

How to Make a Heart Valve: From Embryonic Development to Bioengineering of Living Valve Substitutes

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Cardiac valve disease is a significant cause of ill health and death worldwide, and valve replacement remains one of the most common cardiac interventions in high-income economies. Despite major advances in surgical treatment, long-term therapy remains inadequate because none of the current valve substitutes have the potential for remodeling, regeneration, and growth of native structures. Valve development is coordinated by a complex interplay of signaling pathways and environmental cues that cause disease when perturbed. Cardiac valves develop from endocardial cushions that become populated by valve precursor mesenchyme formed by an epithelial–mesenchymal transition (EMT). The mesenchymal precursors, subsequently, undergo directed growth, characterized by cellular compartmentalization and layering of a structured extracellular matrix (ECM). Knowledge gained from research into the development of cardiac valves is driving exploration into valve biomechanics and tissue engineering directed at creating novel valve substitutes endowed with native form and function.

Cardiac valves maintain unidirectional blood flow during the cardiac cycle. As the heart contracts and relaxes, passive opening and closing of the valves, caused by a transvalvular pressure gradient, results in alternate blood flow from the atria to the ventricles and from the ventricles to the great vessels (Fig. 1A) (Yoganathan et al. 2000; Sacks and Yoganathan 2007). When the ventricles contract, the inlet or atrioventricular (AV) valves (the tricuspid and mitral

valves) prevent backflow from the ventricles to the atria; and when they relax, the outlet or semilunar (SL) valves (the pulmonary and aortic valves) prevent reverse flow from the arteries to the ventricles. Valve malfunction occurs when the valve fails to open properly (stenosis) or does not shut completely (regurgitation).

Heart valve disease, which primarily affects the aortic and mitral valves, is a health-care problem of epidemic proportions because of the in-

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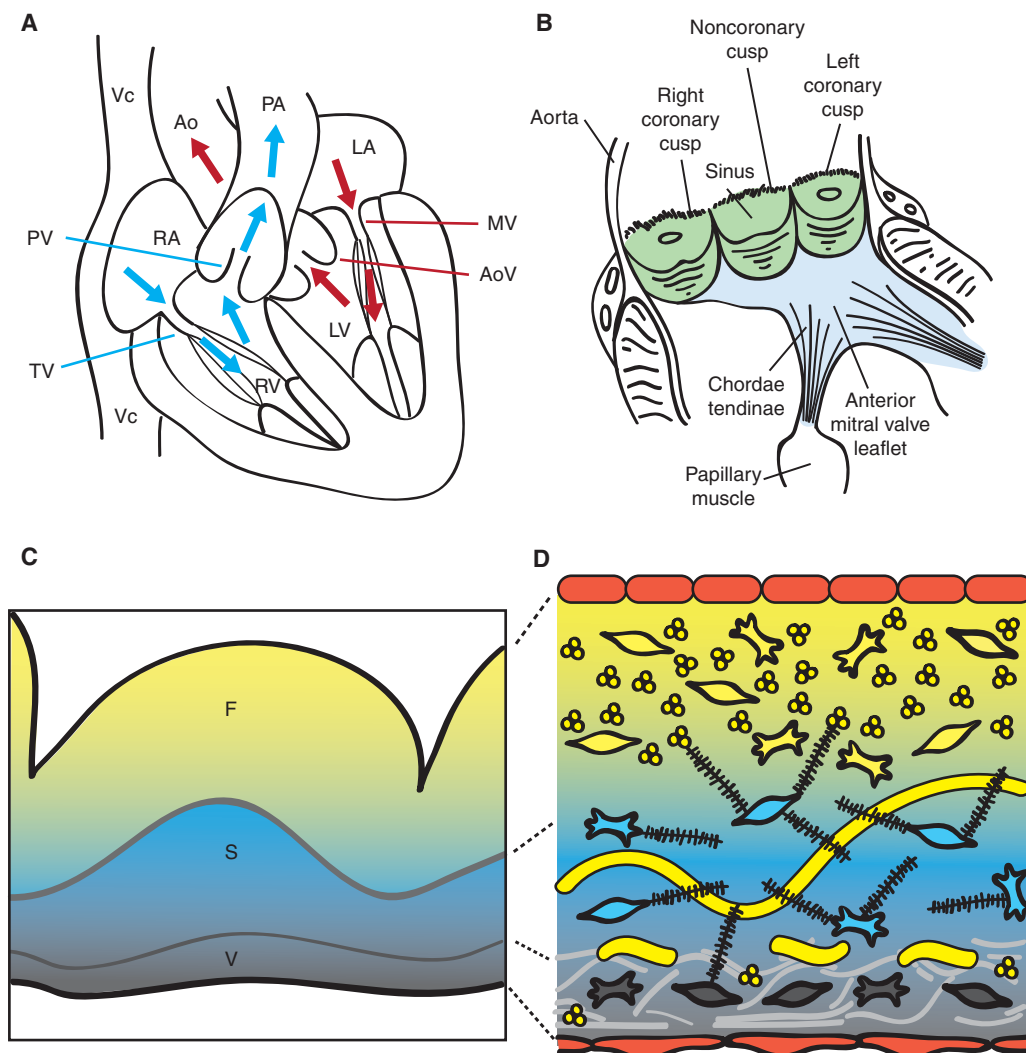


Figure 1. Heart valve structure and function. (A) Oxygen-depleted blood (blue) enters the heart from the vena cava (Vc) through the right atrium (RA) and transits via the tricuspid valve (TV) into the right ventricle (RV). From there, it passes through the pulmonary valve (PV) and reaches the lungs via the pulmonary artery (PA). Oxygen-rich blood from the lungs (red) enters the left atrium (LA) and empties into the left ventricle (LV) by passing through the mitral valve (MV). From the LV, the blood is pumped through the aortic valve (AoV) to the aorta (Ao), and from there it distributes to the rest of the body. (B) Scheme of a longitudinally opened aortic root, showing the close relation between aortic (green) and mitral (blue) valves in situ. The cusps are attached to the annulus fibrosus, a fibroelastic structure entrapping the valves (not shown). The free edge of the AoV leaflets is anchored to the LV wall by papillary muscles via chordae tendinae. (C) Artist rendition of a Movat's pentachrome staining of a cross section of the AoV cusp. (D) Depiction of the detailed architecture. The monolayer of endothelium (red) lining the valves has a cuboidal appearance on the arterial side and a flattened appearance of the ventricular side. Fibroblasts, smooth muscle, and myofibroblasts are interspersed in the different layers. Collagen (yellow) is densely packed and fibrillar and circumferential in the fibrosa (F), loose in the spongiosa (S), and multidirectional in the ventricularis (V). Proteoglycans (black) predominate in the spongiosa, and interact with cells and collagen. Radial elastin (light gray) predominates in the ventricularis.

creasing burden of elderly patients with degenerative heart valve disease and a growing population of young adults with congenital heart disease, involving complex valve anomalies. The gold standard treatment for advanced heart valve disease is surgical replacement, but none of the currently available mechanical and biological heart valve substitutes are ideal solutions. Thus, patients fitted with mechanical valves face the burden of lifelong treatment with anticoagulants, whereas patients with biological valves face the prospect of reoperation because of the limited durability of biological valve substitutes (Schoen and Levy 2005). Tissue engineering seeks to overcome these drawbacks by exploiting living cells to develop a living valve replacement that has the capacity to remodel in response to functional demand and repair inflicted damage. In principle, the living tissue-engineered heart valve (TEHV) could have the capacity to grow, but once it has matured, it might achieve the same functional and durability properties as the native valve. The superiority of a living heart valve replacement is underscored by the outstanding results achieved using living autografts compared with nonliving homografts for aortic root replacement (El-Hamamsy et al. 2010).

Translating knowledge gained from studying the mechanisms that drive valve development to engineering living valve replacements is an ongoing challenge for biomedical engineers. This article describes the anatomy and structure of native adult cardiac valves, summarizes how the endocardial cushions (ECs) form by epithelial-mesenchyme transition (EMT) and extracardiac mesenchyme contribution, and reviews the processes of valve morphogenesis and the ensuing cellular and matrix remodeling that give rise to the mature cusps and leaflets. The article closes by outlining how this knowledge is applied in state-of-the-art of tissue-engineering approaches to generate functional and long-lasting cardiac valve replacements.

