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Embryonic development of bicuspid aortic valves

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ABSTRACT

Bicuspid aortic valve (BAV) is the most common congenital cardiac malformation, frequently associated with aortopathies and valvulopathies. The congenital origin of BAV is suspected to impact the development of the disease in the adult life. During the last decade, a number of studies dealing with the embryonic development of congenital heart disease have significantly improved our knowledge on BAV etiology. They describe the developmental defects, at the molecular, cellular and morphological levels, leading to congenital cardiac malformations, including BAV, in animal models. These models consist of a spontaneous hamster and several mouse models with different genetic manipulations in genes belonging to a variety of pathways. In this review paper, we aim to gather information on the developmental defects leading to BAV formation in these animal models, in order to tentatively explain the morphogenetic origin of the spectrum of valve morphologies that characterizes human BAV.

BAV may be the only defect resulting from gene manipulation in mice, but usually it appears as the less severe defect of a spectrum of malformations, most frequently affecting the cardiac outflow tract. The genes whose alterations cause BAV belong to different genetic pathways, but many of them are direct or indirectly associated with the NOTCH pathway. These molecular alterations affect three basic cellular mechanisms during heart development, i.e., endocardial-to-mesenchymal transformation, cardiac neural crest (CNC) cell behavior and valve cushion mesenchymal cell differentiation. The defective cellular functions affect three possible morphogenetic mechanisms, i.e., outflow tract endocardial cushion formation, outflow tract septation and valve cushion excavation. While endocardial cushion abnormalities usually lead to latero-lateral BAVs and septation defects to antero-posterior BAVs, alterations in cushion excavation may give rise to both BAV types. The severity of the original defect most probably determines the specific aortic valve phenotype, which includes commissural fusions and raphes.

Based on current knowledge on the developmental mechanisms of the cardiac outflow tract, we propose a unified hypothesis of BAV formation, based on the inductive role of CNC cells in the three mechanisms of BAV development. Alterations of CNC cell behavior in three possible alternative key valvulogenic processes may lead to the whole spectrum of BAV.

Contents

Abbreviations: BAV, Bicuspid aortic valve; CNC, Cardiac neural crest; CR, Conotruncal ridge; EMT, Endocardial-to-mesenchymal transformation; FHF, First heart field; IC, Intercalated cushion; L-NC, Fusion of left and non-coronary leaflets; OFT, Outflow tract; R-L, Fusion of right and left leaflets; R-NC, Fusion of right and non-coronary leaflets; SHF, Second heart field; TAV, Tricuspid aortic valve.

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Introduction

Bicuspid or bifoliate aortic valve (BAV) is the most frequent congenital cardiac malformation, with an incidence of around 1%. Although a BAV may be silent during the whole life of a carrier, it entails a high risk for clinically relevant valvulopathies and aortopathies. A BAV may occur isolated or associated with other congenital malformations, such as aortic coarctation or ventricular septal defect, sometimes forming part of complex syndromes like Turner, DiGeorge or Shone syndromes.[1,2]

A normal, tricuspid aortic valve (TAV) consists in three cusps or leaflets, right, left and posterior (dorsal in rodents) or non-coronary, which constitute the mobile element of the valve; three sinuses at the aortic root; a commissure between each two adjacent leaflets; and an interleaflet triangle between each two adjacent sinuses (Figs. 1A,B). Some BAVs show two more or less symmetric leaflets, sinuses, commissures and interleaflet triangles (Fig. 1E), but most BAVs are constituted by two anatomically fused leaflets, with a joint commissure or a raphe and a reduced interleaflet triangle (Figs. 1C,D), giving rise to a wide spectrum of anatomical variation.[3–5] However, three basic anatomical types of BAV are distinguished depending on the anatomical orientation of their constituents (Figs. 1E-G).[3] R-L BAVs are the result of the anatomical fusion of the right and left valve leaflets. They show antero-posterior orientation of the leaflets, and the two coronary arteries normally arise from the common anterior sinus (Fig. 1E). R-NC (Fig. 1F) and L-NC (Fig. 1G) BAVs are the result of the anatomical fusion of the right or the left and the non-coronary valve leaflets, respectively. They show latero-lateral orientation of the leaflets, and each coronary artery normally arises from one, right or left sinus.

During the second half of the last century, numerous studies dealing with the congenital origin of BAV pointed to two possible etiological factors: hemodynamic alterations and cardiac neural crest (CNC) defects during the embryonic development.[6–11] According to some authors, a BAV might be secondary to obstructive vascular defects, such as aortic coarctation, which would alter the aortic and pulmonary blood flow pattern during the perinatal period affecting late valvulogenesis.[6,7] However, this hypothesis could not explain the occurrence of isolated BAVs, i.e., not associated with other congenital cardiovascular malformations. In addition, experimental blood flow alterations in chick embryos frequently led to the development of valve stenosis or atresia, as well as histological defects of the leaflets, but rarely to BAV.[11,12]

The discovery of CNC involvement in normal and abnormal cardiovascular development was a breakthrough for understanding the etiology of many congenital cardiovascular diseases.[13,14] The CNC is a transient ectomesenchymal cell population that migrates into the developing heart from the neuroectodermal junction of rhombomeres 3–8 at the hindbrain.[15] A number of studies demonstrated that abnormal CNC cell behavior causes syndromes such as DiGeorge and 45,X cystic hygroma, including BAV.[8,16] In addition, Kappetein et al.[9] described a significant association between BAV, aortic arch defects and malformations of the head and neck in patients, pointing to defective CNC cell migration as a common etiological cause. All these data offered a strong hypothetical basis for the etiological association between BAV formation and CNC defects. However, experimental evidence of this association was not gained until 20 years later.

The first embryonic evidence of BAV formation was obtained by Shanner in 1963, from four affected pig embryos.[6] Histological reconstructions of the hearts revealed fusions of the right and left developing aortic valve leaflets in three cases and a developing latero-lateral BAV in another case. The author suggested that these defects might be caused by abnormal hemodynamics due to a developing aortic coarctation and by the absence of the non-coronary leaflet primordium, respectively.

New reports on BAV formation were not obtained until the end of the century, when Sans-Coma and collaborators developed a spontaneous hamster model of R-L BAV, in which the trait took the form of a continuous phenotypic spectrum, ranging from a TAV with no commissural fusion to a BAV devoid of a raphe.[17–19] Embryologic studies on this model revealed that BAVs developed early during valvulogenesis, as a consequence of the fusion of the right and left leaflet primordia.[20] Although abnormal behavior of CNC cells was proposed as the main etiological factor, this study did not show evidence on the molecular and developmental mechanisms leading to fusion of the leaflet primordia. Importantly, it was shown that all the phenotypes of the adult spectrum acquired their specific morphology prior to the end of the valvulogenetic process, demonstrating the congenital origin of a morphological spectrum caused by the variable severity of the original defect.

A decade later, new studies on the hamster model demonstrated a direct role of CNC in R-L BAV formation.[21,22] In addition, the developmental mechanisms of R-L and R-NC BAV formation were described, by comparing normal and abnormal valvulogenesis in embryos from the hamster model and from a newly developed *NOS3* (*eNOS*) knockout mouse model.[21,23] The results were a milestone in the study of BAV etiology by demonstrating the different developmental origins of distinct BAV phenotypes, and were followed by numerous clinical and experimental studies in which the anatomical type of BAV was considered as an important factor in BAV disease.

During the last decade, a relatively high number of studies dealing with the etiology of congenital cardiac malformations, using different strategies of genetic manipulation in mice, has included BAV in their screening protocols, resulting in an entire set of new information on the etiology and embryonic development of BAV. In the next sections of this article, we aim to gather the information on the embryonic defects and the cellular and molecular mechanisms involved in BAV formation, according to the studies using animal models (Table 1). One possible limitation of these models for extrapolation to humans has been recently outlined in a review paper on BAV anatomy and etiology in animal models.[24] Although normal and abnormal aortic valve morphology is quite similar and comparable among species (human, mouse and hamster), the incidence of BAV anatomical types (R-L, R-NC and L-NC) and intermediate phenotypes (commissural fusions and raphes) are species-specific and show a reduced variability in mice. Despite these limitations, the combined analysis of the results obtained with the different animal models of BAV development brings out a new and comprehensive understanding of the embryonic mechanisms of BAV formation.

