Embryological Origin of Valve Progenitor Cells

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Abstract The cardiac valves are required for unidirectional blood flow, preventing backflow during diastole. The adult mammalian heart includes four valves: the aortic, pulmonary, mitral and tricuspid valves. Cardiac valves all have common features, notably a stratified structure consisting of three layers of specialized interstitial cells and extracellular matrix. However, the "semilunar" aortic and pulmonary valves and "atrioventricular" tricuspid and mitral valves have notably different embryonic origins. Indeed, several cell lineages, including endocardium, epicardium and neural crest, are valvulogenic. The endocardium, or inner endothelial lining of the heart, makes major contributions to all valves by undergoing endothelial-to-mesenchymal transition. Neural crest and epicardium make secondary contributions to the semilunar and atrioventricular valves, respectively.

The embryonic origins of endocardium, and valve progenitors within, are still not entirely elucidated. The current paradigm stipulates that endocardium is mainly derived from two early embryonic fields, the first and second heart fields. Further delineating the origins of valve progenitors and their specification towards the valve lineages is essential for understanding cardiac congenital defects. Furthermore, it will be essential for developing therapeutic strategies ranging from pharmacological interventions to improving valve replacement. Finally, the biology of valve progenitors is highly relevant to cardiac fibrosis. Indeed, endothelial to mesenchymal transition of endothelium, comparable to that generating valve mesenchyme, is considered to be a major contributor to cardiac fibrosis. However, it has recently been shown that a more likely source of most, if not all, EndoMT derived fibroblasts in heart is EndoMT associated with valvulogenesis.

Keywords Endocardium · Endothelial-mesenchymal transition · Heart · Mouse embryo · Valvulogenesis

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1 Introduction

The heart is the first organ to develop in the embryo. Its function of ensuring that blood is distributed to and from other developing organ systems is essential and requires increasing efficiency as the embryo grows. This efficiency is achieved when cardiac valves develop within the contracting myocardium, preventing backflow as blood is pumped by the myocardium's contractions. Oxygenated blood is pumped from the lungs into the left ventricle through the mitral valve, and from the left ventricle through the aortic valve. Deoxygenated blood enters the right ventricle through the tricuspid valve, and is pumped back to the lungs through the pulmonary valve. Valves are stratified structures that consist of up to three extracellular-rich leaflets organized within a fibrous ring, and are firmly anchored to the myocardium. The semilunar aortic and pulmonary valves present a number of differences with the atrioventricular tricuspid and mitral valves. Notably, in terms of structure, the atrioventricular valves are linked to papillary muscles by chordae tendinae, a structural adaptation that prevents prolapsing.

These well characterized structural and positional differences are in contrast with the more elusive origins of the valve progenitors, as well as the morphogenic and biomechanical cues that guide their specification. Indeed, it is currently believed that several early lineages include valve progenitors, and the timing of the specification of these valve progenitors and relative contributions of different lineages is still an area of intense investigation. Furthermore, although much has been revealed on signaling pathways involved in valvulogenesis, much remains to be elucidated, notably in terms of epigenetics or biomechanics (shear stress).

Valves form between the 5th and 8th week of human fetal life, and between E9.5 and E14.5 in mouse. Up to a third of cardiac congenital diseases are characterized by valve malformations [1]. Valves are under intense use, opening more than 2.5 billion times in the life of an adult person. They remodel throughout life, adapting to wear-and-tear, myocardial growth/remodeling and changes in cardiac hemodynamic load. Hence pathological conditions affecting the myocardium lead to adverse remodeling of the valves. Such abnormalities are present in up to 13 % of patients who are 75 years or older [1, 2].

Current therapeutic approaches rely heavily on the replacement of defective valves with mechanical or bioprosthetic valves. Although this has greatly improved the outcome of heart disease for many patients, there are many limitations to this approach. Notably, in the case of congenital malformations in children, replacement valves cannot adapt to the growing myocardium, requiring further interventions. Furthermore, in adults, long term complications can develop linked to limited durablility and thrombogenicity of prostheses.

