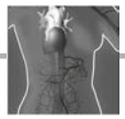
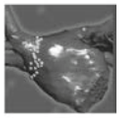


Challenges of biological valve development

The development of mechanical and bioprosthetic valvular prostheses have enhanced the survival and quality of life for countless patients. Despite the benefits provided by prosthetic valves, there are still significant limitations to those currently available. The goal biological valve tissue engineering strives to overcome is the limitations of the currently available prosthetic valves. This review will outline some of the past and present approaches that have been and are being taken in an attempt to create the ideal replacement valve. In addition, some of the challenges associated with this task will be highlighted.

KEYWORDS: aortic valve ▪ bioreactor ▪ decellularization ▪ heart valve ▪ pulmonic valve ▪ recellularization ▪ scaffold ▪ stem cell ▪ tissue engineering

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Cardiac valves are dynamic structures that open and close approximately 3 billion-times over an average lifetime [1]. The ability of cardiac valves to allow unobstructed forward flow of blood and prevent regurgitation depends on the overall structural integrity, mobility and pliability of the valve cusps/leaflets. Valves must continually adapt and undergo structural, as well as functional, changes throughout the life of the individual in order to prevent deterioration and malfunction [2,3]. Pathologic valve failure most commonly results from maladaptive structural and functional changes in response to changing physiologic conditions. The definitive treatment for valve failure is replacement; currently, the options for replacement include mechanical and bioprosthetic valves.

The development of mechanical and bioprosthetic valvular prostheses have enhanced the survival and quality of life for patients with valvular dysfunction. Despite the benefits afforded by prosthetic valves, there are still significant limitations to those currently available. The goal of biological valve tissue engineering is to overcome the limitations of the currently available prosthetic valves. The ideal tissue-engineered heart valve (TEHV) is one that can grow with the patient, remodel in response to physiologic challenges and does not require anticoagulation. This ideal valve would become a living/growing organ within the patient, one that is able to maintain homeostasis in the harsh, ever changing environment of the human cardiac cycle and eliminate the need for subsequent valve replacements owing to growth of the patient or degeneration of the prosthesis.

Current valve replacement options & unmet needs

The two types of prosthetic valves that are currently available are mechanical and bioprosthetic. Mechanical valves have similar properties and have come in four major designs over the years. The ball-in-cage type of mechanical heart valve (e.g., Starr–Edwards) was one of the first developed and is no longer commercially available. The hinged bileaflet type (e.g., St Jude Medical, MN, USA) is currently frequently used. Other types of mechanical heart valves include tilting disk (e.g., Bjork–Shiley, Shiley Corp., CA, USA; and Medtronic-Hall, Medtronic Inc., MN, USA) and trileaflet (e.g., Roscardioinvest). The leaflets of mechanical valves are usually constructed of pyrolytic carbon owing to its excellent biocompatibility, low thrombogenicity and high durability, making it superior to the metals and plastics that have previously been used. The major benefits to using mechanical valves for valve replacement are their excellent durability (20–30 years) and flow dynamics, which are similar to the native valve. The major drawback of mechanical valves is the need for chronic anticoagulation using vitamin K antagonists [4].

Bioprosthetic heart valves are frequently secured in a support frame (stented), although less commonly, they can be without the support frame (stentless). They are available in three main types, determined by the tissue from which they are made. These materials are most commonly cusps from porcine aortic valves, bovine pericardium and cadaveric homografts. Typically bovine and porcine bioprosthetic valves are glutaraldehyde fixed, whereas cadaveric homografts

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are not. The major benefits of bioprosthetic valves are that they have a very low incidence of thromboembolism and lifelong anticoagulation is usually not necessary. In addition, they have flow dynamics similar to native valves and they can be delivered percutaneously in some patients [5]. The major drawbacks of bioprosthetic valves are their limited durability, with a significant failure rate at 10 years in all patients, and an even higher failure rate in pediatric and young adult populations [4,6]. A major reason for the decreased durability of bioprosthetic valves is that they are fixed in glutaraldehyde to eliminate antigenicity. The glutaraldehyde fixation process affects the valve durability owing to devitalization of the tissue and subsequent accelerated calcification.

While there is significant overlap between the valve-replacement needs of adult and pediatric patients, there are also important differences. The most significant difference is the need for growth of the replacement valve in the pediatric population. Pediatric aortic valve disease has many etiologies (e.g., congenital, rheumatic and infectious) with the final common pathway being valuvlar stenosis or regurgitation. In addition, congenital heart disease often requires the replacement of the pulmonic valve in the right ventricular outflow tract, which is uncommon in adult patients. Aortic valve replacement in children has classically been a procedure of last resort, being performed only when multiple other treatments have failed. A major reason for the delay or avoidance of aortic valve replacement in children is the inadequate performance of the valve substitutes currently available. The use of mechanical and fixed-tissue valves in the pediatric population is limited by their inability to grow, repair and remodel, necessitating repeat surgeries to enlarge the valve as the patient grows. Bioprosthetic valves (porcine or pericardial) used in children are limited by their rapid calcification and requirement for early reoperation. Mechanical prostheses, while durable, are susceptible to other complications, including hemolysis and thromboembolism. The use of anticoagulation with vitamin K antagonists, which is required with all mechanical valves, presents significant additional risks and challenges in the pediatric population and in the case of females who are, or desire to become, pregnant [7]. Owing to the limited options in the pediatric population, the Ross procedure is commonly used in children requiring a replacement aortic valve, whereby the autologous pulmonic valve is used to replace the aortic valve and an allogenic pulmonic valve is used to replace the pulmonic valve [8,9]. This procedure has improved outcomes in pediatric patients by placing a homograft in the higher-pressure aortic-valve position. The benefits of using a pulmonary autograft in the aortic position is that the living graft can grow with the child, decreasing the need for reoperation and eliminating the need for anticoagulation that would be required with the use of a mechanical valve. Despite its benefits, this procedure has its complications and is clearly not optimal, as it requires sacrificing a healthy valve to treat the diseased valve and, in the end, requires two valves being replaced to treat a single diseased valve [10,11].

