Morphogenetic Aspects of Mitral Valve Development

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Introductory Remarks

The purpose of this chapter is to provide an overview explaining how genes and genetic pathways control the formation and remodelling of the mitral valve in order to link the clinical and morphological observations on the development of the mitral valve in Chap. 9, with the approaches taken by clinical geneticists expounded in Chaps. 10 and 11.

The mitral and tricuspid valves develop at the junction between the atriums and ventricles from local expansions of sub-endocardial extracellular matrix known as endocardial cushions. Similar swellings are involved in arterial valve formation and outflow tract septation. Whilst the basic underpinning mechanisms that explain how the atrioventricular valves form have been known for some time [1–3], these are less well understood for the arterial valves. It has become clear that, although there are similarities in the development processes that lead to the formation of the atrioventricular and arterial valves, at least some of the cell lineages and morphogenetic mechanisms are different [4]. Notably, for both sets of valves, key gaps in our understanding remain, particularly relating to remodelling of the valve leaflets from their endocardial cushion precursors (Fig. 9.1). Although the majority of the early processes involved in mitral valve development are thought to be very similar to that for the tricuspid valve, subtle differences in embryonic cell lineage contributions, gene expression, and exposure to different haemodynamic forces, may influence their differential maturation and the disorders they incur. In this chapter, we focus on the molecular genetic factors that influence atrioventricular valve development. We highlight reported differences between the mitral and tricuspid valves that may account for their differential development, physiology, and function. By necessity,

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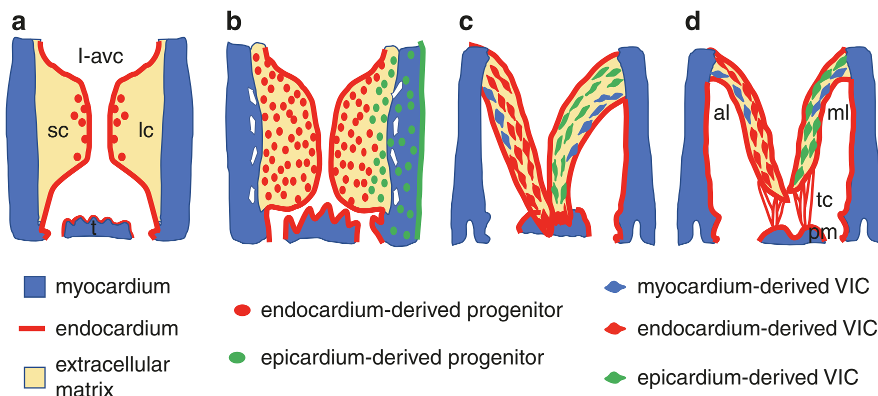

Fig. 9.1 Development of the mitral valve (**a**) At 3 weeks the endocardial cushions—the precursors of the mitral valve leaflets—are first seen in the left side of the atrioventricular canal. At this stage, they are composed of extracellular matrix and undifferentiated mesenchymal cells derived from the endocardium. The myocardium associated with the cushion tissue begins to break down. (**b**) By 4–5 weeks of gestation, these endocardial cushions have expanded and mesenchymal cells derived from the embryonic epicardium have migrated into the atrioventricular myocardium and the lateral cushion. (**c**) Between 10–13 weeks of gestation, the leaflets remodel and their tips associate with the trabeculations—this step is poorly understood. (**d**) By 15 weeks the leaflets have remodelled further and the tendinous cords have become apparent. These are in continuity with the papillary muscles that have remodelled from the trabecular myocardium of the left ventricle. Al = aortic leaflet, l-avc = left atrioventricular canal, mc = mural leaflet; pm = papillary muscle; t = trabeculae; tc = tendinous cords

the majority of this experimental information comes from experiments in the mouse, and occasionally the chicken. The close similarity in cardiovascular development between these organisms and ourselves [5, 6], nonetheless, means that as we begin to analyse human embryos, we are finding that the same genes and processes are involved in all these higher vertebrates.