Normal formation of the aortic valve

Cardiogenesis is initiated by the differentiation of two mesodermal cell populations, the first heart field (FHF) and the second heart field (SHF) into a myocardial scaffold that forms the primitive heart tube.[25] Initially, FHF mesenchymal cells converge in the ventral midline of the

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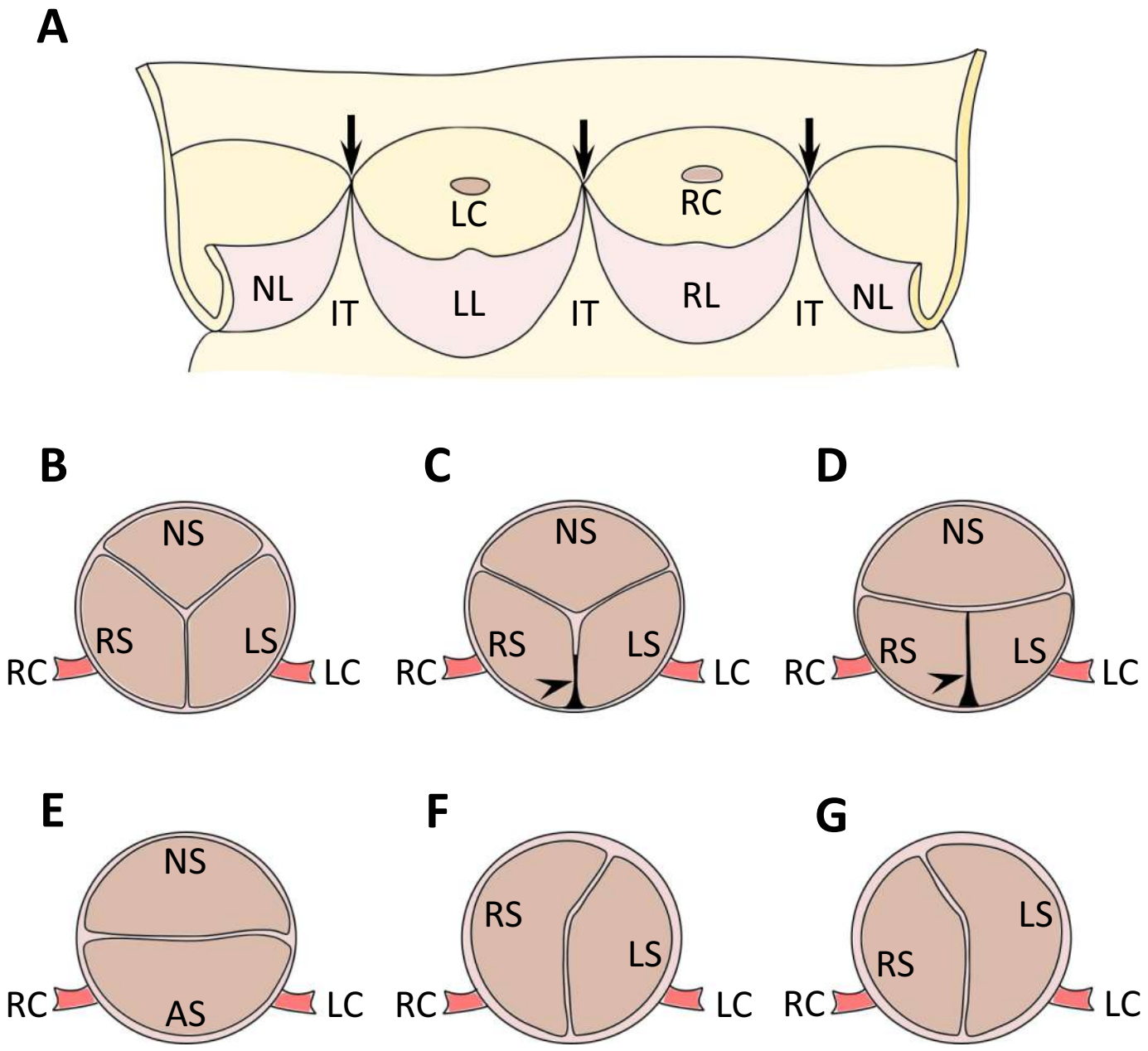

Fig. 1. Schematic representations of different anatomic types of aortic valves. A. Frontal view of a tricuspid aortic valve (TAV) opened through the non-coronary sinus to show the anterior aspect of the valve. The arrows mark the valve commissures. IT: subvalvular interleaflet triangles; LC: left coronary ostium; LL: left leaflet; NL: posterior or non-coronary leaflet; RC: right coronary ostium; RL: right leaflet. B-G. Cranial views of a TAV (B), two intermediate valvular phenotypes with a partial (C) or a complete fusion (D) of the right and left leaflets and with a raphe located anteriorly (arrowhead), and the three types of bicuspid aortic valve (BAV): R-L BAV (E), R-NC BAV (F) and L-NC BAV (G). AS: anterior sinus; LC: left coronary artery; LS: left sinus; NS: posterior or non-coronary sinus; RC: right coronary artery; RS: right sinus.

embryo to form the early heart tube. SHF mesenchymal cells located in the cyrcumpharyngeal mesoderm flanking the FHF are later recruited.[26] A third cell linage, the CNC, is necessary to complete cardiogenesis.[14] Each of these three cardiac lineages participates in the formation of different portions of the adult heart. Whereas the FHF mainly supplies to the myocardium of the left ventricle, the SHF contributes to a large part of the myocardium and endocardium of the atria, right ventricle, and outflow tract (OFT). CNC-derived cells are necessary for the formation of the aortic and pulmonary trunks and the semilunar valves.[14,15,25,26]

Semilunar valve primordia develop in the early single OFT or conotruncus, which is a cylindrical tube located in the anterior portion of the looped heart (Fig. 2). The distal OFT is connected with the aortic sac, where the pharyngeal arch arteries converge. The proximal OFT is in continuity with the right ventricle.[27,28] At the level of semilunar valve formation, the conotruncus is composed of an outer myocardial and an inner endocardial layers, separated by a wide acellular space enriched in hyaluronic acid, glycosaminoglycans and proteoglycans named cardiac jelly (Fig. 3A).[29] The cardiac jelly becomes populated by mesenchymal cells forming four endocardial cushions, which constitute the early semilunar valve primordia (Fig. 3B). These endocardial cushions consist in two conotruncal ridges (CRs) and two intercalated cushions (ICs). The parietal and septal CRs are long, helical, and opposite endocardial cushions that extend through the whole length of the embryonic OFT. The parietal CR is positioned ventro-laterally, while the septal CR is located dorso-medially (Fig. 2, 3B). ICs are small endocardial

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Table 1
Genetic, cellular and developmental defects involved in bicuspid aortic valve (BAV) formation reported in studies using animal models. The anatomical type of BAV and the associated cardiovascular malformations are detailed.

Animal model	Cellular defect	Embryonic defect	BAV type	Additional cardiovascular malformations	Reference
Pbx mutant mouse	CNC	Cushion formation?	L-L	PTA; TF; OA; VSD; BPV	46
Inbred Syrian hamster	CNC	Septation	R-L IPh	Non	21
[a]*eNOS* mutant mouse	EMT	Cushion formation	R-NC	Not addressed	21
Gata5 mutant mouse	?	Cushion formation	R-NC	Left ventricle hypertrophy	47
[a]*Pax3* mutant mouse	CNC	Excavation	Not addressed	DORV	44
Alk2 conditional mutant mouse	MCP	Cushion formation	R-NC IPh	VSD; aortic stenosis	48
Rock conditional mutant mouse	CNC	Cushion formation	R-NC	DORV; VSD; QAV; BPV; QPV; valve dysplasia; Aortic wall anomalies	49
Adamts5; *Smad2* mutant mouse	MCD	Excavation	R-NC L-NC	BPV; valve dysplasia	73
[a]*Brg1* conditional mutant mouse	EMT	Cushion formation	R-NC IPh L-NC	Non	50
[a]*Notch1* conditional mutant mouse	CNC	Septation or Excavation	R-L	Aortic stenosis; underdeveloped aortic sinuses; VSD	45
[a]*Jag1* conditional mutant mouse	CNC	Cushion formation Septation	R-NC R-L	DORV; OA; VSD; valve dysplasia	51
Ift88 conditional mutant mouse	EMT or MCD	Cushion formation or Excavation	R-NC	Not addressed	53
[a]*Npr2* mutant mouse	EMT	Cushion formation	R-NC	Ascending aorta aneurysm	52
[a]*eNOS* mutant mouse	CNC	Cushion formation	R-NC	Not addressed	31
Krox20 mutant mouse	CNC	Excavation	R-NC L-NC IPh	Not addressed	72

[a] Notch pathway involved; BPV: bicuspid pulmonary valve; CNC: cardiac neural crest behavior; DORV: double outlet right ventricle; EMT: endocardial-to-mesenchymal transformation; IPh: intermediate phenotypes; L-NC: bicuspid aortic valve (BAV) with anatomical fusion of the left and the non-coronary valve leaflets; MCD: mesenchymal cell differentiation; MCP: mesenchymal cell proliferation; OA: overriding aorta; PTA: persistent truncus arteriosus; QAV: quadricuspid aortic valve; QPV: quadricuspid pulmonary valve; R-L: BAV with anatomical fusion of the right and left valve leaflets; R-NC: BAV with anatomical fusion of the right and the non-coronary valve leaflets; TF: tetralogy of Fallot; VSD: ventricular septal defect.