In adults, aortic stenosis (AS) is the most common form of degenerative valve disease. The treatment of choice for severe AS is replacement of the aortic valve. The prevalence of AS increases with age and it is seen in 2.8% of all patients over 75 years of age [12]. The frequency of AS continues to increase with the general aging of the population in North America and Europe. Owing to the frequency of AS aortic valve replacement, procedures are commonplace with over 200,000 performed worldwide each year in adults alone [13]. The choice of which prosthetic valve to use in adults usually revolves around the age of the individual and the individual factors associated with chronic anticoagulation use. Owing to the limited durability of bioprosthetic valves, they are frequently reserved for older individuals and those that have contraindications to chronic anticoagulation.

Strategies of biological valve development

Owing to the limitations of the available prosthetic valves, there are a variety of strategies currently under investigation to develop a TEHV. At this point, there is not one clear pathway to success. The variables in play with regards to TEHV development include the material used as a scaffold (biological vs synthetic), the methods used to fabricate these materials into a valve construct and the type of cells used to seed the construct.

When deciding among the various cell types and scaffold materials available for the development of a TEHV, a thorough understanding of the anatomy and histology of the native valve is essential. The architecture of a native heart valve consists of three layers: the ventricularis on the inflow side, the spongiosa in the middle and the fibrosa on the outflow side. Valvular interstitial cells (VICs) are found throughout all three

layers; however, they are more concentrated in the spongiosa. Valvular endothelial cells (VECs) line the surfaces of the valve cusps and are phenotypically distinct on the inflow and outflow surfaces. The fibrosa contains a dense matrix of collagen aligned circumferentially for mechanical strength during diastole. The spongiosa contains glycosaminoglycans (GAGs) for lubrication during flexure. The ventricularis contains collagen and elastin for efficient coaptation during valve closure and distensibility during diastole.

The complex structure of the native semilunar valves leads to complex biomechanical functionality. Achieving similar functionality with a TEHV requires careful cell and scaffold selection, as well as a strategy for directing those components into a functional tissue. Two major paradigms of TEHV development have been described (Figure 1). The first pathway of TEHV design involves an *in vitro* stage of cell seeding, sometimes followed by a conditioning phase in a bioreactor, prior to the *in vivo* phase, which is implantation in a large animal model. The second pathway consists of an unseeded scaffold that is placed directly into a large animal model with the expectation that the scaffold contains biological signals necessary to attract the appropriate cells *in vivo*. The following section will review some strategies that have been previously attempted taking into account the different approaches with regards to scaffold materials, fabrication methods and recellularization, including cell types and timing of cell introduction.

Scaffolds

The role of the scaffold is to provide an initial architecture for cellular attachment. Ideally, the scaffold would provide the appropriate biomechanical and biochemical signals to allow for cell attachment and migration, setting in motion the process of remodeling, repair and growth. Scaffolds can be made of synthetic or biologic materials.

■ Synthetic scaffolds

Synthetic scaffolds can be made of either biodegradable polymers or nonbiodegradable polymers. Synthetic scaffolds ideally should have a highly porous microstructure and a surface that allows for cellular attachment. Ideally, the scaffold would completely bioresorb without producing toxic byproducts as the cells construct their own matrix. The advantages to synthetic scaffolds are that they are easily produced and allow for control of the material structure/properties such as pore size and degradation rate. Disadvantages to using synthetic scaffolds include limited perfusion of nutrients to cells, premature degradation, lack of biocompatibility and difficulty controlling cell adhesion. The most common synthetic scaffold materials in use today are the polymers polyglycolic acid, polylactic acid and poly(-lactic-co-glycolic) acid. In addition, poly (ε-caprolactone), polyhydroxyalkaneoate, poly-4-hydroxybutyrate (P4HB) and polyurethane have been used to fabricate TEHV.

Nonbiodegradable scaffolds persist and, therefore, need to interact favorably with the recipient over an extended period of time. Polyhedral oligomeric silsesquioxane-poly(carbonate-urea) urethane (POSS-PCU) is a new class of nonbiodegradable polymer, and it offers several advantages over previous materials for synthetic heart valve leaflet fabrication [14]. In addition to having suitable mechanical properties, POSS-PCU has been shown to exhibit superior biocompatibility, hemocompatibility, antithrombogenicity, resistance to calcification and resistance to inflammation [15,16]. Biocompatibility and hemocompatibility can be further enhanced by the formation of a viable endothelium on the surface of the leaflets. POSS-PCU has demonstrated the potential for *in situ* self-endothelialization, which can be harnessed by *in vivo* tissue-engineering strategies [16].

■ Biologic scaffolds

Biologic materials include collagen, fibrin or decellularized aortic/pulmonic valve scaffolds, which are a combination of collagen and elastin. In addition, alginate [17,18] and haluronan [19,20] have also been investigated as a possible biologic scaffold material. The potential advantages to biologic scaffolds are that they maintain the architecture of the native tissue and they can maintain biological signaling cues (GAGs and growth factors, among others) that can help guide cellular recruitment, adhesion, migration and differentiation. Some disadvantages to biologic scaffolds include the difficulty of getting cells to migrate into the interior and possible immunogenicity associated with xenogenic transplants [21,22].