Patterning of the Atrioventricular Canal

In human embryos, the heart initially forms at approximately day 18 or Carnegie stage (CS)8, equivalent to embryonic day (E) 7.5–8 in the mouse, as a simple midline tube of endocardial cells surrounded by atrial and ventricular cardiomyocytes. These cardiomyocytes originate from progenitors that originate in an area of anterior embryonic mesoderm known as the first heart field and sometimes called the cardiac crescent. Later, a closely related group of progenitor cells, known as the second heart field (SHF), add on to the forming heart tube and contribute to the atriums, right ventricle, and outflow tract (reviewed in [7–10]). As the atrial and ventricular portions of the initial primitive (or primary) heart tube expand or "balloon" to form recognisable chambers (reviewed in [11, 12]), the cells at their junction give rise to the atrioventricular canal, where the mitral and tricuspid valves will form.

The early genes expressed in the atrioventricular canal promote valve development but repress atrial and/or ventricular chamber identity. Conversely, genes expressed in the atrial and ventricular chambers enhance the development of contractile cardiomyocytes and repress atrioventricular canal identity (reviewed in [11, 12]). This early atrioventricular canal patterning occurs at around day 23 of human development (CS10–11 or E9.5–E10.5 in mouse). It is dependent on the signalling molecule bone morphogenetic protein 2 (BMP2), secreted by the myocardium (Fig. 9.2). Knock out mouse experiments show that the absence of *Bmp2* in the myocardium leads to a failure to specify the atrioventricular canal as being different from the rest of the chamber myocardium [13, 14]. Conversely, if *Bmp2* expression is artificially expanded throughout the primary heart tube, an atrioventricular canal-like phenotype is seen throughout the ventricular chamber [15]. Bmp2 activates several T-box transcription factors that bind to the regulatory regions of target genes and can either activate or repress their expression. *Tbx2* and *Tbx3* act to maintain the primitive myocardial phenotype, suppressing chamber development in both the atrioventricular region and in the developing outflow tract [16–18]. As with loss of *Bmp2*, loss of *Tbx2* results in the failure to form a distinct atrioventricular canal, with the almost complete failure of cushion, and by extension valve, formation. Another T-box family member, *Tbx20*, is strongly expressed in the forming cardiac chambers but is not expressed in the atrioventricular canal. It has opposing actions to *Tbx2*. It activates chamber-specific genes, but is also a *Tbx2* repressor

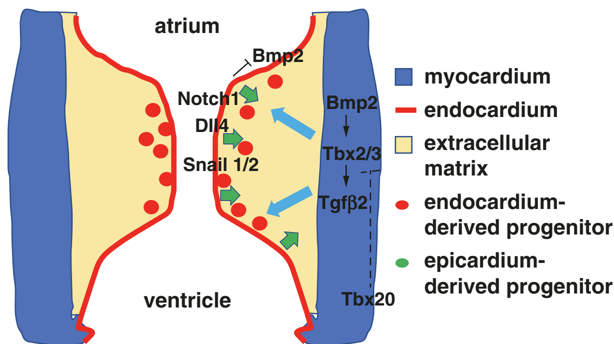

Fig. 9.2 Atrioventricular canal patterning occurs at around day 23 of human development. Bone morphogenetic protein 2 (Bmp2), secreted by the myocardium activates several T-box transcription factors that bind to the regulatory regions of target genes and can either activate or repress their expression. *Tbx2* and *Tbx3* act to maintain the primitive myocardial phenotype and suppress chamber development in both the atrioventricular region and in the developing outflow tract. *Tbx20* is strongly expressed in the forming cardiac chambers but is not expressed in the atrioventricular canal. It has opposing actions to *Tbx2* and activates chamber-specific genes but is also a *Tbx2* repressor, repressing any tendency to develop atrioventricular canal identity. The Notch pathway (including Dll4) plays an essential role in confining expression of *Bmp2* to the myocardium of this region. This activates Snail 1/2, which are required for the transformation of the endocardial cells into mesenchyme that populates the cushion. Genetic disruptions that completely inactivate these genes will either result in a failure to properly pattern the atrioventricular canal or to form the chambers and lead to early embryonic death

[19–22], inhibiting any tendency to develop atrioventricular canal identity. Thus, an adversarial signalling network involving *Bmp2* and *Tbx2/3* is required to specify the atrioventricular myocardium [13, 16], which is restricted from extending into the chambers by *Tbx20*.

