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Defective NOTCH signaling drives increased vascular smooth muscle cell apoptosis and contractile differentiation in bicuspid aortic valve aortopathy: A review of the evidence and future directions☆,☆☆

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ABSTRACT

Bicuspid aortic valve (BAV) disease remains the most common congenital cardiac disease and is associated with an increased risk of potentially fatal aortopathy including aortic aneurysm and dissection. Mutations in the *NOTCH1* gene are one of only a few genetic anomalies identified in BAV disease; however evidence for defective NOTCH signaling, and its involvement in the characteristic histological changes of VSMC apoptosis and differentiation in ascending aortae of BAV patients is lacking. This review scrutinizes the evidence for the interactions of NOTCH signaling, cellular differentiation and apoptosis in the context of aortic VSMCs and provides focus for future research efforts in the diagnosis of BAV aortopathy and prevention of catastrophic complications through NOTCH signaling manipulation.

Introduction

Bicuspid aortic valve (BAV) disease results when the aortic valve forms with just two leaflets (cusps), in place of the normal three. The incidence worldwide is between 0.4–2.25% making it the most common congenital cardiac anomaly in humans, with males more frequently affected at a ratio of 3:1 [1–3]. Since its first documentation over 500 years ago by Leonardo da Vinci, an appreciation has grown of its tendency to predispose individuals to associated cardiovascular disease. BAV patients make up over 40% of the patients who die from or require an operation for aortic valve disease [4]. Furthermore, at least one third of BAV patients will develop complications including valve narrowing (stenosis) or leaking (regurgitation). Consequently, BAV disease accounts for more morbidity and mortality than all other congenital cardiac defects combined [5]. The disease presents a significant financial burden for healthcare systems across the world, and despite increasing research interest, little progress has been made towards defining the pathophysiological mechanisms.

BAV disease is also a major risk factor for ascending aortic aneurysm and aortic dissection (collectively termed BAV aortopathy). The link between valve morphology and ascending aortic pathology was first described by Abbott in 1928 [6]. Microscopic examination of the aortic wall reveals the histological hallmark of BAV aortopathy, first termed 'Erdheim's cystic medial necrosis' on account of the cyst-like appearance of accumulated ground substance [7]. Medial necrosis is accompanied by loss of fibrillin, elastic laminar fragmentation and vascular smooth muscle cell (VSMC) apoptosis [8–10]. Loss of extracellular matrix (ECM) integrity is compounded by overexpression and activity of matrix metalloproteinases (MMPs), which contributes to cell detachment and apoptosis [8,11,12].

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☆☆ **Ethical statement:** We the undersigned declare that this manuscript is original, has not been published before and is not currently being considered for publication elsewhere. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all of us. We confirm that all authors are responsible for the content and have read and approved the manuscript; and that the manuscript conforms to the Uniform Requirements for Manuscripts Submitted to Biomedical Journals published in *Annals in Internal Medicine* We understand that the Corresponding Author is the sole contact for the Editorial process. He/she is responsible for communicating with the other authors about progress, submissions of revisions and final approval of proofs. Signed by all authors as follows: Dr Alexandru C Visan, Mr Oliver J Harrison, Mr Narain Moorjani, Mr Amit Modi, Mr Kareem Salhiyyah, Dr Cristopher Torrents, Mr Sunil K Ohri and Dr Felino R Cagampang.

