatants from SMC and endothelial cells revealed that SMC are not the only cells that synthesize extracellular matrix

proteins in the aortic wall. Also endothelial cells are capable of synthesizing extracellular matrix proteins in aneurysms. Finally, the integrity of the extracellular matrix in the aortic wall of aneurysmal patients is influenced by changes in the ratio between different proteins. Both endothelial cells and SMC from aneurysm patients had synthesis of fragmented collagen I. The complex nature of biosynthesis of extracellular matrix proteins in the aneurysmal aortic wall is in agreement with other studies (LeMaire et al., 2005; Phillippi et al., 2014; Rabkin, 2014). The ability of endothelial-derived Notch signaling to promote collagen synthesis and other markers in SMC has been demonstrated in several studies (Lilly and Kennard, 2008; Lin and Lilly, 2014b). This may be a recapitulation of the developmental program in early vessel development, wherein Notch induces ECM synthesis (Baeten and Lilly, 2017). Apparently, attenuated ECM protein content in SMC of TAA patients is affected by impaired Notch signaling in TAA ECs.

Recent findings show that the gene profiles in TAA patients with BAV and TAV differ, which indicates the various etiologies for TAA (Folkersen et al., 2011; Kjellqvist et al., 2013). Ignatieva et al. demonstrated that SMC from BAV and TAV patients form distinct clusters by expression of Notch-related and proosteogenic genes (Ignatieva et al., 2017). In addition, BAV-derived SMC were more responsive to Notch activation by *ACTA2* transcription (Ignatieva et al., 2017). Our findings regarding different extracellular matrix composition and the migration rate in TAV- and BAV-TAA support the idea that the mechanisms underlying the TAA development are distinct between the two patient groups (Balistreri et al., 2013; Phillippi et al., 2014).

We suggest that the initial process of the vessel formation as well as further healing in response to constant mechanical stress in the aorta could be impaired in TAA patients via Notch-dependent events in particular through inactive feedback loop connecting ECs and SMC and their mutual influence on cellular functional state.

4.2 Notch pathway and endothelial-to-mesenchymal transition.

The most known genetic link in BAV pathology is mutations in *NOTCH1* described in patients with BAV (Garg et al., 2005; McBride et al., 2008; McKellar et al., 2007). Although BAV has strong evidence of a genetic basis, successful identification of disease-causing variants has been limited. Several studies did not find considerable amount of mutations in Notch-related genes in a cohort of patients where previously *NOTCH1* mutations had been described (Bonachea et al., 2014; Campens et al., 2015; Martin et al., 2014).

We have shown that aortic endothelial cells derived from the BAV patients with dilated aorta have impaired EMT induction (Kostina et al., 2016). This impairment could potentially reflect two aspects of the associated aortopathy. First aspect regards the failure to undergo EMT during embryonic development, which is necessary for the proper valve formation (Kovacic et al., 2012). Alternatively, because EMT is described as a stress-induced mechanism in aortic valve, the failure to activate EMT program could lead to valve calcification (Balachandran et al., 2011; Kovacic et al., 2012). A recent work has shown that Notch1 haploinsufficiency disrupts the appropriate endothelial response to shear stress. In contrast to wild-type endothelial cells, shear-exposed *NOTCH1*$^{+/-}$ endothelial cells showed derepression of latent pro-osteogenic and pro-inflammatory gene networks and failed to up regulate anti-osteogenic and anti-inflammatory factors that may be critical for preventing calcification of underlying interstitial cells (Theodoris et al., 2015). Similarly, our results suggest that BAV-derived aortic endothelial cells fail to up regulate Notch-induced EMT. We suggest that this reflects a common stress-defense mechanism, which is impaired in the diseased cells.

We also observed significant down regulation of *NOTCH4* and *DLL4* in the diseased endothelial cells. Dll4 is the first Notch ligand expressed in the forming arteries in the mouse, and *DLL4* expression precedes that of the genes encoding arterial Notch receptors, *NOTCH1* and *NOTCH4* (Chong et al., 2011). Our findings are well in line with recent reports describing fine tuning of Dll4/Notch4 axis and its participation in arterial specification, implying that this axis might be disrupted in BAV-patients and this impaired arterial specification could contribute

to aortic aneurysm development often associated with BAV (Wu et al., 2014; Wythe et al., 2013).

In a recent paper it has been shown that Notch4 agonistic antibody in mice accelerate the healing response increasing amount of SMC in the vessel (Pedrosa et al., 2015). Also this paper demonstrates the proangiogenic role of endothelial Jag1 in adult physiological angiogenesis and the synergistic roles of endothelial Jag1 and Dll4 on vascular maturation. We show that *JAG1* expression induced by NICD was substantially reduced in the BAV-derived endothelial cells and this was associated with downregulation of smooth muscle cell contractile markers in SMC of the aneurysmal wall. We suppose that the response to constant shear stress in the aorta with BAV could be impaired in the BAV patients via Notch-dependent events in particular through inactive feedback loop between Notch1 and Jag1.

In our experiments all the ligands (Dll1, Dll4, Jag1, Jag2) failed to up regulate EMT response in the cells of BAV-patients. However, both Dll1 and Dll4 were able to up regulate *SNAIL* almost to its level in healthy cells. This result demonstrates the complexity of Notch signaling links and also represents Dll1 and Dll4 ligands as potential candidates for the up regulation the Notch system in the diseased cells as it has been already described in other pathologies (Rizzo et al., 2015). However it is clear that the system is extremely dosage-dependent and fine-tuned, thus further studies of endothelial-SMC regulation are needed to understand its function (Xiao and Dudley, 2017).

4.3 Endothelial shear-stress response.

Recent findings suggest that Notch signaling in the valve endothelium is uniquely positioned to mediate the anti-calcific response to shear stress within the valve (Combs and Yutzey, 2009; Masumura et al., 2009; White et al., 2015). Our data support this idea for endothelial cells of the ascending aorta as well. It is clear, that signaling mechanisms do not operate in isolation but that they are integrated into signaling networks. The list of signaling pathways that modulate a given component of the Notch signaling system is already abundant and is likely to be expanded in the coming years (Andersson et al., 2011). In this study we also

show that endothelial cells from patients with TAA along with Notch have changed activity of several important shear stress response pathways such as Wnt/β-catenin and BMP comparing to the cells of healthy donors. In addition, we report attenuation of shear stress response in the ECs of the patients with TAA.

Our data on increased expression of Wnt effectors *GREM1* and *DKK1* and proosteogenic *BMP2* as well as decreased expression of *CYP1B1* in the diseased cells are well in agreement with the data obtained on *NOTCH1+/-* iPS derived endothelial cells (Theodoris et al., 2015).

We observed a strong elevation of Wnt/β-catenin signaling in the diseased ECs. In the vasculature, the Wnt/β-catenin pathway controls vascular stability through remodeling, junction assembly, and pericyte recruitment (Reis and Liebner, 2013). The sequential and parallel interactions between the BMP and Wnt signaling controls mineralization, with intracellular/extracellular fine-tuning of signal duration and strength (Bostrom et al., 2011). *DKK1* expression, a Wnt/β-catenin antagonist, was significantly elevated in the diseased cells. Activation of *DKK1* has been associated with endothelial integrity (Li et al., 2016).

Fluid shear stress is involved in stem cell and mesenchymal progenitor differentiation into vascular ECs and plays an important role in endothelial homeostasis (Obi et al., 2008; Resnick and Gimbrone, 1995; Wang, 2005; Yamamoto, 2004). In response to application of laminar or oscillatory shear stress a differential expression of *DLL4, SNAIL, BMP2, DKK1* and *TCF4* 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 and proper differentiation state of endothelial cells as has been reported by others (Corada et al., 2010; Dejana, 2010; Fernández Esmerats et al., 2016; Morini and Dejana, 2014; Rostama et al., 2014; Wu et al., 2014). *DLL4* was up regulated in response to stress in healthy cells whereas its expression remain low in the patient cells. Activation of Notch, in particular *DLL4* in response to flow, is an important factor for stress resistance (Jahnsen et al., 2015; Pedrosa et al., 2015), and this function seems to be compromised in the cells of TAA patients.

DKK1 expression, in contrary, stayed at high level in the diseased cells and failed to be down regulated at stress suggesting attenuated regulation mechanism.

Activation of Wnt/β-catenin axis has already been described in calcified aortic valves, but not in aortic aneurysms (Bostrom et al., 2011).

A cross talk between Wnt and Notch pathways has been shown to be important for the early endothelial patterning in vertebrate development (Corada et al., 2010; Dejana, 2010; Morini and Dejana, 2014). In our experiments, activation of Wnt/β-catenin by either inhibition of Gsk3 kinase or by introduction of proteolysis resistant S33 β-catenin mutant downregulated the expression of *DLL4*. This was in accordance with data obtained with patient cells where strong activation of Wnt/β-catenin was accompanied by downregulation of *DLL4* and general loss of endothelial properties by the cells of the patients. 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 complex cascades that may be further impacted by the canonical Wnt pathway (Birdsey et al., 2015; Corada et al., 2010; Dejana, 2010; Morini and Dejana, 2014; Yamamizu et al., 2010). Despite the fact that even subtle changes in *DLL4* expression impair vascular development (Duarte, 2004; Krebs, 2004; Trindade et al., 2012), one of the challenges lying ahead is to define the temporal and spatial location of the regulatory mechanisms for fine-tuning of Dll4/Notch signaling *in vivo* during vascular development (Wu et al., 2014).

4.4 Notch pathway and aortic valve calcification.

The mechanisms behind heart valve calcification are not fully elucidated, but they have some similarities with bone ossification. The involvement of dysregulated Notch pathway in calcification is evident. Idiopathic calcific aortic valve disease is associated with hypomethylation of the long noncoding (lnc) RNA H19, which prevents p53 from binding to the promoter region of *NOTCH1*, suppressing transcription (Hadji et al., 2016). This supports recent data showing that the repression of *NOTCH1* leads to calcific aortic valve disease, likely through myofibroblast and osteogenic differentiation (Merryman and Clark, 2016).

Therefore, our data on Notch-related gene expression in valve interstitial cells (VIC) and differences between patients with calcified aortic stenosis and healthy donors suggest that dysregulated expression of Notch genes might contribute to a change of phenotype in these cells (Kostina et al., 2017).

We analyzed the effect of modulating Notch activity by introducing NICD in co-cultures of VIC and endothelial cells (ECs). NICD induced increased downstream signaling from Notch genes and increased expression of proosteogenic genes in co-cultures of ECs and VIC from BAV compared to TAV and controls. NICD induced stronger expression of Notch-responsive genes *HEY1* and *SLUG* in cultures with BAV-derived valve endothelial cells (VEC), whereas expression of key EMT marker *ACTA2* was significantly less in diseased cells compared to control, and VEC of patients displayed failure of EMT process. During embryogenesis the endothelial cells of primordial valve cushions undergo EMT to become interstitial cells (Butcher and Markwald, 2007). EMT also occurs an adult valve (Frid, 2002). Inability of patient VEC to proper EMT may reflect both impaired VIC differentiation during valvulogenesis and attenuated renewal of VIC as part of physiologic valve remodeling. Moreover, insufficient EMT in diseased VEC may result altered stress response (Balachandran et al., 2011).

Our data indicate that BAV-derived cells have increased sensitivity to Notch modulation. This may explain why BAV have higher occurrence of calcification and the process may also go faster. The presented data showing stronger predisposition to calcification in BAV induced by NICD, do suggest that the initiation mechanisms are Notch-dependent and may be different in BAV and TAV. However, our data indicate that the calcification of end-stage disease is similar in calcific BAV and TAV. This is in agreement with studies comparing transcriptomes and serum OPG and OPN level in BAV and TAV patients (Guauque-Olarte et al., 2016; Irtyuga et al., 2017; Padang et al., 2015; Yu et al., 2009).

OPN and POSTIN may be key early players in triggering osteogenic phenotype (Poggio et al., 2014; Zhang et al., 2017), although there are conflicting data regarding the role of OPN (Grau et al., 2012; Passmore et al., 2015; Zhang et al., 2017). Notch activation caused a substantial activation of OPN and POSTIN

transcription in VIC from BAV group comparing to cells from TAV and controls. The exact role of osteopontin in valvular calcification is not clear and its possible regulation by Notch deserves further research (Shen and Christakos, 2005; Xue et al., 2014).

To date, aortic valve calcification based on the concept of diffuse calcification secondary to myofibroblast differentiation of VIC and ossification carried out by osteoblasts (Rutkovskiy et al., 2017). Apparently, the process of valve calcification, particularly BAV, is initiated by impaired Notch signaling in VIC and VEC followed by pathogenic VIC differentiation.

The main feature of OFT is its embryonic complexity due to existing of multiple cellular contributors in OFT development, such as progenitors from SHF, CNC cells, endocardium and endocardium-derived mesenchyme (Hutson and Kirby, 2007). Notch signaling has been implicated in the regulation of cell-fate decisions of OFT contributors during development (High et al., 2007). Thus, OFT pathologies, even those appeared in postnatal life, might have embryonic origin, as initially impaired Notch signaling pathway affects cell-cell communications and further cell fate of OFT components.

In summary, our data provide the evidence that Notch signaling pathway is attenuated in cellular components of aorta and aortic valve from patients with thoracic aortic aneurysm and calcified aortic valve stenosis, respectively. Impaired Notch pathway causes disrupted cell-cell communications and disturbance of aortic and aortic valve tissue homeostasis resulting in failure of aorta and aortic valve to perform their vital functions.

These findings further underline the importance of Notch pathway in cardiovascular system development and maintenance. The obtained data open an additional clinical prospective for searching a therapeutic targets in order to prevent the development of thoracic aortic aneurysm and calcific aortic valve stenosis.

Conclusions

1. Endothelial and smooth muscle cells from thoracic aortic aneurysms of the ascending aorta have downregulated specific cellular markers and altered functional properties, such as growth rate, apoptosis induction, and extracellular matrix synthesis.

2. Notch-dependent endothelial-to-mesenchymal transition is attenuated in the aortic endothelial cells of patients with thoracic aortic aneurysm and bicuspid aortic valve.

3. Shear-stress response is impaired in endothelial cells of the patients with thoracic aortic aneurysm due to altered Notch/BMP/WNTβ/-catenin network.

4. Early events of aortic valve calcification are Notch-dependent and differ in BAV and TAV.

Limitations

The current study has several limitations. First, the unavailability of healthy aortic wall from patients with BAV as the ideal comparative control group to BAV aneurysmal aortic wall and the unavailability of structurally normal BAV as comparative control to calcified BAV. Second, wide variability was observed within each subgroup on qPCR data. This may reflect the fact that each individual sample might be at a different stage of disease. Shear stress *in vitro* experiments with isolated ECs in the absence of SMC layer cannot reflect the complexity of signaling in the whole aortic wall due to cell-cell communication residing in different layers of aorta. Moreover, gene expression and cell behavior can be affected by physical and biochemical environment, and these factors are absent in cultured cells.

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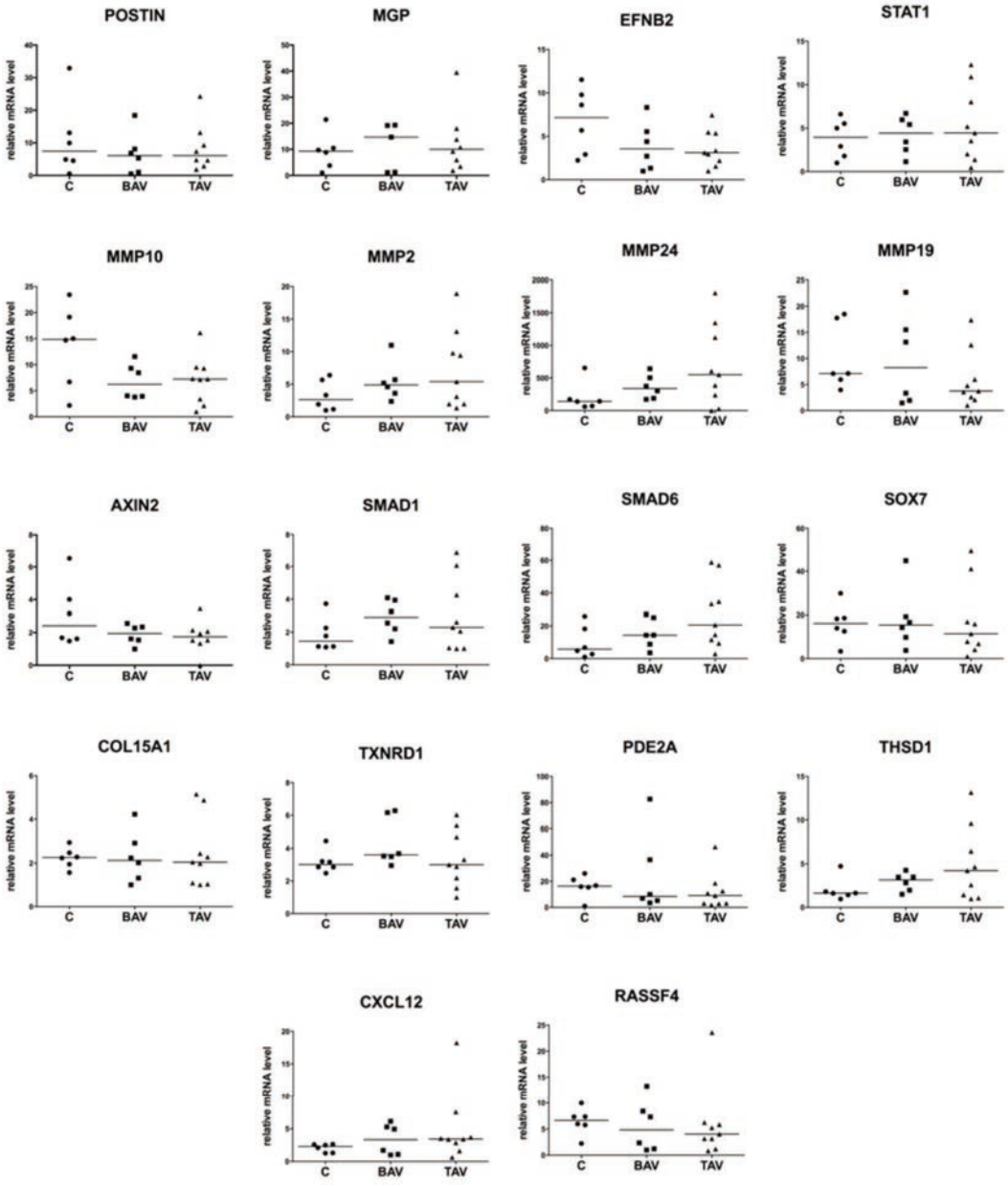
Application



Supplementary Figure 1. Genes dysregulated in the endothelial cells from patients with thoracic aortic aneurysm with either tricuspid aortic valve (TAV, n= 9) or bicuspid aortic valve (BAV, n= 6) and control HAEC (C, n= 6). Groups are compared using Mann-Whitney non-parametric test; line represents the median.

List of publications

1. Kostina, A., Shishkova, A., Ignatieva, E., Irtyuga, O., Bogdanova, M., Levchuk, K., Golovkin, A., Zhiduleva, E., Uspenskiy, V., Moiseeva, O., et al. (2017). Different Notch signaling in cells from calcified bicuspid and tricuspid aortic valves. J Mol Cell Cardiol. 2017 Nov 20;114:211-219. doi: 10.1016/j.yjmmc.2017.11.009. Epub (ahead of print). **IF 5.68**

2. Kostina AS, Uspensky VE, Irtyuga OB, Ignatieva EV, Freylikhman O, Gavriliuk ND, Moiseeva OM, Zhuk S, Tomilin A, Kostareva AA, Malashicheva AB. Notch-dependent EMT is attenuated in patients with aortic aneurysm and bicuspid aortic valve. Biochim Biophys Acta. 2016 Apr;1862(4):733-40. doi: 10.1016/j.bbadis.2016.02.006. Epub 2016 Feb 10. **IF 5.48**

3. Malashicheva A, Kostina D, **Kostina A**, Irtyuga O, Voronkina I, Smagina L, Ignatieva E, Gavriliuk N, Uspensky V, Moiseeva O, Vaage J, Kostareva A. Phenotypic and Functional Changes of Endothelial and Smooth Muscle Cells in Thoracic Aortic Aneurysms. Int J Vasc Med. 2016;2016:3107879. doi: 10.1155/2016/3107879. Epub 2016 Jan 19.

4. Aleksandra Kostina, Hanna Bjorck, Elena Ignatieva, Olga Irtyuga, Vladimir Uspensky, Shohreh Maleki, Alexey Tomilin, Olga Moiseeva, Anders Franco-Cereceda, Mikhail Gordeev, Giuseppe Faggian2, Anna Kostareva, Per Eriksson, Anna Malashicheva. Notch, BMP and WNT/β-catenin network is impaired in endothelial cells of the patients with thoracic aortic aneurysm (Submitted to Atherosclerosis Supplements).

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Original article

Different Notch signaling in cells from calcified bicuspid and tricuspid aortic valves

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ABSTRACT

Aims: Calcific aortic valve disease is the most common heart valve disease in the Western world. Bicuspid and tricuspid aortic valve calcifications are traditionally considered together although the dynamics of the disease progression is different between the two groups of patients. Notch signaling is critical for bicuspid valve development and *NOTCH1* mutations are associated with bicuspid valve and calcification. We hypothesized that Notch-dependent mechanisms of valve mineralization might be different in the two groups.

Methods and results: We used aortic valve interstitial cells and valve endothelial cells from patients with calcific aortic stenosis with bicuspid or tricuspid aortic valve. Expression of Notch-related genes in valve interstitial cells by qPCR was different between bicuspid and tricuspid groups. Discriminant analysis of gene expression pattern in the interstitial cells revealed that the cells from calcified bicuspid valves formed a separate group from calcified tricuspid and control cells. Interstitial cells from bicuspid calcified valves demonstrated significantly higher sensitivity to stimuli at early stages of induced proosteogenic differentiation and were significantly more sensitive to the activation of proosteogenic *OPN*, *ALP* and *POSTIN* expression by Notch activation. Notch-activated endothelial-to-mesenchymal transition and the corresponding expression of *HEY1* and *SLUG* were also more prominent in bicuspid valve derived endothelial cells compared to the cells from calcified tricuspid and healthy valves.

Conclusion: Early signaling events including Notch-dependent mechanisms that are responsible for the initiation of aortic valve calcification are different between the patients with bicuspid and tricuspid aortic valves.

1. Introduction

Calcified valve stenosis, most frequently in the aortic valve, is the third leading cause of cardiovascular disease [1]. The only option for treatment is heart surgery with implantation of valve prosthesis. The mechanisms behind heart valve calcification are not fully elucidated, but they have some similarities with bone ossification.