CARDIAC VALVE ANATOMY

Cardiac valves have a complex anatomy (Yoganathan et al. 2000; Sacks and Yoganathan 2007; Schoen 2008). The central component of each

valve consists of three leaflets, except the mitral valve (MV), which has only two leaflets (Fig. 1B). The AV valves are attached to the annulus fibrosus, a fibroelastic tissue that encircles the AV canal and outflow tract (OFT) and provides structural support to the valves while also separating the ventricular and atrial myocardium. The free edges of the AV valves are tethered to the base of the ventricular walls by tendon-like cords, called chordae tendinae, via papillary muscles (Fig. 1B). This “subvalvular apparatus” maintains the AV leaflets within the ventricular chamber, preventing their prolapse into the atria during valve closure or ventricular contraction. Systolic billowing of one or both leaflets, as seen in MV prolapse, can be caused by myxomatous degeneration, rupture of the chordae tendinae, or, in cases of infarction or ventricular hypertrophy, displacement of the papillary muscles (Guy and Hill 2012). The SL valves do not require a supporting apparatus. Instead, the aortic valve (AoV) cusps are self-supporting and attach to crown-shaped arterial roots via the annulus fibrosus, whereas the pulmonary valve (PV) cusps insert primarily through a freestanding muscular sleeve called the right ventricular infundibulum. AV (or PV) insufficiency is caused by improper coaptation of the valve cusps and results in the movement of blood down its pressure gradient back into the ventricles (Prodromo et al. 2012).

STRUCTURAL BASIS OF VALVE FUNCTION

The composition and distribution of specialized valvular connective tissue types is essential for maintaining normal valve function throughout life (Hinton and Yutzy 2011). The design of replacements that faithfully reproduce native valves depends on elucidating the role played by the extracellular matrix (ECM) in valve development and maintenance.

THE ECM

Traditionally, the cardiac valves have been described as having a trilamellar architecture, which incorporates cellular and ECM components (Fig. 1C) (Latif et al. 2005; Schoen 2008). The layer closest to the sinus side (or atrial side

for AV valves), called fibrosa, is composed of densely packed fibrillar collagens whose circumferential orientation provides tensile strength when the valve is closed. The expression of collagens (mainly type I, and some type II and V) is not restricted to the outflow layer, but suffuses all the valve strata to form a network of thin fibrils, enmeshing and interacting with the other ECM and cellular components. (Fig. 1D) The intervening spongiosa is composed primarily of glycosamino- and proteoglycans and serves as a buffer zone for the bending and stretching imposed by leaflet opening and closing (Fig. 1C,D). Glycoaminoglycans are found throughout the valves and consist of hyaluronic acid (HA), a nonproteoglycan polysaccharide, chondroitin sulfates 4 and 6, and decorin (Latif et al. 2005). Proteoglycans are necessary for the stable assembly of the ECM and functional cell–ECM interactions, whereas decorin and biglycan interact specifically with type I collagen fibrils, modulating the kinetics of fibril formation and the distance between adjacent collagen fibrils. Facing blood flow is a thin ventricularis (or atrialis in the AV valves) composed of elastin and collagen, which provides elasticity through mostly radially oriented elastic fibers (Fig. 1C,D).

ECM Functions

The valve ECM does not merely afford a physical support for cellular growth, it is also a biologically active structure that provides cells with instructional cues and signals (mechanical and humoral), which determine many cellular functions. Valve cells bind to the ECM either through focal adhesions, which connect to actin filaments in the cell, or hemidesmosomes, which connect to intermediate filaments, such as keratin. These cell–matrix interactions are regulated by integrins, which interact with the ECM through associations of their extracellular domains with glycosaminoglycans and laminins bound to fibronectin. This sequence initiates intracellular signaling pathways and associations with the cellular cytoskeleton via adaptor molecules, such as actin. Moreover, collagen-binding glycans, including HA and decorin, can regulate

cell–matrix interactions and growth-factor signal output by sequestering growth factors and receptors, thereby regulating the release and diffusion of signaling effectors (reviewed in Latif et al. 2005).

CARDIAC VALVE CELL TYPES

Cardiac valve cells sense the local environment through poorly characterized mechanotransduction pathways. These pathways mediate connective tissue repair through the synthesis, degradation, and remodeling of the ECM, enabling the cells to maintain homeostasis through continuous adaptation to dynamic strain and shear stress states (Fig. 1C).

Valvular Endothelial Cells

Cardiac valve cusps and leaflets are covered by a monolayer of endothelial cells (valvular endothelial cells [VECs]), which are continuous with the endocardium and endothelium of the aortic and pulmonary arteries (Tao et al. 2012). The VEC population regulates multiple aspects of valve physiology, including platelet aggregation, inflammation, myofibroblast contraction and migration, and valve mechanics (Butcher and Markwald 2007; Sacks and Yoganathan 2007; Schoen 2008; El-Hamamsy et al. 2009). The importance of VECs for these processes is underscored by the fact that VEC dysfunction is strongly associated with valvular dysfunction (Leask et al. 2003). In response to injury or disease, VECs can replenish the pool of valve interstitial cells through a process of EMT (Bischoff and Aikawa 2011). Valve cusp endothelium is morphologically different from vascular wall endothelium and responds differently to fluid shear stress. Vascular endothelial cells realign to be parallel with flow in response to cyclic shear stress, whereas VECs always align perpendicularly to flow, regardless of the underlying matrix orientation (Butcher and Nerem 2004). Transcriptional profiling indicates that, although mechanical stress activates a common set of genes in the endothelia of valves and vessels, each cell type also activates its own unique gene expression program when exposed to the

same mechanical environment (Butcher and Nerem 2006). VECs, too, have different phenotypes on the ventricular and arterial sides of the AoV, being flattened on the ventricular side, but having a cuboidal appearance on the arterial side (Maron and Hutchins 1974; Bischoff and Aikawa 2011). Gene expression analysis of the AoV comparing the disease-prone aortic side to the disease-free ventricular side revealed a procalcific gene program on the aortic side, suggested by lower expression of several inhibitors of calcification, coexisting with relatively antioxidative and anti-inflammatory expression profiles (Simmons et al. 2005). The existence of varying shear stress conditions on the opposite sides of the valve cusps may account for this regional heterogeneity and point to endothelial mechanotransduction as an important contributing factor to the development and progression of calcific valve stenosis (Weinberg et al. 2010).

Valvular Interstitial Cells

Cardiac valves are populated by a heterogeneous collection of smooth muscle cells, fibroblasts, and myofibroblasts interspersed between the ECM layers (Fig. 1C) (Taylor et al. 2003; Liu and Gotlieb 2007). Valvular interstitial cells (VICs) are highly plastic and can reversibly transition from fibroblast to smooth muscle-like cells (Rabkin-Aikawa et al. 2004). Fibroblasts produce and secrete most of the surrounding ECM, whereas smooth muscle cells secrete matrix metalloproteases (MMPs) and tissue inhibitors of MMPs, which, respectively, promote and inhibit ECM breakdown in remodeling valves (Rabkin et al. 2001; Soini et al. 2001; Fondard et al. 2005). Interstitial cells are highly responsive to biochemical and biophysical stimuli produced by their surroundings (Sacks and Yoganathan 2007; Chen and Simmons 2011; Warnock et al. 2011). They are normally quiescent, but can become contractile and migratory in remodeling or diseased valves (Rabkin et al. 2001; Rabkin-Aikawa et al. 2004). Native valves undergo passive loading regimes, including flexures and planar tension forces, and VICs respond by altering their stiffness through in-

creased α -smooth muscle actin expression and biosynthesis and remodeling of ECM components (Weston and Yoganathan 2001; Gupta and Grande-Allen 2006; Ku et al. 2006; Merryman et al. 2006; Balachandran et al. 2009; Gupta et al. 2009b). The degree of matrix stiffness, in turn, modulates VIC response to biochemical cues (Yip et al. 2009). AoV interstitial cells respond differently to shear stress and mechanical loading, depending on whether endothelial cells are present (Butcher and Nerem 2006; Sacks and Yoganathan 2007; El-Hamamsy et al. 2009). Thus, VECs contribute to valve homeostasis and ECM remodeling by regulating the VIC phenotype, and this regulation is enhanced by shear flow. Pulsatile hemodynamic shear stress affects VICs through the transduction of mechanical forces by VECs or through bulk matrix shear stress, which induces tensile forces within the valve matrix. Additionally, these forces might be transmitted via the release of vasoactive substances (serotonin, nitric oxide, etc.) or cytokines, which have been shown to alter the contractile and biosynthetic properties of VICs in vitro (Liu and Gotlieb 2007).

