Valvular heart disease occurs as either a congenital or acquired condition and advances in medical care have resulted in valve disease becoming increasingly prevalent. Unfortunately, treatments remain inadequate because of our limited understanding of the genetic and molecular etiology of diseases affecting the heart valves. Therefore, surgical repair or replacement remains the most effective option, which comes with additional complications and no guarantee of life-long success. Over the past decade, there have been significant advances in our understanding of cardiac valve development and, not surprisingly, mutations in these developmental genes have been identified in humans with congenital valve malformations. Concurrently, there has been a greater realization that acquired valve disease is not simply a degenerative process. Molecular investigation of acquired valve disease has identified that numerous signaling pathways critical for normal valve development are re-expressed in diseased valves. This review will discuss recent advances in our understanding of the development of the heart valves, as well as the implications of these findings on the genetics of congenital and acquired valvular heart disease. (Circ J 2014; 78: 1801–1807)

Key Words: Aortic valve calcification; Bicuspid aortic valve; Heart valve development; Mitral valve prolapse; Valvular heart disease

Valvular heart disease (VHD), which includes both congenital and acquired forms, is an important and growing public health problem. Based on epidemiologic studies in the United States, it has an overall prevalence of 2.5%, and the incidence increases with age. With the projected increases in the elderly population worldwide, acquired forms of VHD are likely to rise in prevalence in all industrialized countries. Similarly, advances in the care of infants born with congenital heart defects (CHD) will add to the growing VHD population as congenital valve abnormalities are identified in over 50% of cases of CHD. Despite the prevalence, pharmacologic therapies for VHD are limited, and progressive valve dysfunction often requires surgical repair or replacement as the primary treatment. In the United States, the annual direct cost for VHD is estimated at $1 billion and the need to develop novel therapeutic strategies is becoming increasingly imperative.

Essential to developing new non-invasive therapies for VHD is the elucidation of the primary etiologic contributors to disease development and progression. Congenital valve malformations are primarily the result of perturbation of the genes that regulate normal heart valve development. For acquired valve disease, exposure to non-genetic risk factors, such as hypercholesterolemia, hypertension, tobacco use, and rheumatic heart disease, are proposed as primary disease contributors. Although the mechanisms of congenital and acquired valve disease are not fully understood, the pathogenesis is thought to stem from the interplay of genetic and environmental influences. However, there is increasing evidence to suggest that genes critical for normal valve development may play roles in the development of acquired VHD.

Development and maintenance of the cardiac valves is a complex process and requires formation of intricate valve structures that must open and close over 100,000 times each day to maintain unidirectional blood flow through the heart. During embryogenesis, endocardial cushions (EC) are the primordia of mature valve leaflets and are composed of a highly organized extracellular matrix (ECM) consisting of 3 distinct layers made of collagen, proteoglycans and elastin that together provide all the necessary biomechanical properties to withstand constant changes in the hemodynamic environment. Turnover of the valvular ECM is tightly regulated by valve interstitial cells (VICs) that are largely quiescent and fibroblast-like in the absence of disease. Overlying valve endothelial cells (VECs) form an uninterrupted protective endothelium over the surface of the leaflets and molecularly communicate with underlying VICs to regulate their behavior in response to changes in the hemodynamic environment (Figure 1). In contrast to healthy valves, disruption of normal valve development or acquired valve dis-
Valve Structure

The mature heart valves, consisting of the atroioventricular (AV) (mitral and tricuspid) and semilunar (SL) (aortic and pulmonic),

Table. Mouse Models* of EC Defects Associated With Gene Targeting of Tgfβ, Wnt, Notch and VEGF Signaling