One common biological scaffold option is the decellularized porcine xenograft. Ideally, human homografts would be used as the source of scaffolding; however, owing to the limited supply of human valves, porcine valves have been used due to their availability and similar anatomy to human valves. A potential benefit

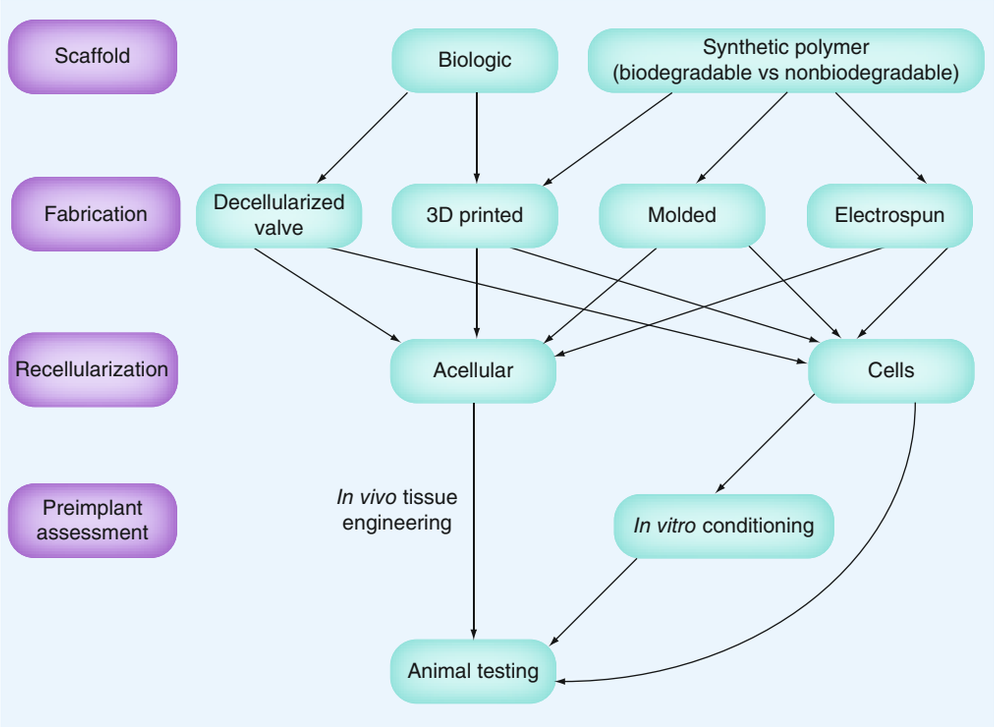

Figure 1. Schema depicting the variables under consideration in the design of a tissue-engineered heart valve. This schema is based upon the literature and highlights the available methods that have been reported.

to using a decellularized scaffold is that there is no additional fabrication necessary and intrinsic molecular cues directing cellular migration and differentiation may be retained. Following the decellularization process, the product should be an intact valve with retained mechanical properties. Studies have shown the ability of recellularization, as well as matrix remodeling and growth potential of decellularized xenografts, in juvenile sheep models [23,24]. Decellularization has been attempted by numerous groups using a variety of protocols. The major agents for decellularization can be categorized in the following groups: chemical agents that include acids/bases, hypotonic/hypertonic solutions, ionic detergents, nonionic detergents, zwitterionic detergents and solvents; biologic agents that include enzymes and chelating agents; and physical and miscellaneous agents including temperature, application of force and pressure. Some common agents used in the decellularization of heart valve tissue include sodium dodecyl sulfate [25–28], triton X-100 [28–30] and the combination of triton X-100 and sodium cholate [31–33]. During the decellularization process, care must be taken to minimize disruption to the extracellular matrix (ECM; i.e., preserve matrix integrity) and remove all remaining decellularization solution. It has been demonstrated by solid phase extraction and HPLC that intensive washing of the scaffold following decellularization can lower the residual decellularization solution to noncytotoxic levels (<50 mg/l) where cellular repopulation is achievable [27]. Despite removal of all cellular material, it is possible that some residual immunogenicity may be present due to collagen and elastin [21]. In addition to the removal of cellular material, ensuring the removal of the major porcine antigen, the α-Gal epitope, has been demonstrated to be important in avoiding hyperacute rejection of untreated porcine valves [34].

■ Methods of scaffold fabrication

The methods used to fabricate the scaffold materials into a valve construct differ depending on the material used. Reported methods of fabrication include molding, electrospinning and bioprinting. Molded valves have been made using fibrin [35–38], polyglycolic acid [39,40], polyhydroxyalkaneoate [41] and P4HB [42]. Molding offers precise morphology; however, it is limited by the fact that the material must be homogenous, which does not allow for multiple cell types in different regions.

Electrospinning is a technique that can be used to produce polymeric fibers with diameters ranging from nano- to micro-meters that can

be intertwined in a meshlike structure. TEHV design has been attempted using electrospinning with poly(ε-caprolactone) [43,44] and poly (ester urethane) ureas [45] and have shown promise in producing the scaffold of a TEHV. These electrospun scaffolds offer the advantage of high porosity and anisotropic mechanical properties closely resembling that of native heart valve cusps.

Butcher and colleagues have demonstrated the potential effectiveness of bioprinting using a 3D printer and alginate/gelatin hydrogel [17,18]. With this process they have reported the ability to print an anatomical architecture with the direct incorporation of two cell types in a regionally constrained manner, which would not be possible with traditional molding. The results of their work demonstrate that anatomically complex heterogeneously encapsulated valve conduits can be produced with 3D bioprinting.

Cells

Native valve cusps consist of two cell types: VECs and VICs (**Box 1**). VICs are the most abundant cell type and are responsible for synthesis, degradation and maintenance of the ECM [46,47]. VICs are a dynamic population of cells that undergo phenotypic transition during the life of the individual depending on the physiological or pathological conditions to which the valve is exposed [48]. The vast majority of VICs in the healthy adult valve are quiescent and fibroblast-like; however, when activated by changes in mechanical stress or disease states, VICs become myofibroblast-like. Recapitulation of a VIC-like cell population is central to the production and maintenance of a functional valve with the ability to grow and adapt. Theoretically, the ideal tissue-engineered valve would be constructed from autologous and easily obtained cell sources that could perform functions of the native fibroblast/myofibroblast VICs.