OVERVIEW OF EARLY CARDIAC DEVELOPMENT

At embryonic day (E) 7.0 in the gastrulating mouse embryo, precardiac precursor cells derived from the epiblast emerge from the primitive streak and move forward bilaterally into the lateral plate mesoderm to form the cardiac crescent (Fig. 2A). At this stage, cardiac precursors are restricted to the splanchnic (visceral) mesoderm, having become specified by signals emanating from the endoderm and surrounding tissues (Harvey 2002; Brand 2003; Abu-Issa and Kirby 2007). By E8.0, folding of mesodermal layers from both sides of the embryo at the ventral midline results in formation of the linear heart tube, consisting of an inner endocardium and outer myocardium. The heart tube is derived from the first heart field (FHF) of the splanchnic mesoderm and contributes cardiac precursors to the atria, atrioventricular canal (AVC), and left ventricle (Buckingham et al. 2005). At E9.0, the tubular heart bends right-

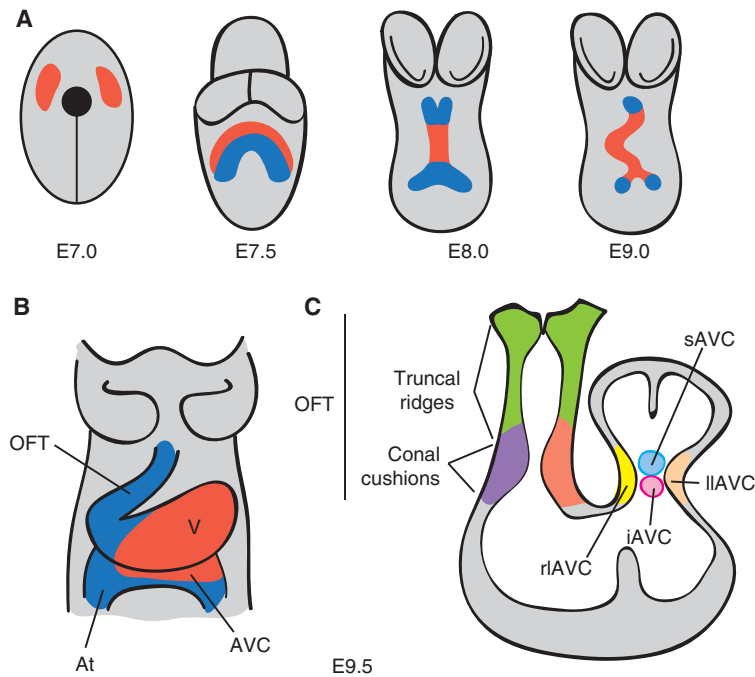


Figure 2. Early stages of heart development. (A) Ventral views of the developing mouse embryo. At E7.0, cardiac progenitors (red) have reached the head folds and, by E7.5, two cardiac lineages can be distinguished: the first heart field (FHF) (red) and the second heart field (SHF) (blue). At E8.0, the FHF progenitors merge to form the heart tube, which elongates at arterial and venous poles by the addition of progenitor cells from the second heart field (SHF). Between E8.0 and E9.0, the heart tube undergoes rightward looping. (B) Ventral view of the E9.5 heart, which consists of four anatomically distinct regions: atrium (At), atrioventricular canal (AVC), ventricle (V), and the outflow tract (OFT). (C) Longitudinal section depicting the prevalvular ECs. Two elongated cushions can be seen in the OFT, consisting of proximal (conal cushions, purple) and distal (truncal ridges, green) sections. The AVC has four cushions: right lateral (rlAVC), left lateral (llAVC), superior (sAVC), and inferior (iAVC). (Figure created from data adapted from Snarr et al. 2008.)

ward to reorient its original anterior portion along the left–right axis of the embryo and, by doing so, brings the atrial region into a position posterior to the common ventricle (Manner 2009). Concomitantly, the heart expands and elongates at both poles by the addition of progenitor cells coming from a second pool of pharyngeal mesoderm cells called the second heart field (SHF) (Kelly and Buckingham 2002; Dyer and Kirby 2009). At the arterial pole, the newly added cells give rise to the OFT, right ventricle, and interventricular septum (Zaffran et al. 2004; Verzi et al. 2005), whereas, at the venous pole, they contribute to the atria and atrial septum through the AV septal complex (Fig. 2B) (Snarr et al. 2008).

PATTERNING OF CARDIAC VALVE TERRITORY AND EMT

Cardiac valves formation in chicken, mice, and humans is restricted to the AVC and OFT regions of the looping heart (Fig. 2B,C). The ECs, that is, the primordia of the valves and septae, are formed in these regions by tissue patterning and EMT. These morphogenetic programs are stepwise processes governed by mechanical forces produced in the hemodynamic environment (Egorova et al. 2011; Riem Vis et al. 2011). Perturbation of the EMT process may lead to hypo- or hypercellularized cushions and, subsequently, to a spectrum of cardiovascular anomalies affecting the valves, septae, and

heart chambers. Endocardial tissue patterning is achieved by establishing developmental domains permissive for valve formation (de la Pompa and Epstein 2012). Specification of the heart-valve-forming region requires signaling from myocardial bone morphogenetic protein 2 (*Bmp2*) to activate *Tbx2* (Yamada et al. 2000; Ma et al. 2005). Outside this territory, cardiogenic signals activate expression of *Tbx20*, which drives expression of *Hey1* and *Hey2* and represses *Tbx2* (Singh et al. 2005; Stennard et al. 2005; Kokubo et al. 2007). *Tbx20* and *Hey1,2*, thus, restrict *Bmp2* and *Tbx2* to valve territory. In the endocardium, *Notch1* represses *Bmp2* via *Hey1*, *Hey2*, and *HeyL* rendering AVC endocardial endothelium competent to form heart-valve mesenchyme (Luna-Zurita et al. 2010).

The first signs of EC formation occur at E9.5 when swellings of proteoglycan-rich ECM secreted by the myocardium appear at the AV and OFT junctions (Eisenberg and Markwald 1995; Person et al. 2005). Over the following day, a subset of endocardial cells lining the AV and OFT cushions undergo EMT (Eisenberg and Markwald 1995; Armstrong and Bischoff 2004; Person et al. 2005). These transforming cells hypertrophy, lose apicobasal polarity, extend filipodia, and migrate into the cardiac jelly (Eisenberg and Markwald 1995; Person et al. 2005). *Snail1* and *Slug/Snail2* are crucial transcription factors for EMT because they down-regulate the expression of vascular endothelial cadherin, an adhesion molecule that maintains intercellular junctions in endothelial tissue (Romano and Runyan 1999; Timmerman et al. 2004; Niessen et al. 2008). Interestingly, in zebrafish, AV valve leaflets form directly through a process of invagination, during which the endocardium does not transform, but instead remains as a single sheet of cells (Scherz et al. 2008).

The signaling pathways that control endocardial EMT have been recently reviewed in depth (Lim and Thiery 2012; von Gise and Pu 2012). EMT induction is regulated by a network integrating *Bmp*, transforming growth factor (TGF)- β , and Notch signaling (Yamagishi et al. 2009; Lencinas et al. 2011; de la Pompa and Epstein 2012; Kruithof et al. 2012). Downstream from TGF- β , β -catenin acts in the AVC endocardium to pro-

mote the acquisition of a mesenchymal phenotype (Gessert and Kuhl 2010). The precise spatiotemporal pattern and levels of vascular endothelial growth factor (VEGF) expression are critical for both the onset and resolution of EMT (Dor et al. 2001; Chang et al. 2004; Stankunas et al. 2010). *Nfatc1* is required downstream from VEGF to regulate the extent of EMT and sustain endocardial proliferation during EMT and post-EMT valve elongation (Chang et al. 2004; Wu et al. 2011; Lin et al. 2012). The ErbB/SHP-2/NF-1/Ras signaling axis promotes mesenchymal migration into the cardiac jelly and proliferation and expansion of cushion mesenchyme (Yutzey et al. 2005; Iwamoto and Mekada 2006; Sanchez-Soria and Camenisch 2010).