<table>
<thead>
<tr>
<th>Family/Gene</th>
<th>Phenotype in mice</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Tgfβ2</td>
<td>Tgfβ2−/−: hypoplastic ECs</td>
<td>Kruithof et al, 2012</td>
</tr>
<tr>
<td>TgfβRI</td>
<td>TgfβRI−/−: hypoplastic ECs because of impaired EMT</td>
<td>Kruithof et al, 2012</td>
</tr>
<tr>
<td>Snai1</td>
<td>Endocardial Tie2cre;Snai1+/−: EMT initiated, but reduced mesenchymal cell proliferation</td>
<td>Tao et al, 2012</td>
</tr>
<tr>
<td>Snai2</td>
<td>Snai2−/−: hypoplastic ECs because of impaired EMT</td>
<td>Niessen et al, 2008</td>
</tr>
<tr>
<td>Bmp2</td>
<td>Myocardial deletion; reduced cardiac jelly and impaired EMT initiation</td>
<td>Kruithof et al, 2012</td>
</tr>
<tr>
<td>BMPRI</td>
<td>Endocardial deletion; hypoplastic ECs because of impaired EMT</td>
<td>Kruithof et al, 2012</td>
</tr>
<tr>
<td>Notch</td>
<td>RBPJk−/−: hypoplastic ECs because of impaired EMT</td>
<td>Reviewed de la Pompa and Epstein, 2012</td>
</tr>
<tr>
<td>Notch1</td>
<td>Notch1−/−: hypoplastic ECs because of impaired EMT</td>
<td>Reviewed de la Pompa and Epstein, 2012</td>
</tr>
<tr>
<td>MAML</td>
<td>Isl1-cre; dom neg MAML: hypoplasia of OFT ECs</td>
<td>Reviewed de la Pompa and Epstein, 2012</td>
</tr>
<tr>
<td>Vegf</td>
<td>Myocardial transgenic; EMT inhibited (50% penetrance)</td>
<td>Dor et al, 2001</td>
</tr>
</tbody>
</table>

*Only relevant review articles are cited.

EC, endocardial cushion; EMT, endothelial-to-mesenchymal transformation; Tgfβ, transforming growth factorβ; Vegf, vascular endothelial growth factor A.
are primarily composed of an outer layer of VECs that surround 3 stratified layers of specialized ECM, interspersed with differentiated VICs. Each ECM layer is organized according to blood flow and they work together to withstand the continual hemodynamic changes of the cardiac cycle. The fibrosa layer, which is predominantly composed of collagen, provides tensile strength to the valve leaflet during opening, while transmitting forces to promote leaflet coaptation when closed (Figure 1). Adjacent to the fibrosa is the spongiosa layer, with a lower collagen abundance and higher proteoglycan prevalence. This composition creates a more compressible matrix, allowing the valve to “flex” and absorb high force. The layer adjacent to blood flow is the atrialis or ventricularis in the AV or SL valves, respectively. It largely consists of elastin fibers that facilitate extension of the leaflets during closure. This is further supported by the fibrous annulus structure between the leaflets/cusps and myocardium and external chordae-tendineae in the AV position. The VIC and VEC populations also play essential roles in maintaining connective tissue homeostasis in the valve leaflets/cusps. Although originally thought to be a homogeneous population of fibroblast-like cells, VICs are now considered highly heterogeneous with quiescent, activated and progenitor-like phenotypes reported in development and disease. In addition to VICs, VECs are also highly responsive to changes in the hemodynamic environment and molecularly communicate with underlying VICs to regulate their behavior. As normal valve function is dependent on the complex arrangement of connective tissue and overall valve morphology, it is not surprising that alterations in localization and/or contribution of matrix components leads to functional VHD.

**Valve Development and Remodeling**

**EC Formation**

The primitive beating heart tube consists of an outer layer of myocardial cells and an inner layer of endothelial cells, separated by cardiac jelly. Following rightward looping, a subset of endothelial cells localized within the AV canal and outflow tract (OFT) regions secrete a hyaluronan-rich ECM to form “swellings” known as ECs. In response to signals emanating mostly from the adjacent myocardium, endothelial cells overlying the cushions lose cell-cell contact, transform into mesenchymal cells, and migrate into the underlying cardiac jelly and proliferate. This process of endothelial-to-mesenchymal transformation (EMT) is essential for generating the pool of precursor cells that will give rise to the mature valves. Complex networks of signaling pathways tightly regulate each step of EMT. Mice with EC defects caused by aberrations in EMT usually die at approximately E10.5, highlighting the requirement of EMT for valvulogenesis. Furthermore, gene-targeting studies have been insightful in identifying the critical regulators of EMT, which include secreted growth factors, transcription factors and signaling molecules (discussed later; shown in the Table).