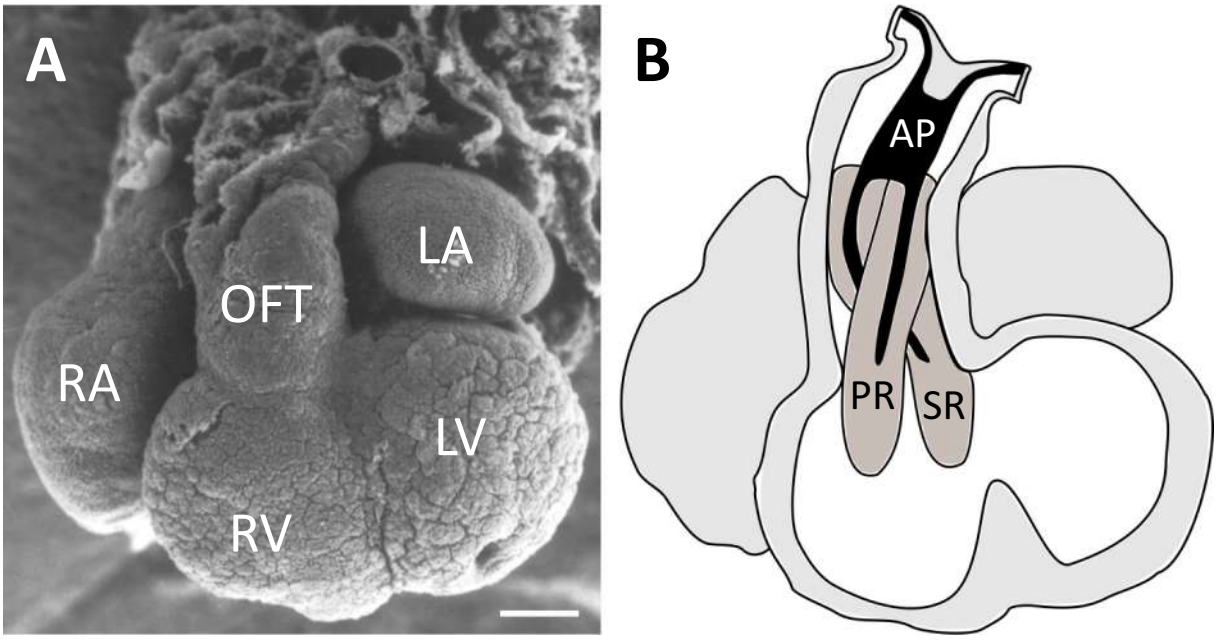

Fig. 2. Hamster embryo at an early stage of the outflow tract septation. A. Scanning electron micrograph of the developing heart. LA: left atrium; LV: left ventricle; OFT: outflow tract; RA: right atrium; RV: right ventricle. Bar = 200 μm. Reprinted from Fernández et al. (2009). Copyright 2009 by the Journal of the American College of Cardiology. Reprinted with permission. B. Schematic representation showing the position of the aorticopulmonary septation complex (AP) and the two opposite ridges arranged helicoidally. The cardiac neural crest cells (represented in thick black line) migrate from the pharyngeal arch arteries towards the OFT, mainly concentrating in two prongs that proximally enter towards the parietal (PR) and septal (SR) conotruncal ridges.

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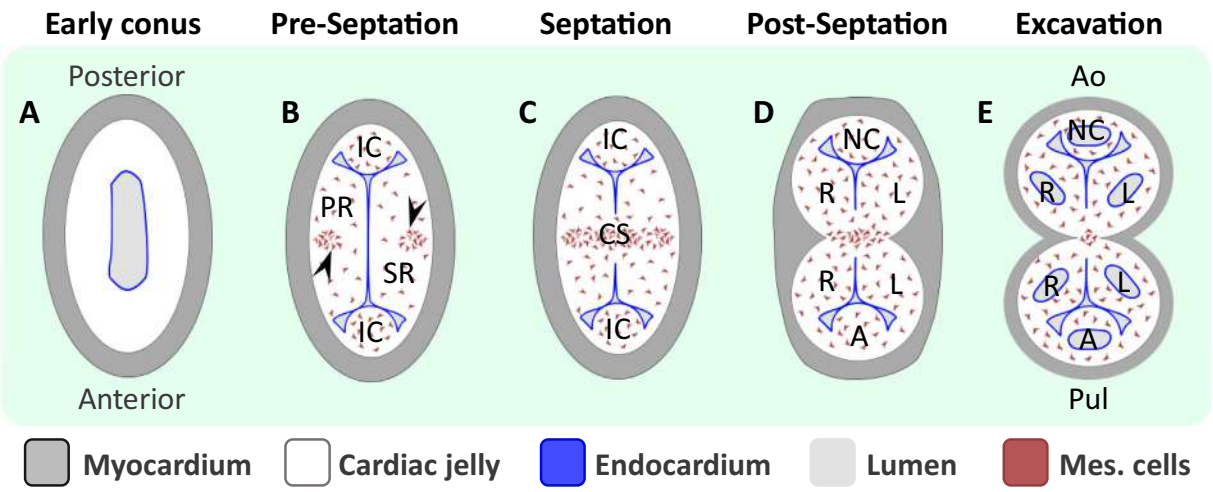

Fig. 3. Schematic representation of the successive stages of normal development of the cardiac outflow tract (OFT). Cranial views. A. Before septation starts, the OFT is composed of the myocardial and endocardial layers, separated by the cardiac jelly. B. Four mesenchymal cushions are formed, consisting of two opposite conotruncal ridges, parietal (PR) and septal (SR), and two intercalated cushions (IC), anterior and posterior. Cardiac neural crest (CNC) cells form two prongs (arrowheads) of condensed mesenchymal cells. C. CNC cells induce the fusion of the central portions of the conotruncal ridges. D. The conotruncal septum (CS) divides the OFT into two independent tracts, aortic and pulmonary. Each of them contains three valve cushions, which are the aortic and pulmonary valve primordia. E. Valve cushions undergo remodeling by an excavation process, giving rise to the definitive aortic and pulmonary leaflets. A: anterior pulmonary valve cushion; L: left aortic or pulmonary valve cushion; NC: posterior or non-coronary aortic valve cushion; R: right aortic or pulmonary valve cushion.

cushions located anterior and posteriorly in the OFT (Fig. 3B). They develop as lateral extensions of the CRs (the posterior IC from the parietal CR and the anterior one from the septal CR), so that, although ICs are connected with their respective CRs proximally, each IC can be individually distinguishable at the distal level.[30,31]

The cellularization of the cardiac jelly to form the endocardial cushions is driven by two mechanisms: local delamination of SHF-derived endocardial cells lining the cardiac jelly by an endocardial-to-mesenchymal transition process (EMT),[32] and CNC cell migration from the cyrcumpharyngeal area through the walls of the arch arteries and aortic sac to the distal OFT.[33,34] While EMT gives rise to a homogeneous mesenchymal cell population in the endocardial cushions, migrating CNC ectomesenchymal cells preferentially concentrate in two prongs that extend deep proximally into the two CRs (Figs. 2, 3B).[34–36]