Hence, the improvement of valve replacement requires these issues to be addressed by using engineered materials with enhanced hemodynamics, mechanical integrity and thromboresistance. Furthermore, cell therapy could also provide a means of giving more functionally integrated replacement valves. Indeed, more in-depth knowledge of the development and characteristics of valve lineages could

enable the generating of various human valve cell-types and pave the way for cell therapy.

Valvulogenesis is initiated in the atrio-ventricular canal (AVC) and outflow tract (OFT). These regions become defined when the heart tube elongates and loops, forming the primary ventricle and atrium. Endocardium plays a primary role in early valvulogenesis in both the AVC and OFT. Endocardial cells are separated from the myocardium by a layer of extracellular matrix (ECM) known as cardiac jelly, which is composed of hyaluronan and chondroitin sulfates [3, 4]. Within the AVC and OFT, the myocardium secretes larger amounts of ECM, leading to the formation of "cushions". In response to signals including BMP2 and TGFβ, endocardial cells lining the cushions undergo an endothelial-to-mesenchymal transition (EMT), whereby they delaminate whilst acquiring mesenchymal properties and migrate into the jelly. The cushions become rapidly populated with valve mesenchymal cells characterized by the expression of genes such as Sox9, Sox5, Sox17, Tbx20 and Msxs [4].

Although the endocardium is the major lineage at the origin of valvulogenesis, other lineages have been shown to be essential for normal development of the OFT and AVC valves. Neural crest, a prominent migratory cell population that emerges from the neural tube, makes significant contributions to the valves forming in the OFT. Epicardium also plays a major role in development of the AVC valves. Epicardium is the protective epithelial layer that covers the heart, and undergoes EMT to give rise to cardiac fibroblasts, pericytes, coronary smooth muscle and possibly a subset of endothelial cells and myocytes. Interestingly, epicardium also makes major contributions to the AVC valves, notably generating mesenchyme in the annulus fibrosis and leaflets.

Here we focus on the development of the heterogeneous lineages that contain valve progenitors, as well as the fates of these cells within the maturing valves. Much remains to be determined concerning how and when these subsets of valve progenitors become specified, a point that will be discussed at the end of this section.

2 Early Cardiogenesis and Valve Progenitor Specification

2.1 Overview of Cardiac Development and Key Concepts

Cardiogenesis involves the mobilization of multiple progenitor populations at distinct stages that contribute to specific cardiac compartments. According to the current model, two main populations of progenitors give rise to cardiac myocytes [5]. During gastrulation, a mesodermal cardiac progenitor population, known as the first heart field, emerges from the anterior part of the primitive streak. These cells migrate to the splanchnic mesoderm to form the cardiac crescent. The crescent then fuses at the midline forming a tube-like-structure which elongates on both the arterial and venous poles *via* the addition of progenitor cells originating from the secondary heart field. The latter lies medially and posteriorly to the crescent and is

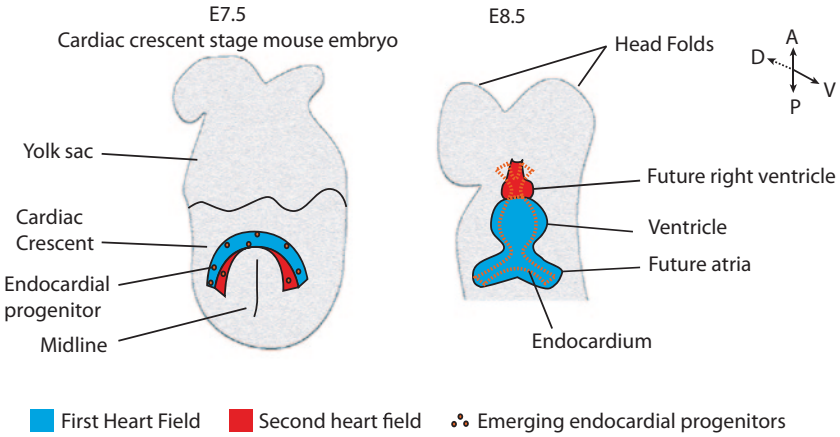

Fig. 1 Emergence of the first valve progenitors occurs in both heart fields. Endocardial cells, including valve progenitors, form by vasculogenesis and give rise to the endocardial tube around which a myocardial layer develops