Differentiated cell types, as well as progenitor/stem cell types, from both humans and other animal sources have been used for the recellularization of TEHV scaffolds (**Box 1**). The differentiated cell types that have been used include: ovine endothelial cells and fibroblasts [49], ovine endothelial cells and myofibroblasts [40,50], ovine bone marrow mononuclear cells [51], nonhuman primate bone marrow mononuclear cells [52], human VECs [53] and human VICs [54]. Progenitor/stem cell types that have been used include: ovine mesenchymal stem cells [51,55,56], ovine peripheral blood endothelial progenitor cells [57,58], human umbilical cord-derived progenitor cells [59] and human bone marrow mesenchymal stem cells [32].

One of the major considerations with autologous cell harvest is determining a site of harvest that is easily accessible. Recent work from the SCIPIO and CADUCEUS trials have laid the ground work for the generation and delivery of autologously-derived cardiac cells and demonstrated the feasibility of this approach in trials of cardiac regeneration [60,61]. Thus, one could consider using cells generated from cardiac biopsies for *in vitro* seeding of TEHVs.

Some groups have seeded cells on scaffolds and then conditioned those constructs in bioreactors prior to implantation in large animal models. Sodian *et al.* reported early attempts to seed polyhydroxyalkaneoate scaffolds with ovine carotid artery vascular cells followed by up to 8 days of bioreactor conditioning [41]. They report cell proliferation and migration, as well as collagen and GAG production. From the same group, Hoerstrup *et al.* improved upon that approach by seeding ovine carotid artery endothelial cells and myofibroblasts onto

Box 1. Comparison between the cell types that make up the native semilunar valve cusps and reported cell types that have been used for recellularization.

Cells found in the native valve cusps

- Valvular endothelial cells:
  - Phenotypically different from vascular endothelial cells that line the adjacent aorta
  - May interact with valvular interstitial cells to maintain homeostasis
- Valvular interstitial cells:
  - Most abundant type of cell in the cusps
  - Responsible for synthesis, degradation and maintenance of the extracellular matrix
  - Dynamic cells that undergo phenotypic transitions during the life of the valve
  - Majority are quiescent and fibroblast-like
  - When activated by mechanical stress of disease states they become myofibroblast-like

Reported cell types/sources used for recellularization

- Ovine cell types:
  - Endothelial cells and fibroblasts [46]
  - Endothelial cells and myofibroblasts [37,47]
  - Bone marrow mononuclear cells [49]
  - Mesenchymal stem cells [48,52,53]
  - Endothelial progenitor cells [54,55]
- Nonhuman primate cell types:
  - Bone marrow mononuclear cells [49]
- Human cell types:
  - Vascular endothelial cells [50]
  - Valvular interstitial cells [51]
  - Umbilical cord-derived progenitor cells [56]
  - Bone marrow mesenchymal stem cells [29]

P4HB-coated polyglycolic acid scaffolds followed by up to 28 days of bioreactor conditioning [40]. They report collagen, DNA and GAG content to be approximately 85, 60 and 60%, respectively, compared with native tissue. Elastin production was not detected. These valves functioned well in a sheep model for up to 20 weeks. Since these pioneering studies, several groups have taken the approach of seeding cells onto a scaffold and conditioning the cell-seeded constructs in a bioreactor. Examples include ovine carotid artery endothelial cells and myofibroblasts seeded onto decellularized porcine pulmonary valves [62], human umbilical cord cells seeded onto P4HB [42], ovine carotid artery vascular cells seeded onto fibrin [63], human dermal fibroblasts seeded onto fibrin [64], ovine bone marrow-derived mesenchymal stem cells seeded onto poly(-lactic-co-glycolic) acid [65], and human VECs and fibroblasts seeded onto polyurethane [66].

Other groups have taken the approach of eliminating the bioreactor and moving directly from a recellularized valve into large animal models. Hoerstrup *et al.* have recently reported success using synthetic scaffolds seeded with autologous cells percutaneously implanted in large animals (ovine and nonhuman primates) without the additional conditioning in a bioreactor [52,67]. Their work has demonstrated the potential feasibility of a transcatheter, stem cell-based TEHV implantation into both the pulmonic- and aortic-valve positions within a one-step intervention.

Still others have taken the approach eliminating all *in vitro* work and placing an acellular valve in a large animal model and allowing the recipient to recellularize the graft after implantation an approach that has been termed *in vivo* tissue engineering. A number of studies have reported success with this approach [23,68–72]. A possible advantage to the *in vivo* tissue engineering approach is that it is less time consuming and has the potential for off-the-shelf availability. A possible concern with the *in vivo* tissue engineering approach of allowing an acellular graft to become recellularized by the host is that pathologic cells may be the cells that attach and repopulate speeding up the eventual degeneration or calcification of the scaffold. The success of this method could theoretically differ on a patient specific basis. For example, one could imagine that the cells repopulating the valves could differ in young versus old patients and in patients with multiple comorbidities (diabetes, hypertension and hyperlipidemia, among others) versus healthy individuals.

Yoo *et al.* has tried to overcome this potential limitation of *in vivo* tissue engineering by attempting to attract a specific cell population through antibody labeling of their decellularized constructs [73]. They have shown early success using CD133 antibody labeling of decellularized porcine pulmonic valves in an attempt to attract an endothelial progenitor cell type following implantation. Their results demonstrated that the decellularized CD133 antibody labeled valves had improved recellularization with endothelial cells when compared with unconjugated valves and valves that were unconjugated and statically seeded with endothelial cells prior to implantation. In addition, the CD133 antibody conjugated valves showed increased interstitial cell and structural protein content after 1 and 3 months.

Finally, a unique approach undertaken by Tranquillo *et al.* has used a combination of the two above methods [38]. They initially use cells in a fibrin mold and allow these cells to produce their own matrix *in vitro*. After a period of time when enough matrix has been deposited the scaffold is then decellurized and implanted in a large animal model and allowed to be recellularized by the animal. Their early results have shown success with this method and they have produced engineered leaflets with similar tensile properties and collagen content compared with native leaflets.