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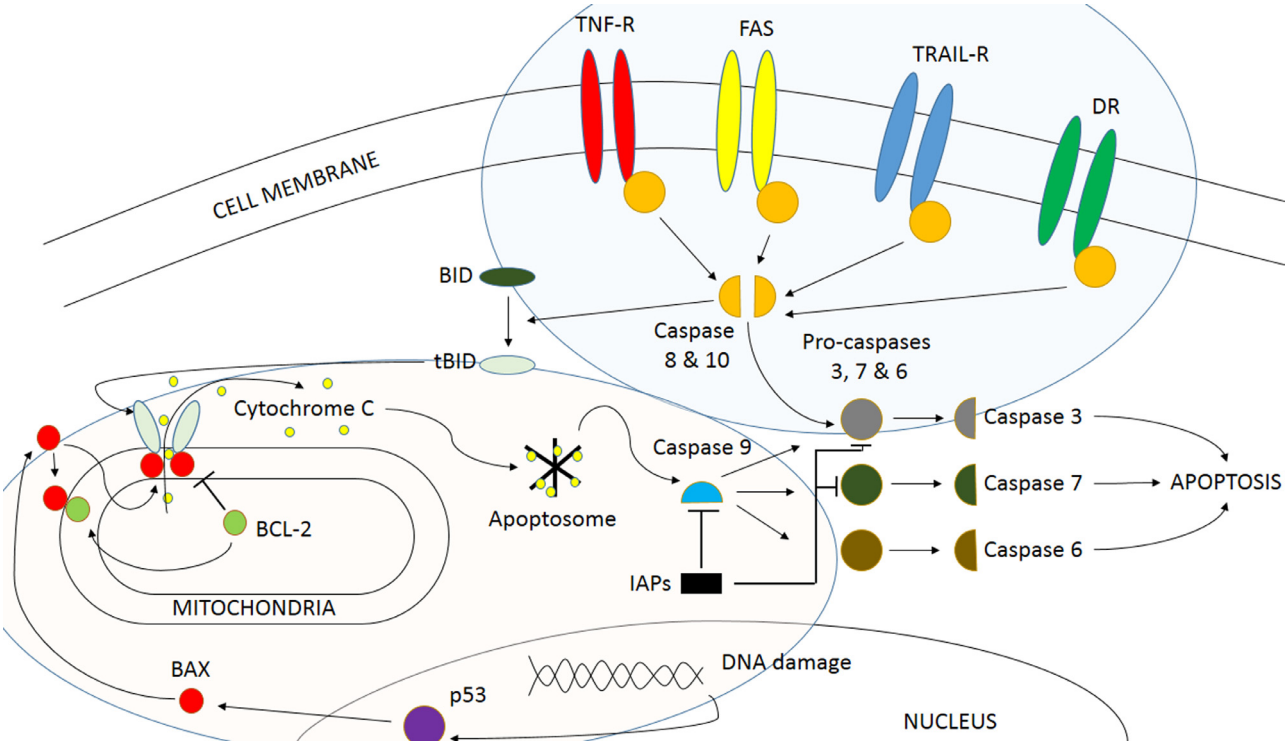

Fig. 1. Schematic summary of the key pathways of apoptosis. Light blue oval = extrinsic (death receptor) pathway; pale red oval = intrinsic (mitochondrial) pathway. BAX = BCL-2-associated X; BCL-2 = B-cell lymphoma 2 protein; BID = BH3 interacting domain death agonist; DR = death receptor; IAPs = inhibitor of apoptosis proteins; TNF-R = tissue necrosis factor receptor; TRAIL-R = TNF-related apoptosis-inducing ligand receptor [105]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Conflicting evidence exists regarding the contribution of hemodynamic stress ('post-stenotic' dilation) and genetics to the pathognomonic changes of BAV aortopathy [13–15]. BAV disease is a largely heritable condition with between 10–35% of first degree relatives being affected in an autosomal dominant fashion [16,17]. BAV commonly occurs in association with other genetic syndromes including Marfan, Ehlers-Danlos and Turner, and with other congenital cardiac abnormalities including aortic coarctation and hypoplastic left heart [18]. BAV is a heterogenous disease and a single gene model has yet to be identified to explain its inheritance. A number of studies utilizing animal models have been implemented in an attempt to identify the genetic pathogenesis of BAV disease. A recent review by Wu et al. identified in excess of 40 genes linked to BAV disease in mouse and hamster models [19]. In humans, the most significant mutational variants have been identified on chromosomes 9q34-35 (*NOTCH1*), 9q22.33 (*TGFBR1*), 3p22 (*TGFBR2*), 13q33-qter, 5q15-21, 17q24 (*KCNJ2*), 10q23.3 (*ACTA2*), 15q25-q26.1, 20q13.13(*GATA5*), 5q34 (*NKX2-5*) and 18q [20,21]. In addition, a recent genome-wide association study performed by Yang et al. involving over 400 BAV patients identified two new protein-altering and regulatory genetic variants near *GATA4*, a key regulator of endothelial to mesenchymal cell transition in valvulogenesis [22].

Mutations in the *NOTCH1* gene have been identified in populations with both familial and sporadic BAV disease and at the present time, are the only proven candidate gene to cause BAV [23,24]. The NOTCH signaling pathway is an evolutionarily-conserved cell signaling mechanism that dictates cell fate decisions [25]. In addition to its implication in the development of BAV disease, NOTCH signaling is identified as a key effector of neural crest cell migration during cardiogenesis coordinating differentiation of the first VSMC population in primitive ascending aorta [26–28]. Thus, it is hypothesized that a common genetic defect may affect both valve and ascending aorta in BAV disease predisposing to aortopathy [29,30].