Normal aortic valve leaflets have two cell types: valve interstitial cells (VIC) and valve endothelial cells (VEC) [2]. It has been suggested that VIC are the main functional units of the valve that undergo mineralization [3]. However, the VEC may also participate in the mineralization process [4–7]. The progressive valve fibrosis and mineralization are thought to be active rather than passive processes. A main risk factor for development of calcified aortic valve disease is bicuspid anatomy of the aortic valve (bicuspid aortic valve, or BAV). Only < 2% of the population has BAV [8], but they represent about 50% of the patients undergoing aortic valve replacement. Calcification also occurs at an earlier age in BAV compared to individuals with the normal tricuspid anatomy (tricuspid aortic valve, or TAV) [9–11]. The average age of surgery for calcified BAV is approximately 27 years younger than in patients with TAV [12]. However, the cellular and molecular mechanisms of valve calcification have been considered to be

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common for BAV and TAV [13–15].

Although the heritability of BAV is well known, the genetic causes of BAV are not elucidated. *NOTCH1* remains the only proven candidate gene [16,17]. Notch is also a key signaling pathway during cardiac valvulogenesis, ensuring cross talk between different types of cells and their physiological differentiation [18]. All Notch receptors (Notch1–4) and ligands (Jag1 and 2 and Dll1, 3, and 4) are expressed in the vascular system. Activation of Notch receptors requires binding to a transmembrane ligand presented by neighboring cells. This binding enables a series of successive cleavage events in the receptor, ultimately resulting in intracellular release of the Notch Intracellular Domain (NICD), which is the transcriptionally active form of Notch. NICD translocates to the nucleus, where it regulates a broad range of target genes, including those involved in calcification [18]. The data regarding the role of Notch in aortic valve calcification are controversial. Notch1 may inhibit osteogenic calcification [19], however, opposite data suggest that Notch1 sustains osteogenic calcification in human VIC [20]. *NOTCH1* haploinsufficiency promotes proosteogenic and inflammatory gene expression [21].

In this work we sought to find the differences of aortic valve calcification between bicuspid and tricuspid aortic valves and to explore if there are Notch-dependent mechanisms of osteogenic transformation of valve cells that differ between the two groups of patients. We show that the expression pattern of Notch genes is altered in the aortic valve cells of patients with calcific aortic stenosis compared to those of healthy persons. The expression pattern is different between VIC derived from BAV and from TAV patients. We also show different sensitivity to proosteogenic stimuli in the cells of BAV versus TAV patients. Collectively, our findings suggest that the mechanisms of the early phase of aortic valve calcification are different between BAV and TAV patients and Notch pathway deregulation in BAV is important for this process.

2. Materials and methods

The clinical research protocols were approved by the local Ethics Committee of the Almazov Federal Medical Research Center and were in accordance with the principle of the Declaration of Helsinki. All patients gave informed consent. Valve interstitial cells (VIC) and valve endothelial cells (VEC) were isolated from aortic valves explanted during aortic valve replacement at the National Almazov Research Centre. Patients with known infective endocarditis and rheumatic disease were excluded from the study. VICs and VECs from normal aortic valves were isolated from healthy valves obtained from explanted hearts from recipients of heart transplantation ($n = 11$). Due to the low incidence of BAV in the population all healthy valves were TAV. Clinical data regarding patients with aortic stenosis in BAV and TAV are shown in Supplementary Table 1 (for qPCR) and Supplementary Table 2 (for plasma osteopontin analysis).

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical cords at the National Almazov Research Centre.

2.1. Plasma osteopontin measurement

Peripheral venous blood was obtained at 08:00 on the morning of surgery. Plasma samples were immediately frozen and kept at − 70 °C until assay. CRP and parameters of lipid metabolism were measured. Osteopontin, a biomarker of calcification (OPN), was measured by the human ELISA kit (BMS2066 eBiosciences, Vienna, Austria) according to the manufacturer's instructions (pmol/l).

2.2. Isolation of primary cell cultures

Valve leaflets were washed in PBS and incubated for 10 min at 37°C in 0.2% collagenase solution (Collagenase type IV, Worthington Biochemical Corporation, USA). The valve was vortexed for one minute to remove VECs, the supernatant was collected and centrifuged at 300*g* for five minutes, and VECs were plated in Endothelial Cell Medium (ECM) (ScienCell) on 0.2% gelatin (Sigma-Aldrich). Then the cells were purified from VICs using magnetic cell separation (MACS) with CD31 + microbeads (CD31 MicroBead Kit, Miltenyi Biotec, Germany) according to the manufacturer's instructions. Purity of the VECs was confirmed by immunocytochemistry with anti CD31 and anti vWF antibodies (Abcam).

To isolate VICs the remaining valve tissue was incubated with 0.2% collagenase solution for 24 h at 37°. Then the tissue was pipetted repeatedly to break up the tissue mass and spun at 300 g for five minutes. The pellet containing VICs were resuspended in DMEM (Gibco) supplemented with 15% FBS, 2 mM L-glutamine, and 100 units/ml penicillin/streptomycin, and plated on T75 flask.

To minimize variations between cultured cells at different passages all population analysis of gene expression by qPCR was done using the cells at the same passages.

Human umbilical vein endothelial cells (HUVEC) were harvested from umbilical vein by enzymatic dissociation. The vein was washed in PBS, filled by 0.1% collagenase solution (Collagenase, Type II, Worthington Biochemical Corporation, USA) and incubated in PBS at 37°C for 10 min. The cell suspension was centrifuged at 300*g* for five minutes. The pellet of cells was suspended and seeded on 35 mm Petri dish covered with 0.2% gelatin in ECM (ScienCell) [22]. Primary cells between passages two and five were used for all experiments. All cultures were maintained in humidified 5% CO2 at 37°C.

2.3. Induction of osteogenic differentiation

The osteogenic potential of VIC was tested by treatment with osteogenic medium (DMEM supplemented with 15% FBS (HyClone), 2 mM L-glutamine, 100 units/ml penicillin/streptomycin, 50 mg/ml ascorbic acid, 0.1 mM dexamethasone and 100 mM b–glycerophosphate) for 21 days. Calcium deposits were demonstrated by Alizarin Red staining. Cells were washed with PBS, fixed in 70% ethanol for 60 min, washed twice with distillated water and stained using Alizarin Red solution (Sigma). The images of calcium phosphate deposition were analyzed for the ratio of differentiated and undifferentiated cell areas with MosaiX software (Carl Zeiss microsystems, Germany).

2.4. Co-culture of endothelial cells and valve interstitial cells

VICs (120×10^3 cells) were plated in 12-well plates coated with 0.2% gelatin. At the same time HUVEC were seeded on a culture dish covered with 0.2% gelatin and transduced with saturating concentration of NICD-bearing lentivirus. After 24 h the HUVEC were reseeded and 120×10^3 HUVEC were added to VICs with fresh DMEM (Gibco) supplemented with 15% FBS, 2 mM L-glutamine and 100 units/ml penicillin/streptomycin. After adhesion of HUVECs, the cell culture medium was replaced by osteogenic medium. ALP staining was performed using Sigma BCIP®/NBT kit (Sigma) 10 days after the initiation of osteogenic differentiation. Cells were washed with PBS and incubated with alkaline-phosphatase working solution for 10–15 min at room temperature. ALP activity appeared as blue deposition and plates were photographed with digital camera. At the same time calcium was measured by Alizarin Red as described above.

2.5. Endothelial-to-mesenchymal transition (EMT) induction

For induction of EMT, 45×10^3 VECs were plated onto 12-well plates and transduced overnight with saturating concentration of the lentiviral concentrate encoding NICD. Early EMT markers such as *SNAIL1*, *SLUG*, *HES1*, *HEY1* were estimated by qPCR after 72 h of EMT induction. αSMA (*ACTA2*) was late responsive and reliable marker of EMT measured by qPCR as well as by ICH staining after 14 days of EMT induction.

2.6. Immunocytochemistry

After growing on cover slides, cells were fixed for 20 min in 1% paraformaldehyde and then for five minutes in methanol at − 20 °C. Fixed cells were permeabilized in 1% BSA/0.1% Triton X 100/PBS for five minutes, followed by blocking in 1% BSA/PBS for 30 min.

Then cells were incubated for one hour with primary antibodies: SMA (sc-32251, Santa Cruz). Secondary antibodies conjugated with Alexa546 (Invitrogen) were used. DAPI was used to visualize nuclei. Microphotographs were taken using an AxioObserver Microscope (Zeiss) at 20 × magnification with AxioVision software.

2.7. Immunoblotting

Cells were lysed with lysis buffer (50 mM Tris (pH 8), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 5 mM EDTA), containing protease inhibitors (Roche). Extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Primary antibodies αSMA (Abcam, USA), beta-actin (ab 6276, Abcam) and calponin (Sigma-Aldrich) were used. Positive bands were quantified by densitometry analyses using a gel documentation system Fusion Fix (Vilber Lourmat) and Fusion-Capt software. Bands were normalized using beta-actin staining.

2.8. qPCR

RNA from cultured cells was isolated using ExtractRNA (Eurogene, Russia). Total RNA (1 μg) was reverse transcribed with MMLV RT kit (Eurogen, Russia). Real-time PCR was performed with 1 μL cDNA and SYBRGreen PCRMastermix (Eurogen, Russia) in the Light Cycler system using specific forward and reverse primers for target genes. Corresponding gene expression level was normalized to *HPRT* or *GAPDH* from the same samples. Changes in target genes expression levels were calculated as fold differences using the comparative ΔΔCT method. All primers sequences will be presented at request.

2.9. Lentiviruses

Lentiviral production was performed as described previously [23]. In brief, 100-mm dishes of subconfluent 293T cells were cotransfected with 15 μg pLVTHM-T7-NICD, 5.27 μg pMD2.G, and 9.73 μg packaging pCMV-dR8.74psPAX2 by calcium-phosphate method. The following day the medium was changed to fresh, and the cells were incubated for 24 h to obtain high-titer virus production. Produced lentivirus was concentrated from 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 HUVEC and primary endothelial cells transduction was 90–95% by GFP.

2.10. Statistics

Discriminant function analysis was performed to determine, which continuous variables discriminate between groups of BAV, TAV and controls. Continuous variables were qPCR gene expression data from ΔΔCT estimation. A stepwise analysis enumerating steps, p-value significance level, and F-test were performed. A discrimination level was evaluated by assessing Wilks' lambda. Significance of an identifying criterion was determined after drawing scatterplots of canonical values and calculating classification value and Mahalanobis squared distance. Discriminant function analysis was performed with Statistica 7.0 software. qPCR data on gene expression and plasma osteopontin content was analyzed using Graph Pad Prism. Values are expressed as means ± SD. Groups were compared using the Mann–Whitney non-parametric test (qPCR) and Students's *t*-test (plasma osteopontin). A value of $P \leq 0.05$ was considered significant.

3. Results

3.1. Expression of Notch receptors and ligands in cells from calcified and healthy valves

We used the cells isolated from the patients with calcific aortic stenosis with either tricuspid (TAV) or bicuspid (BAV) morphology of the aortic valve. The control samples (C) were isolated from healthy tricuspid valves because of the low occurrence of bicuspid aortic valve in the population. We measured the expression of key genes of the Notch pathway, namely *NOTCH1–4* as well as Notch ligands *JAG1*, *DLL1*, *DLL4* in human VIC and VEC. In VIC expression of *NOTCH1*, *DLL1*, and *DLL4* were decreased in BAV compared to both TAV and controls whereas *NOTCH2* was decreased with borderline significance (Fig. 1). *NOTCH3* expression was increased in TAV compared to both healthy valves and BAV. None of these parameters were altered in VEC in either BAV or TAV group comparing to control group (Supplementary Fig. 1).

3.2. Induced calcification in interstitial cells from tri- and bicuspid aortic valves

We compared the sensitivity to osteogenic stimuli of VIC from the patients and healthy controls (Fig. 2). Twenty-one day after stimulation with osteogenic medium calcification occurred as shown by strong Alizarin Red staining, but without any difference between VIC from BAV and TAV (Fig. 2A). Quite little Alizarin Red staining was seen in VIC from healthy valves comparing to what was seen in the cells from the BAV and TAV patients.

Expression of genes that might be involved in calcification was measured in VIC by qPCR after stimulation with osteogenic medium: *BMP2* (bone morphogenetic protein 2), *RUNX2* (runt related transcription factor 2), *POSTIN* (periostin), *CTNNB1* (beta-catenin), *SOX9* (sex determining region Y0-box 9), *OPN* (osteopontin). There were no differences between unstimulated cells derived from either BAV, TAV or controls regarding *BMP2*, *POSTIN*, *CTNNB1*, and *SOX9* in either non-stimulated or stimulated cells (Supplementary Fig. 2). *RUNX2* was lower in unstimulated VIC from BAV patients as compared to healthy controls, while *OPN* expression was higher in non-stimulated VIC from TAV than in BAV and in controls. After 21 days of osteogenic induction only *RUNX2* expression increased in all three groups comparing to non-stimulated cells, but without difference between groups (Fig. 2B).

3.3. Interstitial cells from bi- and tricuspid valves have different levels of markers of fibrosis and smooth muscles

The aortic valve pathology includes mineralization of the valve and also myofibroblast-like transformation of VIC and valve fibrosis [3]. The most common marker for myofibroblasts is smooth muscle actin (α-SMA). Calponin, a marker of fully differentiated smooth muscle cells, has also been reported to be expressed in VIC of stenotic valves [24]. The content of αSMA and calponin estimated by Western blotting was higher in VIC from calcified BAV than from TAV (Fig. 3).

3.4. Bi- and tricuspid valves are two independent groups by gene expression

To identify if VIC from BAV and TAV patients form separate clusters by gene expression we used multivariate discriminant function analysis to discriminate groups by expression of the genes analyzed at the previous steps. Graphical result of the examined groups based on the discriminant analysis (Fig. 4) shows that VIC from BAV and TAV groups form independent and separate clusters. BAV group was more homogenous and well separated from TAV and control groups. VIC from TAV patients were also distinct from healthy controls.

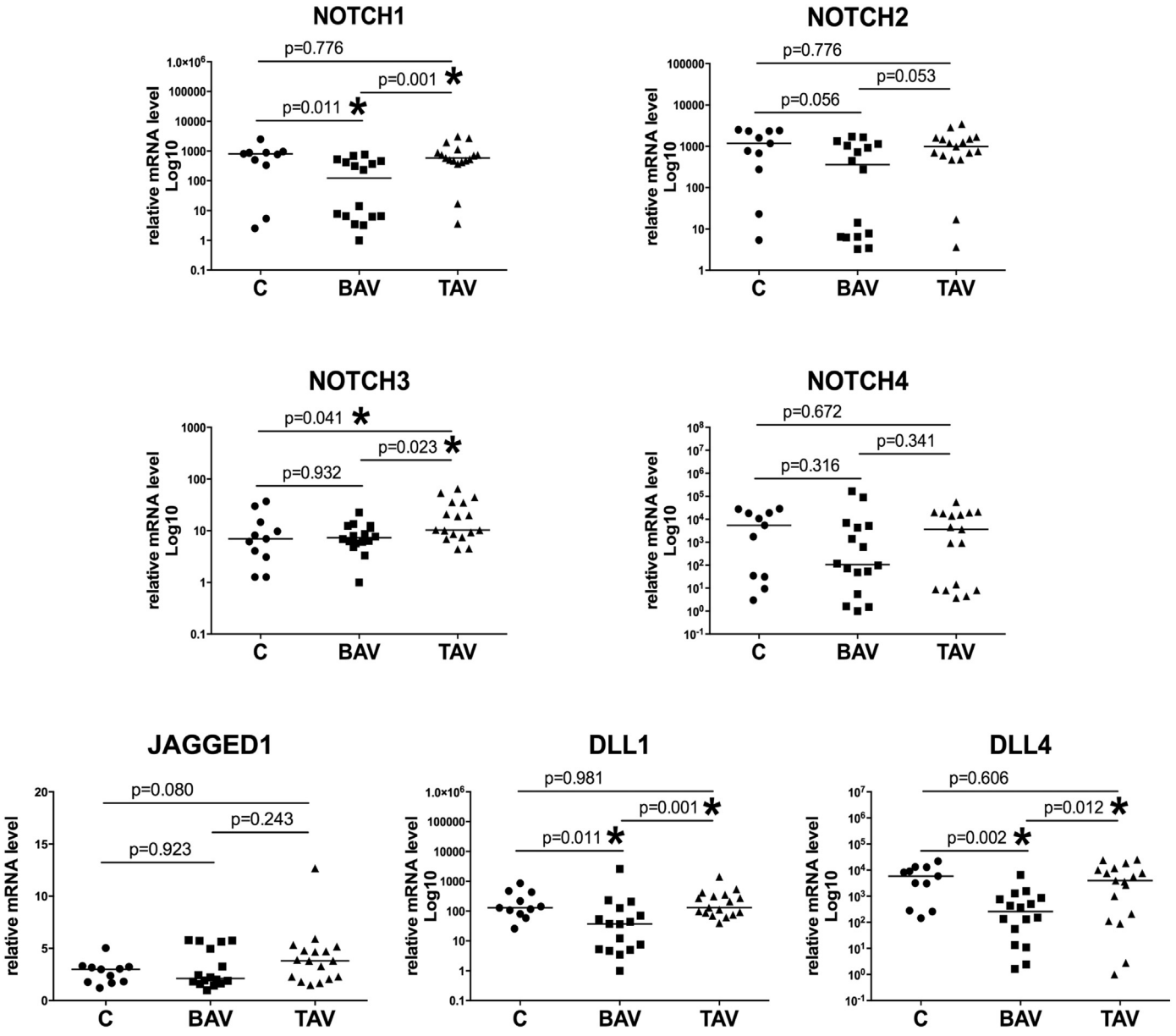

Fig. 1. Expression level of Notch receptors and ligands in aortic valve interstitial cells from the patients with calcific aortic stenosis with bicuspid (BAV, n = 13) and tricuspid (TAV, n = 17) aortic valves and from healthy control valves (C, n = 11). mRNA levels were analyzed by qPCR and normalized to *GAPDH*. Groups were compared using Mann-Whitney non-parametric test; line represents the median; *p < 0.05.

3.5. Patients with calcific aortic stenosis have elevated level of osteopontin in blood

Increased levels of plasma osteopontin are associated with the presence of aortic valve calcification and stenosis [25]. We compared the level of osteopontin in the plasma of BAV and TAV patients and in healthy controls (Fig. 5). Osteopontin concentration was significantly higher in both patient groups compared to control group, however, there was no difference between BAV and TAV (Fig. 5). Interestingly, many patients in both groups had the plasma level of osteopontin overlapping with some healthy donors.

3.6. Notch-dependent initiation of osteoblast differentiation is stronger in BAV

To assess if the initial mechanisms of calcification in BAV and TAV-derived VIC are dependent on Notch signaling we used co-culture of VIC with human umbilical endothelial cells (HUVEC). These co-culture experiments model the situation in vivo when endothelial cells communicate with mesenchymal cells via Notch signaling [26,27]. HUVEC, not VEC were used because they give more uniform endothelial cell cultures. Notch was activated in HUVEC by transduction with lentiviruses bearing Notch Intracellular Domain (NICD). The next day the HUVEC were seeded on VIC (Fig. 6A, left panel). *HEY1*, main Notch target, was elevated in co-cultures and this elevation was more prominent when Notch was activated in endothelial cells (Fig. 6A, right panel). The day after start of co-culture we induced osteogenic differentiation by addition of osteogenic medium. The early expression of Notch target *HEY1* and proosteogenic markers was measured in the co-cultures after three days of exposure to osteogenic medium in the absence (control) or presence of Notch activation (Fig. 6B and Supplementary Fig. 3). Expression of *HEY1* was similar in all three groups without Notch activation. However, when Notch was activated, *HEY1* was the highest in the co-cultures that contained BAV-derived VIC. Co-cultures with VIC from BAV patients demonstrated significantly higher expression of *OPN*, *ALP* and *POSTIN* after osteogenic stimulation.

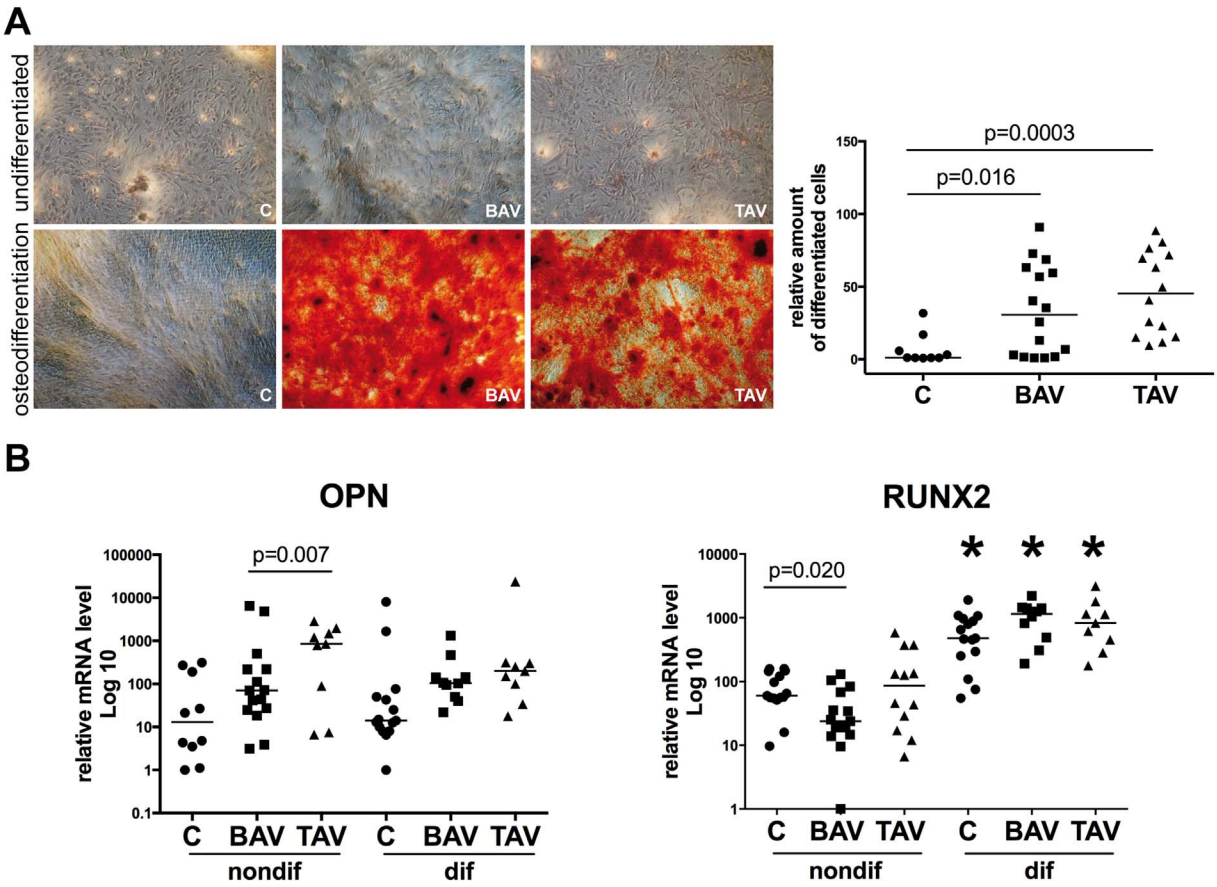

Fig. 2. Sensitivity of aortic valve interstitial cells from the patients with calcific aortic stenosis with bicuspid (BAV, n = 13) and tricuspid (TAV, n = 17) aortic valves and from healthy control valves (C, n = 11) to osteogenic stimulation. Aortic valve interstitial cells (VIC) were cultured in osteogenic medium for 21 days and then stained with Alizarin Red. (A) Left panel shows representative image of VIC differentiation with calcification revealed by red color. Right panel represents digital quantification of calcium deposition. (B) mRNA expression by qPCR of proosteogenic genes in the BAV, TAV and control groups of VIC. The groups were compared using Mann-Whitney non-parametric test, line represents the median. P-value for the differences between the groups is given only for the significant differences ($p < 0.05$). Asterisks indicate the significant differences ($p < 0.05$) of mRNA content between undifferentiated and differentiated cells for a given group (BAV, TAV or control, correspondingly). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Activation of Notch caused even higher elevation of *OPN*, *ALP* and *POSTIN*, which was significantly higher in the co-cultures containing BAV derived VIC. The expression of the other proosteogenic genes was not responsive to changes in Notch activity (Supplementary Fig. 5).