The ECM is a critical regulator of EMT (Schroeder et al. 2003; Lockhart et al. 2011). Disruption of hyaluronan synthase-2 (*Has2*) abrogates normal cardiac morphogenesis and EMT mediated by HA (Camenisch et al. 2002). High molecular weight HA interacts with and activates ErbB2-ErbB3 receptors, initially, to promote EMT and, subsequently, in its depolymerized form to limit the extent of endocardial cell delamination and transformation (Camenisch et al. 2002; Rodgers et al. 2006). Moreover, a similar cardiac phenotype to the HA-deficient embryos occurs in zebrafish harboring a mutated uridine 5'-diphosphate (UDP)-glucose dehydrogenase (*ugdh*) gene (*jeekyll* mutant). This enzyme produces UDP-glucuronate, which is necessary for *Has2* synthesis of HA, suggesting that *Ugdh* may function in valve formation through its requirement for HA synthesis (Walsh and Stainier 2001).

CARDIAC VALVE MORPHOGENESIS

Valvular morphogenesis is intimately tied to developmental processes that lead to heart-chamber septation and connection to pulmonary and systemic circuitries. Multiple types of progenitor cells, originating from both in- and outside the heart orchestrate valve morphogenesis through highly conserved signaling networks. Once again, mistiming or malfunction of the unfolding events cause valvuloseptal de-

fects in patients with congenital heart disease and in experimental animal models (Srivastava 2006; Joziassse et al. 2008; Lin et al. 2012).

Prefusion of Mesenchyme Structures

By E10.5, EMT subsides in the AVC. The major (inferior and superior) AV cushions constitute the bulk of mesenchyme occupying the AVC lumen (Figs. 2B and 3A). Smaller lateral AV cushions start to develop at this stage (Fig. 3A) (Wessels and Sedmera 2003; Snarr et al. 2008). The major cushions take part in the formation of the AV mesenchymal complex and contribute to the formation of the aortic leaflet of the MV and septal leaflet of the tricuspid valve (TV) (Snarr et al. 2008). The lateral AV cushions do not fuse, nor do they participate in AV complex formation. Instead, the right cushion gives rise to the anterosuperior and -posterior leaflets of the TV, whereas the left cushion gives rise to the mural leaflet of the MV (Fig. 3A,B) (Snarr et al. 2008).

By E11.5, the OFT is occupied by spiraling and elongated mesenchymal cushions, referred to as septal and parietal ridges, and two less prominent intercalating ridges (Snarr et al. 2008). The boundary between the proximal conal and distal truncal cushions is marked by the outer curvature of the OFT (i.e., the cono-truncal curvature) and determines the site for SL valve formation (Fig. 2B) (Lin et al. 2012). By E12.5, the fused larger cushions and nonfused Immunocytochemistry (ICC) have given rise to the three arterial valve cusps for each SL valve (Fig. 3A,B). The major cushions participate in forming the aortico-pulmonary septum and contribute to the R–L coronary cusps of the AoV and R–L cusps of the PV. The intercalating cushions do not fuse; instead, the right lateral cushion gives rise to the noncoronary (NC) cusp of the AoV, whereas the left lateral cushion gives rise to the anterior leaflet of the PV. Eventually, the coronary arteries connect to the AoV sinuses immediately proximal to the R–L cusp precursors.

Lineage Tracing of Valve Tissues

Atrioventricular Valves

Labeling of endothelial and endocardial lineages using the *Tie2-Cre*; *ROSA26R* mouse line

shows that the early mesenchyme forming the major cushions is derived almost entirely from endocardial EMT (Rivera-Feliciano et al. 2006; Snarr et al. 2008). β -galactosidase (β -gal) staining is detected in the AV fibrous continuity, valve leaflets, and the chordae tendinae at later gestational stages and postnatally, consistent with these structures being derived from the endocardium (de Lange et al. 2004; Lincoln et al. 2004).

Contributions to AV valve formation by the epicardium and cardiac neural crest also require an EMT process (Fig. 4B) (Lim and Thiery 2012; von Gise and Pu 2012). After undergoing EMT, epicardium-derived cells (EPDCs) give rise to the subepicardial mesenchyme and, subsequently, yield fibroblasts in the myocardial wall and smooth muscle cells of the media in coronary arteries (Lie-Venema et al. 2007; von Gise and Pu 2012). Quail-chick chimera analysis indicates that EPDCs populate the mesenchyme of developing AV valves and contribute to the fibrous heart skeleton (Fig. 4B) (Gittenberger-de Groot et al. 1998; Snarr et al. 2008). EPDC fate analysis using the mouse inducible *Wt1-Cre^{ERT2}*; *ROSA26* Cre reporter line shows an important contribution to the AV sulcus and annulus fibrosus, a fibrous continuity that, in addition to separating atrial and ventricular myocardium, supports the mature valve leaflets (Zhou et al. 2010). At around E12.0, EPDCs migrate through the AV junction to populate the AV cushions. Using the *Wt1/IRES/GFP-Cre*; *ROSA26* Cre reporter line, β -gal positive cells can be readily detected in the remodeled leaflets derived from the postfusion lateral cushions, but there is little contribution to the major AV cushions (Wessels et al. 2012). In the mural MV and TV leaflets, the EPDCs eventually replace the endocardially derived cells to make up most of the mesenchyme. Remarkably, these same leaflets are often implicated in congenital and acquired valve abnormalities, including Ebstein's anomaly, AV valve insufficiency or prolapse, and mitral/tricuspid stenosis (Fig. 4B) (Snarr et al. 2008).

A third source of AV leaflet mesenchyme is a population of cranial neural crest cells (CNCCs) (Fig. 4B). Beginning at E9.5, after undergoing

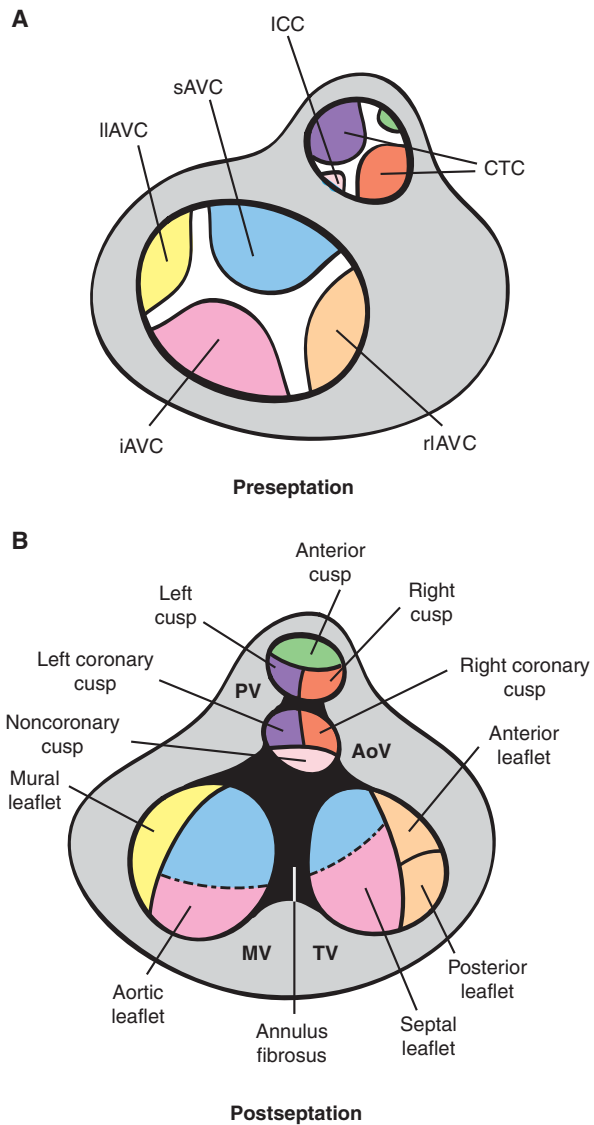


Figure 3. Fate of the cardiac cushions in the mature heart. Top view of the OFT preseptation (A) and postseptation (B). In the AVC, fusion of the major and lateral AV cushions at E13.5 results in formation of inlet valves. The sAVC (blue) contributes mostly to the aortic leaflet of the MV, whereas the iAVC (pink) contributes mostly to the septal leaflet of the TV. The rIAVC (orange) and IIAVC (yellow) contribute, respectively, to the mural leaflet of the MV and anterior and posterior leaflets of the TV. These leaflets are later invaded by epicardially derived mesenchyme. The conotruncal cushions (CTCs) develop laterally right (orange) and left (purple) in the OFT and are separated by smaller anterior (green) and posterior (light pink) intercalated cushions (ICCs). At E12.5, fusion of the CTCs yields the mesenchymal outlet septum, the right and left (R–L) cusps of the PV and R–L coronary cusps of the AoV. The ICCs give rise to the anterior leaflet of the PV and the noncoronary cusps of the AoV. Neural crest precursors invading the distal truncal ridges participate in OFT septation and contribute mesenchyme to the R–L cusps of both PV and AV. (Figure created from data adapted from Snarr et al. 2008 and Lin et al. 2012.)