However, there is little evidence for defective NOTCH signaling in BAV aortopathy, and its contribution to VSMC apoptosis and differentiation has yet to be elucidated [31]. Given the central role of NOTCH signaling in cell fate decisions, and its implication in BAV disease, we hypothesize that changes in NOTCH signaling may underlie increased VSMC apoptosis in BAV aortopathy. Furthermore, the influence of NOTCH signaling on cellular differentiation may underlie the failure of VSMCs to respond to and repair the degenerated ECM, which is also characteristic of BAV aortopathy. In this review, we scrutinize the evidence for the interactions of NOTCH signaling, cellular differentiation and apoptosis in the context of the aortic VSMC and provide focus for future research efforts in the diagnosis and treatment of BAV aortopathy. We propose that manipulation of the NOTCH signaling pathway may represent a therapeutic opportunity to reduce VSMC apoptosis and control cell differentiation and so prevent the catastrophic complications of aortic dissection and rupture.

VSMC apoptosis in BAV aortopathy

VSMCs are the most common cell type found in the healthy aortic media. They are an essential prerequisite for normal development of the ascending aorta and maintenance of ECM homeostasis in the mature vessel. VSMCs provide support to the structure of the vessel wall and in smaller arteries contract and relax to regulate blood flow in response to physiological stimuli. VSMCs are capable of contraction, secretion and maintenance of the ECM components and can undergo apoptosis (programmed cell death), which is a physiological event critical for maintaining vascular wall homeostasis. The consequence of reduced apoptosis is evident in cancer where mutations in tumor suppressor genes (e.g. p53) in-

stigate uncontrolled growth. Conversely, excessive apoptosis is also associated with disease processes, including aneurysm formation.

There are two major pathways of apoptosis in VSMCs, the extrinsic (death receptor) and the intrinsic (mitochondrial) pathways (Fig. 1) [32]. The extrinsic pathway is initiated by activation of membrane-bound 'death receptors' including tissue necrosis factor receptor (TNF-R); TNF-related apoptosis-inducing ligand receptor (TRAIL-R), FAS (first apoptosis signal) ligand receptor and death receptors (DR3, 4 and 5). Subsequent step-wise activation of proteolytic caspases (the caspase cascade) ensues, which cleaves intracellular substrates required for cell survival [33,34]. Caspase-3, a major effector of the caspase cascade, is responsible for the hallmarks of apoptosis including DNA fragmentation, nuclear condensation and apoptotic body formation [35].

Conversely, the intrinsic pathway utilizes mitochondria, and may be activated by either the extrinsic pathway (described above), or by a p53 dependent response to DNA damage [36–38]. Activation of pro-apoptotic BCL-2 (B-cell lymphoma 2) protein family members (BCL-2-associated X, BAX; BCL-2-interacting killer, BIK; and BCL-2 homologous antagonist/killer, BAK; and BID) initiates their translocation to the mitochondrial membrane where they activate mitochondrial membrane channels. This facilitates the movement of cytochrome c into the cytoplasm, activating caspases and triggering apoptosis [39]. BCL-2 family anti-apoptotic proteins are able to bind to these channels and prevent activation. Finally, cytosolic inhibitors of apoptosis proteins (IAPs) bind and inhibit caspases, inhibiting apoptosis independently of the mitochondrial pathways [40]. An example is X-chromosome linked IAP (XIAP), which inhibits caspase-3 and -7 reducing apoptosis through BAX mediated, cytochrome c release pathway [40]. Thus, XIAP does not reduce expression of BAX and cytochrome c but inhibits their action of activating important caspases in the cytoplasm.

VSMC apoptosis was first quantified in BAV aortopathy by Bonderman et al., who identified that apoptotic neural crest-derived VSMCs appeared to be concentrated around areas of medial degeneration (MD) [14]. Moderate grade MD was seen in all patient groups (including TAV patients with non-aneurysmal aortas), however, BAV patients with both aneurysmal and non-aneurysmal aortas had significantly higher apoptotic indices than non-aneurysmal TAV patients. Of note the aneurysmal TAV group had a higher apoptotic index than both BAV groups, reinforcing the observation that MD and VSMC apoptosis is not exclusive to BAV disease. Furthermore, it suggests that the mechanism of BAV aortopathy is active before aneurysm occurs, and that BAV aortas are inherently different from TAV aortas.