Increased alkaline phosphatase 10 days after the initiation of osteogenic stimulation confirmed that Notch activation induced osteogenic phenotype more readily in BAV-derived co-cultures (Fig. 6B) with more intensive staining of the co-cultures containing VIC derived from BAV patients and activated Notch.

3.7. Early endothelial-to-mesenchymal transition activation is more prominent in endothelial cells from BAV

Recently it has been shown that endothelial-to-mesenchymal transition (EMT) precedes the onset of calcification in valve cells [4]. We studied Notch-dependent EMT activation in VEC derived from BAV and TAV patient and healthy valves. For Notch-induced EMT we used a previously described model with introduction of NICD into VEC by lentiviral transduction [23]. Expression of Notch-responsive genes *HEY1* and *SLUG*, which activate the initial stage of EMT, was measured after three days (Fig. 7, left panel). In VEC from BAV expression of both *SLUG* and *HEY1* was higher than in TAV and healthy controls. After 10 days *ACTA2* expression encoding αSMA, the definitive EMT marker, was not different between BAV and TAV, but was significantly less than in control cells. Immunocytochemical staining of control cells for αSMA showed well differentiated cells that undergone EMT and terminated by appearance of true mesenchymal cells with nice actin fibers (Fig. 7, right panel). At the same time endotelial cells from BAV and TAV patients failed to form actin fibers properly that suggested failure of EMT process.

4. Discussion

In this study we show differences in the initial proosteogenic events between the valvular cells derived from the patients with aortic stenosis associated with bicuspid aortic valve versus the cells derived from the patients with tricuspid aortic valve. This is to our knowledge the first study showing that the mechanisms that underlie calcification of BAV and TAV are different. Furthermore discriminant analysis showed that calcified BAV had a distinctly different genetic pattern compared to calcified TAV and noncalcified controls.

4.1. Bicuspid valves and calcification

In the present study Notch-related genes *NOTCH1*, *NOTCH3*, *DLL1*, *DLL4* as well as proosteogenic *RUNX2* showed a different pattern of expression in VIC derived from BAV patients compared to the cells from TAV patients. Furthermore protein content of markers of smooth muscle and fibrosis (αSMA, vimentin, and calponin) were different between BAV and TAV derived cells.

There is accumulating evidence that dysregulated Notch is implicated in calcification. *NOTCH1* haploinsufficiency promotes

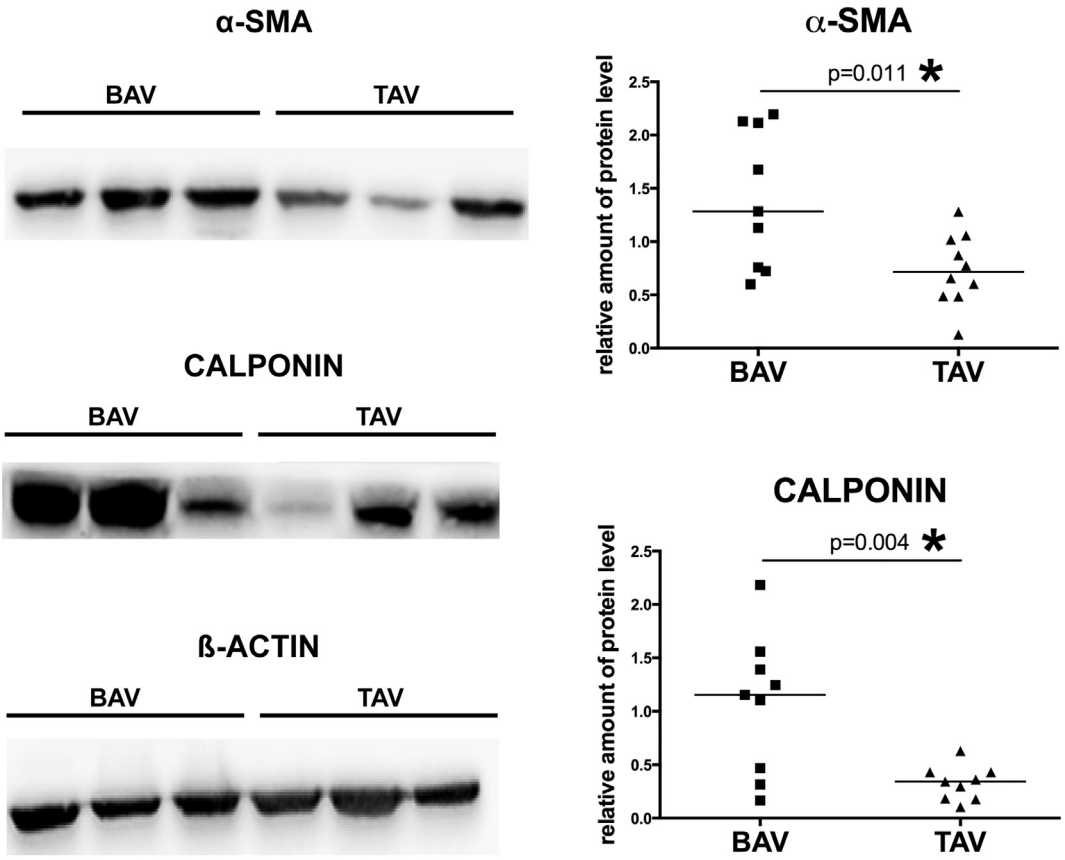

Fig. 3. Protein level of fibrotic/smooth muscle markers in aortic valve interstitial cells from patients with calcified bicuspid (BAV) and tricuspid valve (TAV). Left panel shows representative Western blots for protein level of αSMA and calponin. Right panel represents digital quantification of protein level by densitometry of the corresponding bands. Bands were normalized using beta-actin staining. BAV: n = 9, TAV: n = 10. The groups were compared using Mann-Whitney non-parametric test, line represents the median. P-value for the differences between the groups is given.

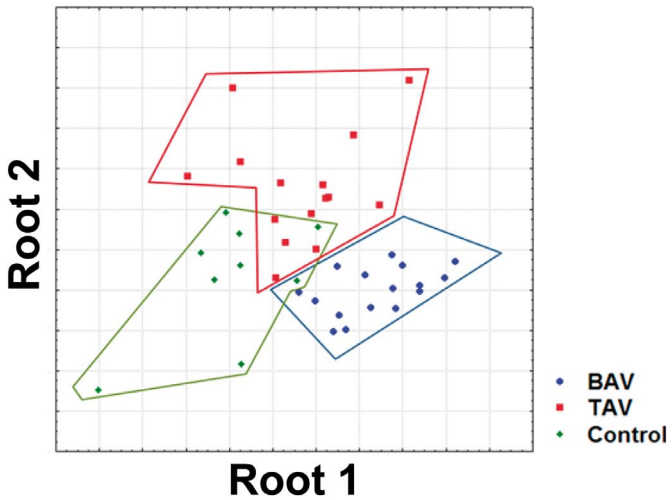

Fig. 4. Partition of the groups based on the results of discriminant analysis of gene expression in aortic valve interstitial cells. Discriminant analysis was used to investigate what parameters in gene expression could divide aortic valve interstitial cells (VIC) derived from patients with bicuspid aortic valve (BAV) or with tricuspid aortic valve (TAV) or healthy control (C) groups. Expression data on a set of the following genes was used: *NOTCH1*, *NOTCH2*, *NOTCH3*, *NOTCH4*, *DLL1*, *DLL4*, *JAG*, *BMP2*, *RUNX2*, *POSTIN*, *CTNNB1*, SOX9, *OPN*, *VIM*, *SPRY1*). Scatter plot demonstrates that VIC from BAV and TAV groups form separated clusters by gene expression, while VIC from control group overlap with both BAV and TAV group.

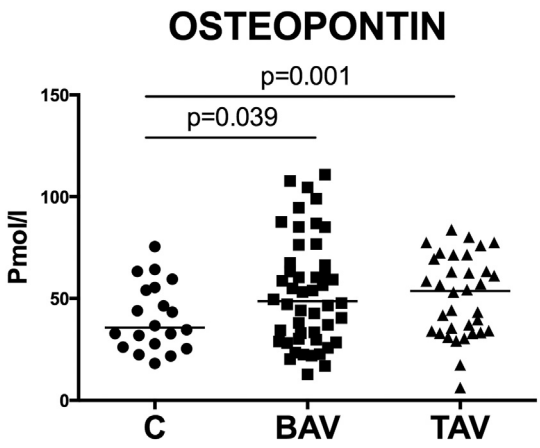

Fig. 5. Level of osteopontin in the plasma of patients with calcified bicuspid (BAV) and tricuspid (TAV) aortic valves and patients with healthy control valves (C). Groups were compared using *t*-test.

proosteogenic gene expression in the cells from BAV patients [21]. Idiopathic calcific aortic valve disease is associated with hypomethylation of the long noncoding (lnc) RNA H19, which prevents p53 from binding to the promoter region of *NOTCH1*, suppressing transcription [28]. This supports recent data showing that the repression of *NOTCH1* leads to calcific aortic valve disease, likely through myofibroblast and osteogenic differentiation [29].

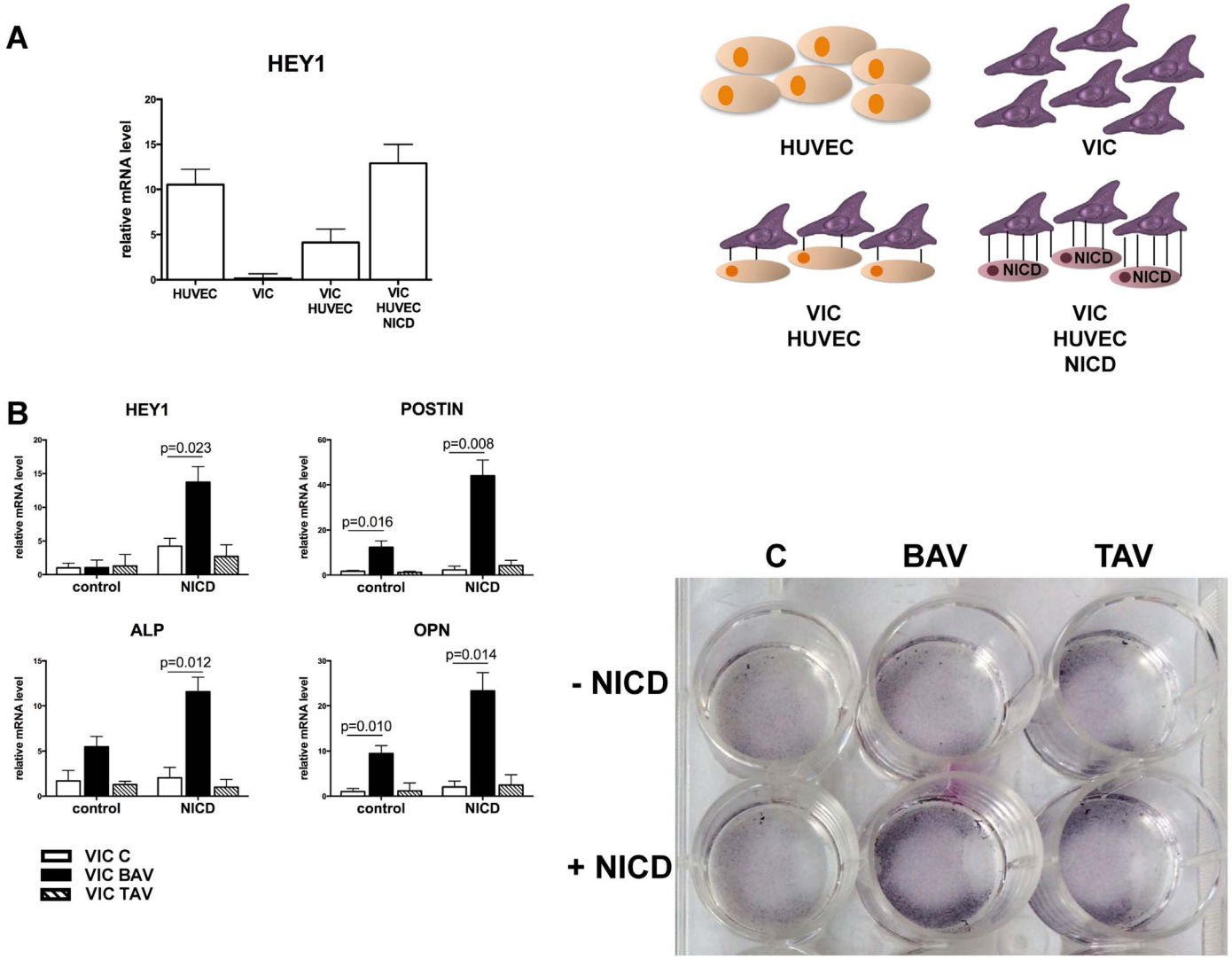

Fig. 6. Notch activity distictly influences osteogenic differentiation of valve interstitial cells (VIC) of patients with calcified bicuspid (BAV) and tricuspid valve (TAV). VIC from TAV, BAV and control groups were cocultured with human umbilical vein endothelial cells (HUVEC) in the presence of osteogenic medium. To test how activation of Notch influences proosteogenic responce HUVEC were transduced with lentivirus bearing Notch1 intracellular domain and cocultured with VIC (n = 5 for each group). (A) Activation of *HEY1* expression in co-cultures of VIC from normal valves with HUVEC with or without activation of Notch. (B) Left panel. The cells were stimulated with osteogenic medium. Level of proosteogenic gene transcription was estimated in by qPCR in the absence/presence of Notch-activation 3 days after inducing osteogenic differentiation. The groups were compared using Mann-Whitney non-parametric test. P-value for the differences between the groups is given. Right panel. To assess the induction of proosteogenic phenotype alkaline-phosphatase activity was verified after 10 days of osteogenic induction.

Notch-signaling is extremely dose sensitive due to the lack of a signal amplification step or utilization of secondary messengers to transmit the signal from the cell surface to the nucleus [30]. By altering the amount of ligands and receptors expressed in a cell, numerous scenarios of Notch activation patterns can be generated. The tissue specific context-dependent action of Notch is poorly understood, but dysregulation in expression of Notch-related genes could contribute to the development of cardiovascular pathology [31,32]. Therefore, our data on Notch-related gene expression in VIC and differences between BAV and TAV and controls suggest that dysregulated expression of Notch genes might contribute to a change of phenotype in these cells.

4.2. Notch and initiation of calcification

We analyzed the effect of modulating Notch activity by introducing NICD in co-cultures of VIC and endothelial cells. NICD induced increased downstream signaling from Notch and increased expression of proosteogenic genes in co-cultures of endothelilal cells and VIC from BAV compared to TAV and controls. NICD induced stronger expression of Notch-responsive genes *HEY1* and *SLUG* in cultures with BAV-derived VEC. Our data indicate that BAV-derived cells have increased sensitivity to Notch modulation. This may explain why BAV have higher occurrence of calcification and the process may also go faster.

The presented data showing stronger predisposition to calcification in BAV induced by NICD, do suggest that the initiation mechanisms may be different in BAV and TAV and that they are Notch-dependent. However, our data indicate that the calcification of end-stage disease is similar in calcific BAV and TAV. This includes both data from cell cultures as well as similar plasma levels of osteopontin from endstage disease with BAV and TAV. This is in agreement with studies comparing transcriptomes of BAV and TAV [33,34]. Both studies conclude that the gene expression profiles of calcified valve tissues are similar, but different from healthy control valves and both studies proposed that the early mechanisms inducing valve calcification might be different between BAV and TAV patients.

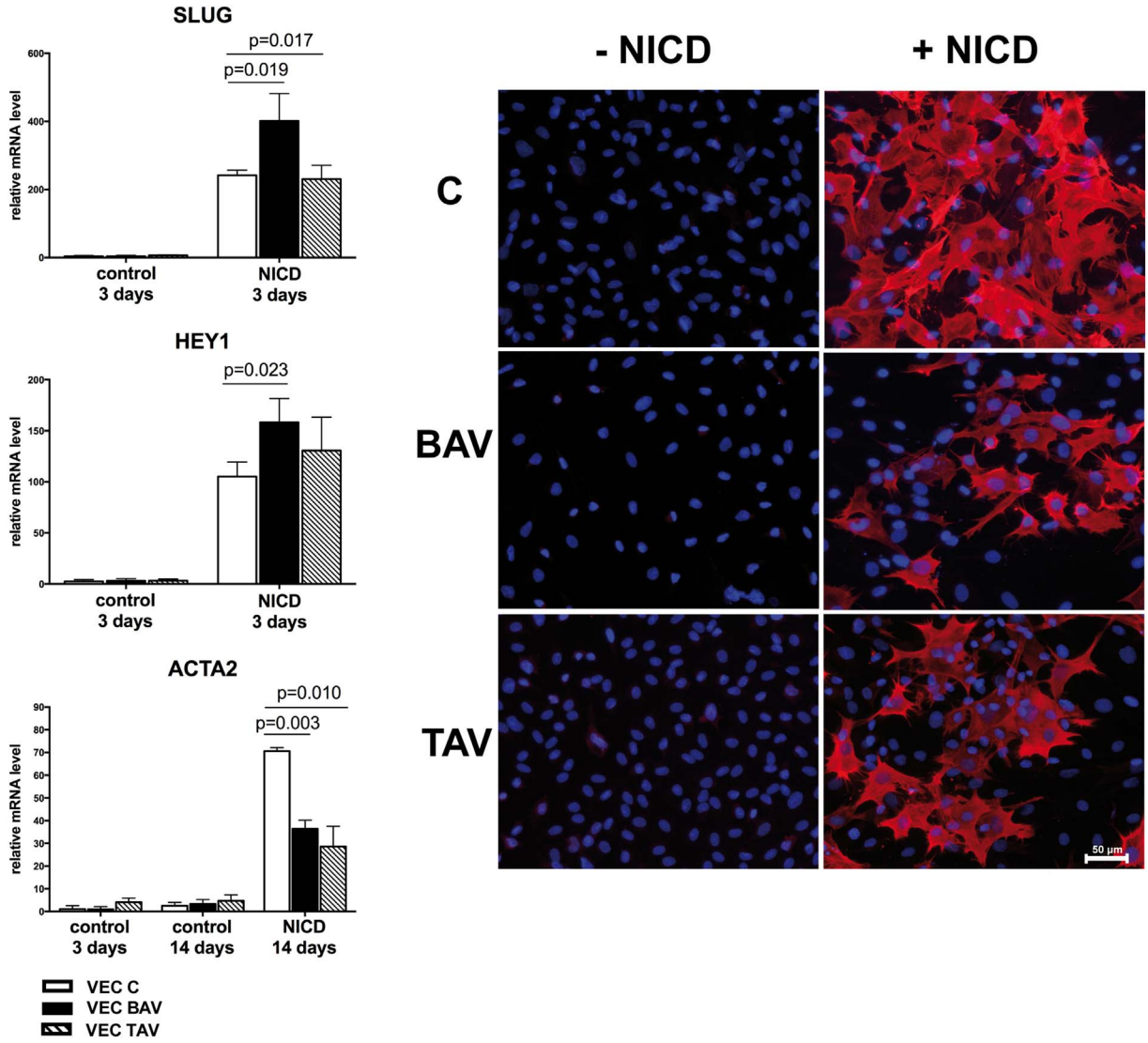

Fig. 7. Endothelial-to-mesenchymal transition (EMT) initiation is more prominent in the valve endothelial cells (VEC) derived from the patients with calcified bicuspid aortic valve (BAV) compared to the cells derived from the patients with calcified tricuspid aortic valve (TAV) or control cells derived from healthy valves (C). VEC were transduced with the lentivirus bearing activated Notch intracellular domain (NICD), n = 5 for each group. *SLUG* and *HEY1* expression was analyzed by qPCR 3 days after the induction of EMT; *ACTA2* expression was analyzed 10 days after the induction of EMT by qPCR (left panel) and by ICH staining of the cells with antibody against αSMA (right panel). Scale bar corresponds to 50 µm. The groups were compared using Mann-Whitney non-parametric test. P-value for the differences between the groups is given.

4.3. Osteopontin and valve calcification

OPN was increased in patients with calcified BAV and TAV without any difference between groups. *OPN* and *POSTIN* may be key early players in triggering osteogenic phenotype [35,36], although there are conflicting data regarding the role of *OPN* [36–38]. Notch activation caused a substantial activation of *OPN* and *POSTIN* transcription in VIC from BAV group comparing to cells from TAV and controls.The co-culture experiments show greater response of BAV VIC to NICD for several genes including OPN. The exact role of osteopontin in valvular calcification is not clear and its possible regulation by Notch deserves further research.

4.4. Markers of fibrosis and smooth muscle

Multiple mesenchymal markers are used to characterize EMT. αSMA is a key factor often used to identify fully differentiated myofibroblasts, which are often termed 'activated fibroblasts'. αSMA not only exerts traction forces that are central in the alteration of tissue architecture during fibrosis, but also plays an important role in myofibroblast differentiation and function [39].