The Notch pathway provides direct signalling between endothelial and myocardial cells and is active in the atrioventricular canal. It has comprehensively been shown that its expression in the endocardium of the atrioventricular canal plays an essential role in confining expression of *Bmp2* to the myocardium of this region [23, 24]. Abnormal activation of Notch1 signalling throughout the entire endocardium of the heart produces a similar effect to ectopic *Bmp2* expression. In contrast, expression of activated Notch1 in the atrioventricular canal myocardium represses *Bmp2* and *Tbx2* and results in failure to form the atrioventricular endocardial cushions [24, 25]. Any genetic disruptions that completely block these genes will either result in a failure to properly pattern the atrioventricular canal or to form the chambers and lead to early embryonic death. If subtly disrupted, however, these genes may still have relevance to mitral valve malformations and disease. For example Follistatin-like 1 (Fstl1) is a secreted glycoprotein that regulates *Bmp2* and *Tgfβ1* (reviewed in [26]). Knock out of this gene in the mouse endocardium resulted in the persistence of *Tgfβ1* and *Bmp2* expression in neonatal valves. With ongoing endothelial-to-mesenchymal transformation, the valves become enlarged and myxomatous, leading to severe mitral regurgitation and death between 2–4 weeks after birth [27]. Importantly, the tricuspid valves were spared.

Extracellular Matrix

The heart tube has a layer of the extracellular matrix, known as cardiac jelly, which separates the inner endocardial layer from the outer myocardial layer. The next stage in the development of the mitral valve is the appearance of localised acellular expansions of this cardiac jelly within the atrioventricular canal. These are the endocardial cushions. Even at this early stage, they act as valves to prevent retrograde blood flow as the ventricles contract. Initially, superior and inferior atrioventricular cushions are seen, which separate the atrium from the inlet component of the ventricular loop. A pair of smaller "lateral" cushions, precursors of the atrioventricular mural leaflets, form subsequent to the development of the right ventricle and expansion of the atrioventricular canal [5, 28]. The extracellular matrix in the atrioventricular cushions is mainly composed of hydrophilic proteoglycan molecules, the most abundant of which are versican and hyaluronan. Mice lacking versican [29] or hyaluronan synthase-2 (Has2), the enzyme required for the production of hyaluronan [30], die shortly after heart looping. This is almost certainly because it is not possible to achieve sufficient cardiac output without adequate endocardial cushion bulk to prevent retrograde flow within the heart (reviewed in [31]). In addition to a mechanical role, hyaluronan and versican, together with a number of other molecules (such as cartilage link protein; [32]) form a scaffold that modulates cell signalling processes. They control the activity of other extracellular matrix molecules by

sequestering latent forms or cleaving them to create activate ones. For example hyaluronan can act together with the epidermal growth factor (EGF) family of growth factor receptors, ErbB2/B3, to stimulate Ras-dependent intracellular signalling. This signalling is required for the formation of the cushion mesenchyme and thus the development of the endocardial cushions [30, 33]. Enzymes that control the breakdown of this extracellular matrix can have profound effects on development, but also on ongoing valve homeostasis. For example ADAMTS5 is a metalloprotease produced by the developing valve endocardium that cleaves aggrecan and versican. *Adamts 5*-knock out mice have enlarged heart valves during the latter stages of foetal life, which manifests as myxomatous valve disease in adult animals. These phenotypes appear to relate to reduced versican cleavage within the developing and mature valve leaflets [34]. In human genetic studies, mutations in *ADAMTS5* have been linked with a range of valve problems, including bicuspid aortic valve [35]. Proteoglycan accumulation is a hallmark of human myxomatous valve disease. Extrapolating from the animal studies, it is possible that ineffective aggrecan/versican cleavage during valve development might underlie later myxomatous disease in adulthood, suggesting that this disease of ageing could have origins in foetal life.

Endocardial-to-Mesenchymal Transformation

The entry of cells into the atrioventricular cushions is required for their development into valve primordiums. The main source of cells is the layer of endocardial cells lining the atrioventricular canal. To invade the cushions, these cells lose attachments with surrounding endocardial cells. This means they change from an epithelial to a mesenchymal phenotype, and hence are able to migrate into the cushions. This important role for endocardial-to-mesenchymal transformation (EndMT) in cushion formation was first described in the last century [36]. Compared to other aspects of mitral valve development, it is relatively well understood [37, 38]. Notably, a very similar genetic cascade is activated in cancer, which is responsible for local tumour invasion and metastasis. As with those molecules that are involved in initial patterning of the atrioventricular canal, marked deficiency or loss of the molecules that control EndMT frequently result in failure to form the endocardial cushions and early embryonic death.