Subsequently, Schmid et al. examined aortic samples from BAV and TAV patients with aortic aneurysms and compared them with donor control tissue [41]. Similarly to Bonderman et al. [14], they demonstrated MD in both TAV and BAV aneurysmal tissue, however MD was more severe in the BAV group. Apoptotic indices were no different between TAV and BAV, but were both significantly higher than control, which is consistent with previous findings. Similarly, assessment of cellularity revealed a significant decrease in cell nuclei number in the TAV group and BAV group (25% and 32%, respectively) compared to healthy control. In addition, expression of pro-apoptotic proteins FAS and Perforin (PRF) were found to be elevated in aneurysmal TAV and BAV tissue versus control. These proteins are associated with activation of the extrinsic pathway of apoptosis typically triggered by extracellular ligands. Interestingly, infiltration of inflammatory cells was seen in both BAV and TAV groups, which suggests a possible role of activated inflammatory cells releasing FAS and PRF to induce VSMC apoptosis.

Della Corte et al. support these earlier findings demonstrating a consistent increase in apoptotic VSMCs in BAV patients when minimal aortic dilation was present [42]. Again, this differed from TAV patients who displayed high variability in apoptotic indices. The authors also quantified VSMC density demonstrating significantly decreased VSMC numbers in normal or mildly dilated BAV aortas, but similar numbers in aneurysmal aortas compared to control. They also measured expression of pro-apoptotic BCL-2-modifying factor (BMF)-binding protein, which triggers apoptosis in response to ECM disruption, and anti-apoptotic BCL-2 mRNA expression as a marker of the molecular tendency to apoptosis. In the BAV non-aneurysmal group, elevated BMF-BCL-2 binding was observed suggesting that cytoskeletal disruption is occurring at an early stage. However, this did not increase further in BAV aneurysmal patients despite marginal increases in the apoptotic index, which suggests differing mechanisms may underlie apoptosis in early and late BAV aortopathy. Differences in apoptotic index, cell density and expression of synthetic VSMC proteins between early and late aortopathy is interesting. A possible explanation is that normal contractile VSMCs are susceptible to flow-induced apoptosis, whereas phenotypically-changed synthetic VSMCs are not [43]. However, evidence for this switch in BAV aortopathy is lacking.

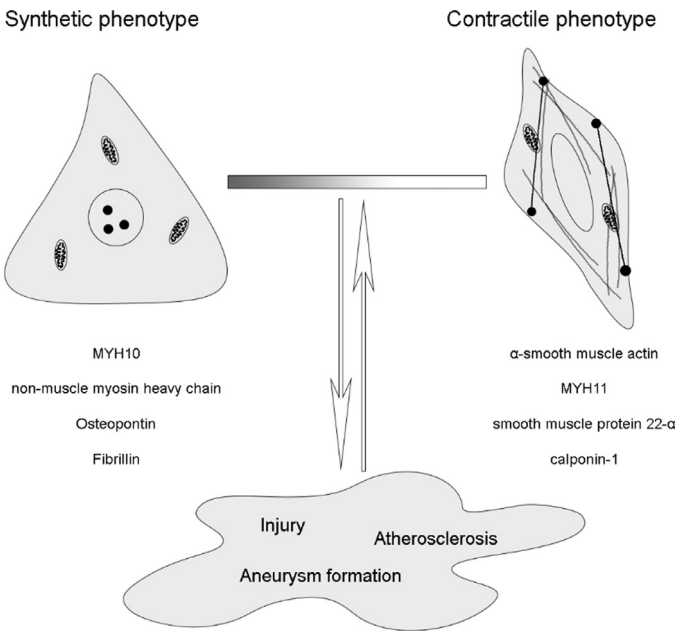

Fig. 2. Phenotypic states of the vascular smooth muscle cell and key proteins specific for each state [105].

VSMC differentiation in BAV aortopathy

VSMCs retain a degree of plasticity allowing them to carry out specialized functions including contraction, proliferation and ECM synthesis [44]. The cells are capable of modifying their phenotype in a continuous and dynamic fashion between a more differentiated 'contractile' phenotype, and a less differentiated 'synthetic' phenotype, which allows them to achieve their function (Fig. 2). The phenotypic state of the VSMC may be defined by the expression of characteristic proteins. α-smooth muscle actin (αSMA), smooth muscle myosin heavy chain (MYH11), calponin-1 (CNN1) and smooth muscle protein 22-alpha (SM22α) are key proteins expressed in the contractile phenotype. Downregulation of these proteins, and upregulation of synthetic proteins (e.g. non-muscle myosin heavy chain; MYH10) is an indication that the differentiation of VSMC is more towards the synthetic phenotype [45]. In reality, VSMC phenotype represents a spectrum between the contractile and synthetic type. Phenotype modification of VSMCs is a key aspect of vascular remodeling implicated in injury, atherosclerosis and aortic aneurysms [46,47].