αSMA content was increased in VICs from BAV compared to TAV according to our data. Increased expression of αSMA has been shown to directly reduce the clonal potential of human mesenchymal stem cells and to guide their differentiation toward osteoblasts. It has been also demonstrated, that αSMA not only identifies osteoprogenitors in mesenchymal populations [40,41], but may be part of the mechanism driving differentiation [42]. In this regard high αSMA content in BAV-derived VIC might also contribute to their higher predisposition to osteogenesis compared to TAV cells.

4.5. Limitations

The current study is limited by the unavailability of structurally normal BAV as the ideal comparative control group to calcified BAV. The availability of healthy valves is in itself a difficulty, and to obtain a material of healthy BAV is virtually impossible. Wide variability was observed within each subgroup on qPCR data. This may reflect the fact that each individual sample might be at a different stage of disease.

5. Conclusion

The present data demonstrated genetic differences between calcification in BAV and in TAV with special reference to Notch signaling, which may be particularly important in the initiation of calcification in BAV. Gene expression in VIC from BAV, TAV, and healthy controls represent different clusters.

Author contributions

A.K. designed and performed experiments, analyzed data, wrote manuscript, A.S., E.I., K.L., M.B. performed experiments, analyzed data, A.G., O. I., E.Z., V.U., O.M. acquired and analyzed clinical data, G.F., J.V., A.K. discussed and wrote the manuscript, A.R., A.M. designed research, performed experiments, analyzed data, wrote the manuscript.

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Notch-dependent EMT is attenuated in patients with aortic aneurysm and bicuspid aortic valve

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ABSTRACT

Bicuspid aortic valve is the most common congenital heart malformation and the reasons for the aortopathies associated with bicuspid aortic valve remain unclear. *NOTCH1* mutations are associated with bicuspid aortic valve and have been found in individuals with various left ventricular outflow tract abnormalities. Notch is a key signaling during cardiac valve formation that promotes the endothelial-to-mesenchymal transition. We address the role of Notch signaling in human aortic endothelial cells from patients with bicuspid aortic valve and aortic aneurysm. Aortic endothelial cells were isolated from tissue fragments of bicuspid aortic valve-associated thoracic aortic aneurysm patients and from healthy donors. Endothelial-to-mesenchymal transition was induced by activation of Notch signaling. Effectiveness of the transition was estimated by loss of endothelial and gain of mesenchymal markers by immunocytochemistry and qPCR. We show that aortic endothelial cells from the patients with aortic aneurysm and bicuspid aortic valve have down regulated Notch signaling and fail to activate Notch-dependent endothelial-to-mesenchymal transition in response to its stimulation by different Notch ligands. Our findings support the idea that bicuspid aortic valve and associated aortic aneurysm is associated with dysregulation of the entire Notch signaling pathway independently on the specific gene mutation.

1. Introduction

Bicuspid aortic valve (BAV) is the most common congenital heart malformation, occurring in 1% to 2% of the population [1]. It has been suggested that the valve defect may arise during development of the aortic valvular cusps and aortic media from neural crest cells [2,3]. BAV patients are at increased risk for aortic dilation, aneurysm, and dissection [4–6]. However, the reasons that cause aortic dilation as well as calcification in BAV patients remain unclear [7] and conservative therapy is currently absent for this disease.

NOTCH1 mutations are associated with BAV and have been found in individuals with various left ventricular outflow tract abnormalities such as hypoplastic left heart syndrome, coarctation of the aorta and aortic valve stenosis, [8,9]. Notch is a key signaling pathway during cardiac valve formation that via Snail1 activation, promotes the endothelial-to-mesenchymal transition (EMT) of endocardial cells to form the valve primordium (10). Combined loss of *Notch1* downstream targets, *Hey1* and *HeyL* causes impaired EMT in mice [11].

Functional studies on the missense *NOTCH1* mutations associated with left ventricular outflow abnormalities have shown reduced receptor signaling associated with defective epithelial-to-mesenchymal transition in HMEC-1 microvascular endothelial cell line used as a model [12]. Recently it has been shown that *NOTCH1* haploinsufficiency dramatically influences the capacity of human iPS-derived endothelial cells to resist shear stress and this may explain the aortic pathology in the case of *NOTCH1* mutation [13]. All these highlights the role of Notch signaling in endothelial cells in maintaining aortic wall integrity and its resistance to continuous stress.

Multiple studies have demonstrated the high heritability of BAV in humans [14–16]. Animal models of BAV also suggest a possible genotype–phenotype correlation related to cusp fusion phenotypes. Despite tremendous advances in gene sequencing technology, the genetic etiology of many congenital heart malformations, including BAV, remains poorly understood [17,18]. We hypothesized that the entire

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0925-4439/

Notch-dependent pathway is impaired in the aortic endothelial cells of BAV patients thus causing endothelial dysfunction and consequent alteration of the aortic wall.

We address the role of Notch signaling in human aortic endothelial cells (HAEC) from patients with BAV and aortic aneurysm (BAV/AoA). We show that HAEC of the patients have down regulated Notch signaling and fail to activate Notch-dependent EMT in response to its stimulation by different Notch ligands and TGF-β. These findings support the idea that BAV/AoA is associated with deregulation of the entire Notch1 signaling pathway independently on the specific gene mutation. This might be especially important finding in terms of searching for a possible therapeutic agent which is currently absent in BAV associated aortopathies.

2. Methods

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

Samples of the aneurysmal wall of the ascending aorta were harvested during aortic surgery at the Almazov Federal Medical Research Center. 12 specimens were sampled from patients with ascending aortic aneurysm associated with bicuspid aortic valve. All samples were from aneurysms with a diameter of >5 cm measured by preoperative transthoracic echocardiography. The patients are described in Table 1. Control aortic specimens were obtained during organ harvesting from organ transplant donors (n = 9) with the authorization of the law of Russian Federation and in accordance with the Declaration of Helsinki. Donors used as controls had no evidence of aneurysmal disease and all had TAV. All tissues were sampled from the outer curvature of the ascending aorta and the tissue was immediately dissected to separate medial layers followed by either freezing or enzymatic digestion.

2.1. Isolation of primary cultures

Human umbilical vein endothelial cells (HUVEC) were harvested from the human umbilical vein by enzymatic dissociation as previously described [19]. Human aortic endothelial cells (HAEC) were isolated according to [20] from tissue fragments of BAV-associated thoracic aortic aneurysm (TAA) patients after surgery for aneurysm corrections. Under sterile conditions tissue fragments were dissected away from the adventitia. After washing in PBS, the tissue fragments were first incubated for 30 min at 37 °C in 0.1% collagenase solution (Collagenase, Type 3, Worthington Biochemical Corporation, USA). Then endothelial layer was removed mechanically by scraper, endothelial cells were washed twice and plated onto fresh 3 cm^2 culture dish covered 0.1% gelatin (Sigma) in EGM2 medium (Promocell) and incubated at 37 °C. On the next day endothelial cells were washed by PBS and culture medium was changed. Then the cells were purified from interstitial cells using magnetic cell separation (MACS) with $CD31^+$ microbeads (Miltenyi Biotec) according to the manufacturer's directions. Purity of the endothelial cells was confirmed by ICH staining with anti CD31 and anti vWF antibodies (Abcam). The $CD31^+$ cells were used in experiments at passages 2–5.

Table 1
Clinical characteristic in the study group.
Values are means ± S.E.M.; CSA/h, ascending aortic cross-sectional area to patient height ratio

	BAV ($n = 12$)
Male gender (%)	59
Age (years)	42–65
Aortic diameter (cm)	5.9 ± 0.2
Aortic CSA/h (cm^2/m)	7.6 ± 0.6
Peak valve gradient (mmHg)	86 ± 11
Mean valve gradient (mmHg)	59 ± 9
Aortic valve area index (cm^2/m^2)	0.38 ± 0.02
Hypertension (%)	81

2.2. Genetic constructs and lentiviruses

Lentiviral packaging plasmids were a generous gift of D. 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. Open reading frame for murine Notch intracellular domain (NICD) was amplified from reversely transcribed mouse ES cells mRNA, using the 5′-*GGCGCGCCTCTGGATCC*AGTGCTGCTGTCCCGCAAG-3′ and 5′-*CCACTAGTGCGGCCGC*TTATTTAAATGCCTCTGGAATGTG-3′ primers; cDNA of murine *Dll1*, *Dll4*, Jaggged1 and Jagged2 were kind gift from Prof. Shigeru Chiba (University of Tsukuba, Japan) and Dr. Katsuto Hozumi (Tokai University School of Medicine, Japan) [21]. The NICD PCR fragment was cleaved with AscI and SpeI, then cloned in frame of the T7 tag, replacing the cm gene within pLVTHM-T7-cm. Similarly, the *Dll1* was cloned at the AscI and SpeI restriction sites of pLVTHM-T7-cm, the *Dll4* and *Jag2* at AscI and EcoRI restriction sites of pLVTHM-T7-cm, and the *Jag1* into BamHI and EcoRI restriction sites of the LeGO-G/BSD (Addgene). Lentiviral production was performed as described previously [22]. The virus titer was defined by GFP-expressing virus; the efficiency of primary endothelilal cell transduction was 90–95% by GFP. The efficiency of transgene expression with NICD-bearing virus and Dll4-bearing virus was verified by ICH-staining with the antibodies to Notch1 and Dll4 correspondingly and also was 90–95%.

2.3. EMT induction

For direct induction 50×10^3 HAEC were plated onto 6-well plates and transduced with saturating concentration of the lentiviral concentrate encoding Dll1, Dll4, Jag1, Jag2 or NICD relatively (not shown).

For induction in co-culture 50×10^3 of HUVEC were plated onto 6-well plates and transduced with Dll1, Dll4, Jag1or Jag2-bearing lentiviruses respectively. After 24 h 50×10^3 HAEC were seeded onto HUVEC in fresh EGM2. EMT was also induced by addition TGF-β1 (5 ng/ml) to the culture media.

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. The thermocycling conditions were as follows: 95 °C for 5 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. A final heating step of 65 °C to 95 °C was performed to obtain melting curves of the final PCR products. 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. Immunocytochemistry

After growing on cover slides, cells were fixed for 20 min in 1% paraformaldehyde and permeablized in 1% BSA/0.1% Triton X-100/PBS for three minutes, followed by blocking in 1% BSA/PBS for one hour. Then cells were incubated for one hour with primary antibodies: SMA (sc-32251, Santa Cruz). Secondary antibodies conjugated with r Alexa546 (Invitrogen) were used. DAPI was used to visualize nuclei. Microphotographs were taken using an AxioObserver Microscope (Zeiss) at ×20 magnification with AxioVision software.

2.6. NOTCH1 mutation screening

Genomic DNA was extracted from peripheral blood of all patients used in the study with a FlexiGene DNA purification Kit (Qiagen, GmbH, Hilden, Germany). We applied a strategy of targeted mutation screening for 10 out of 34 exons of the *NOTCH1* gene as described earlier [23]. Genomic DNA was extracted from peripheral blood using a FlexiGene DNA purification Kit (Qiagen, GmbH, Hilden, Germany). Amplification of exons 10, 11, 12, 13, 20, 23, 24, 29, 30, and 34 was performed using primers (available upon request). Mutation screening in patient and control groups was performed by direct sequencing of amplified fragments with an ABI capillary sequencer (Applied Biosystems, Foster City, CA, USA) using a BigDye Terminator v3.1 mix (Applied Biosystems). Obtained sequences were analyzed and aligned using BioEdit and Geneious software; new and rare variants were checked against the 1000 Genomes and EVS databases.

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).

3. Results

3.1. The baseline level of Notch signaling is altered in the HAEC from BAV patients

Notch signaling has been shown to be decreased in the ascending aorta wall specimens of patients with BAV [24]. Initially, we assessed the levels of expression for key Notch genes – *NOTCH1-4*, *JAG1*, *DLL1*,

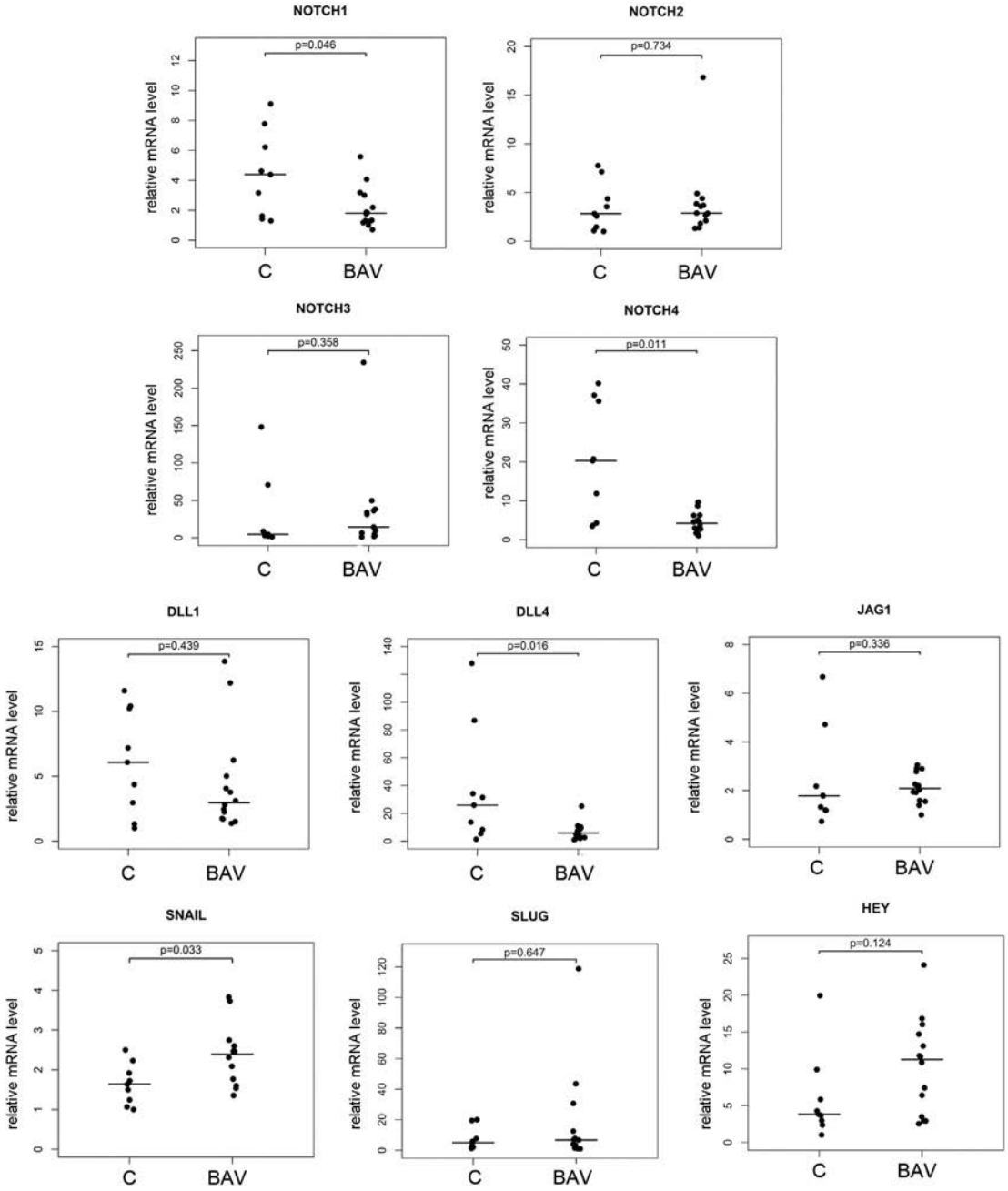

Fig. 1. Expression level of main Notch receptors and ligands in the endothelial cells from the patients with thoracic aortic aneurysm associated with bicuspid aortic valve (BAV), n = 12 and in control endothelial cells (C), n = 9. Groups are compared using the Mann–Whitney non-parametric test.

DLL4 in endothelial cells of BAV/AoA patients and controls (Fig. 1). Patient cells had significantly lower mRNA levels of *NOTCH1*, *NOTCH4* and *DLL4* comparing to controls. However the mRNA level of direct Notch targets *HEY1* and *SLUG*, which is required for EMT during cardiac morphogenesis [25] was not different between patients and controls. *SNAI1* (*Snail*) is one of the master regulators of EMT and Notch1 induces elevation in its expression during EMT [10,26]. Its mRNA level was significantly higher in the endothelial cells of BAV patients compared to control cells. Our data suggest alterations of baseline Notch signaling in aortic endothelial cells of BAV patients.

3.2. Experimental models for EMT induction

To establish an experimental model for estimating EMT efficiency in HAECs of BAV/AoA patients we induced EMT in HUVECs and control HAECs either by introduction of Notch intracellular domain (NICD) with lentivirus or by the addition of TGF-β as it was described earlier [25,27]. Stimulated cells lost cobblestone morphology after addition of NICD-virus or TGF-β suggesting loss of endothelial phenotype and transformation to mesenchymal cells (data not shown).

During cardiac EMT, endocardial cells undergo significant changes in gene expression including Notch-dependent induction of *ACTA2* (αSMA), *SNAII*, and *SNAIL2* [28]. We made additional screening of 11 genes previously described as markers of EMT [29]: *PECAM1*, *VWF*, *ACTA2*, *COL1A1*, *FOXC2*, *CDH2* (N-cadherin), *TAGLN* (SM22α), *TWIST1*, *FSPS100A4* (fibroblast specific protein s100a4), *SNAI2*/*SLUG*, *SNAI1*/*SNAIL* for their up or down regulation at EMT for HAECs and HUVECs after TGF-β or NICD-induction. Among responsive early EMT markers by qPCR were *HEY1*, *HES1*, *FSPS100A4*, *SLUG*, which were up regulated after EMT induction (Fig. 2A). SMA was late responsive and reliable marker of EMT in HAECs and HUVECs by qPCR as well as by ICH staining after 14 days of EMT induction. *HEY1* and *HES1* are among main Notch transcriptional targets [30] and were up regulated at mRNA level in endothelial cells in NICD-induced cultures. Loss of endothelial markers such as *PECAM1*, *VWF* was quite weak (data not shown). Thus, both HAECs and HUVECs activate EMT program in response to Notch1 intracellular domain activation and to a relatively lesser extent in response to TGF-β stimulation.

3.3. EMT is impaired in HAEC of BAV patients

To test the hypothesis that Notch signaling is impaired in aortic endothelial cells of BAV/AoA patients we compared effectiveness of EMT in HAECs from the patients with control cells by gain of mesenchymal markers and loss of endothelial markers.

First we induced EMT in BAV/Ao cells and in controls by introduction of NICD or addition of TGF-β (Fig. 3). We observed significantly impaired NICD-induced activation of *HEY1* and *SLUG* after 3 days of EMT induction by NICD; *HES1*, *FSPS100* and *ACTA2* also were not activated in response to EMT–induction by NICD in the cells of patients.

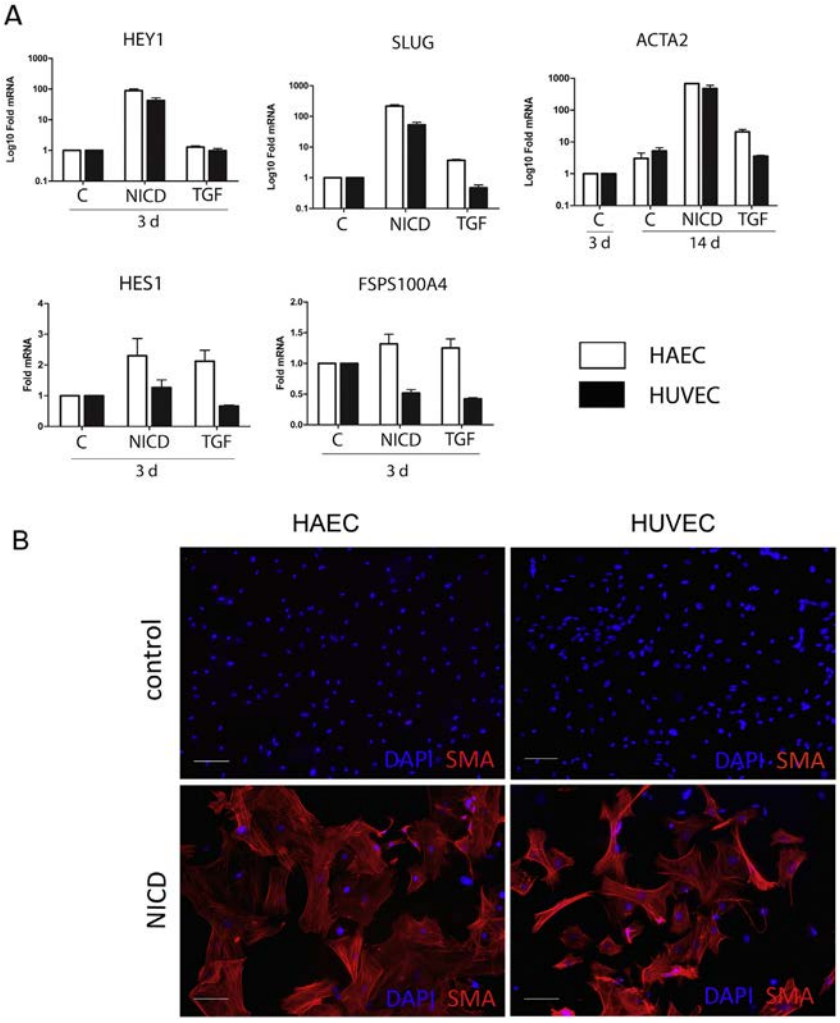

Fig. 2. Induction of endothelial-to-mesenchymal transition (EMT) in endothelial cells derived from human aorta (HAEC) and human umbilical vein (HUVEC) by either introduction of Notch-intracellular domain (NICD) or by TGF-β. (A) expression of responsive genes 3 and 14 days after EMT induction; (B) SMA (alpha-smooth muscle actin, *ACTA1*) induction in HAECs and HUVECs 14 days after EMT induction by ICH staining. Scale bar corresponds to 200 μm.

Similarly TGF-β-induced activation of the key EMT markers was significantly reduced. The loss of endothelial markers was not different between 2 groups (Fig. 3C).

Up regulation of *ACTA2* expression induced by NICD or by TGF was also impaired in the cells from patients. Fig. 3D demonstrates failure to activate NICD and TGF-β–induced EMT program in the cells of BAV patients by SMA staining of the cells.

Notch is known as a complex and lineage-specific signaling with several receptors and ligands [30], therefore we analyzed EMT induction in HAECs from donors and patients in co-culture with the ligand-expressing cells using the set of four Notch ligands (Fig. 4). Our data confirm the findings obtained in EMT stimulation with NICD that the cells of BAV patients fail to activate EMT program in response to activation of Notch ligands.