**Contribution of Non-Endothelial Cell Lineages to Valve Structures**

Formation of ECs requires the contribution of several cell lineages from multiple sources. Early cell lineage studies using Tie2Cre;Rosa26R mice that allowed for marking of endocardial cells and their progenitors suggested that the majority of mesenchymal cells within the AV canal cushions are derived from endocardial cells following EMT. Recent studies have shown that migrating epicardial cells also contribute to the paretial leaflets of the AV valves. In the OFT valves, there appears to be little epicardial contribution but lineage tracing studies using Wnt1-Cre suggest that neural crest-derived cells contribute to the OFT valves and aortopulmonary septum. The valve mesenchymal cell population is derived from multiple sources, and collectively, each may play essential roles throughout valvulogenesis.

**Regulators of EMT**

The number of signaling pathways known to contribute to EC formation is expansive. This review will focus on the most common, including signaling molecules transforming growth factor-β (Tgfβ), Bmp, Wnt, Notch and vascular endothelial growth factor A (Vegf), because they have been linked to human VHD (Table). Tgfβs and their downstream signaling mediators are derived from the myocardium and regulate several EMT-related processes. During avian EC formation, Tgfβ2 and Tgfβ3, via TgfβRII, are required for VECs to initiate EMT and promote migration of newly transformed cells. Mice with deletion of Tgfβ, latent Tgfβ binding protein, or endocardial deletion of TgfβRII (Alk5) develop valve abnormalities including hypoplastic ECs. Tgfβ-mediated activation of the downstream effectors, phosphorylated (p)Smad2/3, is sufficient to induce expression of the transcription factors Snai1 (Snai1) and Snai2 (Slug). Studies have shown that Snai1 and Snai2 are essential for EMT in vivo, and mice with null alleles develop hypoplastic ECs, similar to Tgfβ mutants. That series of studies indicated that Tgfβ receptors, ligands and downstream effectors are important for several of the biological processes required for EC formation, including EMT initiation, breakdown of cell-cell contact, mesenchymal cell motility and proliferation.

As members of the TGF super-family, BMP signaling through canonical SMADs (pSmad1/5/8) also plays a critical role in EMT formation. Numerous studies in primary cells and animal models show that BMPs similar to Tgfβs are a major source of myocardial-derived signals for EMT initiation. Among BMP family members, Bmp2 and 4 are, respectively, most predominantly expressed in the AV and OFT myocardium adjacent to developing ECs. As with Tgfβ, mutations that target decreased BMP signaling in mice commonly result in hypoplastic ECs as a result of impaired EMT. The significance of EC defects observed in BMP mutants is likely the result of compromised pSmad1/5/8 activity, which in healthy hearts is required to sustain BMP signaling from the myocardium to VECs during EMT. Proper BMP signaling is also required for sufficient functioning of Tgfβ, as well as the expression of transcription factors Twist1 and Mx2, which are well-known positive regulators of EMT. These studies highlight the complex transcriptional networks activated from growth factor signaling during EMT and EC formation.

**Wnt**

Tgfβ signaling pathways cross-talk with several signaling pathways including β-catenin, a mediator of canonical Wnt signaling that is an important regulator of EC formation. Endothelial deletion of β-catenin inhibits Tgfβ-mediated induction of EMT in mice, suggesting a requirement for canonical Wnt signaling in EMT. In zebrafish, overexpression of the secreted Wnt inhibitor, Dickkopf1, blocks EC formation, whereas truncation mutants develop hyperplastic EC because of increased cell proliferation. The notion that Wnt signaling regulates VIC proliferation is continued in the avian system where the Wnt receptor Frzb and the ligand Wnt9a promote mesenchymal cell number in the AV cushions. These studies identify a role for β-catenin during the early stages of EMT, whereas the downstream effectors of the Wnt signaling pathway appear to be important for mesenchymal cell proliferation.
and establishing the pool of valve precursor cells.