characterized by the expression of the transcription factor *Isl1* [6]. At this point the heart consists of an outer myocardial layer and an inner endocardial layer (Fig. 1). The latter is formed by de novo vasculogenesis of cells within the cardiac crescent and in the second heart field [7–9]. This process can be recapitulated *in vitro* using human pluripotent stem cells i.e embryonic (HUES) or induced stem cells (iPS), from which both myocardial and endocardial cells can be derived [10, 11].

In order to acquire its definitive form, the heart tube must first undergo rightward looping, whereby the posterior region moves to the anterior allowing segmentation into atrium, an atrioventricular canal, a ventricle and an outflow tract. Intense proliferation of myocardial cells associated with the ventricle and atrium, but not AVC, results in "ballooning" and initial chamber formation [12]. Subsequently, valve formation (valvulogenesis) and septation take place, generating the four cardiac chambers.

2.2 Endocardium Formation

Endocardium forms the inner epithelial lining of the heart, and plays major functions in the development of not only the valves, but also of the formation of the septa, conduction system and trabecular myocardium [13]. In terms of evolution, the development of valves coincides with the separation of cardiac chambers in vertebrates [14, 15]. Although much is known about the development of endocardium, whether the valve progenitors within this lineage i.e. endocardial cells that undergo EMT, represent a distinct population in terms of their embryonic origin is not currently known.

The endocardium develops forming a continuum with the dorsal aorta anteriorly and the cardinal veins posteriorly. Early endocardium is positive for endothelial markers PECAM1, Flk1 and VE-cadherin. However, the origin of endocardial cells

is different from other vascular components, although the origin of all endocardial cells has is not completely understood. Early studies using retroviral tracing in chicken and quail embryos have suggested that endocardial and myocardial cells segregate early from a common progenitor in the cardiac field prior to gastrulation [16]. Indeed, retroviral single cell tracking by Mikawa has shown that a population of cells derives from a specific region of the primitive streak migrates to bilateral heart regions, generating either myocardium or endocardium, but not both [17].

Using single-cell tracking in zebrafish, Lee et al. [18] determined that endocardial progenitors were restricted to a specific area of the cardiac field, but could give rise to endothelium, including endocardial cells. This was in agreement with a previous study in chick where a subset of cardiac progenitors was found to co-expressed endothelial (QH-1) and myocardial markers (N-cadherin), suggesting they could give rise to both the myocardial and endocardial lineages [19]. This view has been backed by various studies in mouse, notably showing that these progenitors included a Flk1+ population [20]. Flk1 has been shown to be expressed by endocardium and myocardium as early as E8.5 in mouse heart (Flk1 Lacz reporter) [21]. Genetic lineage tracing in mouse suggests that common myocardial/endocardial progenitors exist relatively late in development, at the cardiac crescent stage, notably being labeled by Nkx2.5-Cre [22] and Isl1-Cre [6] lineage-tracing. Interestingly, an Nkx2.5 response element was identified in the Ets-related protein 71 (Etsrp71), that targets genes required for endothelial/endocardial cell specification of cardiac progenitor cells [23].

Studies performed *in vitro* have put forward the possibility that multipotent cardiac progenitors, notably Flk1+ or Isl+ cells derived from ESCs give rise to myocardium, endothelial (potentially endocardial) and smooth muscle exist [24, 25]. Interestingly, Wnt and BMP signaling that is shown to induce myocardial enrichment in embryoid bodies also promotes the formation of cells with endocardial characteristics, notably the expression of an Nfatc-nuc –LacZ reporter [20].