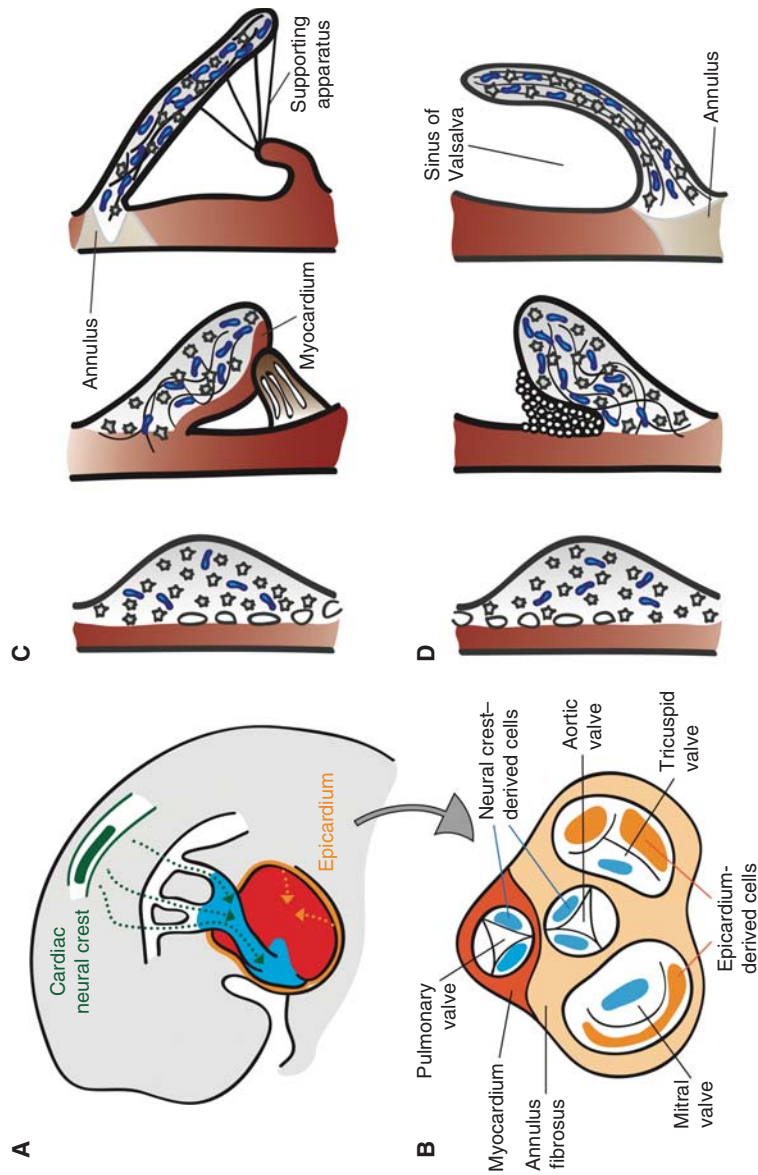


Figure 4. Remodeling of the cardiac valve leaflets and cusps. (A) Contribution of cardiac neural crest (green)- and epicardial (orange)- derived cells (CNCC and EPDCs). CNCCs migrate through the pharyngeal arches and into the OFT to initiate the reorganization of the OFT and formation of SL valves. EPDCs contribute to the formation of the coronary arteries, interstitial cells in the myocardium, and the AV valves. (B) Top view of the septated heart, depicting the relative contributions of CNCCs and EPDCs to cardiac valve leaflets and cusps. (C) Morphogenesis of the AV valves. From E13.5 onward, the leaflets and tensile apparatus (light brown) of the AV valves form predominantly by delamination of the inner layers of the inlet zone from the ventricular septal wall. The chordae tendinae, annulus fibrosus, and the leaflet itself are derived from the endocardium. The septal leaflet of the TV (in contrast to the aortic leaflet of the MV), together with its supporting tendinous cords, remains connected to the myocardium until E17.5. (D) Morphogenesis of the SL valves. The excavation of the cusps takes place initially by solid ingrowth of the endothelium at the arterial face of the cusp and, subsequently, by lumenation. Elongation and remodeling of the primordia into mature valve structures are associated with regionalized cell proliferation and matrix alignment guided by hemodynamic forces. Myocardium is depicted in dark red, proteoglycans in blue, and mesenchyme in gray.

an EMT in the dorsal neural tube, CNCCs migrate into the pharyngeal arches and surround the thymus and thyroid as they form. A subset of CNCCs continues into the arterial pole of the heart to initiate the reorganization of the OFT and formation of SL valves (Hutson and Kirby 2007; Snider et al. 2008). Earlier mouse studies concluded that there was little CNCC contribution to the AV cushions (Jiang et al. 2000; de Lange et al. 2004). More recent studies with *Wnt1-Cre; R26R* and *P0-Cre; R26R* Cre reporter mice detected β -gal positive cells migrating into the AV canal and valves at E12.5 (Nakamura et al. 2006). These cells were found almost exclusively in the septal leaflet of the TV and aortic leaflet of MV, with the mural leaflets almost devoid of staining. Moreover, these cells persisted postnatally in the septal leaflets, as revealed by positive staining for the melanocyte marker TRP1 (Fig. 4B) (Nakamura et al. 2006).

Lineage analysis using a *cGATA6-Cre; R26R* Cre reporter line indicated that AVC cardiomyocytes contribute to the developing tricuspid mural and posterior leaflets and the mitral septal leaflet, and persist in the corresponding mature leaflets. The presence of β -gal-positive cells at the atrial border of the annulus fibrosus suggests that AVC cardiomyocytes contribute also to the electrical isolation of the atrial and ventricular chambers (Gaussin et al. 2005).

SL Valves

At E10.5, the OFT cushions are populated by mesenchyme originating from two distinct lineages (Snarr et al. 2008; Wu et al. 2011). Tracking studies using the *Tie2-Cre; ROSA26R* line showed that the contribution of endocardially derived cells is restricted to the proximal portion of the conal cushions and distal part of the truncal ridges (Snarr et al. 2008; Wu et al. 2011). Cell fate analysis using *Wnt1-Cre; ROSA26R* reporter mice showed that CNCCs are found mostly in the distal portion wherein they form two prongs of mesenchymal cells (Fig. 4B) (Jiang et al. 2000; Nakamura et al. 2006). By E11.5, the interface of endocardium and NC-derived mesenchyme at the conotruncal junction delineates a boundary corresponding to the site of

SL valve development in humans (Anderson et al. 2003). Early fate mapping studies indicated that CNCCs present in the truncal cushions are selectively eliminated as development proceeds. As a result, it was thought that CNCCs ultimately made little substantial contribution to the valves in late-gestation embryos and postnatal animals (Jiang et al. 2000; de Lange et al. 2004). More robust fate mapping using *Wnt1-Cre; R26R* and *P0-Cre; R26R* reporter lines, subsequently, found CNCCs persisting throughout late development and into adulthood (Nakamura et al. 2006). Remarkably, β -gal staining in this latter study concentrated in the leaflets adjacent to the aorticopulmonary septum, that is, R–L coronary cusps of the AoV and R–L cusps of the PV (Fig. 4A). This finding is intriguing in light of the hypothesized neural-crest origin of bicuspid aortic valve (BAV) with fusion of the R–L coronary cusps (Fernandez et al. 2009; see below).

The SHF might constitute a third source of OFT mesenchyme. Lineage tracing using *Isl1-Cre; R26R* mice showed that SHF cells are added to the myocardial wall as the OFT elongates before septation and contribute to endocardial lineages within the OFT (Cai et al. 2003; Yang et al. 2006). Moreover, immunohistochemical detection of *Isl1* confirmed that *Isl1*⁺ cells are present in the OFT myocardium and cushions (Sun et al. 2007; Snarr et al. 2008). Recent lineage tracing analysis suggests that the *Isl1*⁺ cells are not derived solely from the SHF, but may originate also in the neural crest (Engleka et al. 2012).