**Notch** Notch signaling is an activator of EMT, predominantly through Notch1, which is expressed in VECs. As ligands (Delta and Jagged) and receptors (Notch) in the Notch signaling pathway are membrane-bound proteins, Notch sets up an efficient intercellular local signaling system that binds to the DNA via the corepressor RBPJ or recruits the coactivator, MAML1, to regulate transcriptional activity of multiple downstream targets. Deletion of Notch1 or RBPJ in mice leads to defective ECs that lack significant numbers of transformed mesenchymal cells. In contrast to reduced Notch signaling, over-expression of constitutively active Notch1 in the endocardium delays EC formation and ectopic EMT is observed in the ventricular chambers. Target genes affected by reduced Notch signaling in developing ECs include Sna2 and the Hey family of transcription factors. In the chick and in mice, Hey1 and Hey2 are highly expressed in the VECs and are important for restricting the expression of Bmp2 and Tbx2 in the AV canal region. These studies highlight the essential role(s) that Notch-related genes play in EMT and EC formation.

**VEGF** VEGF is a potent cytokine that is highly expressed by the myocardium and VECs before EC formation. However, expression of the ligand and its receptors (VEGFR) is restricted to the endocardium once EC formation is initiated. Collectively, studies in multiple animal models have demonstrated a tightly controlled role for VEGF in EC formation, as too much VEGF inhibits EMT, while too little attenuates VEC proliferation, thereby decreasing VEC availability for transformation. Recently, it was shown that VEGF signaling through Vegfr2 in endocardial cells is required for transformation into mesenchymal cells in the OFT, but not the AV cushion. This is one of the few studies to identify discrepancies in the EMT regulation between the OFT and AV valves. Nfatc1 (nuclear factor of activated T-cells cytoplasmic 1) is a downstream target of VEGF in both developing and mature VECs and promotes cell proliferation. Although Nfatc1-/- mice undergo successful EMT, a new role has recently emerged for an Nfatc1 enhancer region in the fate decisions of VEC during transformation stages.

**Valve Remodeling, Maturation and Maintenance**

Once EC formation is complete, the overlying VECs stop undergoing EMT, regain their cell-cell contacts and form an uninterrupted endothelium. Around this time, the ECs fuse and elongate into mitral and tricuspid valve primordia in the AV region, and into primitive aortic and pulmonic structures in OFT (Figure 2). The mechanisms of elongation are unclear, but cell proliferation is localized to the distal tips of developing valves, and VEGF has been suggested to play a role. As the primordia develop, mesenchymal cells within the developing valves proliferate less and lose expression of mesenchymal markers but maintain smooth muscle α-actin (SMA) expression, suggesting a VIC-like phenotype. These embryonic VICS actively remodel the ECM by expressing matrix degradation enzymes to break down the hyaluronan, and secrete new ECM that will later form the 3 stratified layers. Compared with EMT, much less is known about valve remodeling, but some of the key regulators of EMT have differential roles at this stage. Bmp2 has been shown to be important for promoting expression of proteoglycans, including versican and hyaluronan, in the remodeling valves. In addition, Sox9 and Scleraxis regulate expression of other ECM components associated with the fibrosa and spongiosa layers, respectively. In mice, Sox9 is required during the remodeling stages for expression of known cartilaginous downstream target genes Col2a1 and Cartilage Link Protein within the leaflets. Scleraxis is both sufficient and required for proteoglycan expression and is positively regulated by canonical Tgfβ2 with cross-talk from MAPK signaling.

It is not until the postnatal stages that the elastin fibers are laid down in the atrialis/ventricularis layers and the valve becomes tri-stratified. At this time, VICS downregulate SMA and become quiescent and fibroblast-like and remain this way throughout life in the absence of injury/disease. Little is known about the pathways that maintain tissue homeostasis and integrity in the postnatal valve, but disturbances in VEC or VIC function are proposed to have detrimental effects on valve ECM organization and therefore biomechanical properties.

**Genetics of Valve Disease**

The sequencing of the human genome and advances in genetic technologies have contributed to the discovery of VHD-causing genes. Congenital anomalies of each of the 4 heart valves have been described, and these malformations disrupt normal valvular function. Not surprisingly, the same genes and molecular pathways implicated in heart valve development have been found to be mutated in humans with congenital VHD and are being disrupted...
found to play a role in acquired VHD. Here, we will focus on the genetic basis of 2 of the most common congenital valve anomalies – bicuspid aortic valve (BAV) and mitral valve prolapse (MVP) – and in addition discuss the genetic contributors to the most common form of acquired VHD, calcific aortic valve disease (CAVD). The genetic contributors to less common types of VHD are reviewed elsewhere.44