Fate mapping and cell tracking within quail embryos suggests that some endocardial cells are derived from non-cardiogenic progenitors situated in the second heart field. However, evidence that endocardium represents a distinct lineage from endothelium comes from studies of zebrafish mutants *Cloche* [26] and *Faust*, and hence likely derives from a distinct progenitor pool than other endothelium.

Overall, these studies, looking at expression of precursor markers such as Flk1, or constitutive Cre genetic labeling systems, present limitations. Notably, some of the markers/cre drivers used may not be specific to one lineage throughout cardiac development. Conflicting results, such as the lack of a consensus on the timing of specification of the endocardial progenitors i.e. the existence of early versus late common progenitor for myocardium and endocardium, could be reconciled in the case endocardium is derived from multiple progenitor populations in distinct waves, including a primary wave from vasculogenesis in the first heart field, and subsequent contribtutions from second heart field and possibly other lineages. Future studies could employ approaches such as retrospective clonal analysis, used to provide solid evidence for the presence of two distinct cardiac progenitor populations contributing to specific aspects of the developing heart [5, 27, 28]. Such methods, although time

consuming, could provide novel insight into the development of the endocardial lineage. Finally, although controversies have arisen over the extent of the heterogeneity of endocardium, it currently seems likely that endocardial cells share a similar heterogeneous origin to that of the other major early cardiac lineage, the myocytes.

Hence, the embryonic origins of endocardial cells have not been fully elucidated. Key questions remain, including whether distinct developmental origins confers EMT competence to endocardium within the OFT and AVC.

2.3 Epicardium

Epicardium is the protective outer epithelial layer of the heart. In humans, the epicardium is multi-layered and has a sub-epicardial adipose tissue layer, whereas it is formed by a single layer in mouse (and chick). Impaired epicardial development leads to defects in valve development, cardiac myocyte proliferation and alignment as well as conduction system defects.

Epicardial development begins at E9.5 in mouse heart, with the emergence of the pro-epicardium at the venous pole of the heart, a "cauliflower-like" structure (Fig. 2). From E9.5 onwards, pro-epicardial cells begin to migrate and form a sheath

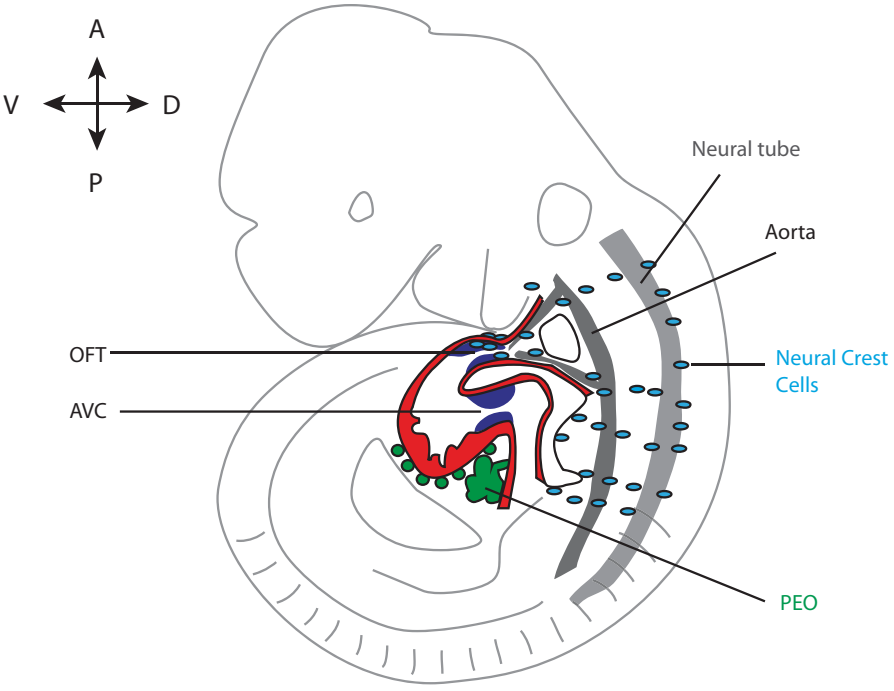

Fig. 2 Illustration of an E9.5 embryo showing the locations of the neural crest and proepicardium/early epicardial cells. Neural crest cells invade the distal parts of the OFT cushions at this stage, whereas epicardial EMT contribution to the AVC cushions/valves occurs at latter embryonic stages