To verify if any of the BAV/Ao patients had mutations in *NOTCH1* gene we sequenced DNA from the patient blood samples. We applied a strategy of targeted mutation screening for 10 out of 34 exons of the *NOTCH1* gene as described previously [23]. The choice of these specific exons was based on previously published reports on the implication of *NOTCH1* mutations in cardiac malformations, including BAV [8,31–33]. We defined that none of the patients included in the study had mutations in these exons of *NOTCH1*.

3.4. Notch1-Jagged1 feedback loop is inactivated in HAECs of BAV patients

Notch functions as a part of positive feedback loop in which Notch receptor activation promotes Notch ligand expression in surrounding cells thus relaying a signal, a process known as lateral induction [34]. Jagged1 has been shown to be a direct transcriptional Notch target [35]. Therefore we tested if this feedback loop is active in the cells of BAV patients. We transduced HAECs with NICD-bearing lentiviruses and verified up regulation of Notch genes in control donor cells and in the cells from the patients (Fig. 5). Our data show that *JAG1* expression induced by NICD was substantially reduced in the cells of BAV patients.

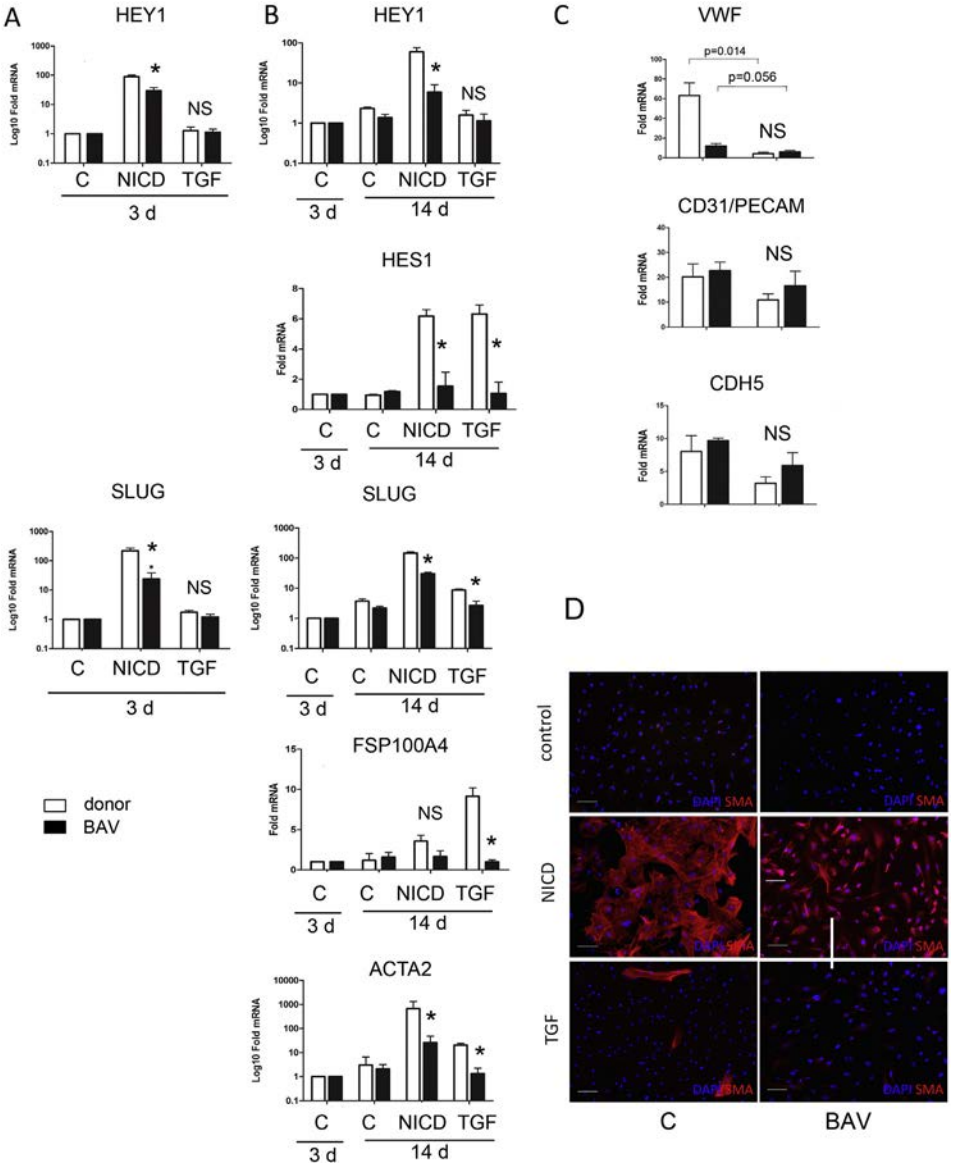

Fig. 3. Comparison of EMT induction effectiveness between HAECs from the patients with thoracic aortic aneurysm associated with bicuspid aortic valve (BAV) and control HAECs (donor) by either NICD or TGFβ. (A) Expression of mesenchymal markers estimated by qPCR 3 days after the induction; (B) Expression of mesenchymal markers estimated by qPCR 14 days after the induction; (C) Expression of endothelial markers 7 days after the induction (D) Expression of SMA by ICH staining 14 days after the induction. Scale bar corresponds to 200 μm. The initial level of a given gene was normalized between different samples and the fold change was estimated. Arrows (*) indicate differences between control (C) and BAV groups. P indicates the differences between non-stimulated and stimulated samples.

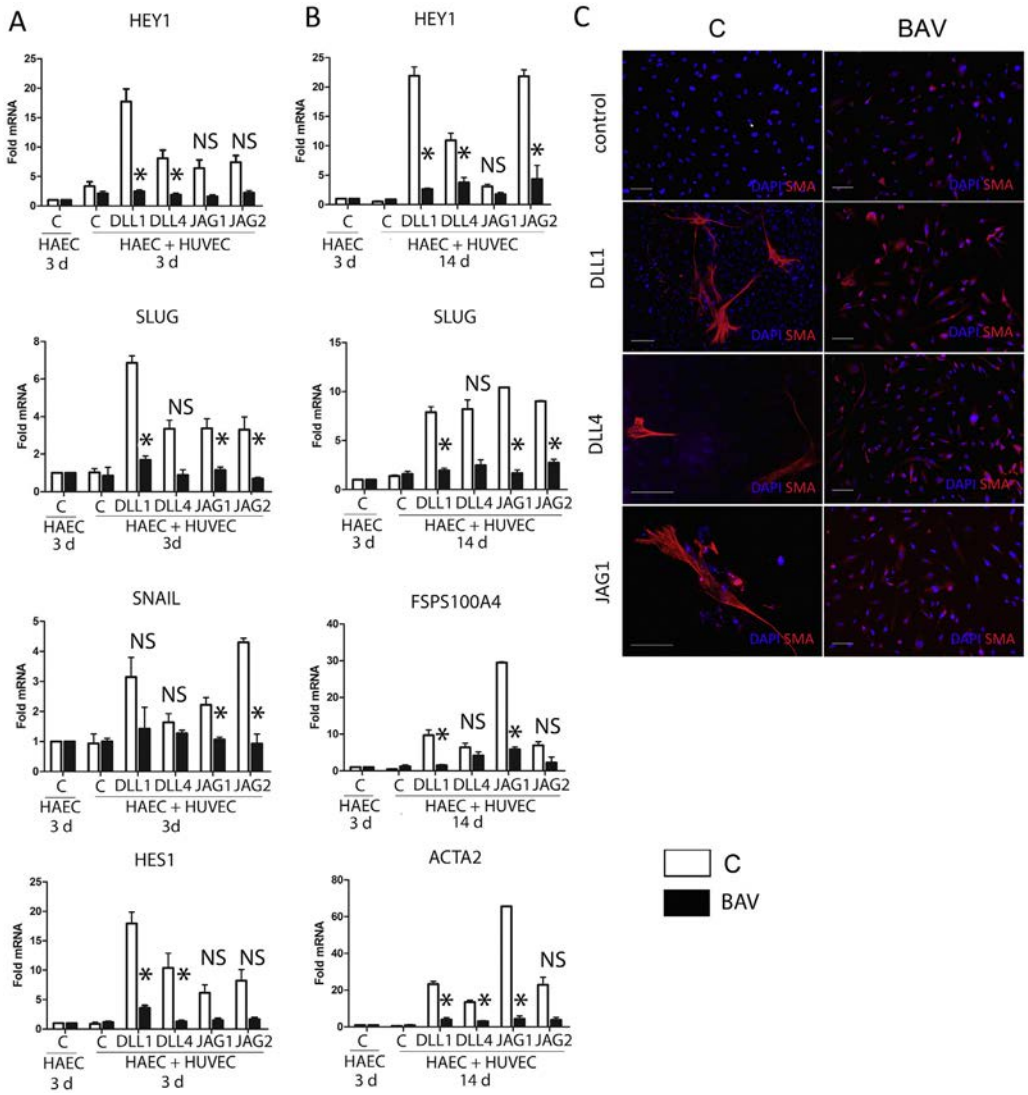

Fig. 4. Comparison of EMT induction effectiveness between HAECs from the patients with thoracic aortic aneurysm associated with bicuspid aortic valve (BAV) and control HAECs (donor) by co-culture with HUVECs expressing one of the ligands Dll1, Dll4, Jag1 or Jag2. (A) Expression of EMT-responsive markers estimated by qPCR 3 days after the induction; (B) Expression or EMT-responsive markers estimated by qPCR 14 days after the induction; (C) expression of SMA by ICH staining. *$P < 0.05$, Mann–Whitney non-parametric test. Scale bar corresponds to 200 μm.

4. Discussion

The etiology of a bicuspid aortic valve accompanied by aortic dilation remains poorly understood. Whether this is a consequence of a constant stress due to valve configuration or it is a consequence of a developmental mistake is widely discussed. A role of the endothelial cells in the developing of aneurysms is largely unknown, although a recent study suggests that aneurysms from BAV patients may be associated with endothelial dysfunction [36]. The most known genetic link in BAV pathology is mutations in *NOTCH1* described in patients with BAV [8,9,31–33]. Although BAV has strong evidence of a genetic basis, successful identification of disease-causing variants has been limited. It has been suggested by many researchers that the mutations not only in *NOTCH1* itself but also in related genes could be responsible for left ventricular outflow malformations. However, several recent papers do not support such a correlation [16,37,38]. Surprisingly, the authors did not find considerable amount of mutations in Notch-related genes in a cohort of patients where previously *NOTCH1* mutations had been described.

Our study provides the first direct functional evidence that primary endothelial cells derived from aortas of BAV/AoA patients have attenuated Notch signaling, irrespectively of *NOTCH1* mutation. We have shown that aortic endothelial cells derived from the BAV patients with dilated aorta have impaired EMT induction. This impairment could potentially reflect two aspects of the associated aortopathy. First aspect regards the failure to undergo EMT during embryonic development, which is necessary for the proper valve formation [39]. Alternatively, because EMT is described as a stress-induced mechanism in aortic valve, the failure to activate EMT program could lead to valve calcification [39]. A recent work has shown that Notch1 haploinsufficiency disrupts the appropriate endothelial response to shear stress. In contrast to wild-type endothelial cells, shear-exposed *NOTCH1*$^{+/-}$ endothelial cells failed to up regulate anti-osteogenic factors that may be critical for preventing calcification of underlying interstitial cells [13]. Similarly, our results suggest that BAV-derived aortic endothelial cells fail to up regulate Notch-induced EMT. We suggest that this reflects a common stress-defense mechanism, which is impaired in the diseased cells.

We also observed significant down regulation of Notch4 and Dll4 in the diseased endothelial cells (Fig. 1). Dll4 is the first Notch ligand expressed in the forming arteries in the mouse, and *Dll4* expression precedes that of the genes encoding arterial Notch receptors, *Notch1* and *Notch4* [40]. Our findings are well in line with recent reports

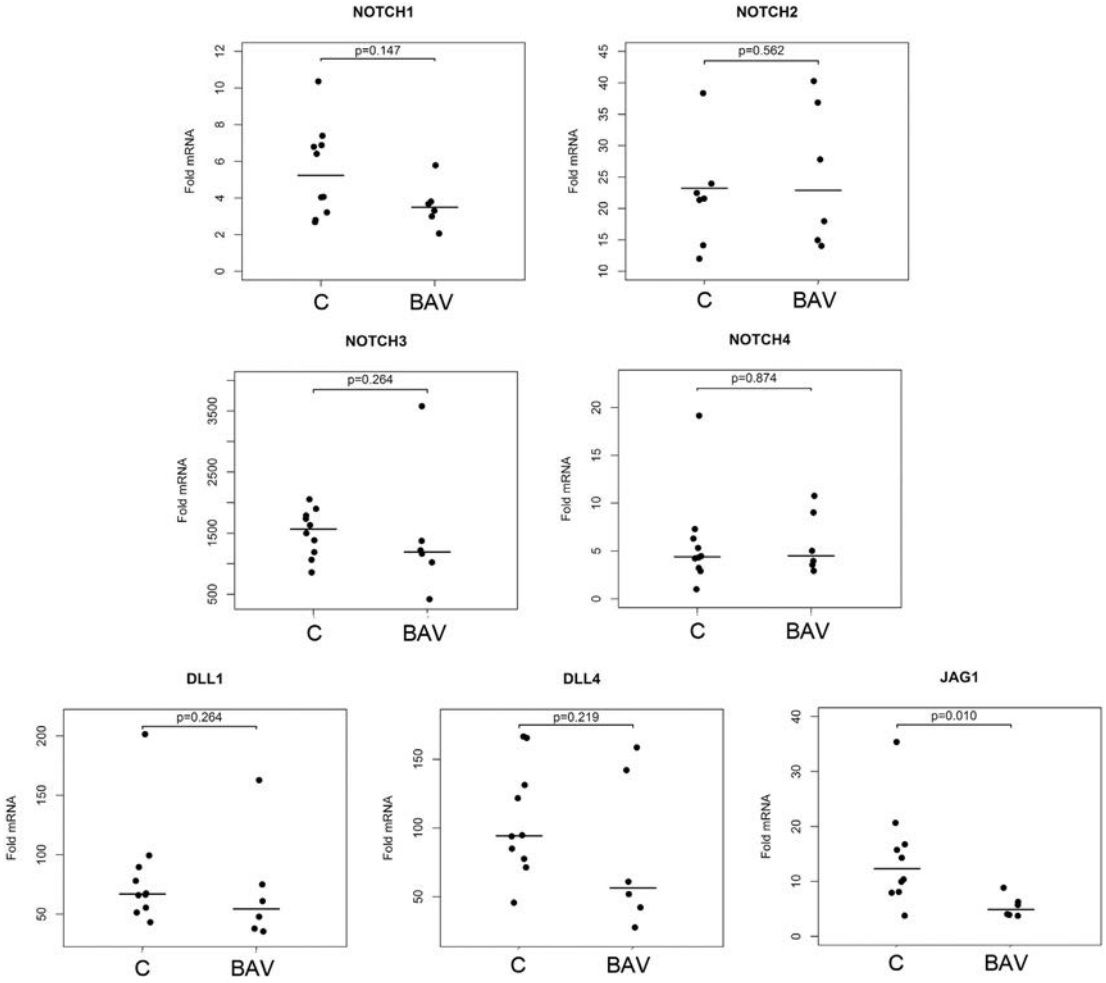

Fig. 5. Lateral induction of Notch genes by Jag1. HAECs from the patients with thoracic aortic aneurysm associated with bicuspid aortic valve (BAV), n = 6 and control HAECs (donor), n = 9, were transduced with lentivirus bearing Jag1 and the expression of Notch family genes was verified after 72 h by qPCR. Groups are compared using the Mann–Whitney non-parametric test.

describing fine tuning of Dll4/Notch4 axis and its participation in arterial specification [41,42], implying that this axis might be disrupted in BAV-patients and this impaired arterial specification could contribute to aortic aneurysm development often associated with BAV.

In a recent paper it has been shown that Notch4 agonistic antibody in mice accelerate the healing response increasing amount of SMC in the vessel [43]. Also this paper demonstrates the proangiogenic role of endothelial Jagged1 in adult physiological angiogenesis and the synergistic roles of endothelial Jag1 and Dll4 on vascular maturation. We show that *JAG1* expression induced by NICD was substantially reduced in the BAV-derived endothelial cells and this was associated by down regulation of smooth muscle cell contractile markers in SMC of the aneurysmal wall. We suppose that the initial process of the vessel formation as well as further healing in response to shear stress is impaired in the BAV patients via Notch-dependent events in particular through inactive feedback loop between Notch1 and Jagged1.

In our experiments all the ligands (Dll1, Dll4, Jag1, Jag2) failed to up regulate EMT response in the cells of BAV-patients. However, both Dll1 and Dll4 were able to up regulate *SNAIL* almost to its level in healthy cells. This result demonstrates the complexity of Notch signaling links and also represents Dll1 and Dll4 ligands as potential candidates for the up regulation the Notch system in the diseased cells as it has been already described in other pathologies [44]. However it is clear that the system is extremely dosage-dependent and fine-tuned, thus further studies of endothelial-SMC regulation are needed to understand its function.

The list of signaling pathways that modulate a given component of the Notch signaling system is already abundant and is likely to be expanded in the coming years [30]. One of the challenges lying ahead is to define the temporal and spatial location of the regulation in vivo and to determine the relative importance of all the interacting signaling pathways that impinge on Dll4/Notch activity. In addition, very little is known about the complex genetic program triggered by Notch activation and how it changes in time and with relation to the cellular status. Notch is clearly of central importance for physiological and pathological angiogenesis and a deeper knowledge of its molecular regulation in different contexts will allow the development of better therapeutic strategies.

In summary, our data provide the evidence that Notch signaling pathway is attenuated in BAV/AoA-derived endothelial cells resulting in non-effective EMT process. This finding further underlines the importance of Notch pathway in cardiovascular system development and maintenance. The obtained data open an additional clinical perspective for searching a therapeutic target in order to prevent the development of aortic aneurysm/stenosis associated with bicuspid aortic valve.

Author contributions

A.S.K. conducted experiments, analyzed data, and wrote the manuscript, V.E.U., O.B.I., N.D.G., O.M.M. acquired data, E.V. I., O.A.F., S.V.Z., A.N.T. conducted experiments, A.N.T., A.A.K. wrote the manuscript, and A.B.M. designed research, analyzed data, and wrote the manuscript.

Conflict of interest

The authors have declared that no conflict of interest exists.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Research Article

Phenotypic and Functional Changes of Endothelial and Smooth Muscle Cells in Thoracic Aortic Aneurysms

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Thoracic aortic aneurysm develops as a result of complex series of events that alter the cellular structure and the composition of the extracellular matrix of the aortic wall. The purpose of the present work was to study the cellular functions of endothelial and smooth muscle cells from the patients with aneurysms of the thoracic aorta. We studied endothelial and smooth muscle cells from aneurysms in patients with bicuspid aortic valve and with tricuspid aortic valve. The expression of key markers of endothelial (CD31, vWF, and VE-cadherin) and smooth muscle (SMA, SM22α, calponin, and vimentin) cells as well extracellular matrix and MMP activity was studied as well as and apoptosis and cell proliferation. Expression of functional markers of endothelial and smooth muscle cells was reduced in patient cells. Cellular proliferation, migration, and synthesis of extracellular matrix proteins are attenuated in the cells of the patients. We show for the first time that aortic endothelial cell phenotype is changed in the thoracic aortic aneurysms compared to normal aortic wall. In conclusion both endothelial and smooth muscle cells from aneurysms of the ascending aorta have downregulated specific cellular markers and altered functional properties, such as growth rate, apoptosis induction, and extracellular matrix synthesis.

1. Introduction

Thoracic aortic aneurysms have altered cellular composition and degeneration of the extracellular matrix in the aortic wall. There are several different etiologies of thoracic aortic aneurysm such as monogenic syndromes (Marfan and Loeys-Dietz syndromes), aneurysm associated with bicuspid aortic valves, and idiopathic aneurysms. The pathogenesis of aneurysm formation in the monogenic syndromes has been extensively studied [1], whereas the molecular and cellular mechanisms of the other forms, which constitute the majority of thoracic aortic aneurysms, remain largely unknown.

Most studies of the cell and molecular biology of thoracic aortic aneurysm have used entire wall specimens [2–6]. Consequently, the biology of the different cell types in the aneurysmal wall remains largely unknown. Single studies comparing smooth muscle cells (SMCs) from aneurysm patients with the cells from patients with acute aortic dissection revealed differences in expression of some SMC specific genes. In addition, SMCs from aneurysms were demonstrated to have significantly shorter telomeres, reduced metabolic activity, and impaired proliferation and migration rates [7]. Comparison of SMC derived from either bicuspid aortic valve (BAV) or normal tricuspid aortic valve (TAV) in thoracic aortic aneurysms showed expression difference in several markers including osteopontin and tissue inhibitor of metalloproteinase 3 which may reflect different etiologies

of TAV- and BAV-associated aneurysms [5–10]. However all above-mentioned studies did not address the functional properties of endothelial cells. As a consequence, a possible role of the endothelium in the developing of aneurysms is largely unknown, although a recent study suggests that aneurysms at least from BAV patients may be associated with endothelial dysfunction [11].

Endothelial cells have a substantial influence on smooth muscle cell differentiation [12]. Recent studies show that endothelial cells could directly influence smooth muscle cell phenotype [13, 14]. The major role of mature differentiated vascular smooth muscle cells is to maintain blood vessel tone and to regulate blood pressure through constriction or relaxation. This is achieved through the expression of a complement of regulatory and contractile genes that provide the machinery for this response [15]. The differentiated contractile phenotype is largely characterized by expression of coordinately regulated smooth muscle-specific markers that include smooth muscle (SM) α-actin (*ACTA2*), and SM22α (*TAGLN*) and some other proteins [15].

We sought to investigate if SMCs from aortic tissue of the patients with thoracic aneurysm undergo phenotypic and functional change such as growth, apoptosis, and extracellular matrix synthesis and whether this change is also accompanied by endothelial cell changes in the cells of the patient tissues compared to the cells from healthy tissue. Our data demonstrate downregulation of smooth muscle as well as endothelial cell specific markers in the patient cells and also changes in functional state of both SMC and endothelial cells.

2. Materials and Methods

The clinical research protocol was approved by the Local Ethics Committee of the Almazov Federal Medical Research Center 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 at the Almazov Federal Medical Research Center. Thirty specimens were sampled from patients with BAV ($n = 17$) or TAV ($n = 13$) (Table 1). Patients with connective tissue disorders were excluded. Control aortic specimens were obtained from organ transplant donors ($n = 11$) and all had TAV. Donors were all men with mean age 48 ± 11. All tissues were sampled from the outer curvature of the thoracic aorta.