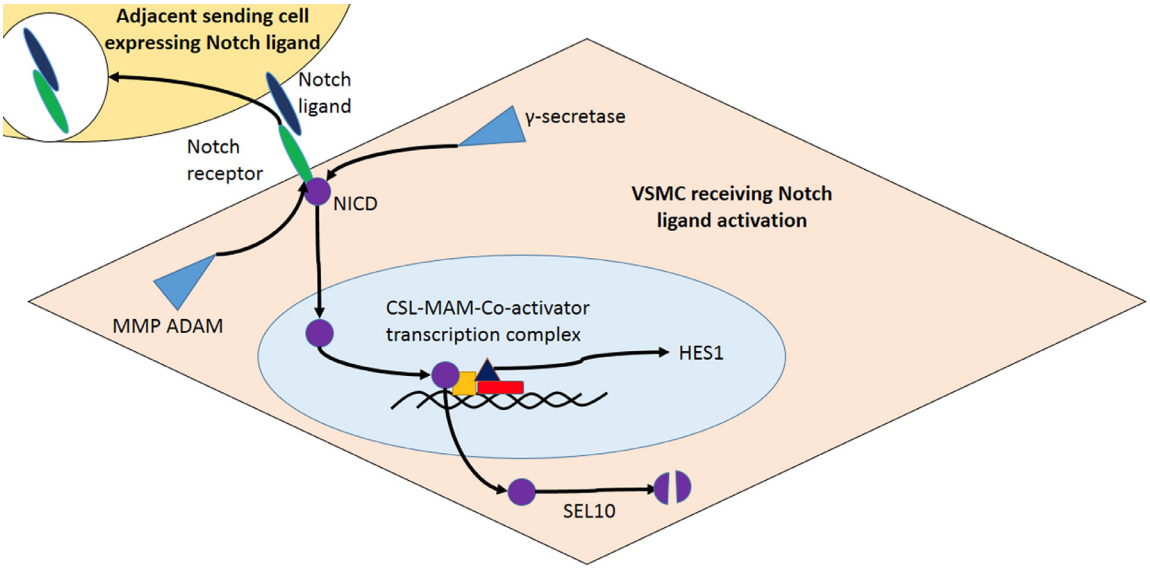

Fig. 3. NOTCH signaling pathway. NICD (NOTCH intracellular domain), CSL (CBF1, Suppressor of Hairless, Lag1), MAM (Mastermind), HES (Hairy enhancer of splice), MMP ADAM (a disintegrin and metalloproteinase), SEL10 (a E3 ubiquitin ligase) [105].

Mutational variants in the *FBN1* gene (Fibrillin-1) have been associated with BAV disease in the presence of aortopathy, but not when aortopathy is absent [48,49]. The main signaling pathway of Fibrillin-1 is via TGF-β, which leads to the phosphorylation and activation of the canonical SMAD 2/3 (via activin-like kinase 5) and SMAD 1/5/8 (via activin-like kinase 1) transcription factors, through the binding of its active form, TGF-β1, to the cell membrane receptor TGFRB1 [50]. In normal aortic VSMCs, phosphorylation of the canonical SMAD pathways induces a contractile phenotype [51]. Conversely, activation of SMAD-independent intracellular pathways (via PI3K/AKT, MAPK, or NF-kB) promotes overexpression of MMP, thus leading to ECM degradation and cellular apoptosis [52,53]. Therefore, TGF-β plays a key role in VSMC apoptosis and differentiation and ECM homeostasis. Moreover, in *FBN1* knock-out mice, increased activation of TGF-β signaling and angiotensin II type I receptor (AT1R) is observed [54,55]. Research interest has grown for a role of losartan, a competitive AT1R antagonist, in the treatment of aneurysm in Marfan syndrome through its interaction with TGF-signaling [56–59]. Doxycycline may also be beneficial in reducing aortic dilation in patients with Marfan syndrome via inhibition of matrix metalloproteinase-2 and -9 [60]. However, a recent retrospective study in BAV patients concluded there was no difference in aortic enlargement rate for BAV patients taking ACE inhibitors [61].