In vitro conditioning & testing

■ Purpose of a bioreactor

A cell-seeded scaffold provides a starting point for the generation of a TEHV. Additional *in vitro* tissue remodeling may be necessary before the valve is ready for successful *in vivo* function, remodeling and maintenance. A bioreactor can be used to provide biomechanical and biochemical stimuli to a TEHV in a controlled environment in order to direct *in vitro* tissue formation [74,75].

One goal of a bioreactor is to develop proper tissue architecture. This requires positioning the proper cells and generating the proper ECM components in the various regions of the tissue. When subjected to appropriate stimuli, cells may proliferate, migrate, differentiate and align in a manner that distributes the desired cell types throughout the tissue. In addition, cells may resorb, synthesize and align ECM in a manner that distributes and properly orientates the desired ECM constituents throughout the tissue. The ECM, in turn, provides signaling cues to the cells, which further direct tissue formation. Furthermore, turnover of the original scaffold

material and replacement with autologous ECM may be critical to the acceptance of the TEHV upon implantation.

Another goal of a bioreactor is to achieve proper tissue function. The functionality of a TEHV depends upon its biomechanics, which in turn depends upon the tissue's underlying structure. Thus, it is critically important for a TEHV to achieve and maintain proper morphology and ECM architecture throughout its entire service life. Degeneration, thickening and calcification of the cusps alter their biomechanics and can lead to catastrophic valve failure *in vivo*.

■ Functions of a bioreactor

Bioreactors can expose TEHV to a variety of biomechanical stimuli, including stretch, flexure, shear and pressure. Cyclic stretch has been shown to increase cell proliferation [76], collagen synthesis [76–79], stiffness [78] and GAG content [80]. Cyclic flexure has been shown to increase collagen content [81,82], stiffness [81,82] and cell migration [81]. Oscillating fluid shear stress has been shown to increase strength [83], alignment [84,85], inflammation resistance [84,85], protection from calcification [84,85], GAG content [86] and protein content [86]. Pressure has been shown to increase collagen synthesis [87–89] and increase GAG synthesis [88,89].

Bioreactors can also expose TEHV to a variety of biochemical stimuli, including growth factors, nutrients and dissolved gasses. These factors can stimulate cells to proliferate, migrate, differentiate and synthesize ECM. For example, FHGF was shown to increase endothelial cell proliferation [90], TGF-β1 and insulin were shown to increase elastin and collagen production [91], HGF was shown to promote cell adhesion [92], bFGF was shown to increase cell proliferation [93,94] and migration [94], as well as collagen production [65], ascorbate was shown to increase collagen synthesis [93], VEGF was shown to promote endothelial cell proliferation [95,96] and TGF-β1 was shown to induce endothelial cell differentiation [95].

An additional benefit of bioreactors is the improved nutrient diffusion created by convection. Many bioreactor designs incorporate perfusion or mixing of culture medium, which helps to evenly distribute nutrients around and within a tissue. This makes it possible for cells deep within the tissue to survive owing to nutrient diffusion alone.

■ Current bioreactor designs

Various cardiac-valve bioreactor designs have been described in the literature and they tend to fall into three main categories based on their objective: flow-based whole-valve conditioning (Table 1); strain-based whole-valve conditioning (Table 2); and isolated cusp stimulation (Table 3). The first bioreactor type seeks to condition a TEHV by simulating conditions similar to physiological systole and diastole. This is performed by pulsing media through the valve lumen in a manner that opens and closes the cusps with the proper pressure gradients. The second bioreactor type seeks to condition a TEHV by simulating conditions similar to physiological diastole. This is performed by cyclically pressurizing media around the valve, such that the tissue strains but the cusps remain closed and there is no flow through the valve. The third bioreactor type allows isolated cusps or cusp segments to be studied for their response to specific biomechanical stimuli.

Flow-based whole-valve conditioning bioreactors typically achieve desired flows and pressures by utilizing a computer-controlled pump, a capacitance element and a resistance element [97]. Other common elements include a valve chamber, a media reservoir and a gas exchanger (Table 1). More advanced systems include sensors for flow, pressure, temperature, pH, pO_2, pCO_2, glucose, lactate and valve deformation. These sensors are used for system monitoring [63,98–101], feedback control [99,102,103] and tissue monitoring [102–106]. Videos or photographs of the valve may also be captured using a digital camera [100,101,105,107], endoscope [108] or microscope [109].

The first TEHV bioreactor design was described by Hoerstrup *et al.* [110]. Their design utilized a diaphragm pump to generate 50–2000 ml/min of flow through the valve. The resulting pressures ranged from 10 to 240 mmHg, but no independent pressure control was possible. The group pioneered key concepts including flow control, sterilizability, a media reservoir and housing the system within an incubator. They were able to generate functional TEHVs using this system in their subsequent studies [40–42].

Independent pressure and flow control were achieved by Dumont *et al.* by utilizing a variable capacitance element and a variable resistance element [111]. The capacitance is varied by pumping air in or out of a chamber that is filled partially with circulating culture medium. The resistance is varied by tightening or loosening a clamp on a segment of tubing that carries circulating culture medium. Together, these two elements create an afterload on the valve by mimicking the compliance of the large arteries and the resistance of the arterioles and capillaries. A flow probe and a

Table 1. Flow-based whole-valve bioreactor designs reported in the literature.