Endocardial cushion formation is followed by division of the single conotruncus in two independent OFTs by means of the so-called septation process. CNC cells in the wall of the aortic sac induce the formation of a mesenchymal septum that grows proximally and contacts the distal CRs. This mesenchymal septum and the conotruncal prongs constitute a single structure with an inverted-U-shape, called aorticopulmonary septation complex (Figs. 2B, 3B), which progresses proximally to divide the conotruncus in two (right and left) independent OFTs.[30,33,36] Although the cellular mechanisms involved in conotruncal septation are still poorly understood, the process requires contact and fusion of the CRs (Fig. 3C). The fusion takes place by the disappearance of endocardial cells covering the central portion of the opposite CRs, probably mediated by a process of EMT likely induced by CNC cells of the aorticopulmonary septation complex.[15,33,37,38] After fusion, CNC cells aggregate at the center of the coalesced CRs forming a cellular condensation called conotruncal septum (Fig. 3C,D). At later stages, some CNC cells of the conotruncal septum die by apoptosis and others form part of the walls of the aortic and pulmonary roots.[14,39]

Conotruncal septation results in the formation of two separated OFTs, each containing three mesenchymal cushions called valve cushions, which constitute the aortic and pulmonary valves primordia (Fig. 3D). The unfused margins of the CRs are the primordia of the right and left aortic and pulmonary valve leaflets, whereas the ICs form the aortic posterior or non-coronary and the pulmonary anterior valve leaflets (Figs. 3B-D).[21,35] The valve cushions have an inverted pyramid shape and acquire the adult semilunar morphology by a remodeling process known as excavation (Figs. 3E, 4).[20,40–42] Excavation consists of thinning and elongation of the distal margins of the valve cushions, a poorly understood process that includes cell proliferation and cell death.[40,43–45] During excavation, valvular interstitial cells differentiate from a homogeneous population of mesenchymal cells with two different lineage origins, the SHF and the CNC. The latter regulates mesenchymal cell differentiation, apoptosis, and production of extracellular matrix, which continue during the post-natal life.[15,41,42,44]

Mechanisms of BAV formation

According to the studies that have examined the anatomy and/or the morphogenesis of the aortic valve in animal models of cardiovascular malformations (Table 1), a BAV may develop as a consequence of defects during three possible valvulogenetic stages: endocardial cushion formation (Fig. 5), OFT septation (Fig. 6) and valve cushion excavation (Fig. 7). For a better understanding, we will refer to these stages as the key morphogenetic processes involved in BAV formation. The defects in these morphogenetic processes and the alterations in the cellular and molecular mechanisms involved are outlined below.

1. Endocardial cushion formation defects

As explained in the previous section, the primordia or anlagen of the aortic valve appear early during OFT formation, by the development of the endocardial cushions, two long CRs and two small ICs (Fig. 3). Several studies using genetic manipulation in mice have shown that mutations in genes belonging to different molecular pathways cause abnormal endocardial cushion formation (Fig. 5), leading to latero-lateral BAVs.[21,31,46–52] The morphological defect consists in the development of three instead of four endocardial cushions, breaking the symmetry of the OFT transversal plane (Fig. 5B, J). In most of these embryos, the parietal (more frequently) or septal CR appeared merged or coalesced with the posterior IC, forming a big common mesenchymal cushion. We avoid here the term fusion or fused because, although it properly describes the morphological appearance of the defective cushion, there is no active union of two previously separated structures, i.e., in these embryos, invasion of the cardiac jelly by mesenchymal cells results in three instead of four endocardial cushions.

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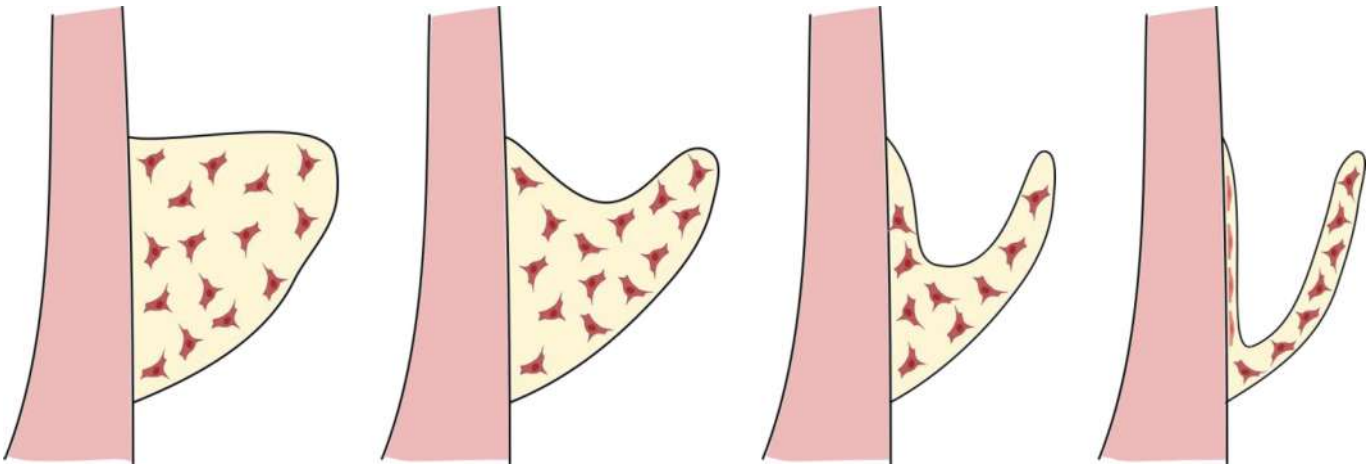

Fig. 4. Schematic representation of the sagittal view of a remodeling valve cushion. Each valve cushion consists of a mesenchymal core covered by the endocardial endothelium. During the excavation process, from an initial inverted pyramid (or semiconical) shape, the valve cushion progressively acquires the definitive semilunar shape.

Some of the studies reporting abnormal endocardial cushion formation and BAV development described in detail the division of the OFT during consecutive developmental stages.[21,31,46,49] It was found that conotruncal septation was not altered in these embryos (Figs. 5C,K). Normal septation in OFTs with abnormal endocardial cushions led to the formation of a right outflow with three normal pulmonary valve cushions, and a left outflow with only two aortic valve cushions (Figs. 5D,L). The excavation of these abnormal aortic valve cushions led to the formation of latero-lateral BAVs with R-NC or L-NC morphology (Figs. 5E,M).

Mesenchymal cells in the endocardial cushions belong to two different embryonic cell lineages, the SHF-derived endocardium, which populate the OFT by a local process of EMT, and the CNC-derived ectomesenchyme that migrates into the OFT from the neural tube through the extracardiac cyrcumpharingeal region. Alterations in these two cellular mechanisms, EMT and CNC cell migration, have been adduced as the possible cause of abnormal endocardial cushion formation leading to R-NC or L-NC BAVs.[21,31,46,49–52] However, some studies reporting R-NC BAVs caused by defects in the process of endocardial cushion formation did not detail the cellular mechanism involved.[47,53]

1.1 Alterations in EMT

The *eNOS* knockout mouse was the first animal model in which BAV was found to result from defects in the formation of the endocardial cushions.[21] In affected embryos, the dorsal margin of the parietal CR and the posterior IC developed as a unique cushion (Fig. 5B). This morphology was described as if these two cushions were fused, what led to later misinterpretations. Actually, there is no active fusion of the CR and IC. The detailed observation of cardiac OFT development in *eNOS* mutant mice revealed that the outflow cushions form abnormally from their onset, i.e., mesenchymal cells defectively populate the cardiac jelly.[21] A defect in the process of EMT during endocardial cushion formation was proposed as the cause of the malformation, given the regulatory role of eNOS in this developmental mechanism.[54,55] Why the defect localized specifically in the right-posterior aspect of the conotruncus, corresponding to the area of development of the parietal CR and the posterior IC, remained unsolved. Recently, it was shown that each IC is formed as an extension of a specific CR.[31] The posterior IC is connected proximally with the parietal CR. Thus, an EMT defect that alters the formation of the posterior IC would preferentially result in the coalescence of these two endocardial cushions.

Some years later, Liu and collaborators[56] found that lack of eNOS during embryonic development leads to morphologically abnormal and regurgitant mitral and tricuspid valves due to a defect in EMT. In endocardial cells, eNOS regulates *Tgfβ1*, *Bmp2* and *Snail1* expression through a cGMP-dependent pathway to promote EMT.[56,57] In addition, it was shown that eNOS regulation of EMT is dependent on NOTCH activation of the PI3K/AKT pathway.[58] In fact, the embryonic NOTCH1-BMP2-SNAIL1 axis promotes and regulates the extent of EMT.[59]

Two additional studies have demonstrated the involvement of the NOTCH pathway in EMT defects causing abnormal endocardial cushion development and BAV formation.[50,52] Blaser and collaborators[52] found that heterozygous loss of NPR2 leads to low penetrant R-NC BAVs in mice. The authors suggested that CNP/NPR2 signaling in the endocardial cushions may induce synthesis of cGMP to promote EMT in both a NOTCH-dependent and NOTCH-independent manner. Akerberg et al.[50] showed that endocardial BRG1 deficiency in mutant mice causes BAV formation by disruption of EMT in endocardial cushions, which is partially compensated by increased proliferation of CNC cells. BRG1 is a chromatin-remodeling enzyme that regulates NOTCH ligand expression.[60,61] Interestingly, the authors suggested that the severity of the EMT defect determines the anatomical type (R-NC or L-NC) of the resulting BAV.