The crucial signals for initiating EndMT are Bmp2 and transforming growth factor-beta 2 (TGFβ2), secreted by the atrioventricular myocardium (reviewed in [39, 40]). Notch1 signalling in the endocardium, via its receptor Delta-like 4 (Dll4; [41, 42]), works together with Bmp2 to regulate *Snail* and *Slug* transcription factors. It is *Snail* and *Slug* (*Snail* 1 and 2) that down-regulate the expression of vascular endothelial cadherin (VE-cadherin), an adhesion molecule that maintains intercellular junctions in endothelial tissue and allows cells to escape the endocardial monolayer [41, 43–45]. It also upregulates the expression of genes required for cell migration and invasiveness (reviewed in [46]). The ingress of cells by EndMT must be controlled. Thus, whilst vascular endothelial growth factor (VEGF) is important in initial induction of EndMT, it facilitates replication of endothelial cells and then

controls its resolution [47, 48]. VEGF does this by activating *Nfatc1* (another transcription factor) to limit the extent of EndMT [49]. *Nfatc1* maintains proliferation of the endocardium during EndMT and valve sculpting, but also suppresses the expression of the *Snail* transcription factors [50]. The extent of signalling crosstalk between these various pathways is poorly characterised. This remains an important unexplored aspect of the regulation of both arterial and atrioventricular valve development. Low-level EndMT is also thought to be essential for maintaining valve integrity throughout the life. Evidence of such activity can be found in 1–2% of valve endocardial cells in healthy adults [51]. These cells may be required to renew the valve interstitial cell population and thus replenish the extracellular matrix that is needed in mature valves for their durability. Hence subtle defects in the regulation of EndMT may be relevant to human mitral valve disease. Maladaptive EndMT has been shown to occur subsequent to myocardial infarction, in conjunction with thickening of the mitral valve leaflets [52].

Sources of Mitral Valve Interstitial Cells

A number of different cell lineages clarified using Cre-driven enhancer mouse lines (Fig. 9.3; reviewed in [53]), have been shown to contribute to the developing atrioventricular valves.

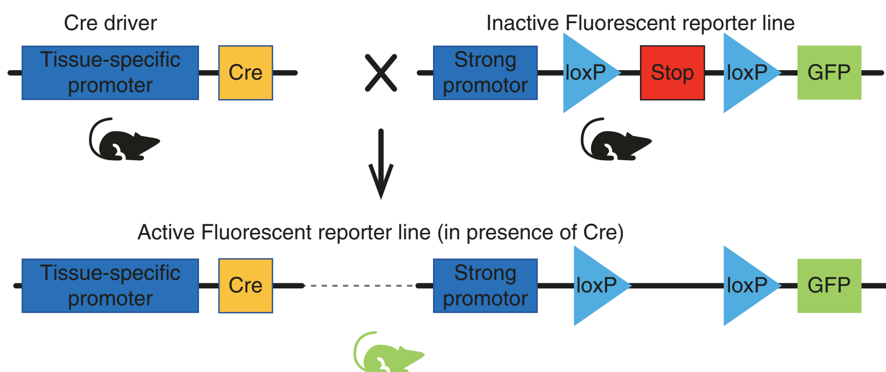

Fig. 9.3 Cell lineage tracing using Cre-lox mice. Distinct cell lineages can be tracked using genetically modified mouse strains. One line of mice is created in which the enzyme Cre recombinase is linked to a tissue-specific gene promoter. This results in the expression of Cre only in the tissue of choice. In a second mouse line, a gene that encodes a fluorescent marker, for example green fluorescent protein (GFP) is placed downstream of strong promoter. The presence of a Stop sequence, flanked by loxP sites (the recognition sites for Cre recombinase), prevents the GFP from being expressed. When these two lines of mice are crossed together, Cre recombinase is expressed only in the cells/tissue where the tissue-specific promoter is expressed. This results in recombination of DNA at the loxP sites and removal of the Stop sequence. This allows the strong promoter to drive expression of GFP only in the cells (and their progeny from later divisions) where the tissue-specific promoter is expressed

Endocardial Cells

Endocardium, the endothelium of the heart, originates from at least two sources. The majority of the endocardium found in the atrioventricular region, which undergoes EndMT to enter the valve interstitium [54, 55], derives from the first heart field. A small population, in both the endocardial and interstitial components, comes from the second heart field [SHF; [56]]. It is not known if these different origins are of biological importance. A particularly interesting subset of cells are restricted to the cushion/valve endocardium and are labelled by the *Nfatc1en-Cre* driver [50]. The labelled endocardial cells do not undergo EndMT to enter the valve. Although it remains uncertain, it has been suggested that this population is essential for maintaining the integrity of the valve endocardium itself.