2.1. Primary Cultures. To obtain SMC cultures the cells were isolated as previously described [16]. The cells were used in experiments at passages 2–5. Human aortic endothelial cells (HAEC) were isolated from tissue fragments of patients after surgery for aneurysm corrections. Under sterile conditions tissue fragments were dissected away from the adventitia. After washing in PBS, the tissue fragments were first incubated for 30 min at 37°C in 0.1% collagenase solution (Collagenase, Type 3, Worthington Biochemical Corporation, USA). Then endothelial layer was removed mechanically by scraper, and endothelial cells were washed twice and plated onto fresh 3 cm^2 culture dish covered with 0.1% gelatin (Sigma) in EGM2 medium (Promocell) and incubated at 37°C. The next day endothelial cells were washed by PBS and culture medium was changed. The cells were used in experiments at passages 2–4.

2.2. Cell Migration Assay. Cell migration was determined using a "scratch" wound assay as described previously [17]. SMCs were grown to confluence on 6-well plates; after the cells formed a monolayer, the medium was exchanged for serum free medium containing 10 mM hydroxyurea and 10 ng/mL PDGF_BB growth factor to inhibit proliferation and to stimulate migration and the cell monolayer was scraped with a 200P pipette tip to create a cell-free zone. The number of cells which migrated into the wounded area was counted after six and 24 hours. Experiments were performed in duplicate and then repeated three times.

2.3. Apoptosis Assay. For estimation of apoptosis SMCs were seeded at a density of 10×10^3 cells/cm^2, and 10×10^{-3} M hydrogen peroxide (H_2O_2) was added to the culture medium 48 hours later. After two hours the cells were removed and labeled with FITC-conjugated annexin V (Sigma). The number of annexin V labelled cells was estimated by flow cytometry using Calibur II (BD).

2.4. Reverse Transcription-PCR. Total RNA was extracted from SMCs or endothelial cells using Trizol reagent (Invitrogen) according to the instructions of the manufacturer. Reverse transcription was performed using kits (Eurogen, Russia). Real-time PCR was performed in the LightCycler system with SYBR Green detection (Fermentas) using specific primers. The mRNA levels were normalized to GAPDH or HPRT mRNA. Changes in target genes expression levels were calculated as fold differences using the comparative ΔΔCT method. The primer sequence is available upon request.

2.5. Immunoblotting. Proteins were extracted from medial tissues or SMCs. Specimens were homogenized in a lysis buffer (50 mM Tris (pH 8), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 5 mM EDTA), containing protease inhibitors (Roche). Extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Primary antibodies used are SM22α (ab14106, Abcam), SMA, vimentin (M072529, DAKO), beta-actin (ab 6276, Abcam), collagen I, fibrillin, and elastin. Positive bands were quantified by densitometry using a gel documentation system Fusion Fix (Vilber Lourmat) and Fusion-Capt software. Bands were normalized using beta-actin stainings.

2.6. Immunocytochemistry. Primary antibodies used are SMA (sc-32251, Santa Cruz), SM22alpha (ab14106, Abcam), vimentin (sc-6260, Santa Cruz), VE-cadherin (MAB938, RandD), von Willebrand factor (ab20435, Abcam), and calponin (ab700, Abacam). Secondary antibodies conjugated with Alexa488 or Alexa546 (Invitrogen) were used. DAPI was used to visualize nuclei. Microphotographs were taken using

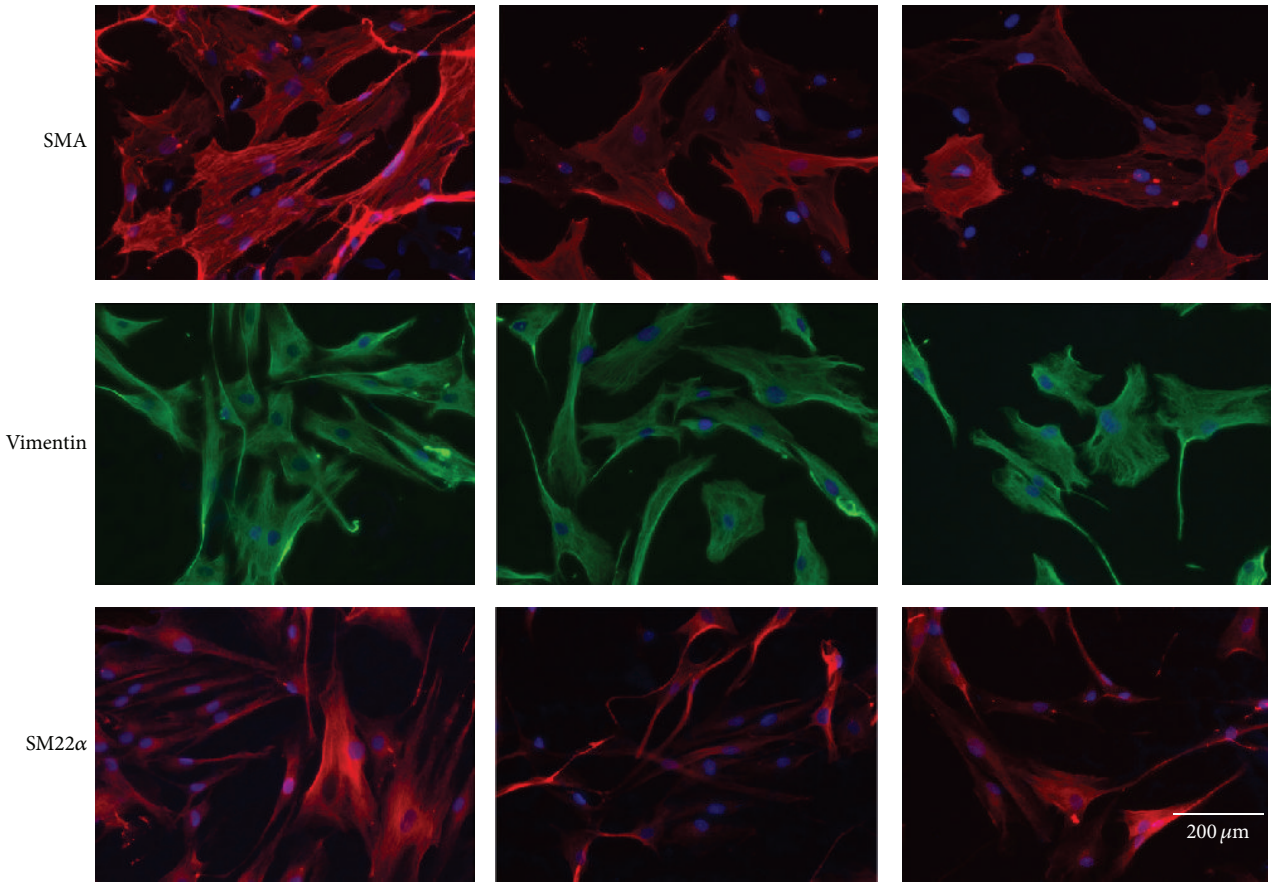

Figure 1: Expression of smooth muscle cell (SMC) markers in SMC from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and controls (C) determined by immunohistochemical staining of vimentin, α-smooth muscle actin (SMA), and SM22α (magnification ×20).

AxioObserver Microscope (Zeiss) at ×20 magnification with AxioVision software.

2.7. Zymography. MMP activity was assayed by a modified gelatin zymography method [18]. Activity and content of MMP-2 and MMP-9 were expressed in QuantiScan arbitrary units.

2.8. Statistical Analysis. Values are expressed as means ± SD. Groups were compared using the Mann-Whitney nonparametric test. A value of $P \leq 0.05$ was considered significant.

3. Results

3.1. Expression of Smooth Muscle Cell Markers in Smooth Muscle Cells from Aneurysms of the Thoracic Aorta. SMCs from aneurysms of the thoracic aorta and from control aortas were analyzed regarding the expression of SMC markers like α-smooth muscle actin (SMA), vimentin, and SM22α. Figure 1 shows typical immunofluorescent staining of SMC from control aortas and from aneurysms in patients with BAV and TAV. Both TAV- and BAV-derived SMCs appeared to have decreased level of SMA, vimentin, and SM22. However, there were no visible differences between SMC from patients with BAV and TAV.

At both mRNA and protein level expression of SMA and vimentin was reduced both in the BAV- and TAV-derived SMC and in the aortic media (Figure 2). However, although SMA was lower in aortic media of aneurysms from patients with BAV than in controls, it was still higher than in patients with TAV. SM22 expression was decreased only in SMC and aortic media from patients with TAV. In aneurysms from patients with BAV the expression of SMA was higher than in TAV patients both in SMC and in aortic media (Figure 2).

3.2. Expression of Endothelial Markers in Endothelial Cells from Aneurysms of the Thoracic Aorta. Figure 3 demonstrates primary cultures of endothelial cells and ICC staining of the cells including staining for SMA to confirm that the cultures were not contaminated with SMC. Endothelial markers appeared to be reduced in endothelium in aneurysms from patients with both TAV and BAV (Figure 3).

We compared also the mRNA level of SMA, CD31/PECAM, VE-cadherin, and vWF in endothelial cells derived

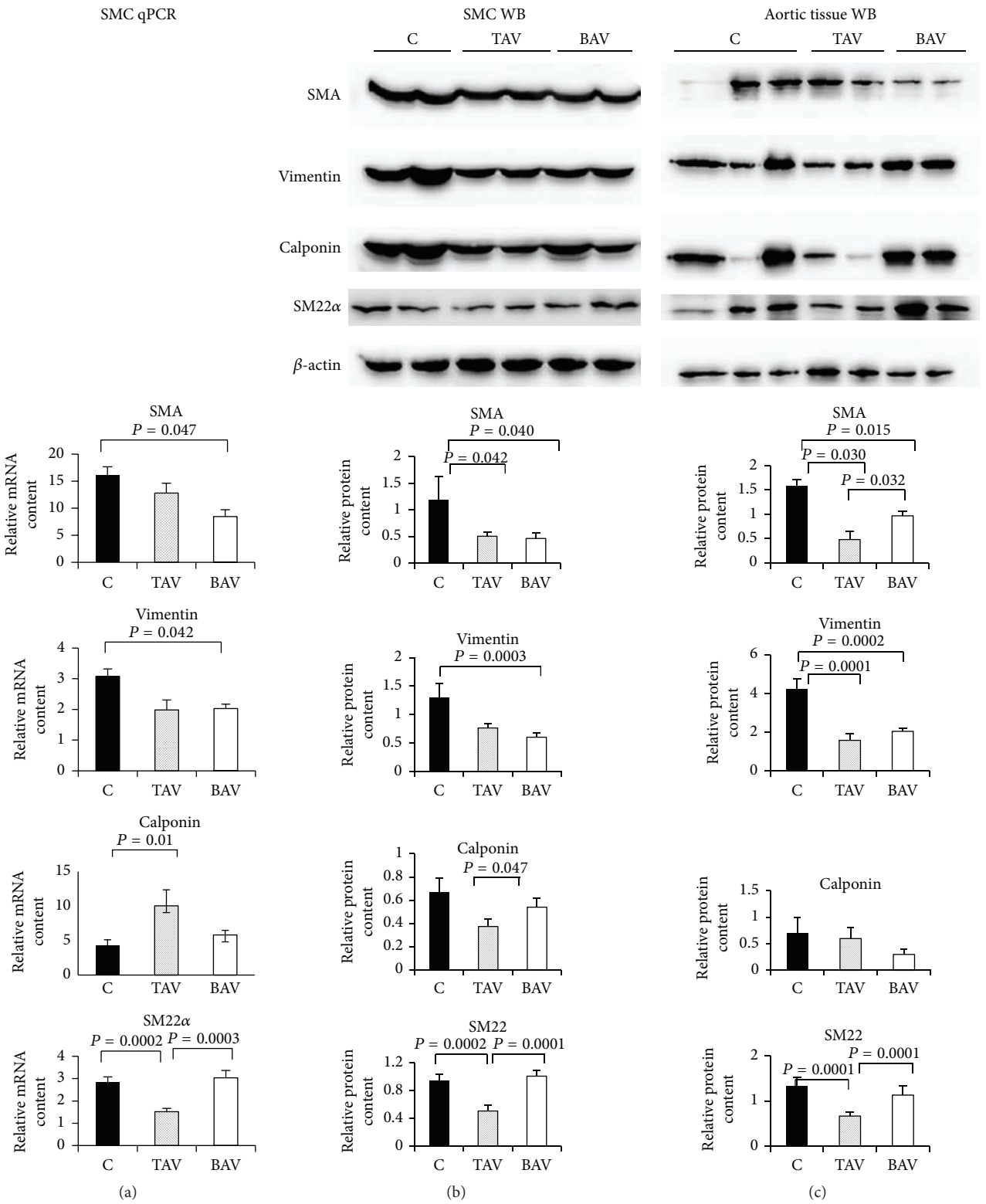

Figure 2: Expression of smooth muscle cell (SMC) markers in SMC from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and controls (C). mRNA level was determined by qPCR; protein level was determined by Western blot. The diagrams represent the results of densitometry. The bands were normalized by β-actin. (a) mRNA level in SMC, C: $n = 10$; TAV: $n = 13$; BAV: $n = 11$. (b) Protein level in SMC, C: $n = 10$; TAV: $n = 13$; BAV: $n = 11$. (c) Protein level in aortic media, C: $n = 11$; TAV: $n = 13$; BAV: $n = 17$.

Table 1: Clinical characteristics in the study groups.

	TAV ($n = 13$)	BAV ($n = 22$)
Male gender (%)	46	59
Age (years)	71.3 ± 2.53 (range 55–84)*	62.1 ± 1.87 (range 42–79)*†
Aortic diameter (cm)	5.6 ± 0.18*	5.9 ± 0.16*
Aortic CSA/h (cm^2/m)	6.6 ± 0.6*	7.6 ± 0.6*
Peak valve gradient (mmHg)	83 ± 9	86 ± 11
Mean valve gradient (mmHg)	55 ± 7	59 ± 9
Aortic valve area index (cm^2/m^2)	0.39 ± 0.02	0.38 ± 0.02
Hypertension (%)	84*	81*
Medication		
Angiotensin receptor blockers (%)	38*	18
Statins (%)	0	41*†
Aspirin (%)	31	14

Values are means ± SEM. *$P < 0.05$ compared with donors; †$P<0.05$ compared with TAV; CSA/h, ascending aortic cross-sectional area to patient height ratio.

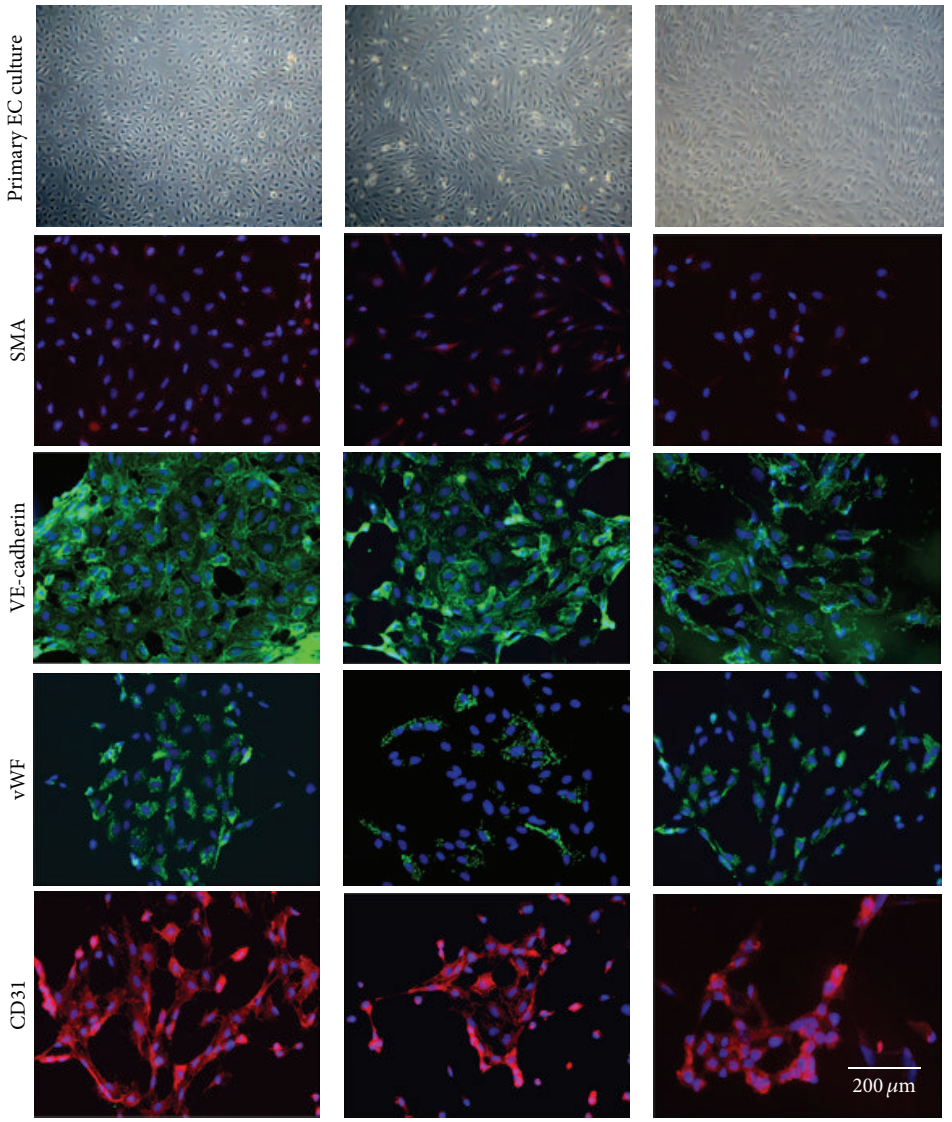

Figure 3: Characterization of the aortic endothelial cells from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and controls (C). Upper panel represents typical aortic endothelial cell cultures from control and aneurysmal aortas. The SMA staining confirms the lack of medial SMC contamination in the endothelial cell culture. Vascular endothelial-cadherin (VE-cadherin), von Willebrand factor (vWF), and CD31/PECAM staining confirm endothelial nature of the isolated cells.

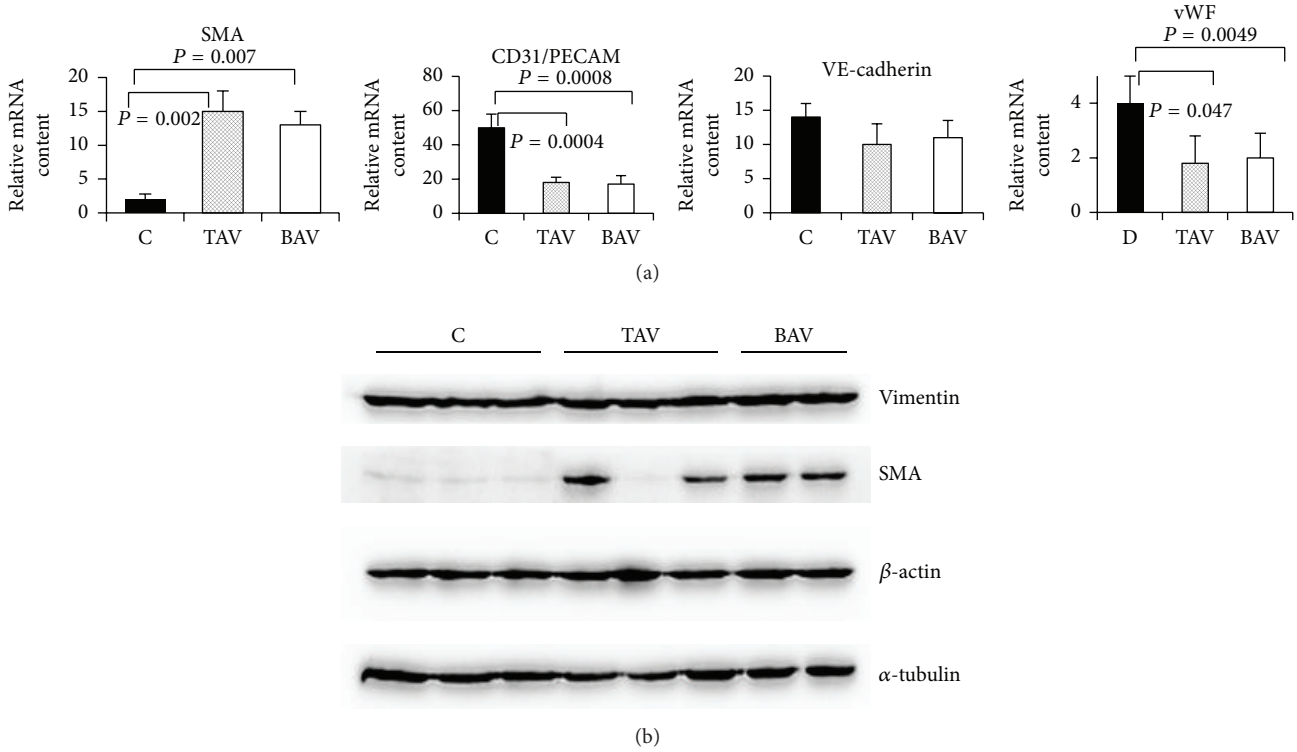

Figure 4: Expression of smooth muscle cell (SMC) markers and endothelial cell markers in endothelial cells from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and controls (C). mRNA level was determined by qPCR; protein level was determined by Western blot. The diagrams represent the results of densitometry. The bands were normalized by β-actin. (a) mRNA and protein level in endothelial cells: controls: $n = 5$; TAV: $n = 5$; BAV: $n = 5$; (b) representative Western blot picture for SMA and vimentin protein level in endothelial cells.

from control aortas and from aneurysms. The expression of SMA mRNA was elevated in both TAV- and BAV-derived endothelial cells (Figure 4). Typical SMA microfilament staining was not observed in our endothelial cultures (Figure 3); thus the elevation of SMA mRNA level was not due to contamination with SMC. Expression of the endothelial markers vWF and CD31/PECAM was substantially decreased in endothelial cells from aneurysms (Figure 4); the level of VE-cadherin mRNA was not changed.

3.3. Proliferation and Migration of Cells from Aneurysms. Specific degenerative processes associated with reduced cellularity are observed in the aneurysm wall [19]. To evaluate the possible contribution of SMC and endothelial cells to these changes, we compared cell proliferation and migration in healthy donors and patients with aneurysms of the thoracic aorta. SMC proliferation rate (Figure 5(a)) in both BAV and TAV aneurysm was lower compared to healthy donors, but without any difference between the two types of aneurysms. Endothelial cells from aneurysm patients had also lower proliferation than controls, but endothelium from patients with BAV had lower proliferation rate than endothelium from aneurysms of patients with TAV (Figure 5(b)).