BAV

With an estimated prevalence in the population of 1–2%, BAV is one of the most common valve malformations.45 Although the normal aortic valve has 3 cusps, BAV is the result of fusion of 2 of the leaflets during development. BAV is associated with significant long-term morbidity from aortic valve dysfunction, primarily through calcification (discussed later) and is thought to have a strong genetic etiology.46 BAV can be categorized by the type of cusp fusion, which is proposed to have different embryologic origins.47 Fusion of the right-left cusps is related to abnormal septation of the OFT and right-non-coronary fusion to abnormal OFT EC development, and these subtypes are associated with different clinical outcomes.48

The first gene found to be mutated in human BAV was NOTCH1, a member of the Notch signaling pathway (discussed earlier). In families with autosomal dominant BAV, heterozygous loss-of-function NOTCH1 mutations are found to segregate with disease. Subsequently, NOTCH1 mutations have been found in other individuals and families with aortic valve disease.49,50 Further support of this link was the identification that mice haplo-insufficient for Notch1 in the setting of endothelial nitric oxide synthase (Nos3) deletion display a near 100% penetrance of BAV as compared with the lower incidence (~2%–30%) of BAV in Nos3+/− mice.51 Recently, deletion of Gata5, a zinc finger transcription factor, in the valve endoderm was found to result in a partially penetrant right-non-coronary BAV phenotype in mice, with associated dysregulation of the Notch signaling pathway in the developing OFT.52 Examination of humans with BAV has identified rare non-synonymous variants in GATA5, but individuals harboring mutations were found with both right-left and right-non-coronary BAV.53,54 Although these findings support a genetic association between NOTCH1 and GATA5 and BAV, the underlying mechanisms remain unclear, hindering the ability to link genetic etiologies to long-term morbidities.

MVP

MVP, which affects approximately 2–3% of the population, occurs when there is systolic displacement of a thickened mitral valve leaflet into the left atrium. MVP is associated with valve regurgitation, congestive heart failure, arrhythmias, and infective endocarditis.55 It is often identified in adulthood and is characterized by fibromyxomatous degeneration of the leaflets, which results in abnormally thickened and lengthened leaflets that bulge into the atrium, leading to mitral regurgitation.56 Diseased myxomatous valves have an expanded spongiosa layer, which is the result of excess proteoglycan deposition. This pathological remodeling is also associated with diminished collagen fibers, elastin fragmentation, myofibroblast activation, and overexpression of proteolytic enzymes such as MMP-1, MMP-2, and MMP-13.43,56

The best-studied etiology of MVP is mitral valve disease associated with Marfan syndrome (MFS), a connective tissue disorder affecting multiple tissues. Mutations in Fibrillin-1 (FBN1), a key component of ECM microfibrils, cause MFS.57 As has been reported with ascending aortic aneurysm associated with MFS, the molecular basis of MVP has been demonstrated to be increased Tgfβ signaling, which is a critical regulator of valve development (discussed earlier).58 A mouse model of MVP that carries a human disease-causing Fbn1 mutation, displays thickened valves soon after birth associated with increased Tgfβ expression, which can be rescued by pharmacologic inhibition of Tgfβ signaling during embryogenesis. Further supporting a role for Tgfβ signaling in the pathogenesis of MVP is the identification of mutations in Tgfβ receptors 1 and 2 (TGFBR1 and TGFBR2) as a cause of Loeys-Dietz (LDS), which has a similar MVP phenotype.59

Additional human genetic evidence linking abnormal Tgfβ signaling with MVP was identified when investigating families with an X-linked form of valvular dysplasia, which affects the mitral and aortic valves. There were mutations in Filamin A (FLNA), which encodes a widely expressed protein that interacts with ECM bound cell-surface integrins to regulate the actin cytoskeleton.60,61 Mouse null for Flna display a spectrum of valve abnormalities and defects in cardiac and OFT septation, and loss of endothelial Flna in mice leads to myxomatous mitral valve disease.62 Similar to Fbn1, Flna was shown to regulate the Tgfβ signaling pathway through its interactions with the Smad proteins.60,63 These studies suggest a critical role for Tgfβ signaling in the development of syndromic forms of MVP and also suggest a role for Tgfβ in the remodeling and maintenance of the mitral valve. Further studies are needed to determine if similar mechanisms are responsible for non-syndromic MVP.