Study (year)	Pump	Res	Cap	Mount	Materials	Sterilization	Tissue	Comments	Ref.
Konig *et al.* (2012)	Pneumatic	No	No	Suture	Acrylic	Formaldehyde	Valve	Endoscopic visualization	[108]
Kaasi *et al.* (2011)	Pneumatic	Yes	Yes	Suture	n/s	Autoclave	Valve	Ventricular assist device	[101]
Sierad *et al.* (2010)	Pneumatic	Yes	Yes	Suture	Acrylic	Autoclave	Valve	Can accommodate various valve types	[100]
Ruel and Lachance (2010)	n/a	Yes	Yes	n/a	n/a	n/a	n/a	Windkessel model, RRC configuration is best	[97]
Ziegelmueller *et al.* (2010)	Pneumatic	No	No	n/s	n/s	etOH	Valve	Optical monitoring	[107]
Durst and Grande-Allen (2010)	None	No	No	Suture	n/s	Autoclave	Valve	Valves mounted to actuating pistons	[128]
Lee *et al.* (2009)	Gear	Yes	Yes	Suture	Polycarb	n/s	Valve	Laminar flow through valve	[58]
Ruel and Lachance (2009)	Pneumatic	Yes	Yes	O-ring	n/s	n/s	Valve	Windkessel RC model	[129]
Migneco *et al.* (2008)	Peristaltic	Yes	No	Clamp	n/s	n/s	Valve	Heated reservoir	[130]
Flanagan *et al.* (2007)	Pneumatic	No	No	Suture	Plexiglass	Gas plasma	Valve	Column of fluid for afterload	[63]
Morsi (2007)	Pneumatic	No	No	n/s	PMMA	n/s	Valve	Multiple valve types, laser Doppler validation	[131]
Lichtenberg *et al.* (2006)	Piston	No	Yes	n/s	n/s	n/s	Valve	Cell seeding inlets	[98]
Karim *et al.* (2006)	n/s	No	No	n/s	Glass	n/s	Valve	For decell and recell	[132]
Warnock *et al.* (2005)	Pneumatic	No	Yes	n/s	n/s	Autoclave	Valve	Silicon tubes for gas exchange	[133]
Hildebrand *et al.* (2004)	Pneumatic	Yes	Yes	Suture	Polycarb	etOH	Valve	First sophisticated system	[99]
Narita *et al.* (2004)	Pneumatic	Yes	Yes	n/s	Acrylic	etOH	Valve	Digital camera	[106]
Schenke-Layland *et al.* (2003)	Pneumatic	No	No	n/s	n/s	Autoclave	Valve	Similar to Zeltinger except valve below bladder	[62]
Dumont *et al.* (2002)	Pneumatic	Yes	Yes	n/s	PMMA	etOH	Valve	First resistor and capacitor	[111]
Zeltinger *et al.* (2001)	Pneumatic	No	No	Staple	n/s	Electron-irradiation	Valve	More pressure control	[134]
Hoerstrup *et al.* (2000)	Pneumatic	No	No	Suture	PMMA	etOH	Valve	First system	[110]

Cap: Capacitance element; etOH: Ethanol; n/a: Not applicable; n/s: Not specific; PMMA: Poly(methyl methacrylate); Polycarb: Polycarbonate; RC: Resistor–capacitor; Res: Resistance element; RRC: Resistor–resistor–capacitor.

pressure transducer were used to demonstrate a wide range of physiological flows and pressures.

A very sophisticated system capable of achieving a range of flow and pressure conditions, including both pulmonary and systemic conditions, was described by Hildebrand *et al.* [99]. Their design included a computer-controlled resistance element, a capacitance element and sensors for pressure and flow. A feedback control loop was employed to adjust the driving pressure and the resistance in order to automatically maintain the mean pressure and mean flow. Proportional pressure regulators were used to generate highly detailed driving pressure waveforms at prescribed stroke volumes and beat frequencies.

As examples of more recent designs, Kaasi *et al.* [101] and Sierad *et al.* [100] introduced systems utilizing pneumatic pumps, capacitance elements and resistance elements to achieve various flow and pressure conditions. Both designs require the valves to be mounted by suturing, which is tedious and increases the possibility for contamination; however, a more simplistic and reliable method has yet to be described. In addition, both designs are capable of achieving only pulmonary circulation conditions, underscoring the difficulty of achieving systemic circulation conditions even with modern designs.

The second category of bioreactor designs is the strain-based whole-valve conditioning approach (Table 2). This approach was pioneered by Mol *et al.* with their diastolic pulse duplicator [39]. The valve is cyclically pressurized in its closed state in a manner mimicking diastole. This approach is hypothesized to accelerate tissue growth and development since the valve experiences the largest strain during diastole [112]. This group was able to demonstrate successful tissue conditioning using the diastolic pulse duplicator approach [39,113].

Precise control of strain is important for the success of the strain-based approach; however,

Table 2. Strain-based whole-valve bioreactor designs reported in the literature.

Study (year)	Pump	Res	Cap	Mount	Materials	Sterilization	Tissue	Comments	Ref.
Vismara *et al.* (2010)	Peristaltic	No	No	n/s	PMMA	etOH	Valve	Strain-based, compliance monitoring, DPS	[103]
Syedain and Tranquillo (2009)	Piston	No	No	n/s	n/s	n/s	Valve	Radial cyclic stretch	[105]
Kortsmit *et al.* (2009)	Pneumatic	Yes	No	n/s	n/s	n/s	Valve	Strain-based, deformation feedback control	[102]
Kortsmit *et al.* (2009)	Pneumatic	Yes	No	n/s	n/s	n/s	Valve	Strain-based, volumetric deformation measurement	[104]
Mol *et al.* (2005)	Pneumatic	No	Yes	n/s	Polycarb	etOH	Valve	Strain-based, DPD	[39]

Cap: Capacitance element; DPD: Diastolic pulse duplicator; DPS: Diastolic pulsed stimulation; etOH: Ethanol; n/s: Not specific; PMMA: Poly(methyl methacrylate); Polycarb: Polycarbonate; Res: Resistance element.

this is made difficult by the changing biomechanical properties of the developing tissue. One solution is to design feedback control algorithms based on measured estimates of the strain in order to consistently achieve the desired strain value. One such feedback control system was described by Kortsmit *et al.*, who estimated strain based on the volume of fluid pushed into the valve during each cycle [102,104]. Another feedback control system was developed by Vismara *et al.*, who utilized pressure as the control variable [103].