NOTCH1 missense variants have been identified in patients with left ventricular OFT malformations including BAV.[62] Riley and collaborators[63] showed that these genetic variants cause defective EMT in human endothelial cell lines through impaired induction of different SNAIL transcription factor family members. The authors raised the interesting proposal that slight reductions in NOTCH1 dosage can partially affect EMT induction in endocardial cushions, predisposing to the development of a variety of OFT malformations, including BAV.

1.2 Alterations in CNC migration/aggregation

As explained above, multiple evidence points to a direct role of CNC cells in BAV formation. However, the specific molecular, cellular, and morphogenetic mechanisms involved have remained elusive for decades. Recently, Phillips and collaborators[49] showed that mice with conditional disruption of *Rock* in CNC cells develop a variety of aortic and pulmonary valve defects, including BAV. ROCK is a highly conserved RHO kinase involved in multiple cellular processes such as contraction, adhesion, migration, apoptosis, and proliferation.[64] ROCK is required for cell–cell adhesion and communication of CNC cells populating the cardiac OFT, and its disturbance causes abnormal cell-cell contacts.[49] As a consequence, defective aggregation of CNC cells results in abnormal positioning of the OFT endocardial cushions, so that well-defined, discrete, outflow cushions do not form, or are misplaced. Interestingly, Phillips et al.[49] noticed a greater contribution of CNC cells to the posterior IC,

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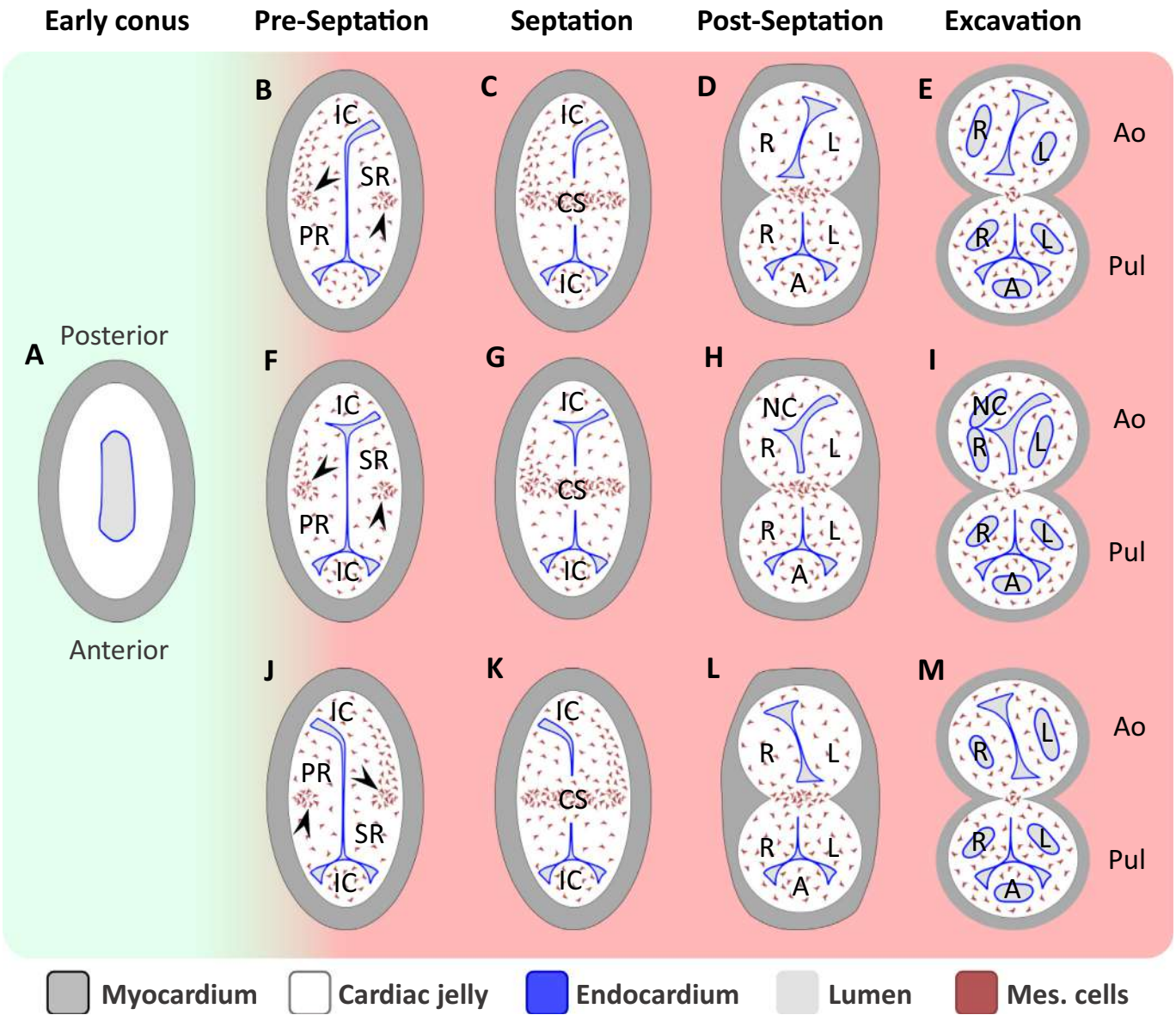

Fig. 5. Schematic representation of the development of latero-lateral bicuspid aortic valves (BAV) by defective formation of the endocardial cushions. Cranial views. A. Before septation starts, the OFT structure is normal, as described in Fig. 3A. B-E. Formation of a R-NC BAV by coalescence of the parietal conotruncal ridge (PR) with the posterior intercalated cushion, which is indistinguishable (panel B). The septal conotruncal ridge (SR) and the anterior intercalated cushion (IC) are normal. The conotruncal septum develops as usually (panels C and D), but generates abnormal primordia of the aortic valve (panels D and E). F-I— Formation of a R-NC BAV with an intermediate phenotype by partial coalescence of the PR with the posterior IC (panel F). The SR and the anterior IC are normal. The conotruncal septum develops as usually (panels G and H), but generates abnormal primordia of the aortic valve, with a partial anatomical fusion of the right and leaflet developing leaflets (panels H and I). J-M. Formation of a L-NC BAV by coalescence of the SR with the posterior IC, which is not individually distinguishable (panel J). The PR and the anterior IC are normal. The conotruncal septation occurs as usually (panels K and L), but resulting in a pulmonary tract with three normal pulmonary valve cushions and an aortic tract with two aortic valve cushions (panels L and M). In panels B, C, F, G, J and K, the mesenchymal cells (in red) are drawn not homogeneously distributed in the PR or the SR ridges (compare with Figs. 3 B,C), representing the abnormal migration of cardiac neural crest cells detected in some mouse models of latero-lateral BAV development. A: anterior pulmonary valve cushion; CS: conotruncal septum; L: left aortic or pulmonary valve cushion; NC: posterior or non-coronary aortic valve cushion; R: right aortic or pulmonary valve cushion. Arrowheads mark the two prongs of condensed mesenchymal cells, as described in Fig. 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

what could explain the increased incidence of BAV, particularly of the R-NC type, compared with L-NC BAV and bicuspid pulmonary valve in their study. This revealed the existence of areas in the OFT with increased susceptibility to CNC disturbance.

Homeodomain transcription factors such as PBX are well known regulators of CNC cell behavior.[65] Genetically modified mouse embryos with different combinations of *Pbx* mutations (*Pbx-1*, *Pbx-2* and/or *Pbx-3* null alleles in homozygosis or heterozygosis) display a spectrum of cardiac OFT malformations including persistent truncus arteriosus, tetralogy of Fallot, overriding aorta and BAV.[46] Interestingly, the different phenotypes of mutant mice were correlated with *Pbx* gene dosage. It was suggested that aberrant expression of different PBX isoforms alters CNC distribution in the developing OFT leading to BAV formation, although the authors did not detail whether the CNC alterations caused abnormal endocardial cushion formation or defective OFT septation.