Second Heart Field-Derived Mesenchyme

As development proceeds, the atrial and ventricular septal structures fuse with the atrioventricular cushions at the crux of the heart. An additional second heart field-derived structure, the vestibular spine or dorsal mesenchymal protrusion), fuses with the atrioventricular cushions to bring about atrial and atrioventricular septation [57, 58]. Deficiency of the spine, brought about by disruption of the SHF, is associated with atrioventricular septal defects. A subset of atrioventricular septal defects exhibit abnormal valve development, the most severe of which have a solitary atrioventricular valve orifice with bridging leaflets. The development of this abnormality is a current topic of research, as it remains unclear how much of the valve abnormality is the result of a shared requirement of SHF cells for the development of the vestibular spine and the mitral valve, and how much is a secondary consequence of the atrioventricular septal defect.

Epicardial-Derived Cells

The heart is covered with a layer of cells (the epicardium) that mainly originate from the region of the developing diaphragm (reviewed in [59, 60]). These cells attach to the heart surface and invade, being at this stage known as epicardial or epicardium-derived cells, to provide most of the cardiac fibroblasts, along with the smooth muscle cells of the coronary arteries. In the mouse, the epicardium and epicardial-derived cells can be specifically labelled by *Wt1*^{ERT2}*-Cre* or by *Tbx18-Cre* drivers. Studies have shown that in the mural leaflet of the mitral valve, which is derived from the lateral cushions, epicardially derived cells give rise to the majority of the valve interstitial cells, replacing the original cells that entered by EndMT [61–63]. In contrast, epicardially derived cells make only a minor contribution to the aortic leaflet of the mitral valve. The biological relevance of this difference, if any, remains unknown.

Neural Crest Cells

These multipotent progenitor cells migrate from the neural tube early in development. They are important in forming the peripheral nervous system, pigment cells, and many of the bones in the head. They are also essential for septation of the outflow of the heart, making a major contribution to the outflow tract cushions and valves [64]. They make only a minor contribution, however, to the cells populating the atrioventricular valves [65, 66]. A specific role in the mitral valve remains unclear, although their persistence in adult valves suggests that they may have one.

Bone Marrow-Derived Cells

Studies in humans after bone marrow transplantation surprisingly revealed that cells of haematopoietic lineage appear in adult heart valves [67]. Recently this has been investigated in mice [68]. Lineage tracing has shown that, whereas at birth only ~5% of atrioventricular valve cells are of the leukocyte lineage, this rises to close to 20% within the mitral valve by two months of age [69]. Moreover, their gene expression profile changes considerably during the postnatal period [70]. The specific function of these bone marrow-derived cells remains unclear, but they appear to be macrophages and dendritic cells rather than endocardial cells or valve interstitial cells. Recently, it has been shown that they play critical roles in valve remodelling [71]. Importantly, these bone marrow-derived cells are increased in myxomatous mitral valves in both mice and humans [72, 73]. Very little is known about their function either during development, normal homeostasis, or in pathological situations. It is clearly important to evaluate whether any of these specific progenitor populations have specific roles in health and disease.

Mitral Valve Growth and Maturation

Once the endocardial cushions have formed, and been populated by interstitial cells, they undergo growth and remodelling in order to acquire the structure of mature valve leaflets. The gross morphological changes that occur as the valve develops are described in Chap. 8. Maturation and thinning of the endocardial cushions to form valve leaflets is characterised by both downregulation of cell proliferation and transition from an undifferentiated mesenchymal phenotype to differentiated valve interstitial cells (VIC). Higher levels of cell proliferation may persist at the distal ends of the valve primordiums. The active growth in these regions may be important to form sculpted leaflets [54]. Growth and fusion of the superior and inferior atrioventricular endocardial cushions are driven by proliferation of newly formed mesenchymal cells in response to signals from the endocardium [74]. Although the processes coordinating cushion growth and fusion, as opposed to early cushion formation, are not well understood, studies in mice have shown that transcription