Calcific Valve Disease

Among the acquired valve diseases, calcification of the aortic valve and the mitral anulus have been described. CAVD and mitral annular calcification (MAC) are characterized by calcification deposition on the AV cusps and MV annulus, respectively, and they share environmental risk factors similar to atherosclerosis. Human genetic studies, by evidence of familial clustering and genome-wide association, have demonstrated a genetic component to the development of CAVD and MAC.64,65 Recently, a large genome-wide association study including individuals with both CAVD and MAC led to the identification of a single nucleotide polymorphism (SNP) in the lipoprotein(a) (LPA) locus that was linked to only CAVD in multiple ethnicities, highlighting the differences for these acquired calcific valve diseases.66 As the SNP correlated with Lp(a) levels, it is a potentially exciting finding that may lead to novel therapies for CAVD.

CAVD is associated with endothelial dysfunction, lipid accumulation and inflammatory cell infiltration.6 Environmental factors are thought to increase the risk for development of CAVD by a process initiated by endothelial cell dysfunction that results in activation of VICS leading to the expression of bone development genes, including Runx2, Osteopontin and Osteocalcin, as found in calcified human aortic valves.6 The first suggestion of this concept came from human genetic studies of BAV families discussed before, which suggested a role for NOTCH1 in CAVD, because some family members harboring a NOTCH1 mutation did not have BAV but developed CAVD.66 Constitutively active Notch1, along with Hey1 and Hey2, inhibited Runx2 activation in cell culture assays. Further in vivo evidence of this inhibitory role has been demonstrated in mice heterozygous for Notch1, Rbpj, Jagged1 and Hey2, which develop aortic valve calcification.67–69 Those studies demonstrated that valve development genes may potentially play a role in this adult-onset disease and the mechanisms by which loss of Notch signaling leads to calcification are being investigated.70 Sox9, which encodes an SRY-related transcription factor
well known for its requirement in cartilage development, is also implicated in CAVD.\(^{71}\) Sox9 is required early for EC development.\(^{42}\) Although later, targeted reduced function in mice leads to early onset CAVD by 3 months of age and this is associated with increased expression of osteogenic genes seen in human calcified valves, partly because of lost repression of the target gene, Osteopontin (Spp1).\(^{7,24}\) In addition to increased calcification, heart valves from Col2a1-cre;Sox9\(^{46}\) mice show reduced expression of cartilage-associated ECM proteins that are highly expressed in healthy valves. This imbalance between osteo- and cartilage-like phenotypes likely occurs because Sox9 is known to transcriptionally activate chondrogenic genes and repress osteogenic markers, and therefore this regulatory hierarchy is lost in Sox9-mutant mice.\(^{42,77}\)

Similar to Notch1 and Sox9, additional valve developmental pathways have been linked to the development and progression of CAVD. Wnt/\(\beta\)-catenin signaling has been implicated in CAVD because Lrp5, a coreceptor in this pathway, is overexpressed in diseased valves.\(^{74}\) For instance, Notch1 and Sox9 are known to transcriptionally activate Notch target genes, Sox9 is required early for EC development and progression remains unclear why the mitral and aortic valves are susceptible to differential pathogenic programs, which highlights the complexity of VHD that involves the interplay of genetic, hemodynamic and environmental factors.

Conclusions and Future Perspectives

In summary, advances in our understanding of the molecular pathways that regulate valve development are beginning to have broad implications. As expected, identification of genes important for valve morphogenesis has assisted in the discovery of genes responsible for congenital valve malformations, and will accelerate gene discovery in the future.\(^{76}\) Ultimately, this increased understanding will affect the long-term morbidities associated with common forms of VHD, including BAV and MVP. Specifically, the importance of Tgfb signaling in MVP offers a therapeutic target to halt the progression of disease, as has been demonstrated for ascending aortic aneurysms in MFS.\(^{77}\) Human genetic studies of CAVD and the identification of embryonic pathways active in pathogenesis are beginning to offer insights into the development of therapies, but to date, it remains unclear why the mitral and aortic valves are susceptible to differential pathogenic programs, which highlights the complexity of VHD that involves the interplay of genetic, hemodynamic and environmental factors.

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