Transplant repopulation experiments suggest that bone marrow (BM)-derived cells can contribute to the valve leaflets postnatally. Valves of chimeric mice transplanted with EGFP-labeled hematopoietic stem cells are EGFP⁺/CD45⁺ and have fibroblast-like properties characterized by collagen type I synthesis (Hajdu et al. 2011). Postnatal valve cellularization by BM-derived cells might be important for valve homeostasis, for example, by providing a population of synthetic VICs responsive to injury. Tissue bioengineering in situ takes advantage of the capacity of BM-derived cells to engraft by cell homing, and constitutes an

attractive alternative to the classical paradigm of bioengineering ex vivo (Fig. 5).

FUSION OF THE ECs

From E11.5 onward, the ECs expand by mesenchyme proliferation driven by Bmp/TGF- β , Egf, Nf1/ras, and Wnt signaling (Gitler et al. 2003; Iwamoto et al. 2003; Jackson et al. 2003; Krenz et al. 2005; Alfieri et al. 2010) and the combined activities of highly expressed mesenchyme-specific transcription factors, including Msx1/2, Runx2, Sox9, Twist1, and Tbx20 (Gitler et al. 2003; Lincoln et al. 2007; Shelton and Yutzey 2007; Chakraborty et al. 2008, 2010). Septation of the OFT and AV junctions into separate R–L ventricular inlets is mediated by fusion of the EC structures. In the OFT, the fusion of the larger cushions at midline yields separate outlet lumens, connecting the left and right ventricles to the AoV and PV, respectively. The primary

ECs in the AVC form a partition between atria and ventricles and determine the alignment of the R–L AV valves and proper relationship of the great arteries to the ventricular chambers (Wessels and Sedmera 2003; Snarr et al. 2008).

The mechanism(s) by which the EC fuse remain largely unknown (Hay and Low 1972). However, aberrant fusion events cause membranous ventricular septal defects and extrafusion of the AoV cusps leads to BAV. In humans, R–L fusion is the most common, followed by R–NC (Siu and Silversides 2010). A comparative study of inbred Syrian hamsters with R–L morphology and Nos3-knockout mice with R–NC morphology suggests that the etiologies of these phenotypes are different (Fernandez et al. 2009). R–L fusion may be the result of defective OFT septation, implicating the neural crest, whereas R–NC fusion may be caused by defective lateral cushion formation, suggesting inadequate EMT. The BAV is usually detected in

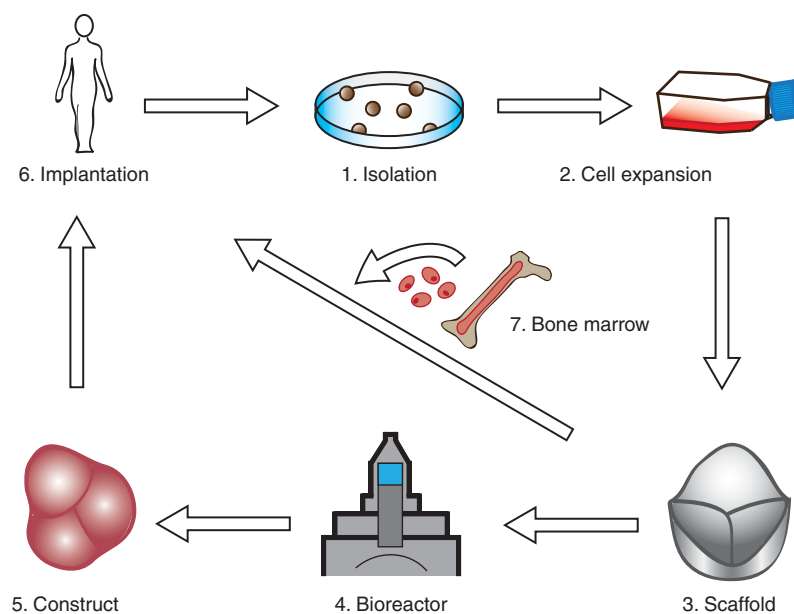


Figure 5. Valve tissue–engineering paradigm(s). (1)–(3) Conventional valve bioengineering entails a scaffold that is seeded with cells to grow a valve construct ex vivo in a bioreactor (dynamic conditioning) (4), followed by implantation in the anatomic site in vivo (5). In the modified protocol, the appropriate scaffold (3) is implanted in vivo (6) and cellularization is achieved by recruitment of circulating endothelial and mesenchymal cells from bone marrow (7). The scaffold can be engineered to incorporate molecules that stimulate recruitment, adhesion, migration, proliferation, differentiation, and cell function within the scaffold. (Figure created from data adapted from Weber et al. 2012.)

isolation, but can also coexist with other cardiovascular malformations, suggesting a multigenic etiology (Siu and Silversides 2010). Consistent with this notion, family-based linkage analyses suggest linkage to loci on chromosome 18q, 5q, and 13q, but the genes within these regions remain to be identified (Martin et al. 2007). To date, only mutated *NOTCH1* alleles on chromosome band 9q34-35 have been found to be causative in familial BAV in the context of calcific AoV disease (Garg et al. 2005).

POSTFUSION MORPHOGENESIS

During late AV valvulogenesis, the septal leaflet of the TV remains in contact with the septum until it delaminates at E17.5 (Fig. 4C) (Lamers et al. 1995; de Lange et al. 2004; Gaussin et al. 2005). The mural leaflets of both AV valves are supported by AV myocardium at their ventricular side. In comparison, the mitral septal leaflet is never supported by myocardium, but is in contact with the AV-myocardium-derived mitral gully at its anterior and posterior margins (de Lange et al. 2004; Gaussin et al. 2005). As the primordial leaflets distend into the lumen, thin strands of elongated muscle remain attached to the valve leaflet until E17.5. Programmed cell death yields a mobile leaflet and remnants that contribute to the chordae tendinae and papillary muscles (de Lange et al. 2004; Lincoln et al. 2004). Postfusion SL valve morphogenesis is less well characterized. SL valves undergo progressive excavation from E12.5 until E15 when they achieve their typical morphology (Fig. 4D). This process is driven by “selective endothelial growth” of the free edges of the cusps on their arterial face, producing an epithelial ridge or groove between the emerging cusps and the arterial wall. The groove eventually becomes luminated to yield the sinus of Valsalva (Hurle 1979; Hurle et al. 1980).

CARDIAC VALVE REMODELING

The later phases of valve development are characterized by the gradual transition from undifferentiated mesenchyme to specialized VICs. Remodeling of the primitive ECM into a highly

organized and stratified ECM is strongly influenced by hemodynamic stimuli. The developmental mechanisms that coordinate VIC specialization and ECM organization during valvulogenesis remain uncertain (de Vlaming et al. 2012).

Lineage Diversification

Cell proliferation, density, and turnover, substantial in early valvulogenesis, become less pronounced in remodeling valves (Aikawa et al. 2006; Hinton et al. 2006). Apoptosis in the OFT cushions is substantial at later fetal stages, consistent with ongoing remodeling processes (Poelmann and Gittenberger-de Groot 2005; Aikawa et al. 2006; Jain et al. 2011). Early overlapping gene expression patterns become restricted (Lincoln et al. 2004; Chakraborty et al. 2008). The transition from EC growth to remodeling requires calcineurin/NFATc1 signaling in the endocardium (de la Pompa et al. 1998; Ranger et al. 1998; Chang et al. 2004), notably through the regulation of *RANKL* and *Cathepsin K* expression (Lange and Yutzey 2006). Interstitial cell progenitors show transcriptional profiles normally associated with cartilage and tendon lineages (Lincoln et al. 2006b; Chakraborty et al. 2008). Expression of *Sox9* is necessary for early interstitial cell proliferation and, subsequently, expression of cartilage matrix proteins (Akiyama et al. 2004; Lincoln et al. 2007), and scleraxis is required for tendinous cord specification (Levay et al. 2008). In the AV valves of avian embryos, *Sox9* and *aggrecan* are predominantly expressed in the leaflets, whereas scleraxis and tenascin C are expressed in the supporting AV structures, including the chordae tendinae (Lincoln et al. 2004). In contrast, SL valves express genes associated with both lineages and diversify into cusps with an internal supporting apparatus (Zhao et al. 2007). A delicate balance of BMP and fibroblast growth factors (FGF) signals is required for lineage diversification. In cultured early valve precursors, *Sox9* and *aggrecan* are induced by BMP2, whereas *scleraxis* and *tenascin* are regulated by FGF-4, but it is unclear how these activities coordinate spatiotemporally to

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direct VIC diversification (Lincoln et al. 2006a; Zhao et al. 2007).