SMC migration rate was higher in aneurysm patients with TAV, but not in BAV patients compared to controls (Figures 5(a) and 5(b)).

3.4. Apoptosis in Smooth Muscle Cells from Aneurysm Walls. Reduced cell number has been shown in aortic tissue of thoracic aortic aneurysm patients [20] and may reflect increased apoptotic level in the vessel wall cells. Indeed, the number of cells that are positive for DNA double strand breaks (an apoptotic marker) is increased in the media of the wall of thoracic aneurysms [20]. Therefore we studied apoptosis in SMC cultures from aneurysm patients and controls (Figure 6(a)). The number of annexin V positive cells was significantly higher in SMC cultures from patients with both BAV and TAV.

Oxidative injury might be a cause of increased wall weakness in both abdominal and thoracic aortic aneurysm [21]. The ability of oxidative stress to cause apoptosis might be altered in SMC from aneurysms. To test this hypothesis H_2O_2 was added to SMC. We measured apoptosis as a residual between the percentage of annexin V positive cells after H_2O_2 treatment and the amount of annexin V positive cells in normal cultures ("baseline"). H_2O_2-induced apoptosis was reduced in SMC from aneurysms compared to SMC from nonaneurysm aortic walls (Figure 6(b)).

3.5. Matrix Metalloproteases and Matrix Protein Content. To evaluate SMC contribution to extracellular matrix protein synthesis (elastin, fibrillin, and collagen I) in the aortic wall we estimated the protein content in aortic media specimens

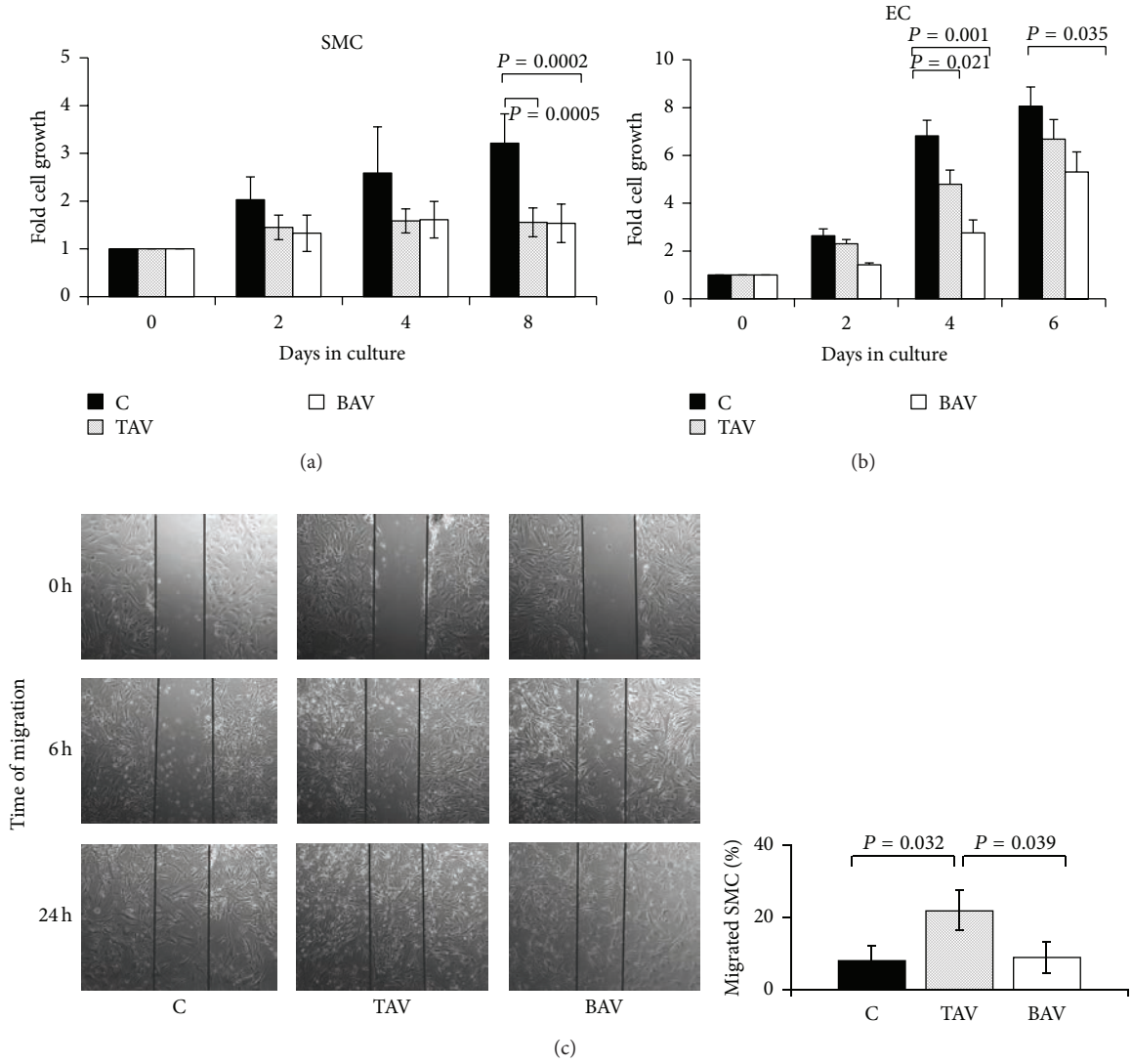

Figure 5: Proliferation and migration characteristics of SMC and endothelial cells from patients with aortic aneurysm with either tricuspid aortic valve (TAV, $n = 5$) or bicuspid aortic valve (BAV, $n = 5$) and controls (C, $n = 5$). The cells were seeded at an equal density and counted each two days for proliferation assay. The migration was estimated via scratch-assay (see Section 2). Migrated cells were counted after 6 h and 24 hours. (a) SMC proliferation. (b) Endothelial proliferation. (c) SMC migration.

and in SMC protein extracts (Figure 7). The elastin and fibrillin content was reduced in aortic media from aneurysms in both BAV and TAV patients (Figure 7(a)).

Collagen I content was higher in aortic media from both types of aneurysm patients but was not significantly changed in SMC from aneurysm patients. However, the amount of collagen I was higher in SMC of aneurysms from patients with TAV only (Figure 7(a)).

The culture media from endothelial cells were analyzed for elastin, collagen I, and fibrillin content (Figure 7(b)). Our data show that aortic endothelial cells are capable of synthesizing these proteins and synthesizing fragmented collagen I.

MMP expression may be increased in thoracic aneurysms [2, 22], but their role in the etiology of aneurysms is not clarified. The exact type of cells in the aortic wall that synthesizes MMPs is still unknown [23]. We did not detect increase in MMP2 or MMP9 activity in total aneurysm medial tissue samples (Figure 8), whereas MMP-9 activity was significantly increased in SMCs and SMC culture media from aneurysm patients with both BAV and TAV.

4. Discussion

The functional studies of the cells from TAA patients are still rare. However the ultimate target of any therapy approach is a cell, whose healthy properties are changed in the pathology. That is why, it is important to know not only a set of genes changed at a definite pathology, but also cellular properties that are the cause of a pathology and thus to find a possible

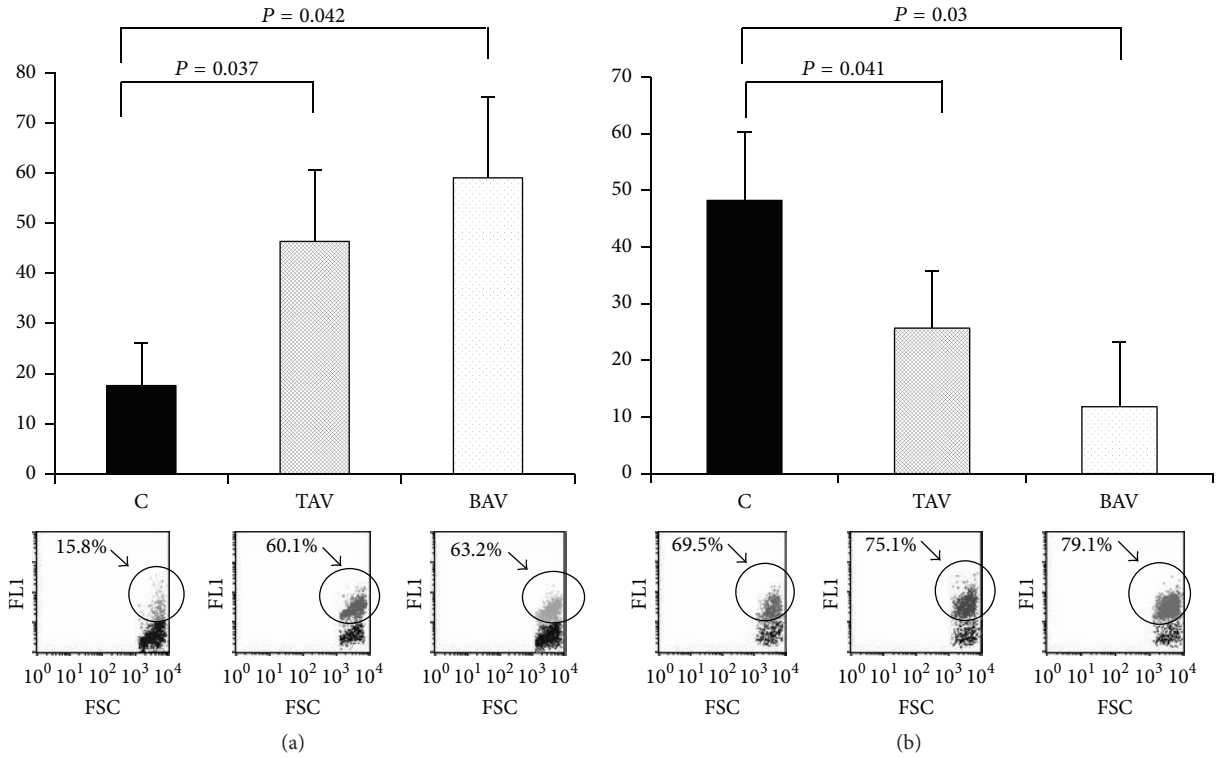

Figure 6: Apoptosis level in cultured SMC from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and controls (C). (a) The level of "baseline" SMC apoptosis in culture. The diagram shows the percentage of annexin V positive SMC in vitro estimated by flow cytometry. The lower panel shows representative plots from the analysis of live SMC in culture. C: $n = 5$; TAV: n=5; BAV: $n = 5$. (b) Apoptosis induction by H_2O_2. The diagram shows the residual between the percentage of annexin V positive cells after H_2O_2 treatment and the level of annexin V positive cells in untreated cultures. The lower panel shows representative plots from the analysis of SMC treated with H_2O_2. C: $n = 5$; TAV: $n = 5$; BAV: $n = 5$. Arrows mark annexin V positive cells.

tool for a correction. This study aimed to characterize two major aortic cellular populations, SMC and EC, from TAA patients and healthy donors.

In the present study we demonstrated that both SMC and endothelial cells from thoracic aortic aneurysms have impaired functional properties in terms of proliferation, contractile and extracellular protein expression, and apoptosis with significant difference between BAV- and TAV-associated aneurysms. Of most importance, we revealed the primary changes of endothelial cells. Our findings are in agreement with a recent publication describing endothelial dysfunction in BAV patients [11].

SMC from thoracic aneurysms demonstrated decreased expression of key SMC proteins such as SMA and SM22α both at mRNA and at protein level. Endothelial cells from thoracic aneurysms also demonstrated downregulation of specific markers and impairment in growth. Our data suggests that the cells from the aneurysm aortic wall are in less differentiated state comparing to normal aortic wall. This observation is well in line with the findings that endothelial cells influence differentiation and functions of underlying SMC [13, 14]. Thus, endothelial changes may contribute to impairment of the aortic wall structure built by SMC.

Cultured SMC from aneurysm patients demonstrated surprisingly high amounts of annexin V positive cells suggesting a high level of apoptosis in the aortic wall of aneurysm patients [3]. This may also be partly a consequence of the cell culture, but nevertheless there were differences between the different groups. Surprisingly the SMCs were relatively resistant to apoptosis induced by H_2O_2. This may be an important finding, but we have no good explanation. Philippi and coauthors showed that SMC from aneurysms in BAV patients had the poorest resistance to oxidative stress [10], but they did not show a baseline level of apoptosis in their SMC cultures but counted the viability of the cells under oxidative stress. Apoptosis/proliferation rate is a very important parameter of a cellular population turnover. We suggest that both decreased proliferation rate and increased apoptosis contribute to the loss of cells in the aortic wall in aneurysms. The mechanisms of apoptosis resistance/susceptibility in aortic SMC population may be important in the development of aneurysms.

We compared the content of some extracellular matrix protein in aortic tissue samples, SMC and supernatants from SMC and endothelial cells. The observed results suggest that SMC are not the only cells that synthesize extracellular matrix

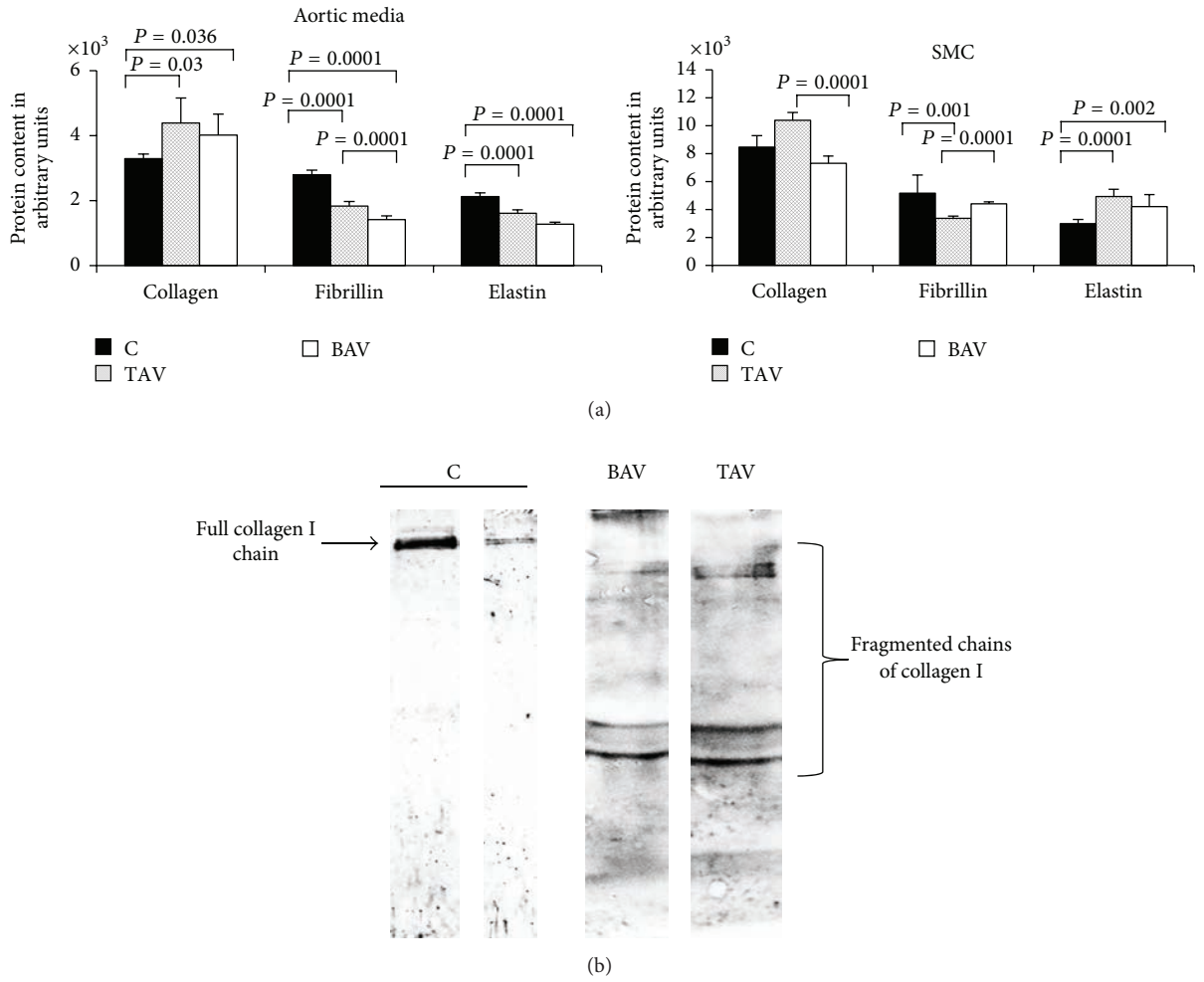

Figure 7: Matrix protein level in SMC and endothelial cells from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and controls (C) determined by Western blot. (a) Matrix protein level in SMC. The diagrams represent the results of densitometry. The bands were normalized by β-actin. C: $n = 10$; TAV: $n = 13$; BAV: $n = 17$. (b) Collagen I protein level in endothelial cell culture media.

proteins in the aortic wall. Also endothelial cells are capable of synthesizing extracellular matrix proteins in aneurysms and the extracellular matrix composition is different between aneurysms from patients with TAV and BAV TAA. Finally the integrity of the extracellular matrix in the wall of aneurysms is influenced by changes in the ratio between different proteins. Fibrillin content was different between aneurysms from TAV and BAV patients. Both endothelial cells and SMC from aneurysm patients had synthesis of fragmented collagen I. The complex nature of biosynthesis of extracellular matrix proteins in the aneurysm wall is in agreement with other recent studies [22, 23].

The role of different MMP in thoracic aneurysm pathogenesis has been discussed [22, 23]. There are a number of papers describing mainly the elevated gene expression levels for some MMPs in the aneurysm wall [2, 22–24]. In our study we analyzed the enzyme activity of MMP2 and MMP9. The activity for MMP2 and MMP9 was elevated in SMC and in supernatants from SMC cultures. This is in accordance with previously reported elevated gene expression of MMP2 and MMP9 in the wall of thoracic aneurysms [22, 23].

This study has several important limitations. The study is heterogeneous because from every tissue sample it is not possible to get SMC and endothelial cell cultures; the cells are in culture for limited amount of passages that limits the possibilities to study them. For the endothelial cells it is difficult to get cultured cells and it is not possible to get enough cells from many patients for different kind of analysis. Another limitation is that we have studied only two types of cells whereas the aorta consists of more types of cells including stem cells, macrophages, and fibroblasts. A very important limitation is that gene expression and cell behavior in dilatative aortopathy can be affected by hemodynamics as well as by physical and biochemical environment, other than by their interaction with the genetic background and these factors are absent in cultured cells.

Further research of the cell alterations in the wall of thoracic aneurysms might lead to a deeper understanding

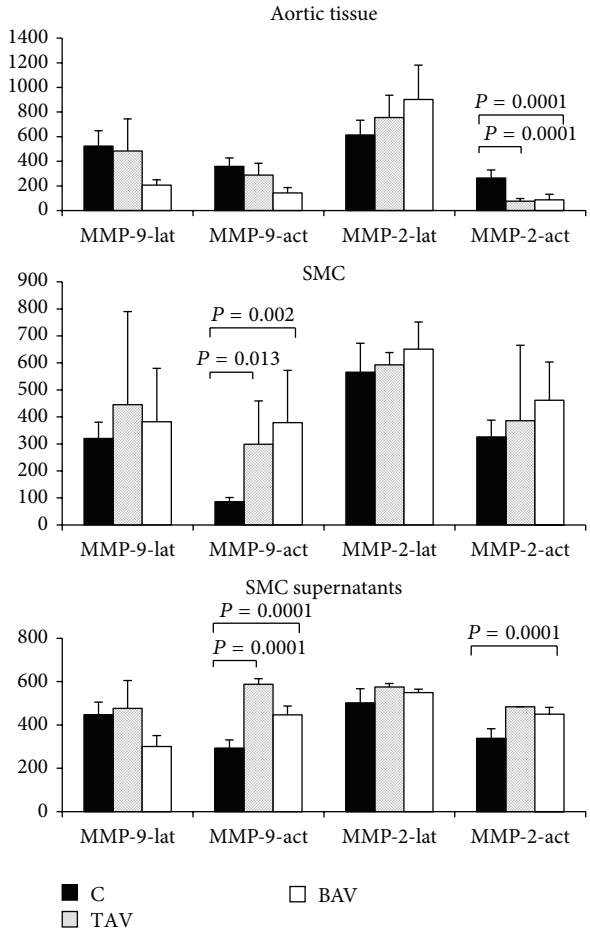

Figure 8: The level of MMP2 and MMP9 activity in SMC from patients with aortic aneurysm with either tricuspid aortic valve (TAV) or bicuspid aortic valve (BAV) and controls (C) determined by different groups of samples determined by zymography. C: $n = 5$; TAV: $n = 5$; BAV: $n = 5$. MMP-2-lat, MMP-9-lat: latent forms of MMP2 and MMP9, correspondingly. MMP-2-act, MMP-9-act: active forms of MMP2 and MMP9, correspondingly.

of pathogenesis and pathology and thus to find a potential therapeutic tool.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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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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ABSTRACT

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.

Keywords: thoracic aortic aneurysms, endothelial cells, Notch, Wnt, shear stress

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 (Davis et al., 2014). The 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 (Verstraeten et al., 2017).

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 (Folkersen et al., 2011; Kjellqvist et al., 2013; Malashicheva et al., 2016). So far, a few genes such as *NOTCH1* (Garg et al., 2005; McBride et al., 2008; McKellar et al., 2007; Mohamed et al., 2006) and *GATA5* (Padang et al., 2012) 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 (Garg et al., 2005; McBride et al., 2008; Mohamed et al., 2006).

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 (High et al., 2008; Kostina et al., 2016; Malashicheva et al., 2016). We have previously shown that primary endothelial cells derived from aortas of BAV/TAA patients have attenuated Notch signaling, irrespective of *NOTCH1* mutation (Kostina et al., 2016), 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 (Ducharme et al., 2013; Irtyuga et al., 2017). 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 analysed 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.

MATERIALS AND METHODS

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.

Primary cultures

Human aortic endothelial cells (HAEC) were isolated from tissue fragments of patients after surgery for aneurysm corrections as described (Kostina et al., 2016). The cells were used in experiments at passages 2–5.

In vitro flow model

HAECs were plated on gelatin-coated teflon-bordered cell culture slides (75x25x10 mm, Flexcell International Corp.) and cultured for 40 hours 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 dynes/cm^2 for 6 hours 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.

qPCR analysis

Total RNA (1 μg) was reverse transcribed with MMLV RT kit (Eurogen, Russia). Real-time PCR was performed with 1μL 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.