The complexities of a feedback control algorithm were avoided by Syedain and Tranquillo who described a strain-based approach that generates consistent cyclic radial distension by mounting a TEHV inside a latex tube [105]. Consistent strain is achieved, since the stiffer latex tube dominates the mechanical response. Distention is achieved by means of a syringe pump that cyclically pressurizes culture media within the tube. This approach also introduces the possibility of simulating somatic growth by increasing the radial strain.

The third category of bioreactor designs focuses on exposing isolated cusps or cusp segments to specific mechanical cues (Table 3). Rather than conditioning a whole valve for the purposes of tissue engineering, these bioreactors enable researchers to study the effects of biomechanical stimulation on isolated tissue specimens. These bioreactors have been designed to expose tissue specimens to strain [79,109,114,115], flow [116–118], pressure [119] or a combination thereof [120].

■ Benefits of bioreactor conditioning

Both the biomechanical and the biochemical stimuli are thought to contribute to the conditioning of a TEHV in a bioreactor. The benefits of conditioning a TEHV in a bioreactor have been demonstrated in numerous studies. For example, some groups have demonstrated improved cell distribution throughout the matrix due to increased proliferation and migration [39,41,42,62,63,66,82,106,113,117]. Other groups have demonstrated improved ECM composition and alignment [39,41,42,62–66,79,106,113,117]. In addition, other groups have demonstrated improved mechanical properties, such as stiffness and strength [39,62,113]. These benefits can be realized after days or weeks of conditioning within a bioreactor.

Table 3. Isolated cusp stimulation bioreactor designs reported in the literature.

Study (year)	Pump	Res	Cap	Mount	Materials	Sterilization	Tissue	Comments	Ref.
Metzler *et al.* (2012)	None	No	No	n/s	Polycarb	Autoclave	Cusp	Cyclic stretch, confocal imaging	[109]
Sun *et al.* (2011)	Peristaltic	No	No	Suture	Polycarb	n/s	Cusp	Side-specific shear stress	[118]
Schipke *et al.* (2011)	Pneumatic	No	No	None	n/s	n/s	Cusp	Cyclic pressure	[119]
Barzilla *et al.* (2010)	None	No	No	n/s	Polycarb	etOH, UV	Cusp	Splashing rotating system	[117]
Engelmayr *et al.* (2008)	Paddle	No	No	Spiral	Polycarb	etOH	Strip	Flex, stretch, flow	[120]
Balachandran *et al.* (2006)	None	No	No	Suture	Polysulfon	Gas plasma	Cusp	Cyclic stretch	[79]
Engelmayr *et al.* (2003)	None	No	No	Pins	PMMA	etOH	Strip	Flex bioreactor	[114]
Weston and Yoganathan (2001)	Peristaltic	No	No	Suture	Polycarb	n/s	Cusp	Parallel plate flow chamber	[116]

Cap: Capacitance element; etOH: Ethanol; n/s: Not specific; PMMA: Poly(methyl methacrylate); Polycarb: Polycarbonate; Res: Resistance element; UV: Ultraviolet.

Nevertheless, some groups have reported success implanting their TEHVs into animal models without any bioreactor conditioning [52,67,73], and it remains unclear how necessary bioreactor conditioning is to the success of certain types of TEHVs. Potential benefits need to be weighed against drawbacks of bioreactor conditioning, such as the additional development time, increased costs, added procedural complexity and the risk of contamination.

Independent of their role in conditioning TEHVs, bioreactors may also be used for *in vitro* testing of function and durability prior to testing in an animal model. This requires a bioreactor capable of achieving physiological flow and pressure conditions, as well as other key physiological parameters. It may even be possible to test the valve's response to simulated somatic growth [105].

Preclinical testing

Regardless of the roles that bioreactors will ultimately play in valvular tissue engineering programs, there will come a point in which the tissue-engineered construct will need to undergo further evaluation in a large animal model. The most common model for the testing of valves is the ovine model [121]. This has long been considered the gold standard by which prosthetic valves are studied owing to the exuberant fibrotic response and rapid calcification that it produces [121,122]. The theory has been that if a valve can withstand the more demanding conditions of the ovine model, then it will be able to withstand the less harsh environment of the human cardiac cycle. This exaggerated response does, however, raise some questions with regards to the translatability of the results into humans. A particular case that highlights this concern was with the preclinical testing of the Sulzer Carbomedics PhotoFix®-α pericardial valve. This valve performed well in the ovine model but then developed severe abrasions to the leaflets when implanted in humans [122]. Eventually the leaflet abrasion problem was attributed to a design flaw that was not noticed in animal testing owing to the exuberant fibrotic response. Owing to the differences seen in the ovine model, some groups have begun to use nonhuman primates for more extensive testing following the ovine experiments. A rigorously validated animal model that is known to correlate with human outcomes will be essential to demonstrating safety and efficacy prior to future human studies [123].

Another question regarding animal models is where anatomically (aortic vs pulmonic position) is the best site to test the tissue-engineered construct? This may depend on the ultimate planned site of human implantation. Many groups have chosen to use the pulmonic position owing to milder hemodynamic conditions.

Finally, what is the required duration of large animal experiments that will give us enough information about the long-term durability? To date, studies have used a variety of time points from days, months to over a year. The optimal time point likely depends on what is specifically being evaluated, but to truly understand the long-term durability, the optimal time point has likely not been established.

Challenges for the translation of engineered tissue valves: preclinical to clinical studies

Despite the limitations, the function and durability of the current generation of prosthetic heart valves have set the bar high for the durability and performance requirements of a TEHV. A major question for the translation of TEHVs is when should a construct be taken from the preclinical testing in large animals and evaluated in humans? Two clinical trials that resulted in very poor outcomes have highlighted the importance of this question.