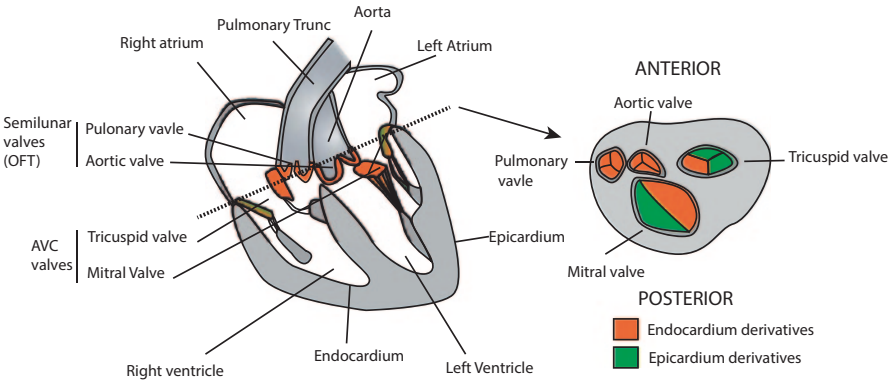

Fig. 3 Illustration summarizing the major contributions of valvulogenic lineages to the adult valve leaflets. Endocardial derivatives make the major contributions to the definitive semilunar valves and the septal and aortic leaflets of the tricuspid and mitral valves. Epicardial derivatives contribute more extensively to the mural leaflets of the tricuspid and mitral valves compared with the septal and aortic leaflets

covering the heart, the epicardium. Early studies by Mikawa [29, 30] using cell-tagging of the propepicardium in avian embryos, show that epicardial cells give rise to independent lineages of coronary smooth muscle and fibroblasts. Subsequent studies using chicken-quail chimeras showed a contribution of epicardial-derived cells (EPDCs) to the AVC cushions and valves, demonstrating that, similarly to endocardium and neural crest, the proepicardium contained valve progenitors [31]. More recent studies using genetic lineage tracing in mouse have provided further details on this contribution from epicardium to the AVC valve leaflets. Interestingly, epicardially-derived mesenchyme has been shown to invest the mural aspects of the AVC valves [32] (Fig. 3). It is probable that this preferential contribution results from the relative proximity of the mural leaflets to the epicardium.

2.4 Neural Crest

The neural crest is a heterogeneous population of cells that originates from the dorsal aspect of the neural tube. These cells arise all along the neural axis and undergo EMT, generating cells that migrate to various locations undergoing ectodermal and mesodermal fates (Fig. 2). These include neurons, glial cells, melanocytes and, at the cephalic level, mesenchymal cells [33].

The cardiac neural crest, a specific subpopulation, plays a key role in morphogenesis of the outflow region of the heart. Initially, understanding the contribution of neural crest to various structures was performed in avian embryos. In particular, neural crest contribution to outflow tract morphogenesis has been well characterized. Removal of a specific portion of the dorsal neural tube between the first and third occipital somites results in a single outflow vessel, or persistent truncus arteriosus, as

well as other defects of the pharyngeal arch arteries [34]. Fate mapping, using quail-chick chimeras, showed that neural crest cells first migrated ventrally, covering the caudal pharyngeal arch ateries, and subsequently projected into the aortic sac where they form the aorticopulmonary septum, required for the separation of pulmonary and aortic structues [34, 35]. More recently, genetic lineage tracing in mouse models has confirmed that cardiac neural crest cells first populate the aorticopulmonary septum and conotruncal cushions before septation and contribute to remodeling [36]. This study also demonstrated that very few neural crest derivatives were present in mature semilunar valves, suggesting that the requirement for this cell population is transient.