Remodeling of the ECM

The elongation of the valve leaflets and stratification of the ECM begins late in gestation and continues postnatally (Aikawa et al. 2006; Hinton et al. 2006; Kruihof et al. 2007; Peacock et al. 2008; Stephens et al. 2010). Initial ECM patterning along the AV leaflet axis occurs between E15.5 and E18.5 through a transient increase in cell density called condensation (Kruihof et al. 2007). In mature valves, the ECM is found in alignment with blood flow, suggesting that stratification and remodeling are driven by hemodynamic forces acting via the endothelium (Combs and Yutzey 2009). Postnatally, additional leaflet elongation is thought to occur by physical pulling of the tendinous cord attachments by the rapidly growing ventricles.

As remodeling proceeds, collagen fibrils become densely packed locally at the outflow side of the leaflet and loosely arranged and interweaving elsewhere (Aikawa et al. 2006; Hinton et al. 2006; Peacock et al. 2008; Tan et al. 2011). Fibrillogenesis is promoted by periostin, a multifunctional fascilin-domain-containing protein (Norris et al. 2009). Periostin promotes the differentiation of endothelial and epicardially derived mesenchyme while blocking other cell types, in particular, cardiomyocytes, and is required for fibrous maturation of the AV leaflets and their supporting apparatus (Norris et al. 2008; Snider et al. 2008). Valve remodeling and organization is dependent on the coordinated actions of MMPs and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families of zinc metalloproteinases (Lockhart et al. 2011). These pericellular secreted proteases target the ECM and mediate structural changes required for cell migration and proliferation. For example, proteolytic cleavage of versican, a chondroitin sulfate proteoglycan, by ADAMTS1 plays a significant role in the maturation of the AV cushions and remodeling of the OFT by facilitating the dense packing of mesenchyme in the elongating valves (Kern et al. 2006).

Dysregulation of the ECM appears to be a general feature of valve disease regardless of etiology; for example, BAVs from pediatric patients have increased collagen and proteoglycan content, whereas myxomatous MVs have loose collagen, increased proteoglycan, and reduced elastin content with altered fiber orientation in all layers (Rabkin et al. 2001; Hinton et al. 2006; Gupta et al. 2009a). These structural changes are associated with the aberrant reexpression of early valve mesenchymal and chondrogenic progenitor markers and may be related to the reawakening of fetal transcriptional programs (Wirrig et al. 2011; Cheek et al. 2012).

HEART-VALVE TISSUE ENGINEERING

General Overview

In the classical tissue-engineering paradigm, cells are harvested from a donor (which can be the recipient) and are subsequently expanded to obtain a sufficiently large number of cells for seeding on a biodegradable starter matrix (scaffold) (Fig. 5). The porous starter matrix is shaped like a heart valve and supports the formation of neotissue (Hoerstrup et al. 2000). In the bioreactor, the construct is subjected to biochemical and mechanical stimuli to enhance the production of ECM proteins in an effort to create a valve that has sufficient strength and durability to meet in vivo hemodynamic requirements (Mol et al. 2005a; Kortsmits et al. 2009). Particular attention has been focused on replicating the anisotropic architecture of the native leaflet (Mol et al. 2006; Neidert and Tranquillo 2006; Balguid et al. 2007; Rubbens et al. 2009; Cox et al. 2010; Sander et al. 2011).

An alternative approach is autologous tissue formation, initiated by intraperitoneal implantation of a heart valve-shaped scaffold (De Visscher et al. 2007) or mold (Hayashida et al. 2007; Yamanami et al. 2010). The mold will elicit a foreign body response causing deposition of autologous ECM on the mold surface. The resulting construct is then transplanted to the heart as a valve replacement. However, the volume fraction of cellular phenotypes currently achieved with this approach is unbalanced,

which may result in inadequate remodeling of the valves (De Visscher et al. 2007).

Cell Sources for In Vitro Heart-Valve Tissue Engineering

A large variety of cell types has been investigated for in vitro seeding of synthetic or biological scaffolds. Early experiments comparing the in vivo response to allogenic and autologous cells showed that seeded cells from an autologous source were desirable to minimize the immune response (Shinoka et al. 1995). However, autologous cells were recently shown to provoke an immune response caused by proinflammatory cytokines originating from cell death, or damage-associated molecular pattern (DAMP) molecules released by dying cells within the autologous tissue (Badylak and Gilbert 2008).

Vascular-derived myofibroblasts and endothelial cells are the gold standard in heart-valve tissue engineering because of their excellent ECM production capacity. These cells can be harvested from the recipient's saphenous vein. Alternative sources are progenitor cells derived from bone marrow, adipose tissue or umbilical cord blood, and circulating endothelial progenitor cells, all of which have shown potential to provide interstitial and endothelial function and have been used to generate heart valves in vitro (Hoerstrup et al. 2002; Schmidt et al. 2007; Sales et al. 2010). One of the advantages of progenitor cells is that, unlike vascular-derived cells, they can be harvested without additional surgical intervention. In this regard, bone marrow-derived mesenchymal stem cells (MSCs) are attractive candidates and have been used to seed decellularized matrices (Vincentelli et al. 2007; Iop et al. 2009) and synthetic scaffolds (Hoerstrup et al. 2002; Sutherland et al. 2005). MSCs are remarkably similar to VICs (Latif et al. 2007), and have antithrombogenic (Hashi et al. 2007) and immunosuppressive properties (Uccelli et al. 2006). MSCs are able to differentiate into endothelial cells, fibroblasts or myofibroblasts, and smooth muscle cells (Iop et al. 2009). Their accessibility, ease of handling, and potential for allogenic applications make MSCs suitable for routine clinical use (Pittenger and Martin 2004).

In addition to supporting proliferation, differentiation, and ECM production *ex vivo*, MSCs can induce the homing and differentiation of autologous host cells through paracrine signaling, involving an array of cytokines and growth factors (Roh et al. 2010). Despite the encouraging results obtained with preseeded scaffolds in sheep and baboons (Vincentelli et al. 2007; Weber et al. 2011), the antithrombogenic properties of MSCs (Hashi et al. 2007) and their ability to stimulate in vivo endothelialization (Mirza et al. 2008) should preclude future requirements for preseeding with endothelial cells.

Scaffolds for Heart-Valve Tissue Engineering

Different types of scaffold materials, or so-called starter matrices, have been used for heart-valve tissue engineering. These include biodegradable synthetic polymers, natural materials, such as fibrin and collagen, and xenogenic or allogenic decellularized heart valves. A decellularized xenograft or homograft is an obvious choice of scaffold material because these grafts closely resemble native human valve geometry and structure and have excellent mechanical and hemodynamic properties. The microstructure of the allograft favors proliferation, differentiation, and survival of reseeded cells (Mirza et al. 2008). The use of allografts, however, is limited by donor availability. Because of its anatomic similarity to human valves, the porcine heart valve makes an attractive alternative. Clinical application of decellularized xenografts has, however, resulted in early failure (Simon et al. 2003; Roh et al. 2010; Ruffer et al. 2010; Hibino et al. 2011). A major concern with xenogenic materials is infection with endogenous porcine retroviruses, prions, or other zoonotic vectors. Improved decellularization techniques recently allowed the preparation of nonimmunogenic decellularized xenograft valves (Bloch et al. 2011). Complete removal of cells from the tissue is crucial, as residual cells and cell remnants within the matrix might lead to calcification (Human and Zilla 2001). It is also important to minimize structural alteration of the ECM to preserve the biomechanical characteristics of the native heart valve. A promising alternative

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is the use of decellularized TEHVs, yielding off-the-shelf available homologous heart valves (Dijkman et al. 2012).