The extent to which VSMC differentiation is a feature of BAV aortopathy, and how this links to apoptosis remains to be elucidated. A few groups have highlighted differences in protein expression between BAV and TAV aortic samples which may indicate VSMC phenotype modification. Significantly elevated levels of osteopontin (OPN) and tenacin C (TNC), produced by synthetic VSMCs, have been demonstrated in TAV versus BAV aneurysms in a number of studies [62–64]. Folkersen et al. showed significantly higher expression of TNC and SPP1 (osteopontin) genes in dilated aortas from TAV samples compared to BAV samples suggesting VSMCs in BAV aortopathy remain in the contractile state [63]. Non-dilated aortic samples from both TAV and BAV patients showed little difference in gene expressions suggesting changes in VSMC phenotype occur over time. Contrary to these findings, Cotrufo et al. found elevated TNC, and significantly decreased laminin (LAM) expression (a protein known to promote the contractile phenotype) in BAV patients versus healthy TAV controls [65].

In summary, there is some evidence to support a role of altered VSMC differentiation in BAV aortopathy albeit conflicting. Furthermore, no studies to date have compared differences in phenotypic markers between non-aneurysmal and aneurysmal aortic specimens from BAV and TAV patients. It is likely that changes in the state of VSMC differentiation occur as aortopathy progresses, which could provide further insight into the underlying mechanisms. In addition, previous studies have not looked specifically at the expression of the contractile genes MYH11 and CNN1 as markers of differentiation in BAV and TAV patients. Since it is these proteins that provide the machinery necessary to function as well-differentiated contractile VSMCs, it seems appropriate to focus specifically on these genes when making inferences about the phenotypic state of VSMCs in the aortic wall.

The NOTCH signaling pathway

NOTCH signaling was first described in the laboratory of Thomas Hunt Morgan in 1913 [66,67]. During the late 1980s and early 1990s, evidence emerged to suggest that NOTCH works as an intercellular signaling mechanism via a transmembrane protein, with large extracellular and intracellular domains [68–70]. Activation of this protein triggers transcriptional intracellular changes leading to a variety of effects, including cell proliferation, differentiation and apoptosis [71–73]. Therefore, mutations in the NOTCH protein have been demonstrated to underlie a number of developmental disorders, whilst a dysregulation of the NOTCH signaling mechanism appears to result in tumor development in a number of tissue types [74]. In comparison to other signaling mechanisms, NOTCH signaling does not occur in a paracrine way, mediated by ligands secreted distantly, but in a juxtacrine manner, the process only taking place between two adjacent cells and requiring the cells to be in direct contact (Fig. 3) [75,76].

Four NOTCH receptors (NOTCH 1–4) have been described in humans and represent large multidomain type I transmembrane proteins [75,77]. Along with these receptors, three Delta-family ligands (Dll1, Dll3 and Dll4) and two Serrate-family ligands (Jagged1 and Jagged2) have been found in mammals. These are also type I transmembrane proteins but have a large extracellular domain with a short intracellular domain [75,78]. The NOTCH receptor

binds to the ligands expressed on the adjacent cell. This activates a proteinase, γ-secretase, which cleaves the NOTCH intracellular domain (NICD), releasing it. Following the NICD cleavage, the extracellular domain of the NOTCH receptor is endocytosed by the sending cell. The NICD then translocates to the nucleus where it interacts with a DNA binding transcription factor CSL (CBF1, Suppressor of Hairless, Lag1), and a coactivator Mastermind (MAML1-MAML3) [79,80]. This cascade leads to the disassembling of the corepressor complex and derepression of the gene targets, with activation of transcription complexes [81–83]. The NICD is then phosphorylated by kinases (CDK8) [84] followed by polyubiquitination via E3 ubiquitin ligases like SEL10 or FBXW7 [85], leading to degradation of NICD and termination of the signal, thus preventing continuous signal activation [86–88].

NOTCH signaling is thought to play a central role in the orchestration of aortic valve development. A key stage in formation of the primitive endocardial cushions is infiltration of migrating neural crest cells (NCC). During migration, a proportion of these cells differentiate into VSMC, which populate the wall of the developing ascending aorta, aortic arch, and head and neck vessels. Together with cells of the secondary heart field and mesenchyme, the NCC orchestrate many important aspects of cardiac outflow tract and ascending aortic development drawing many to hypothesize that a common defect may be responsible for BAV disease and the associated aortopathy [26]. In support of this, it is NCC-derived VSMCs that undergo increased apoptosis in BAV aortopathy [14].