In the embryonic heart, the NOTCH pathway regulates not only EMT, but also CNC cell behavior.[66,67] MacGrogan et al.[51] demonstrated that alterations of the NOTCH signaling pathway in different cell types and at different development time points cause a variety of heart defects including BAV. The authors found that endocardial JAG1 signaling through

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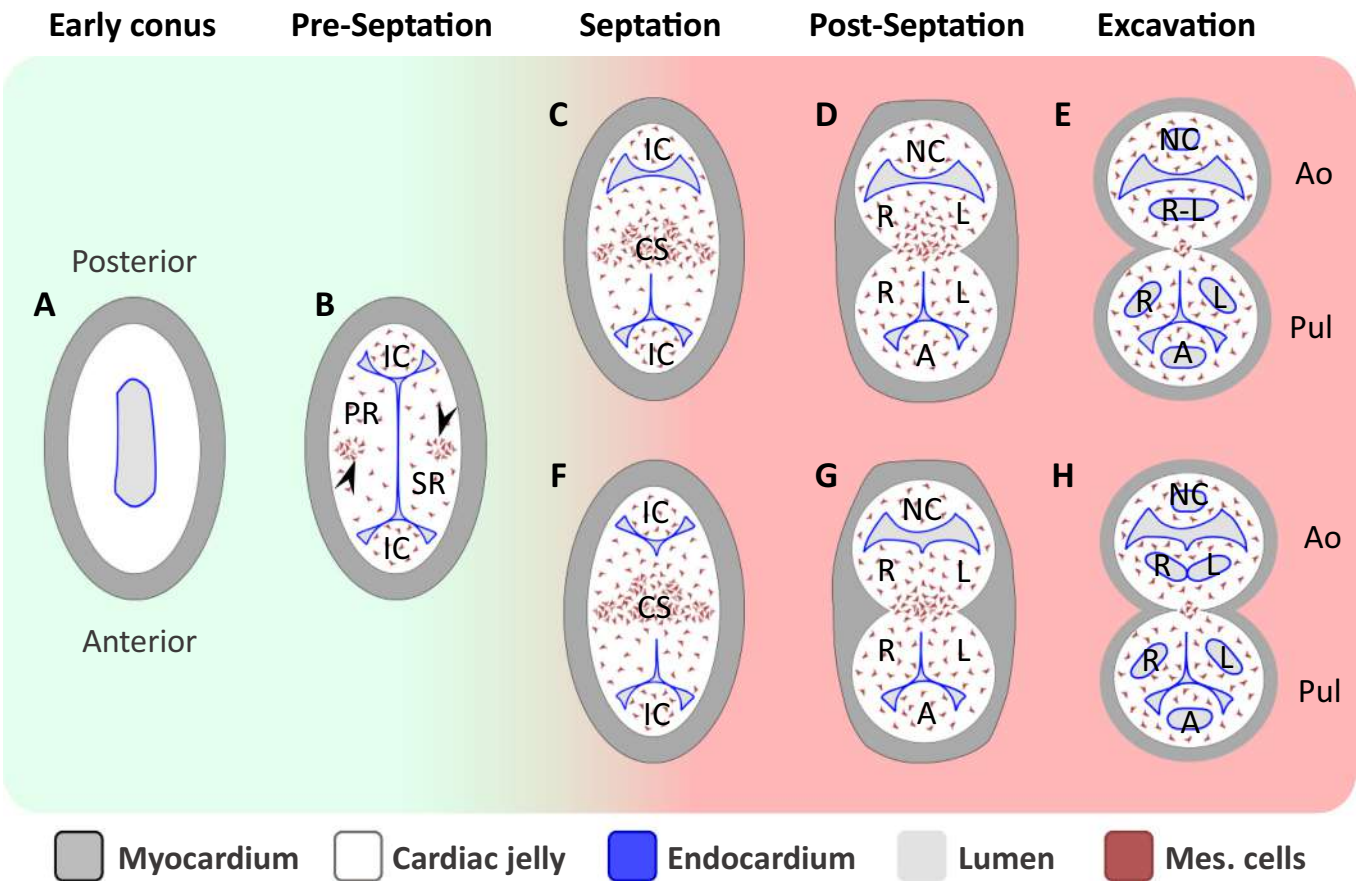

Fig. 6. Schematic representation of the development of antero-posterior (R-L) bicuspid aortic valves (BAVs) by defective septation of the outflow tract. Cranial views. A-B. Early stages of the process occur normally, as described in Figs. 3A,B. C—H. Altered behavior of cardiac neural crest cells responsible for the formation of the conotruncal septum (panels C and F) causes an extrafusion of the posterior margin of the conotruncal ridges, leading to variable degree of fusion of the right and left aortic cushions (panels D and G). While three normal pulmonary valve cushions develop in the pulmonary tract, the extension of the fusion results in a phenotypic continuum of aortic valve morphologies, including R-L BAVs (panels E and H). In panels C, D, F and G, the mesenchymal cells (in red) are drawn not homogeneously distributed in the contruncal septum, (compare with Figs. 3 C,D) representing the abnormal migration of cardiac neural crest cells detected in the spontaneous hamster model of antero-posterior BAV development. A: anterior pulmonary valve cushion; CS: conotruncal septum; L: left aortic or pulmonary valve cushion; NC: posterior or non-coronary aortic valve cushion; R: right aortic or pulmonary valve cushion. R-L: primordium of the anterior valve cushion of a R-L BAV. Arrowheads mark the two prongs of condensed mesenchymal cells, as described in Fig. 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

NOTCH1 is required for post-EMT and suggested that this pathway regulates CNC patterning in the OFT, ensuring endocardial cushion positioning.

Another relevant study in this context is that of Peterson et al.,[31] who proposed that eNOS influences endocardial cushion formation not only by regulating EMT, but through a still little-known effect of this enzyme on cell lineage patterning in the OFT. The abnormal CNC and SHF cell distribution in the endocardial cushions was adduced as the cause of BAV formation in eNOS deficient mice. It was suggested that eNOS expressed by endocardial cells have an active role in guiding CNC and SHF through the OFT. Although Peterson et al. did not detail the molecular pathway by which eNOS may influence cell migration, NOTCH is a good candidate,[68] given the role of eNOS in NOTCH activation[69] and the direct involvement of NOTCH in CNC patterning.[51,66,67] Taken together, the studies by Fernández et al.[21] and Peterson et al.[31] show that eNOS disruption in the OFT may lead to BAV by defects in two different cellular mechanisms, i.e., EMT[21] and CNC cell migration.[31] Both papers basically coincide in the resulting morphological alterations in the outflow endocardial cushions, i.e., coalescence of the parietal CR with the posterior IC, giving rise to a big right aortic valve cushion (Figs. 5B). These apparently discrepant data may be reconciled by the finding that migrating CNC cells in the OFT instruct the endocardium to initiate EMT, while endocardial signaling then instructs CNC relocalization in the endocardial cushions.[38] Thus, alterations in each of these cellular mechanisms, endocardial EMT and CNC cell migration might interchangeably result in OFT endocardial cushions with the same aberrant morphologies.

Another morphogenetic mechanism that has been proposed to cause abnormal cushion formation and BAV development consists in the absence of the posterior IC. This would lead to formation of only two (right and left) aortic valve cushions and latero-lateral BAV. Thomas et al.[48] found that endocardial specific deletion of *Alk2* causes a significant reduction of mesenchymal cell proliferation in the endocardial cushions, leading to the formation of latero-lateral BAVs. This mechanism seems consistent with the one exposed above and represented in Fig. 5. However, Thomas et al.[48] detailed that in most defective embryos, 'a small third cushion in the non-coronary position was still present in mutant aortic valves'. Thus, in this model BAV seems not to result from the coalescence of the parietal CR and the posterior IC cushion, but from the underdevelopment of the IC. This defect leads to a spectrum of abnormal morphologies affecting the right and non-coronary leaflet, including TAVs with a small non-coronary leaflet and functionally BAV with a very small third sinus.[48]

2. Outflow tract septation defects

Septation of the cardiac OFT relies on the fusion of the two opposite CRs and formation of the conotruncal septum by CNC cells (Fig. 3C).