factors including *Sox9, Twist1,* and *Tbx20* [75–77] are active in this process. They drive the high levels of cell proliferation found in the remodelling valves. Deletion of these factors in the mouse embryo results in failure to form proper valve primordiums. Deletion of EGFR and HB-EGF in mice results in enlargement of the atrioventricular cushions due to excessive proliferation in cushion mesenchyme [78], suggesting that HB-EGF-EGFR signalling is also required to modulate mesenchymal proliferation [79, 80]. Jagged1 signalling via Notch1 is also thought to limit the extent of mesenchymal proliferation in the endocardial cushions by positively regulating HB-EGF/EGFR [42].

Other pathways have been implicated in valve remodelling because of human syndromes associated with mitral valve malformation. The BMP and/TGFβ signalling pathway, as previously discussed, seems to be particularly important in growth and remodelling of the leaflets. This pathway has been shown to be abnormal in both Loeys-Dietz and Marfan syndromes. Mice lacking the Bmp-specific inhibitor Smad6 or the BMP antagonist Noggin [81, 82], have enlarged valve leaflets associated with increased proliferation. In contrast, loss-of-function models for BMP and TGF family members and their receptors have reduced cell proliferation in the atrioventricular cushions and hypoplastic valves (reviewed in [40]). Whilst mutations in *TGFBR2* and *SMAD3* are directly implicated in Loeys-Dietz syndrome (reviewed in [83]), the fibrillin (*FBN1*) mutations causing Marfan syndrome may indirectly cause mitral valve prolapse. Fibrillin is a large structural protein that contributes to the functional integrity of connective tissue and normally sequesters latent TGFβ binding proteins. Thus, *FBN1* mutations may interfere with this regulation of TGFβ signalling. Ras signalling has also been implicated in regulating cushion mesenchyme. Mitral valve prolapse is a common feature in Noonan's syndrome, which is caused by gain-of-function mutations of *PTPN11*. Conversely, loss-of-function mutations in Ras-pathway components such as *Nf1* (the gene mutated in Neurofibromatosis type I), result in hypercellular valves, suggesting that the Ras signalling pathway negatively regulates cushion mesenchyme proliferation (reviewed by [33]).

As in earlier stages of development, therefore, a number of molecules are implicated in regulating the proliferation of cells in the atrioventricular cushions and are essential for their normal development. As with EndMT, the interplay between these genes and gene products is underexplored. It is likely to be important for understanding how mitral and tricuspid valves develop and respond to postnatal insults, stresses, and ageing.

Release of the Leaflets and Formation of the Tension Apparatus

This is the area of mitral valve formation that is probably least well understood. After the period of endocardial cushion expansion, the primordium of the mural leaflet has to remodel in order to be freed from the ventricular wall. The fused major cushions must also remodel to achieve their mature flexible forms. Whilst the lateral cushion is initially adhered to the myocardium, this changes over time (between the

10th and 13th week of human development) to leave a free, mobile leaflet attached to the ventricular wall by tendinous cords and papillary muscles [84–86]. At least in the mouse heart, it has been suggested that this process involves programmed cell death in the region between the myocardial wall and the cushion, which creates space between them, freeing the developing leaflet from the myocardium [55]. It is clear that the papillary muscles are myocardial in origin [55] and form by compaction of the pre-existing trabeculations which form from the innermost layer of the myocardium [87]. The processes by which the tendinous cords create the union between the papillary muscles and the leaflets currently remains unknown (see Chap. 4).