3 Valve Maturation

The mature SL, tricuspid and mitral valve cusps are complex stratified structures with three layers, each containing composed of specific extracellular matrix (Fig. 4). Notably, the ventricularis (SL)/atrialis (AV) layer is particularly rich in elastin, the intermediate spongiosa rich in proteoglycans and the fibrosa rich in collagen [4]. This constitution provides specific biomechanical properties to the different layers directly in contact with blood flow (ventricularis/atrialis) or providing support. Chordae tendinae provide extra support to the mitral and tricuspid valves, although less prominent equivalent structures also provide support to the SL valves [37].

Valvulogenesis begins in the lumen of the atrio-ventricular canal (AVC) and proximal outflow tract (OFT), where local tissue swellings, termed endocardial cushions, are formed by the accumulation of abundant extracellular matrix in between the endocardium and myocardium. The development of the various leaflets is better characterized in the AVC compared with the OFT. The mural leaflets of the tricuspid and mitral valves i.e. those associated with the ventricular free wall, are generated by the protrusion of atrioventricular myocardium [38, 39]. Myocytes, lost by apoptosis are progressively replaced by mesenchyme that initially forms at the surface. The septal and aortic leaflets of the tricuspid and mitral valves are derived from the inferior and superior AVC cushions, and this is also reportedly the case for the chordinae tendinae [39].

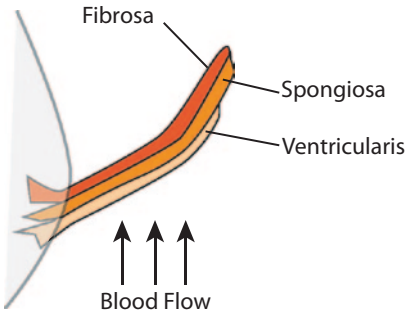

Fig. 4 Illustration depicting the three layers composing the semilunar valves. Each layer has a specific composition in terms of ECM. conferring specific biomechanical properties. The mitral and tricuspid valves are also formed of three layers, with an equivalent of the ventricularis, the atrialis, facing the atrial chambers

Epithelial to mesenchymal transition, a biological process by which a cell loses its epithelial characteristics and acquires mesenchymal markers and morphology [40, 41], is a key event in early valvulogenesis. EMT of endocardial endothelial valve prospective cells (VECs) is restricted to the cushions, and is brought on by signaling from the underlying myocardium. Key signaling pathways include BMP2 and 4, TGFβ and vascular endothelial growth factor (VEGF) [42]. TGFβs are key modulators of EMT and signaling depends on downstream smads. Notch signaling is required for cells to undergo EMT, and deficient notch signaling results in a lack of EMT [43]. Mechanical forces also play a key role in regulating cushion formation and valve maturation [8]. EMT of VECs gives rise to distinct cell lineages required for valve formation and maturation, including fibroblasts, chondrocytes and more tendinous cells [7, 45].

Other non-endocardial derived cells also contribute to cushion formation. The OFT cushions are specifically populated by mesenchymal cells originating from the neural crest [39]. As the cushions undergo remodeling, epicardium-derived cells (EPDCs) also contribute to the maturing leaflets [32]. EPDCs arise from epithelial-to-mesenchymal transition (EMT) of the epicardium, part of the protective epithelial sheet, or mesothelium, which covers the internal organs. As the valves remodel, more mesenchymal cells are recruited from the hematopoietic lineages [45]. The atrium and ventricle undergo septation in order to form the four cardiac chambers and the AVC divides into left and right ventricular inlets. The OFT separates into left and right ventricular outlets, that are connected to the aorta and pulmonary trunk, respectively. In addition, the AVC endocardial cushions develop into atrioventricular (mitral and tricuspid) valves, whereas the OFT endocardial cushions give rise to semilunar (aortic and pulmonic) valves.