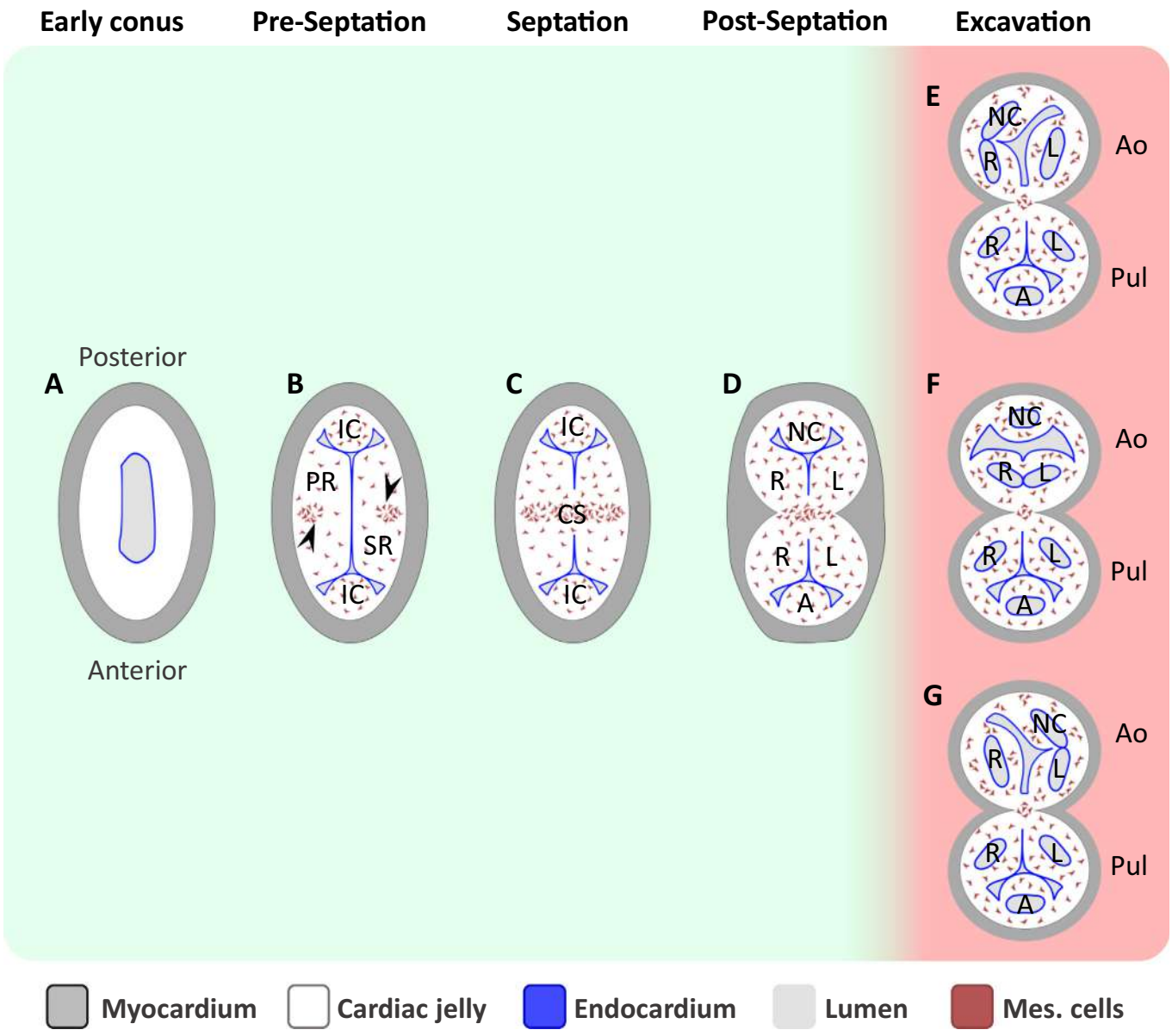

Fig. 7. Schematic representation of the development of antero-posterior and latero-lateral bicuspid aortic valves (BAVs) by defective excavation of the valve cushions. Cranial views. A-D. The outflow tract formation and septation process occur normally, as described in Figs. 3A-D. E-G. Alterations during the excavation and remodeling of the aortic valve cushions may cause fusion of developing leaflets, leading to the formation of R-NC BAVs (panel E), R-L BAVs (panel F) and L-NC BAVs (panel G). In panels E-G, the mesenchymal cells (in red) are drawn not homogeneously distributed in the aortic valve cushions, representing the abnormal distribution of cardiac neural crest cells detected in some mouse models of defective excavation. In these panels (E-G), the defects are represented as partial fusions of developing leaflets, which are frequently found in mutant embryos, but pure BAV morphologies, similar to those represented in Figs. 5E,C and 6H were also detected. A: anterior pulmonary valve cushion; CS: conotruncal septum; L: left aortic or pulmonary valve cushion; NC: posterior or non-coronary aortic valve cushion; R: right aortic or pulmonary valve cushion. Arrowheads mark the two prongs of condensed mesenchymal cells, as described in Fig. 3. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

During normal septation, the fusion is restricted to the central portion of the CRs, so that the symmetrical distribution of the endocardial cushions allows the formation of the aortic and pulmonary valve cushions, symmetrically positioned in the resulting right and left OFTs (Fig. 3D). However, when the fusion is not strictly localized in the center of the CRs, but extends to the dorsal margins of the ridges (Fig. 6C), then fused right and left aortic valve cushions results (Fig. 6D) and antero-posterior or R-L BAVs are formed (Fig. 6E).

This morphogenetic defect was first described in the spontaneous hamster model of BAV, in which alterations in the migration of CNC cells were shown to be associated with the excessive fusion of the CRs.[20,21] Importantly, research in this model demonstrated that the severity of the original embryonic defect, i.e., the extension of the fusion of the CRs (Figs. 6C,F), causes a variety of aortic valve morphologies in the form of a phenotypic continuum.[19–21,24] This continuum includes BAVs without raphe, BAVs with a raphe of variable size and TAVs with different degrees of fusion of the right and left leaflets. Similar phenotypes are now recognized in the anatomical spectrum of human BAVs.[5]

As in humans, BAVs in the hamster model show a complex inheritance with a strong environmental influence,[19,70] but the genes involved are still unknown. It seems clear that alterations in the behavior of CNC cells are responsible for the abnormal septation of the OFT,[21,22] but the defective cellular mechanism has not been described yet. The JAGGED/NOTCH signaling pathway seems to be an important regulator of the process. *Jagged1* and *Notch1* conditional mutant mice show R-L BAVs, probably resulting from defective CNC patterning that

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leads to abnormal CR fusion.[51] Interestingly, the authors demonstrated that reduction of NOTCH1 activity impacts valve development only during a limited temporal window, which lies around the beginning of the septation process. Wang et al.[45] also showed R-L BAV formation in *Notch1* conditional mutant mice, but they could not detail whether the embryonic defect occurs during OFT septation or during valve cushion excavation.[45,71]

In summary, R-L BAVs can develop as a consequence of the abnormal, excessive fusion of the CRs during the process of septation of the embryonic cardiac OFT (Fig. 6). Fusion is the result of the disappearance of endocardial cells covering the opposite CRs. Currently, no study has provided definitive empirical results supporting a mechanistic hypothesis on the process of CR fusion. However, comparisons with fusion events in other embryonic structures suggest that CR fusion results from a process of EMT localized in the central portion of the CRs, induced by CNC cells of the aorticopulmonary septation complex.[15,37,38] The involvement of NOTCH in R-L BAV formation is consistent with this hypothesis, given the central role of this pathway in the EMT process. In addition, the capacity of CNC cell derived SEMA3C to promote EMT via NRP1[38] is consistent with a regulatory role of the CNC.