Characterizing NOTCH signaling in BAV aortopathy is of particular interest because *NOTCH1* mutations are implicated in the pathogenesis of BAV disease [23,24]. To date however, relatively few studies have examined NOTCH signaling changes in human aortic aneurysms. Decreased expression of both NOTCH1 and NOTCH3 are reported in aortic samples from abdominal aortic aneurysms versus control, in parallel to decreased expression of contractile VSMC phenotype markers [89,90]. Conversely, upregulation of NOTCH1, NICD and HES1 was reported in the wall of descending thoracic aortic aneurysms, but decreased expression of these proteins was shown when VSMC populations are examined in isolation [91]. To the best of our knowledge, only one study has quantified NOTCH signaling in ascending aortic tissue. Sciacca et al. demonstrated significantly decreased mRNA and protein expression of several regulators of NOTCH signaling in BAV versus TAV aortic tissue (including *NOTCH1* & *HES1*), although no reference to aortic dimension is given [31]. In summary, changes in NOTCH signaling may be a significant factor in the development of BAV aortopathy. Such changes may impact on VSMC apoptosis and/or differentiation, however, more evidence is needed to confirm this.

The role of NOTCH signaling in altered VSMC apoptosis and differentiation in BAV aortopathy

Given the pivotal role of NOTCH signaling in aortic valve and ascending aortic development, there is remarkably little evidence to link NOTCH signaling with increased VSMC apoptosis and differentiation seen in BAV aortopathy. However, a few studies have investigated the effect of NOTCH signaling on apoptosis and differentiation in cultured VSMCs. Overexpression of *NOTCH1* and *NOTCH3* in rat VSMCs resulted in a significant decrease in cell apoptosis in association with a decrease in *BAX:BCL-xL* mRNA expression ratio [92,93]. This observation concurs with the work of Sciacca et al. who demonstrated decreased NOTCH1 signaling in BAV aortas, which is consistent with the observation of increased VSMC apoptosis [31]. Similar findings from T-cell hybridoma work demonstrated that NOTCH1 receptor activation upregulated anti-apoptotic *BCL-2* expression [94]. Liu et al. demonstrated NICD upregulated X-linked inhibitor of apoptosis protein (XIAP) in Jurkat T leukemia cells by direct interaction with the protein [95]. NICD appears to bind and prevent ubiquitin-dependent degradation of XIAP thereby potentiating its effect of inhibiting apoptosis. Incidentally, significantly reduced *XIAP* mRNA expression has also been demonstrated in patients with BAV and Turner syndrome versus those with TAV [96]. It is not clear whether this occurs in a NOTCH-dependent manner but may contribute to increased VSMC apoptosis in the ascending aorta. Increased expression of NOTCH3 but not NOTCH2 in human aortic VSMCs promoted cell survival genes *BCL-2, BIRC5* and *CFLAR* (cFLIP) [97]. Supporting these findings, Boucher et al. demonstrated reduced proliferation of human aortic VSMC when *NOTCH2* was activated, via upregulation of the cell cycle regulatory gene p27 [98]. Together these observations suggest NOTCH1 & NOTCH3 activation are pro-survival, and NOTCH2 activation is pro-apoptotic. Given the observation of decreased NOTCH1 signaling in BAV aortas, we hypothesize that defective NOTCH1 signaling in BAV patients may contribute to increased apoptosis and ascending aortic aneurysm formation.

NOTCH signaling may also play a key role in cell differentiation. Endothelial cell-induced activation of NOTCH signaling in VSMCs is central to normal cardiovascular development, promoting VSMC development and maturation [99]. *In-vitro*, simulated activation of NOTCH signaling with Jagged1 ligand promotes the contractile phenotype in cultured human aortic VSMCs, as indicated by upregulation of αSM actin, SM22α and CNN1 [100]. Lin et al. co-cultured vascular endothelial cells with human aortic VSMCs and demonstrated similar upregulation of contractile phenotype transcripts and cell quiescence related to upregulation of the *NOTCH3* mRNA expression [101]. However, they also showed upregulation of synthetic markers Caldesmon-1 (CALD1), Retinol binding protein-1 (RBP1), and Vimentin (VIM). Furthermore, inhibition of NOTCH signaling with the γ-secretase inhibitor DAPT blocked endothelial-induced contractile differentiation of VSMCs and decreased the expression of NOTCH3 mRNA, suggesting NOTCH activation is key to this process. Interestingly however, NOTCH inhibition did not affect synthetic phenotype transcript expression suggesting that other factors may be responsible for promoting this phenotype. Consistent with these findings, Liu et al. demonstrated that repression of NOTCH3 in culture human aortic VSMCs stimulates proliferation, apoptosis and cell migration [102]. Conversely, Proweller et al. demonstrated inhibition of myocardin-induced VSMC differentiation in rat aortic VSMC transfected with constitutionally activate NOTCH1, as represented by decreased expression of αSM actin, SM22α and SM MyHC [103]. Myocardin has been identified as an essential co-factor for maintenance of the differentiated (contractile) VSMC phenotype [104]. Therefore, as for apoptosis, opposing effects of different NOTCH receptors on VSMC differentiation are seen, with NOTCH3 promoting the well-differentiated (contractile) phenotype and NOTCH1 promoting the de-differentiated (synthetic) phenotype. Whether or not these observations hold true for VSMCs in BAV aortas remains to be elucidated. There is a clear lack of evidence for the role of defective NOTCH signaling in apoptosis and differentiation in human aortic smooth muscle cells and BAV aortopathy.