Extracellular Matrix (ECM) and Valve Remodelling

The cells present within the matrix are important for the regulation of ECM biosynthesis and its turnover within the valve leaflets. Indeed, the ECM of the remodelling valves is very similar to that found in developing cartilage and bone (reviewed in [88]). It shares many of the same transcriptional regulators, such as *Sox9* and *Scleraxis*, and modulating proteins, for example ADAMTS5. These genes play slightly different roles than at earlier stages. For example whilst *Sox9*, a master regulator of cushion mesenchyme and cartilage formation, is necessary for early interstitial cell proliferation, later it promotes the expression of cartilage matrix proteins such as aggrecan in the remodelling leaflets [54, 75]. Similarly, NFATc1/Calcineurin signalling is important for the transition from growth to remodelling in the cushion/valve endocardium [49, 89] and regulates expression of RANKL and Cathepsin K. These proteins are required for transcriptional activation of bone matrix remodelling enzymes during osteoclast differentiation in the bone. They presumably play a similar role in the highly similar ECM of the developing valves [90]. Periostin promotes the differentiation of both endothelial- and epicardial-derived mesenchyme while blocking the emergence of other cell types, especially cardiomyocytes. It is also required for fibrous maturation of the atrioventricular leaflets and their supporting apparatus [91, 92]. The transcriptional regulator, *Scleraxis*, appears to be particularly important for regulating ECM in the remodelling valves [93]. It is first expressed in the atrioventricular endocardial cushions just prior to remodelling. It becomes more widely expressed as remodelling progresses, and is also retained in the adult valves [54]. *Scleraxis* is specifically expressed in the developing tendinous cords of the valve leaflets in the chicken embryo, although this does not seem to be the case in the mammalian heart [54, 93]. *Scleraxis* regulates the expression of a number of ECM molecules found in the remodelling atrioventricular valve leaflets and its loss in mice results in thickened atrioventricular valve leaflets. Abnormal ECM deposition is characterised by an increase in cartilage-associated proteins such as Sox9, cartilage oligo matrix protein, and cartilage link protein, and a downregulation of tendon-associated proteins such as Collagen XIV [93]. *SCHLERAXIS* is upregulated in human myxomatous mitral valve leaflets, suggesting that these pathways are clinically relevant.

In diseased valves, there is aberrant recruitment of endocardial and interstitial cells, along with the transition of a subset of interstitial cells into myofibrobasts expressing alpha-smooth muscle actin. These cells, together with the expression of matrix metalloproteases and proinflammatory cytokines, result in a degraded and disarrayed matrix in the leaflets and tendinous cords, which can become calcified (recently reviewed in [94, 95]). These structural changes are associated with the aberrant re-expression of early valve mesenchymal and chondrogenic progenitor markers. They have been related to the reactivation of foetal transcriptional programmes [96–99]. These data may go some way to explain why cardiac valves appear to be predisposed to abnormal accumulation of ECM proteins associated with myxomatous degeneration and calcification. Indeed, matrix Gla protein, encoded by the *MGP1* gene, is downregulated in developing bone in order to allow calcification but is maintained in developing heart valves [100]. Loss of *MGP1* in mice results in calcification of the heart valves in the neonatal period, perhaps showing that prevention of calcification of the maturing leaflets is an active process.

The Lamellar Mitral Valve Structure

In addition to the deposition of cells and ECM proteins, the emergence of a lamellar structure is seen in human valves before birth [101], and probably begins in the second trimester [102]. In the mouse heart, lamination starts between E15.5 and E18.5 (reviewed in [103]), but in both cases, there is still considerable remodelling after birth. Extracellular proteins are generally secreted by the valve interstitial cells. Turnover of these molecules continues throughout life. The relative thicknesses of the layers vary between the leaflets of the mitral valve. They also vary within each leaflet from their hinges to the free edge [104].

During the stratification process, collagen fibrils (mainly types I and III) become circumferentially oriented and densely packed at the ventricular side of the leaflet to form the fibrous layer that provides tensile strength to the leaflet [105, 106]. Notably, the valve interstitial cells are connected to the extracellular matrix, including the collagen, via integrin receptors on their surfaces. Disruption of these interactions can result in valve calcification, although it remains unclear whether this results from direct anti-calcification roles for integrins or is secondary to valve-extracellular matrix interactions (reviewed in [107]). Filamin A is a non-muscle actin-binding protein that organises filamentous actin into orthogonal networks and stress fibres. It anchors membrane proteins to the actin cytoskeleton, and provides a scaffold for cytoplasmic and nuclear signalling proteins. Mutations leading to a dysfunctional FLNA protein have been identified in myxomatous valvar dystrophy [108]. They may affect signalling pathways that modulate cellular migration and mechanical stress responses during development [95].