A comprehensive revision of the mechanisms of OFT development and BAV formation exposed above allows to highlight several relevant associations: 1) CNC cell defects are responsible for alterations in both, endocardial cushion formation and OFT septation, leading to latero-lateral and antero-posterior BAVs, respectively; 2) EMT is involved in both developmental alterations, promoting cellularization of endocardial cushions and fusion of CRs; 3) CNC cells are able to induce EMT. At this point, it is tempting to speculate that defective CNC cell behavior alters EMT early during endocardial cushion formation, or later during OFT septation, leading to latero-lateral and antero-posterior BAVs, respectively. The moment at which CNC cells abnormally induce EMT causing valve maldevelopment may depend on specific genetic mutations, genetic modulators, and epigenetic factors, as previously suggested.[24]

3. Valve cushion excavation defects

Although the mechanisms responsible for the excavation of the valve cushions are largely unknown, it is assumed that they include the differentiation of mesenchymal cells with two distinct lineage origins, i.e., the mesoderm (SHF) and the ectoderm (CNC). Using a *Krox20* deficient mouse model,[72] Odelin and collaborators demonstrated that BAVs can develop due to an odd maturation of the aortic valve cushions after normal OFT formation and septation (Fig. 7). In this model, absence of KROX20 causes an alteration in the number and distribution of CNC cells in the aortic valve cushions, which specifically affects the insertion zone of the valve leaflets and the interleaflet triangle between leaflets. As a consequence, modifications in the extracellular matrix production in these areas during cushion excavation widen the insertion areas of contiguous cushions, leading to fused developing leaflets and BAV development. Interestingly, recent studies indicate that eNOS is a KROX20 target, suggesting that reduction in eNOS activation is a contributing mechanism to BAV in Krox20 mutant mice.[68]

A similar mechanism of BAV development was previously proposed by Jain et al.[44] and Wang et al.[45] studying *Pax3* and *Notch1* conditional knockout mice, respectively. In both models, genetic manipulation affected the NOTCH signaling pathway and resulted in alterations of the proliferation and/or apoptosis of mesenchymal cells in the valve cushions, causing abnormal valve cushion remodeling and BAV. In the case of *Pax3* mutant mice, NOTCH inhibition in the SHF affected CNC patterning and altered extracellular matrix production in the remodeling valve cushions.[44] In the case of *Notch1* mutant mice, the possibility that OFT septation and not cushion excavation was the affected morphogenetic process cannot be excluded.[45,71]

Dupuis et al.[73] showed that *Adamts5* and *Smad2* deficiency in mutant mice causes insufficient versican cleavage during valvulogenesis, leading to R-NC or L-NC BAV formation. Versican is a proteoglycan with an important role in maintaining the integrity of the extracellular matrix by interacting with hyaluronan and fibulin-2. It was proposed that versican clearance is required for the organization of fibrous extracellular matrix in the forming commissures. Alterations in versican clearance would affect mesenchymal cell differentiation and collagen formation during excavation, leading to BAV. Alternatively, however, it was proposed that lack of versican clearance might expand endocardial cushion size, leading to fusion of the aortic valve cushions at a post-septation stage.

Toomer et al.[53] found that conditional loss of the interflagellar transport protein Ift88 causes shortening of axonemes in cushion mesenchymal cells suppressing cell differentiation. It was proposed that deficient mesenchymal cell differentiation widens the insertion zone of adjacent valve cushions leading to BAV formation. However, it cannot be excluded that abnormal primary cilia in this model cause BAV due to early defects in the formation of endocardial cushion, given the regulatory role of these cilia in the EMT process.[74]

In summary, BAVs can also result from anomalies during the excavation phase of valvulogenesis. In most cases, alterations in matrix production and proliferation/apoptosis rate of CNC-derived mesenchymal cells in the insertion areas of valve cushions result in the coalescence of two adjacent cushions during excavation and remodeling and BAV formation (Fig. 7). Raphes and commissural fusions were described in a high proportion of the embryos with defective valve cushions after abnormal excavation,[44,45,72,73] suggesting that intermediate aortic valve phenotypes are a common outcome of this type of BAV developing mechanism.

Some of the studies describing defective aortic valve development have shown that the embryonic alteration leads to a spectrum of abnormal morphologies of the developing aortic valve cushions.[20,21,48,50,72] This suggests that the severity of the original embryonic defect may cause a variety of aortic valve morphologies, including BAVs, TAVs and intermediate phenotypes in the form of a phenotypic spectrum (Figs. 5-7).

While most defective embryos in these studies showed asymmetrical aortic valve cushions (one normal and one big valve cushion), adults usually showed two symmetrical (equally sized) valve leaflets.[21,47,48,50,52] Although the process of excavation and maturation of the aortic valve cushions in BAV developing embryos has not been studied in detail, it can be assumed that the size of two aortic valve leaflets and sinuses is compensated during valve maturation.

Conclusions and hypothesis

During the last 20 years, the development of spontaneous and genetically modified rodent models of cardiovascular disease has significantly advanced our knowledge on BAV etiology (Table 1). Some of the genes whose pathways were experimentally altered in mutant mice resulting in BAV formation, including *Notch1*, have been shown to be associated with human BAV disease.[75] The compilation of the information obtained in these studies allows to clearly define several mechanisms of BAV formation. These mechanisms affect three key processes: 1) endocardial fusion formation; 2) OFT septation; and 3) valve cushion excavation.

1) Defective EMT or CNC cell migration may cause abnormal endocardial cushion formation, leading to coalescence of adjacent OFT endocardial cushions that, after normal OFT septation, results in the development of two instead of three aortic valve primordia, the remodeling of which leads to R-NC or L-NC BAVs (Fig. 5).

2) Alterations in the behavior of CNC cells that colonize the cardiac OFT may induce excessive fusion of the CRs during OFT septation, leading to coalescence of the right and left aortic valve cushions that results in R-L BAV formation (Fig. 6).

3) After normal endocardial cushion formation and septation, abnormal distribution of CNC cells, proliferation of mesenchymal cells and/or

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matrix production in the aortic valve cushions may cause defective remodeling of the insertion areas of contiguous cushions, leading to BAVs with different degrees of severity (Fig. 7).

The embryonic defects cited above may cause a wide range of valve morphologies, which represent well the BAV anatomic spectrum described in human patients.[5] This spectrum seems to be the result of the variable severity of the original embryonic defect. Nevertheless, it should be noted that there exist inter-specific differences between humans and laboratory mice with respect to the phenotypic expression of aortic valve defects.[24] Indeed, it has been shown that the same genetic defect may cause different BAV types in human and mice.[47,76] Therefore, one should be cautious when extrapolating specific experimental results on BAV etiology from mouse to human.

Our literature review on BAV embryonic development highlights a variety of molecular pathways, cell mechanisms and developmental processes involved in the formation of different anatomical types of BAV. However, a comprehensive analysis reveals strong connections among these pathways, mechanisms, and processes. Most molecular alterations leading to BAV formation affect endocardial EMT and/or CNC cell migration, being the NOTCH pathway a crucial regulator of both cellular mechanisms. In addition, the three developmental processes whose defects cause BAV, i.e., endocardial cushion formation, OFT septation and valve cushion excavation, are regulated by the behavior of CNC cells at three consecutive developmental stages. First, CNC cells are able to induce EMT in the OFT for endocardial cushion formation.[15,38] Second, fusion of CRs leading to OFT septation most probably relays on EMT induced by CNC cells.[15,37,38] Third, aortic valve excavation defects leading to BAV often result from alterations in CNC patterning.[44,72] Thus, we propose a unified developmental mechanism of BAV formation based on CNC cell behavior alterations at three alternative developmental stages. At early stages, defective CNC cell migrating into the cardiac OFT may alter local EMT and/or mesenchymal cell distribution causing abnormal endocardial cushion formation. At a later stage, during OFT septation, CNC cells may abnormally induce the excessive fusion of the CRs. Later, during aortic valve cushion excavation, abnormal distribution and differentiation of CNC cells may alter the morphology of the remodeling valve cushion. The specific genetic defect would determine the precise CNC cell function alteration and the stage and location in which it takes place, what would in turn determine both the embryonic process affected and the anatomical type of the resulting BAV. The specific cell function alteration, stage and location are most probably tuned by genetic modifiers.[24,77]

Some of the studies cited in this review already suggested BAV developmental hypotheses in line with the embryonic model of BAV formation proposed here. First, the fact that one mutation can cause different BAV types through distinct morphogenetic alterations[21,24,31,51] gives support to the idea that CNC defects constitute a unifying event in a variety of developmental mechanisms leading to BAV. Patients with the same gene mutation can show different BAV types, pointing to distinct morphogenetic defects caused by the same etiological factor.[24] Riley et al.[63] already pointed out that "NOTCH signaling and the resulting reduction in the efficiency of EMT could serve as a unifying event whereby *Notch* mutations contribute to diverse left ventricular OFT malformations", what fits well with the present model given that BAV forms part of these OFT malformations. Experimental evidence gives support to a central role of the NOTCH pathway in this unified hypothesis of BAV development (see asterisk in Table 1). As already discussed, different valve phenotypes result from defects in distinct developmental mechanisms when mutations in genes involved in the NOTCH pathway are induced at different developmental time points.[51]

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Declaration of Competing Interest

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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