4 Valvulopathies, Cardiac Fibrosis and Aortic Stenosis

Cardiac valves are affected in 30% of cardiac congenital diseases and later in life in ageing people. 2% of elderly people feature aortic valve undergoing fibrosis and further calcification. Several pathologies often associated with ageing lead to valve fibrosis and calcification. These include hypertension, diabetes, and hypercholesterolemia. The process of calcific aortic stenosis has been the focus of research for more than 60 years [46]. In calcifying valves, the cups slowly thicken and feature fibrosis with a remodeling of the extracellular matrix and ultimately calcification. The mechanical consequence of this pathological process is an increase in valve stiffness and thus a loss in elasticity, which impairs the opening/closing cycle of the valve. The severity of adverse effect can be correlated with the degree of valve calcification.

Valve fibrosis and calcification are linked to myocardial fibrosis. Indeed the loss in valve elasticity imposes an overload to the myocardium that tries to maintain cardiac output. This leads to ventricular hypertrophy as an adaptative phenomenon and then to a decompensation of the myocardium and ventricular fibrosis;

The molecular and cellular mechanisms of valve fibrosis have been also extensively investigated for a few decades. However, both the cells at the origin of fibrosis and/or calcification and the signaling pathways remain incompletely understood. This is somehow reminiscent of the lack of information as to the same processes that occur during development.

While a subset of endothelial cells that might be specifically competent for activation and subsequent osteogenesis do undergo de novo EMT [47, 48], resident valvular interstitial cells (VIC) can also be activated and transformed into myofibroblasts [49] giving rise to osteogenic cells (Fig. 5). The first steps of VIC activation involves an inflammatory response and lipid deposition as suggested by an increase in C-reactive protein in patients with aortic stenosis [50]. The inflammatory cells likely participate in the remodeling of the extracellular matrix of the valve leaflet. This process is very similar to what has been observed in atherosclerosis.

Besides this local cell activation, circulating hematopoietic cells have been proposed to contribute to some osteogenic progenitors [51]. Circulating endothelial