Biodegradable synthetic materials have been widely used as scaffold material for tissue engineering (Bouten et al. 2011). Commonly used biomaterials are different copolymers and derivatives thereof, whereas, more recently, elastomeric materials have gained attention (Courtney et al. 2006; Sales et al. 2007; Stella et al. 2010). Compared with decellularized xenogenic and allogenic matrices, these materials avoid the risk of disease transfer and immunological complications. In addition, synthetic materials have the advantage of unlimited supply and their biological, mechanical, and degradation properties can be tailored to the specific application. Synthetic scaffold materials have also been combined with natural substrates, such as ECM components (e.g., collagen) and fibrin. Fibrin, in particular, has been frequently used as scaffold material (Ye et al. 2000; Syedain et al. 2008; Flanagan et al. 2009), either alone or in combination with a synthetic scaffold (Mol et al. 2005b). After seeding the scaffold with the selected cell source, the construct is subsequently exposed to biochemical and mechanical cues in a bioreactor to stimulate ECM formation. When seeded with autologous cells and then cultured in vitro, these materials have been shown to be suitable for heart-valve tissue engineering, with functionality shown in vitro and in vivo (Hoerstrup et al. 2000; Sutherland et al. 2005; Flanagan et al. 2009; Gottlieb et al. 2010; Schmidt et al. 2010).

In Situ Tissue Engineering Using Synthetic Scaffolds

An emerging alternative is tissue engineering in situ using synthetic biodegradable scaffolds and endogenous cells (Roh et al. 2010), either recruited from the bloodstream or harvested on the fly (Weber et al. 2011). This approach is supported by recent findings that dramatically alter the view of the role of the seeded cells in relation to in vivo cell recruitment. These findings show that bone marrow-derived mononuclear cells (BM-MNCs) seeded onto a biodegradable scaffold

are rapidly replaced by host cells when implanted either as a vascular graft in severe combined immunodeficiency (SCID)/beige (bg) mice (Roh et al. 2010) or a heart valve in a nonhuman primate model (Weber et al. 2011). The evidence suggests that the seeded BM-MNCs play a paracrine-signaling role during in vivo tissue formation. This further suggests that, even without preseeding, mature tissue might be obtained via an inflammation-mediated process in which infiltration of circulating monocytes into the scaffold is stimulated and controlled by the release of specific cytokines (e.g., monocyte chemoattractant protein [MCP]-1). All implanted biomaterials trigger an inflammatory host response, which is essential for the colonization of starter matrices by blood-derived cells. The nature and differentiation of the infiltrating cells are of pivotal importance to the delicate balance that determines generation of a fibrotic or functional ECM (Roh et al. 2010; Hibino et al. 2011). The inflammatory process and subsequent fibrosis or regeneration are tightly controlled, both spatially and temporally, by a plethora of cytokines that regulate secondary cellular homing, infiltrated cell differentiation, and ensuing ECM production. An important cytokine guiding the inflammatory process toward regeneration is MCP-1, a chemokine secreted by macrophages to attract additional inflammatory cells, resulting in rapid and homogenous infiltration of the starter matrix with blood-derived cells. MCP-1 has, moreover, proved to be an important chemokine in the regulation of macrophage polarization toward a reparative phenotype (Roh et al. 2010). Stromal cell-derived factor (SDF)-1 α is critical for attracting blood-derived tissue-producing progenitor cells and controlling valvular cell phenotype (De Visscher et al. 2010). TGF- β , during the initial inflammatory phase, contributes to polarization of macrophages toward the reparative phenotype, but because of its profibrotic actions, it should be suppressed during later inflammatory phases (De Visscher et al. 2010).

The mechanical and biological requirements to the scaffold materials used for tissue engineering in situ are quite different from those used for the tissue engineering in vitro

(Bouten et al. 2011, 2012). In addition to meeting generic biocompatibility requirements, the implanted scaffold must be durable and able to carry the hemodynamic load so that its degradation properties align with the time scale of in vivo synthesis of ECM proteins.

Challenges in Heart-Valve Tissue Engineering

Preclinical experiments in sheep using in vitro engineered heart valves have highlighted two main problems, thickening and retraction of the leaflets, leading to unacceptable valve regurgitation (Schmidt et al. 2007; Flanagan et al. 2009; Gottlieb et al. 2010; Syedain et al. 2013). Furthermore, both the radial and circumferential length of the leaflets decreased over time. The retraction phenomenon appears to be independent of the scaffold material and cell source because it was observed in fibrin-based scaffold using fibroblasts (Syedain et al. 2013) or myofibroblasts (Flanagan et al. 2009), polyglycolic acid (PGA) coated with a poly-4-hydroxybutyrate (P4HB) scaffold and myofibroblasts (Schmidt et al. 2010), and a PGA-PLLA [PGA-poly(L-lactic acid)] scaffold with BM-MNCs (Gottlieb et al. 2010). Cell-mediated retraction of the leaflets might be compensated for by creating longer leaflets (Neidert and Tranquillo 2006) or adding a slowly degrading additional support scaffold next to the fibrin scaffold (Flanagan et al. 2009). Attempts have been made to reduce cell-mediated retraction by adding the myosin inhibitor blebbistatin (Syedain et al. 2013), but although this reduced cell-mediated retraction initially, leaflet retraction remained apparent beyond 4 weeks. The passive and active contribution of cells to the generated traction forces in tissue-engineered strips have been identified independently (van Vlimmeren et al. 2012). Active cell traction forces were eliminated by treatment with cytochalasin D to disrupt the actin cytoskeleton, and inhibition of the Rho-associated kinase pathway. The passive contribution of the cell can be examined by decellularizing the tissue construct. Cell-mediated retraction accounted for 85% of the observed retraction, with the remaining 15% attributed to residual stresses in the matrix; this

validates the use of decellularization to substantially reduce cell-mediated retraction in TEHV (Dijkman et al. 2012). Subsequently, reseeding of decellularized valves with bone marrow-derived MSCs does not appear to reintroduce cell-mediated retraction in vitro (Dijkman et al. 2012; Syedain et al. 2013).

Decellularized xenografts and homografts are in clinical use. In animal models, decellularized valves recellularize slowly and only partially in vivo (Goldstein et al. 2000; Elkins et al. 2001; Leyh et al. 2003; Erdbrugger et al. 2006). In humans, in vivo recellularization of xenografts remains uncertain (Sayk et al. 2005), and clinical failures have been reported (Simon et al. 2003; Ruffer et al. 2010; Lepage et al. 2012), including complete destruction of a porcine xenograft (Hiemann et al. 2010). Decellularized homografts (Cebotari et al. 2006), because they are less thrombogenic and carry a lower risk of infection, may be more appropriate for human application (Rieder et al. 2005). Recellularization has been observed in a cryopreserved aortic homograft 2 years after implantation (Miller et al. 2006). Freshly decellularized homografts for PV replacements showed improved intermediate performance compared with traditional bioprosthetic valves and cryopreserved homografts in children (Cebotari et al. 2011). However, limited donor availability may inhibit the widespread use of the decellularized homografts, in particular, for young patients.

CONCLUSIONS AND PERSPECTIVES

Significant advances in valve development genetics over the past decade have helped to decipher the underlying causes of valve disease in newborns and adults. The unifying concept that has emerged is that most, if not all, valve disease has its origin during embryogenesis, either as the manifestation of developmental processes gone awry or the aberrant reexpression of fetal gene programs normally quiescent in adulthood. The manipulation of these processes in transgenic lines and the availability of the 3D collagen explant approach have led to the identification of many signaling pathways critical for EMT. However, much remains to be under-



stood about post-EMT valve morphogenesis. Attributable to their iterative use, most signaling pathways critical for EMT are also critical for post-EMT developmental processes and result in early embryonic lethality when genetically removed. Lack of adequate *in vivo* models of post-EMT events is related to the absence of valve-specific mesenchyme enhancer(s); the creation of conditional or inducible models specifically affecting valve mesenchyme will go some way to providing answers.

Progress in heart valve bioengineering requires further understanding of the molecular cascades active during cardiac valve formation to enable more efficient *ex vivo* differentiation of valve progenitor cells. Recent advances in stem-cell biology, such as the discovery of induced pluripotent cells and improved protocols for differentiating endocardial and endothelial lineages, may provide solutions for the design of constructs that more closely match the patients' genetic makeup. Another important advance will be the design of biomaterials, which permits control of cell behavior in engineered valves *in situ*, thereby promoting more efficient recruitment of endogenous cells.

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