Summary and future directions

NOTCH signaling is key to cell survival and differentiation, and mutations in the *NOTCH1* gene are implicated in BAV disease, a condition associated with abnormal apoptosis and differentiation of VSMCs. Yet a pathophysiological association between NOTCH signaling, apoptosis and differentiation in VSMCs from BAV aortas has not been established. Given the limited evidence available, we hypothesize that inherent defective NOTCH1 activation in neural crest cell-derived VSMCs of the BAV ascending aorta promotes pro-apoptotic and inhibits anti-apoptotic protein expression. This imbalance drives VSMC apoptosis, and in turn disrupts the extra-

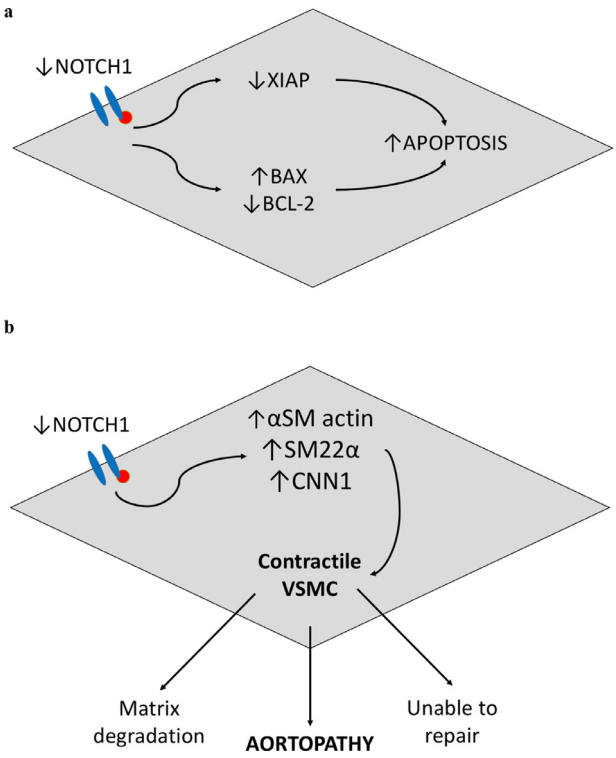

Fig. 4. Summary of the hypothesized mechanism for defective NOTCH signaling causing BAV aortopathy through increased apoptosis (a) and promotion of the contractile VSMC phenotype (b). XIAP (X-linked inhibitor of apoptosis protein), BAX (BCL-2-associated X), BCL-2 (B-cell lymphoma 2), αSM actin (α-smooth muscle actin), SM22α (Smooth muscle protein 22-alpha), and CNN1 (Calponin 1).

cellular matrix homeostasis, fueling catabolic degeneration of the ascending aortic wall, which over time thins and weakens predisposing to aneurysm and dissection. This process is perpetuated by defective NOTCH1 activation simultaneously promoting the contractile, well-differentiated VSMC phenotype which when driven to quiescence, fail to appropriately upregulate extracellular matrix synthesis and repair the thinning aortic wall. A summary of these hypotheses is shown in Fig. 4.

There is a need to design and implement meaningful basic research to further quantify NOTCH signaling in the ascending aorta of BAV patients and include consideration of differing aortic dimensions. Concurrent quantification of key apoptotic gene and protein expression (e.g. BAX and BCL-2) should also be made, together with markers of VSMC differentiation (e.g. MYH11, CNN1, MYH10). Furthermore, VSMCs should be isolated from the ascending aortas of BAV patients and subject to inhibition and activation of NOTCH signaling, and the effect on apoptotic and differentiation gene expression quantified. Therapeutic modulation of the NOTCH signaling pathway may provide a means to reverse the pathological mechanism of increased VSMC apoptosis and control cell differentiation, preserving the ascending aortic wall integrity, and preventing the potentially fatal complications of aneurysm and dissection.

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