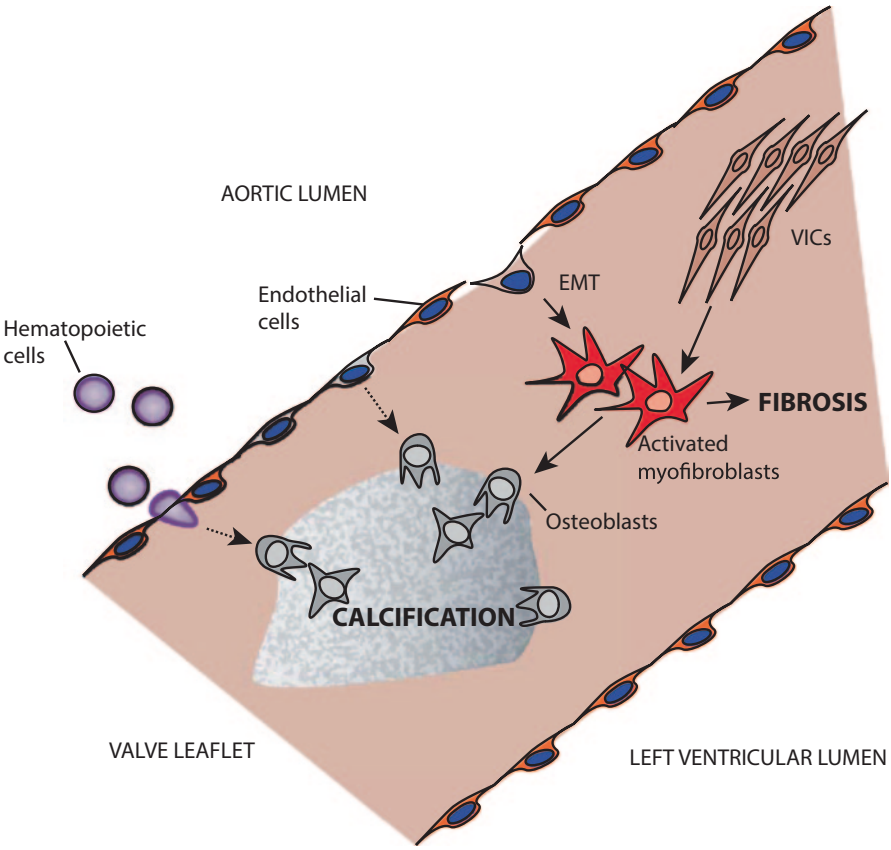

Fig. 5 Cell populations contributing to the calcification of valve leaflets

progenitor cells expressing both endothelial (CD34, KDR) and osteogenic (osteocalcin) markers have been recently found in human calcifying valve [52]. The questions that arise are that of the origin of the progenitors and the signaling pathways that stimulate them. Again the same signaling components active during embryogenesis contribute to the mobilization of progenitor cells. BMP2 and BMP4, secreted by endothelial cells under shear stress [53] and in turn the smad pathway is one of the major morphogens that induces EMT of endocardial cells and a key component of valve calcification.

TGFβ is one of the primary agonist to induce fibrosis in many pathological situations including skin wound healing, liver fibrosis, kidney fibrosis, myocardial fibrosis and an activator of smads.

However TGFβ [54] released together with TNFα and IL1β [56] by T cells infiltrating the endothelium during the inflammatory process does not play a prominent role in valvular fibrosis and calcification. *In vitro* studies have shown that VICs challenged by TGFβ undergo calcification [48, 56]. FGF2 has been shown to counteract TGFβ mediated VIC conversion into myofibroblast [57]. This TGFβ-mediated effect depends on stiffness of the substrate or of the matrix [58, 59]. TGFβ, released by endothelial cells under shear stress, acting through both its canonical signalling pathway in leukocytes and through both canonical and non-canonical signalling in aortic valves of Reversa mice (hypercholesterolemic $Ldlr^{-/-}Apob^{+/+}Mttp^{fl/fl}/Mx\text{-}1Cre^{+/+}$) fed on a western diet, promotes valve calcification. Wnt/beta catenin signaling is also activated in the process of valve calcification [60].

Wnt signaling might be specifically activated by lipid deposition; indeed intranuclear β-catenin has been observed in hypercholesterolemic mice featuring valve calcification [61]. Wnt is specifically important to drive differentiation of myofibroblasts. Finally, other pathways such as the proinflammatory pathway NFκb and the Runx2/Notch pathways both mediate the calcification process [62, 63].

5 Conclusions

During embryogenesis, the cell lineages at the origin of the cellular components of the valves are still not very well known. This lack of information can also be found in adult as to the cell types that contribute to valve fibrosis and calcification. The same signaling pathways (BMP, TGFβ, Wnt, NFκb…) promote EMT in the AVC and OFT for the formation of cardiac cushions as well as they induce de novo EMT and activate progenitor cells in the adult valve undergoing fibrosis and calcification. Thus developmental biology studies should help in a better understanding of valve disease and should pave the way towards therapeutic approaches. The absence of fibrosis and calcification of the mitral valve in contrast to the aortic valve is still questionable. This could be related to a mechanical issue, the aortic valve being submitted to greater stress than the mitral valve. Another and non-mutually exclusive hypothesis could be a different cellular participation and in turn extracellular matrix composition in both valves as it could be anticipated from different embryological

origins of cells that either early contribute or migrate into each valve. That further points to the requirement of more lineage tracing studies in the mouse embryo.

Fibrosis is a general phenomenon observed in many diseases. Aortic fibrosis and myocardial fibrosis are interrelated phenomenon. Any therapeutic approach against valve fibrosis should thus help in reducing myocardial fibrosis. The embryonic origin, the activation process and the cellular physiology and function of valvular fibroblasts are key phenomenon that require to be fully understood in order to develop anti-fibrotic and calcification therapies.

Great progress has been accomplished in the last decades to better understand valve biology and function. There is still much to investigate to get a clear understanding of valve formation and diseases.

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