■ Clinical trials using TEHVs

The initial trial used the Synergraft™ valve (Cryolife Inc., USA) that was described as the "first tissue-engineered decellularized porcine heart valve" [124]. It was approved and received the CE mark in Europe in 2000 and was introduced as an alternative to conventional biological valves. The decellularized porcine constructs were either aortic composite grafts or whole pulmonary roots and were supposedly rendered cell free by a proprietary process. In 2001, four valves were implanted in children (aged 2.5–11 years) in the right ventricular outflow tract as a root. The results of these implantations was that three children died, two suddenly with severely degenerated valves (6 weeks postoperation and 1 year postoperation), and the third child died on the seventh postoperative day owing to an acutely ruptured valve. Owing to the poor results the forth graft was explanted prophylactically 2 days after implantation. Upon histological evaluation, all grafts showed severe inflammation starting on the outside (day-2 explant) leading to structural failure seen in the day-7 explant and severe degeneration of the leaflets and wall seen in the 6-week and 1-year explants. Significant calcific deposits were seen at all stages of valve harvest and no

cell repopulation of the porcine matrix occurred even at the 1-year explant time point. Tragically, preimplant samples revealed incomplete decellularization and calcific deposits.

The second clinical trial also involved the implantation of xenogenic decellularized tissue-engineered pulmonary valve conduits in patients undergoing reconstruction of the right ventricular outflow tract [125]. Between 2006 and 2010, 93 patients underwent right ventricular outflow tract reconstruction using Matrix P™ and Matrix P Plus™ valves (AutoTissue GmbH, Berlin, Germany). A total of 33 patients (35.5%) experienced conduit failure, and conduit dysfunction occurred in 27 (29%) of the patients. The most common reason for conduit failure was stenosis in 20 cases (60%). Histological examination showed inflammatory giant-type cells and poor autologous recellularization in all explanted valves.

These two trials highlight the importance of rigorous *in vitro* and preclinical animal studies prior to human trials. They also suggest that when using a decellularized construct self repopulation by the recipient with circulating cells, without any preimplant, recellularization is not likely to be effective in humans. While ensuring the quality control of constructs seems simple, there has not been a universally accepted definition of what a safe tissue-engineered valve should consist of or how it should function prior to implantation in humans. In addition, individual patients may respond differently to a tissue-engineered valve, which may make predicting the outcome of a replacement valve more difficult then with the currently available prosthesis. It is possible that we may see dramatic differences in how the valve is recellularized or integrated into the host depending on individual factors associated with the recipient. One could imagine the pediatric population having a more exuberant response to the valve, which could be either beneficial or detrimental to its ultimate function. Older patients with multiple comorbidities could theoretically have a more difficult time repopulating the valves with healthy cells that could recapitulate the function of the native VICs. Because of these factors, the patient population that the valve is going into may determine the type of cells and source of cells used for recellularization. Thus, the field may not focus on creating one perfect tissue-engineered valve to be used in all patients, but multiple valves with different design strategies that could be individualized for different patient populations. In order to predict the success of a TEHV, a means of identifying important patient-specific factors (i.e., genetic characteristics or biomarkers) that could reliably predict patient-specific outcomes are essential. In addition, the development and validation of *in vivo* imaging/monitoring to ensure appropriate valve development and function would be useful tools for helping physicians predict outcomes and tailor therapy to optimize valve function [123,126].

At this point, it is clear that an optimal translational approach has not been identified to help move from preclinical studies into clinical trials using TEHVs. Owing to the high standards set by the currently available prosthesis, particularly in adult patients, it is important that these challenges be clearly delineated and solutions be outlined prior to subjecting patients to unnecessary risks. In addition, the surgical community has suggested that for a tissue-engineered valve to see routine clinical use, particularly in the adult population, it must show that the 15-year lifetime of conventional prosthetic valves can be greatly exceeded [127].

Conclusion

Tissue engineering is an exciting field with the potential to make major advances in the treatment of a variety of diseases. Great progress has been made over the past decade in TEHV development, and from this progress the challenges that lie ahead continue to be highlighted. As of today, it is evident that there has not been one clear path to success identified. Each type of scaffold material and fabrication type has it own inherent benefits and limitations and the ideal cell type has yet to be defined. The potential role for bioreactors in the development and conditioning of TEHV constructs has been demonstrated, but the optimal use has yet to be established.

Future perspective

While it is clear there is a need for superior prosthetic valves, particularly for the pediatric population, it is important that this is done in a systematic way with clearly defined objectives/end points and methods by which to measure that these objectives/end points have been met prior to human implantation. The field has two examples of what can happen when this does not occur in a clearly defined fashion. While it is clear that there is a lot at stake for the person/group that develops the 'perfect TEHV', this field would be wise not to treat the development of an ideal TEHV as a so-called 'arms race'. Collaboration and scientific rigor are paramount in order to ensure that the needs of the patient come first. Important lessons have been gleaned from the previous human implantations and the field is moving toward successful clinical

trials in the future of TEHV development. Over the next 10 years, further advances in the design, testing and clinical performance/durability of TEHVs will be made.

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Executive summary

Current valve replacement options & unmet needs

- Current replacement options include mechanical and bioprosthetic valves.
- Mechanical valves are limited by the need for anticoagulation.
- Bioprosthetic valves are limited by their durability.
- The most pressing unmet need is for improved valve replacement options in the pediatric population.

Strategies of biological valve development

- Multiple strategies of valve development are underway involving different scaffolds, fabrication methods and cell types.
- There is not one clear pathway to success.

In vitro *conditioning & testing*

- Bioreactors have been developed to provide biomechanical and biochemical stimuli to direct *in vitro* tissue formation.
- It remains to be determined how necessary bioreactor conditioning is to the success of tissue-engineered heart valves.

Preclinical testing

- The ovine model has been considered the gold standard to test tissue-engineered heart valves.
- Questions remain as to the translatability of the results seen in the ovine model to humans.

Challenges for the translation of tissue-engineered valves from preclinical to clinical studies

- Previous implantation of decellularized scaffolds into humans without recellularization have had very poor results.
- The poor results of the previous human trials highlight the importance of rigorous *in vitro* and preclinical animal studies.

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