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 stabilized 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 (Kostina et al., 2016). 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%.

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 (Korinek et al., 1997) 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, США). Samples were normalized by protein content using Pierce BCA Protein Assay Kit (Thermo Scientific).

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 (Ritchie et al., 2015).

RESULTS

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.

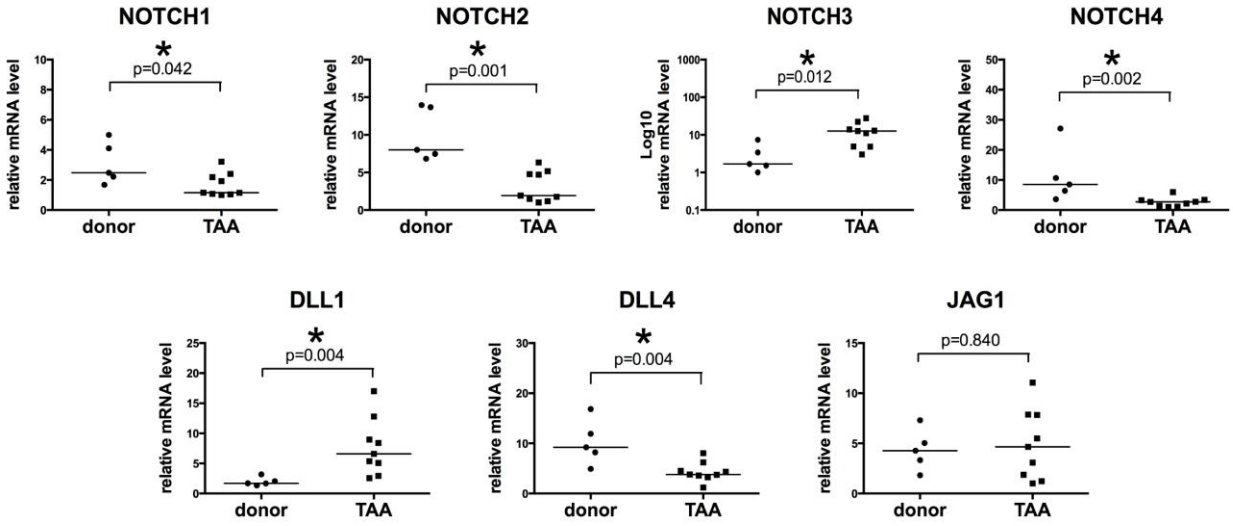


Fig. 1 Expression levels of Notch receptors and ligands in the aortic endothelial cells from the patients with thoracic aortic aneurysm (TAA), n=9 or control cells (donor), n=5. Groups are compared using Mann-Whitney nonparametric test; line represents the median; * $p<0.05$

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.

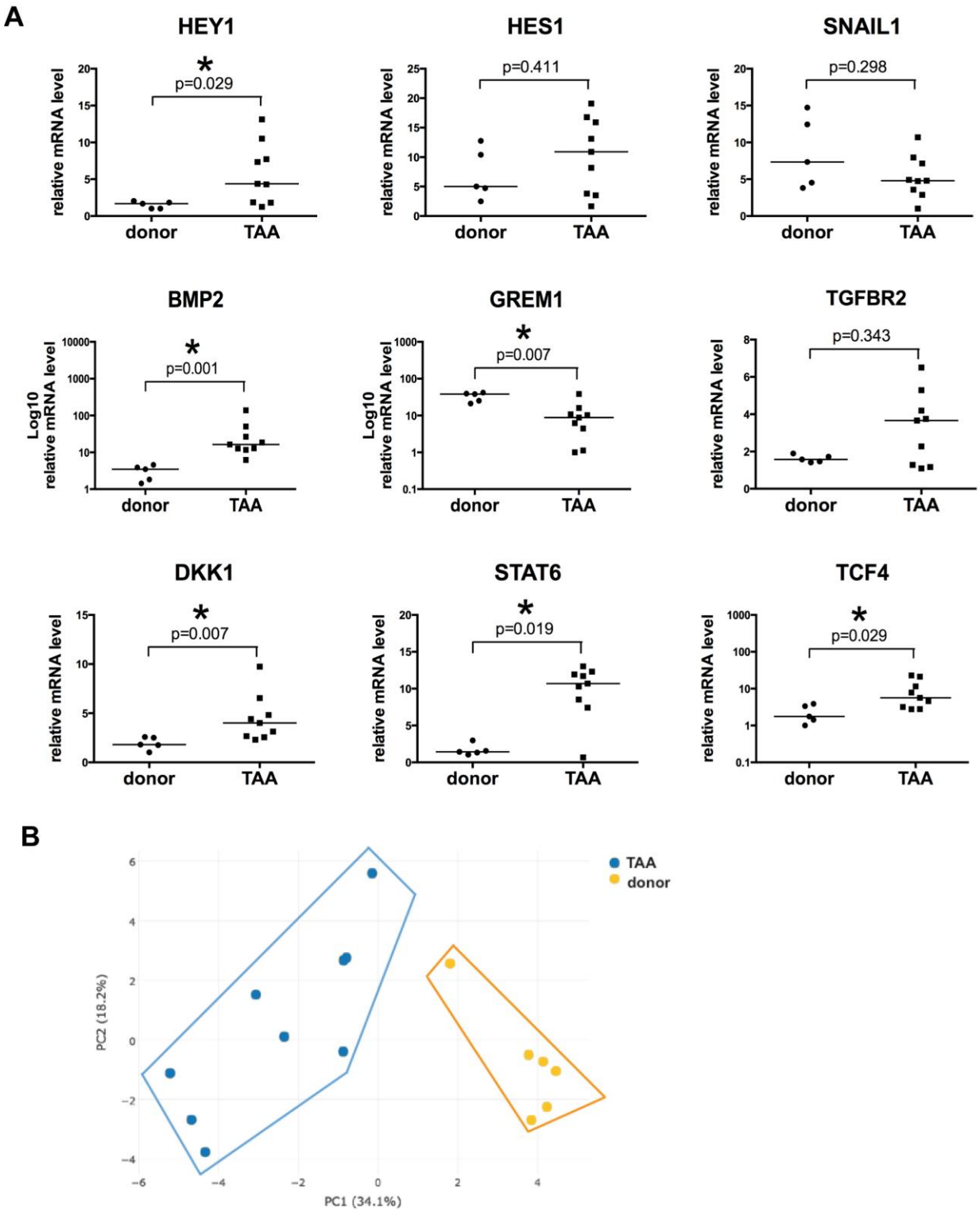


Fig. 2 Genes dysregulated in the aortic ECs from the patients with thoracic aortic aneurysm (TAA), n=9 or control cells (donor), n=5.A. Notch target genes (*HEY1*, *HES1*, *SNAIL*) as well as the expression of genes belonging to pathways that cross talk to Notch such as TGF-β/BMP (*BMP2*, *GREM1*, *TGFRB2*) 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.

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 (Shao et al., 2005). 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.

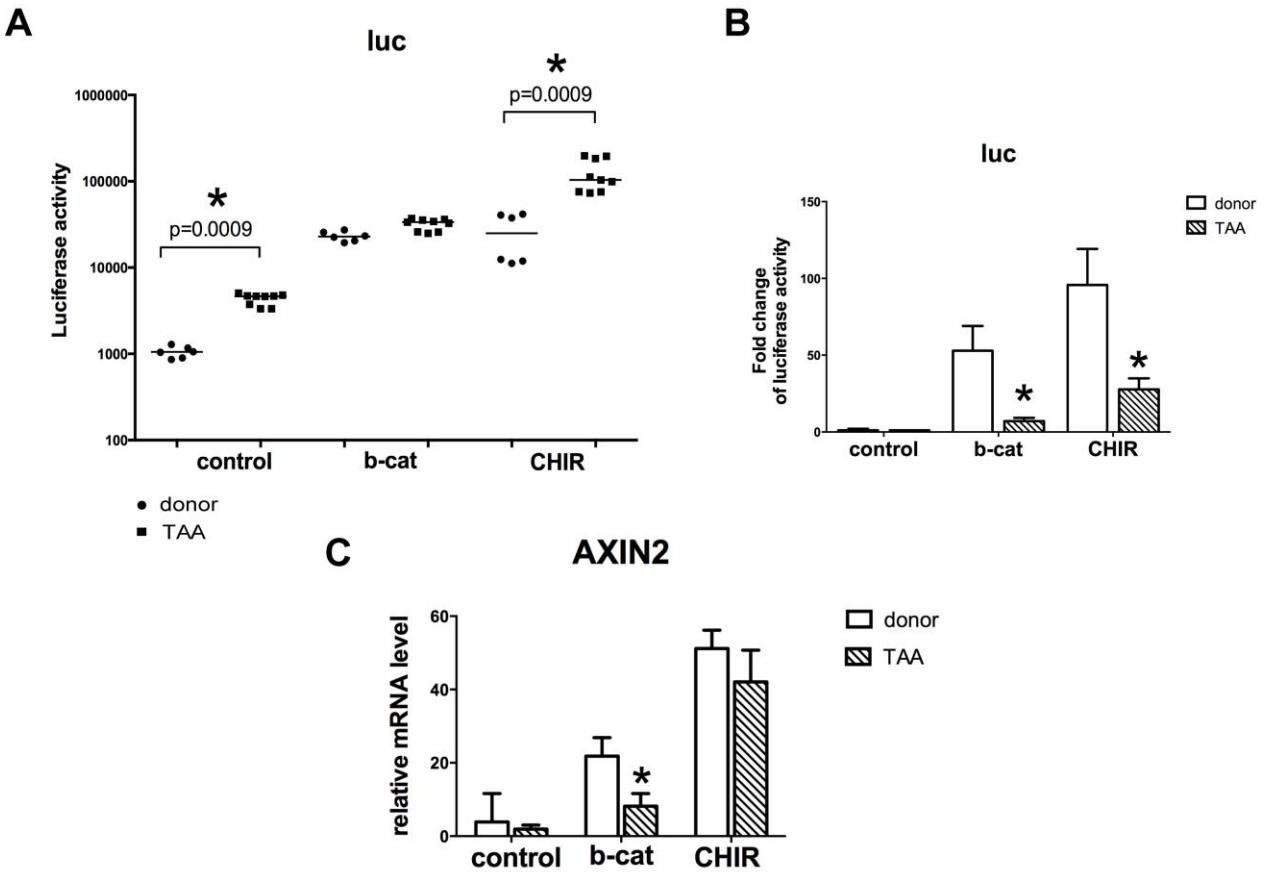


Fig. 3 Wnt activity in the HAEC of patients with TAA. Wnt was activated by a lentiviral transduction of proteolysis resistant S33A mutant of β-catenin into the cells or by addition of specific Gsk3 inhibitor CHIR99021. Wnt-activity was estimated with TOPFlash reporter construct as well as by the expression 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. * $p<0.05$

WNT/β-catenin pathway has been reported to modulate endothelial Notch/Dll4 signaling in mouse development (Corada et al., 2010). 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. 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).

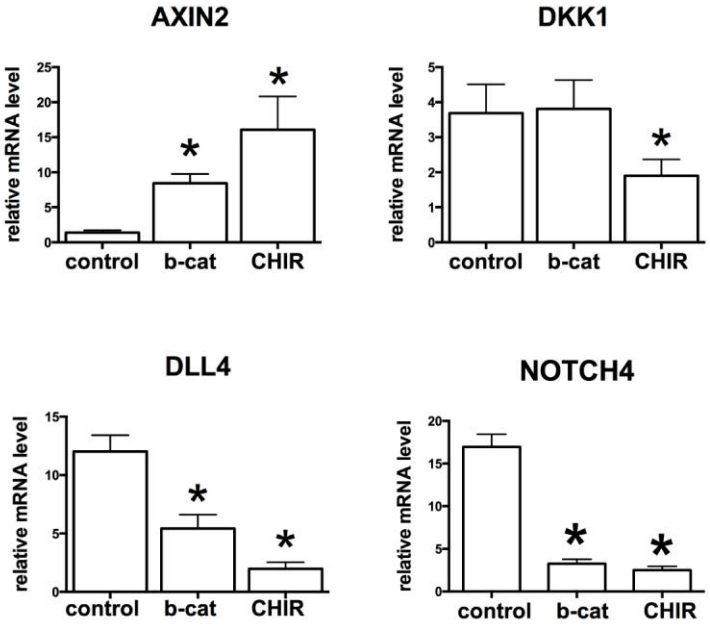


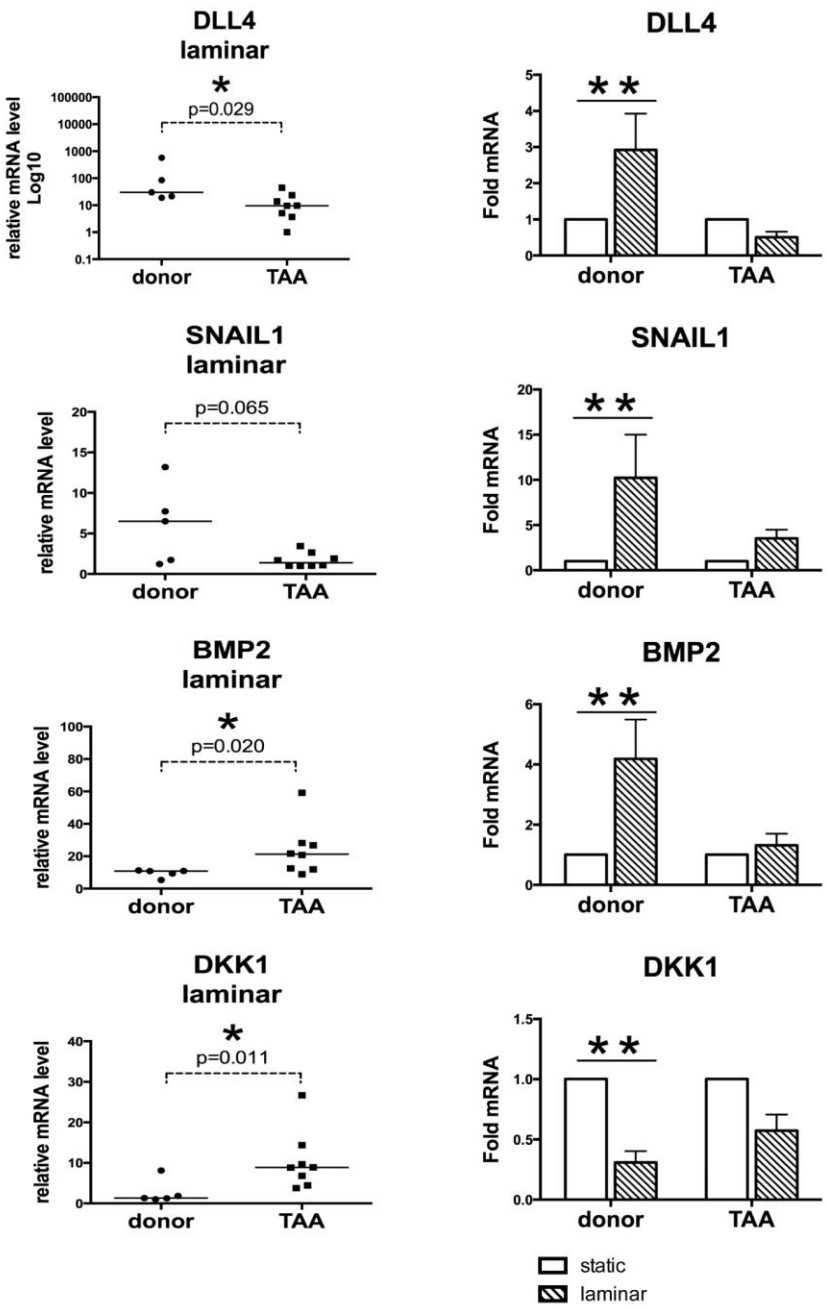
Fig. 4 Cross-talk between Wnt and Notch in adult human aortic endothelial cells (HAECs). Cells were transduced with S33A mutant β-catenin-bearing lentivirus or cultured in the presence of Gsk3 inhibitor CHIR99021. Groups are compared using Mann-Whitney non-parametric test. * $p<0.05$ for the difference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

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 (Theodoris et al., 2015). 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.

A


DLL4
laminar
relative mRNA level
Log10
p=0.029
donor
TAA
DLL4
Fold mRNA
donor
TAA
SNAIL1
laminar
relative mRNA level
p=0.065
donor
TAA
SNAIL1
Fold mRNA
donor
TAA
BMP2
laminar
relative mRNA level
p=0.020
donor
TAA
BMP2
Fold mRNA
donor
TAA
DKK1
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relative mRNA level
p=0.011
donor
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DKK1
Fold mRNA
donor
TAA
static
laminar

B

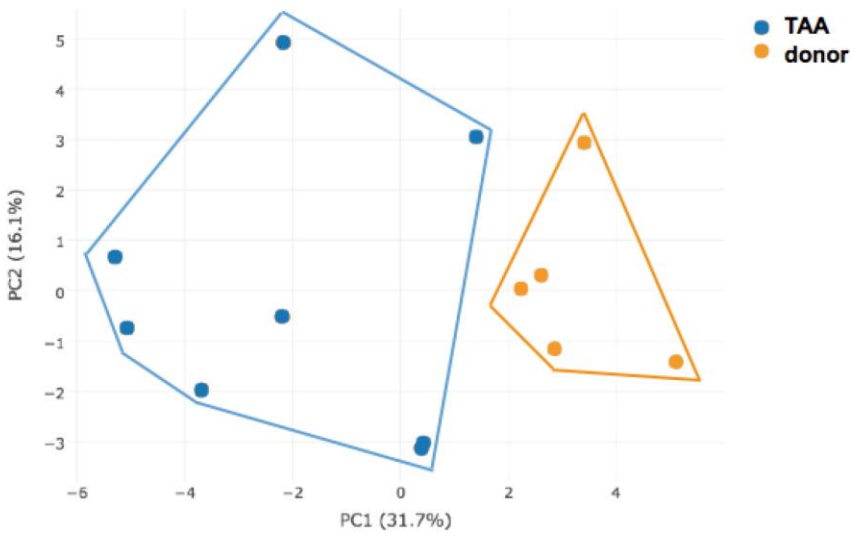
TAA
donor
PC2 (16.1%)
PC1 (31.7%)

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.

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 (Briot et al., 2016). We have recently shown that ECs from BAV/TAA have impaired Notch-dependent EMT irrespective of *NOTCH1* mutations (Kostina et al., 2016). 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 (Krebs et al., 2004) 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 (Luxán et al., 2016). 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 (Pedrosa et al., 2015). This is compatible with our earlier reports in which we demonstrated a decreased expression of contractile markers in aortic SMC of TAA patients (Malashicheva et al., 2016), 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 (Reis and Liebner, 2013). 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 (Boström et al., 2011). 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 (Li et al., 2015). Our data is in accordance with previously published report (Malashicheva et al., 2016) 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 (Resnick and Gimbrone, 1995). 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 (Corada et al., 2010; Dejana, 2010; Rostama et al., 2015; Wu et al., 2014). *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 (Pedrosa et al., 2015) 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 (Corada et al., 2010; Dejana, 2010; Morini and Dejana, 2014). 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 (Corada et al., 2010; Morini and Dejana, 2014). Despite the fact that even subtle changes in *DLL4* expression impairs vascular development (Pedrosa et al., 2015), the regulatory mechanisms for the fine-tuning of Dll4/Notch signaling during vascular development in vivo still remain to be defined (Wu et al., 2014).

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.

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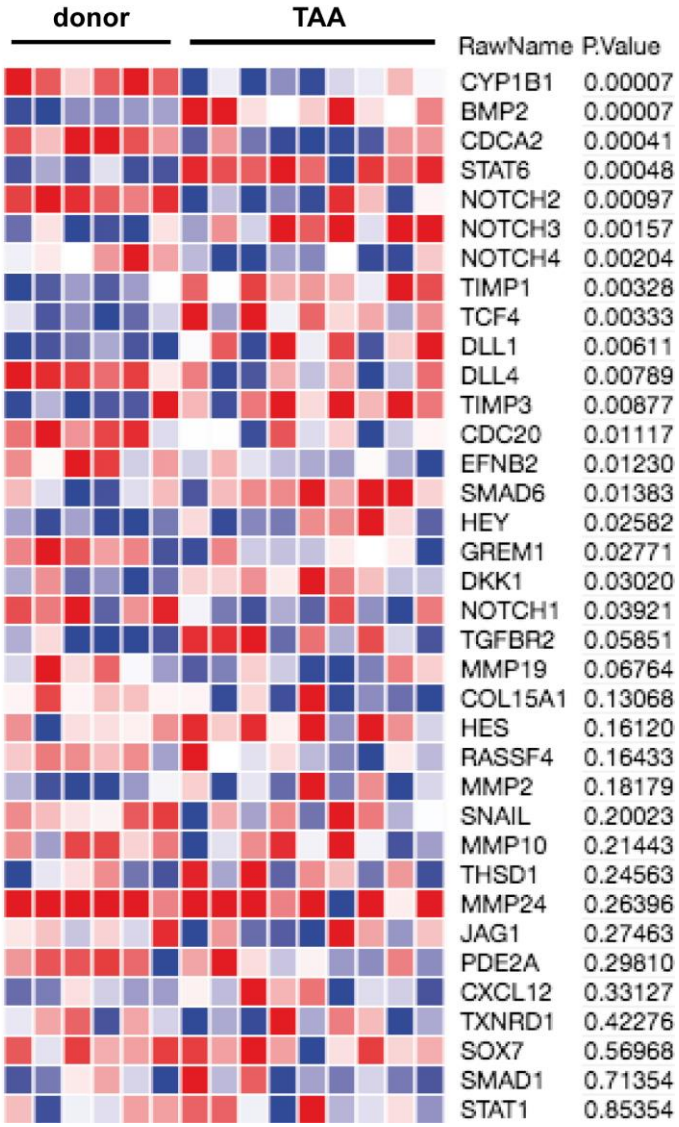
Table 1 Clinical characteristics in the study groups

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

SUPPLEMETARY FIGURES

Supplementary figure 1. Differential expression of genes related to major antiosteogenic, antioxidant, antiatherogenic and proinflammatory pathways in the human aortic endothelial cells derived from either healthy donors (donor) or patients with thoracic aortic aneurysms (TAA). RNA was isolated from the cells and mRNA level for indicated genes was analyzed.



Supplementary figure 2. Differential expression of genes related to major antiosteogenic, antioxidant, antiatherogenic and proinflammatory pathways in the human aortic endothelial cells derived from either healthy donors (donor) or patients with thoracic aortic aneurysms (TAA) after shear stress. The cells were subjected to shear stress, subsequently RNA was isolated from the cells and mRNA level for indicated genes was analyzed.