Proteoglycans, particularly hyaluronan, versican, biglycan, and decorin, are the main components of the middle spongy layer of the leaflet. The distribution of these molecules differs in regions exposed to different stresses. Hyaluronan and versican are abundant in the compressive regions of the leaflets. Biglycan, in contrast, is most

abundant in the centre of the aortic leaflet, which is a tensile region. It is less abundant in the tendinous cords, also tensile, and the free edge of the aortic leaflet, which is compressive. It is least abundant in the compressive mural leaflet [109]. The atrial side of the mitral valve leaflets is largely made up of elastin. Different compositions of glyocosaminoglycan (GAG) side chains attached to the core protein molecules are also found in different parts of the valve and its tension apparatus, correlating again with compressive and tensile regions. Notably, the relative amounts of these molecules change as the valves age [109, 110]. Dysregulation and imbalance of the ECM components appear to be a general feature of valve disease regardless of aetiology. For example myxomatous disease is characterised by loose collagen, increased proteoglycan, and reduced elastin content with altered fibre orientation in all valve layers (reviewed in [95]).

Both gene expression and cell differentiation are involved in establishing the laminar structure of the valve tissue. For example Wnt/β-catenin signalling primes the cushion mesenchyme to respond to patterning cues that promote the proteoglycan-rich spongiosa layer and restrain the boundaries between the tendinous cords and the leaflets [111]. As well as genetic regulation of the stratification process, it has also been suggested that mechanical stimuluses are important for the alignment of collagen fibres in the developing and maturing leaflets [112]. The patterning of the extracellular components of the leaflets align with blood flow, which may suggest that hemodynamic forces acting via the valve endocardium are driving the process.

Cilia and Cell Polarity in the Mitral Valve

Recently, there has been a major interest in the role of cilia in valve development and maintenance, largely because of the association between mutations in cilia-associated genes and valve defects, including mitral valve prolapse. This has come about because of the identification of several cilia-related genes, including Daschous1 (*DCHS1*), Desert hedgehog (*DHH*), and *DZIP1* [113–115] in familial cases of mitral valve prolapse. Primary cilia are small projections of membrane with a microtubule core that are implicated in cell signalling and mechano-sensation. There is considerable evidence to show that the presence of cilia is temporally and spatially regulated during valve formation and that they are maintained on the surface of the valve interstitial cells, although not on the endocardium, during adult life. This absence from the valve endocardium suggests that their role is unlikely to be related to the detection of shear stress. *DCHS1* is a component of a cellular signalling pathway that regulates cell polarity and migration. *DCHS1* deficiency in patient cells, and in cells carrying one mutated allele of the *Dchs1* gene in mice, was sufficient to result in altered migration and cellular arrangement of valve interstitial cells [113]. These studies suggest that interstitial cell organisation, and the pathways that regulate this process, are critical determinants of valve development and that disruption may result in disease [95]. Although the precise and potentially multiple roles of cilia in valve development and maintenance remain unclear, they have been implicated in modulating the extracellular matrix, restraining calcification and

responding to inflammatory signals. Mouse models of *Dzip1* have suggested that the mitral valve prolapse seen in adult mice is a result of developmental defects, apparent from mid-embryogenesis, that result in abnormalities is extracellular matrix production and dysmorphic valve leaflets [114]. Whilst several families have been described with mutations in these genes, they currently explain only a small proportion of cases of mitral valve prolapse, with the causes of the majority of sporadic or familial forms of mitral prolapse remaining unclear.

Conclusions and Perspectives

Despite many important advances to our understanding of the aetiology of valve disease, treatment still relies primarily on surgical intervention. There are currently no available curative or palliative medicines. Any future opportunities for therapeutic intervention will require a better knowledge of the mechanisms leading to congenital malformation of the atrioventricular valves that predispose to adult disease. Developmental transitions from proliferation and expansion of the endocardial cells, to remodelling and elongation of the valves leaflets and supporting tension apparatus, likely involve extensive crosstalk between canonical developmental pathways, for example BMP/TGFβ, Wnt, Notch, and mechanotransduction pathways elicited by blood flow, that remain relatively poorly understood. The generation of novel models using conditional null-mice and delineation of the individual contributing valve cell types will help unravel the mechanisms involved in the post-EMT development of the AV valves. Exome sequencing and studies of structural variation in predisposed individuals may also be important for future pharmacological strategies aimed at maintaining physiological heart function to slow, and maybe eventually prevent, disease.

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