LIVING ARTIFICIAL HEART VALVE ALTERNATIVES: A REVIEW

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Abstract

Conventional replacement therapies for heart valve disease are associated with significant drawbacks. The field of tissue engineering has emerged as an exciting alternative in the search for improved heart valve replacement structures. One of the principles behind this concept is the transplantation of living elements, embedded in a suitable scaffold material, to the diseased site where the structure becomes integrated with patients' tissue to restore natural function. Significant progress has been made in the last ten years in the development of a living artificial heart valve alternative (LAHVA), with the identification of potential replacement sources for valve cells, scaffolds to maintain the cells in a three-dimensional environment, and signals to promote tissue development. This review addresses the need for a tissue-engineered alternative to current prostheses and provides a detailed account of normal heart valve structure – the blueprint for LAHVA fabrication. The research efforts to create a viable LAHVA, including recent developments, are discussed. Particular attention is focused on the choice of cell source for LAHVA construction, the use of biodegradable natural and synthetic polymeric scaffolds as extracellular matrix derivatives, and exogenous stimulation of tissue growth. The critical challenges involved in LAHVA development and possible future areas of investigation are also discussed.

Key Words: Heart valve, tissue engineering, cardiovascular system, extracellular matrix, heart valve prosthesis, interstitial cell, endocardial cell, biodegradable scaffold, bioreactor, growth factor.

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Introduction

Heart valve disease has a devastating impact worldwide; the American Heart Association reports an estimated 87,000 heart valve replacement procedures in 2000 (American Heart Association, 2002), while approximately 275,000 procedures are performed globally each year (Rabkin and Schoen, 2002). Cited as the cause of death in 19,737 cases during 2000 in the US alone, heart valve disease was a contributing factor in an estimated 42,300 further cases (American Heart Association, 2002). Heart valve disease occurs when one or more of the four heart valves can no longer perform their function adequately as gateways in the circulation, failing to maintain a competent unidirectional flow of blood through the heart. Two principal types of valve disease can develop which prevent the valves from opening or closing properly. The first type, valvar stenosis, is characterised by a marked narrowing of the valve opening. The second type, valvar insufficiency, occurs when the valve does not form a tight seal upon closure, resulting in regurgitation of blood. Both disease types burden the heart with an increased work rate to maintain stroke volume, leading to heart muscle dysfunction and eventually heart failure (Carabello and Crawford, 1997). Although the treatment of choice for many years has been surgical valve repair (Alvarez and Murday, 1995), complete valve replacement is warranted in the most advanced of cases.

Current Treatment and Associated Limitations

Exploration into heart valve replacement began in the 1950s, with the first successful human valve implantation being performed in 1952 (Hufnagel et al., 1952). Subsequent decades saw the development of more than 80 designs of prosthetic heart valves (Vongpatanasin et al., 1996); these remain the most common treatment for advanced heart valve disease (Schoen and Levy, 1999). Prosthetic heart valves may be either mechanical, consisting entirely of synthetic components, or may be fashioned from biological tissue (bioprosthetic). 55% of implanted valves worldwide are mechanical, with the remaining 45% bioprosthetic (Butany et al., 2003a); while both types prolong life as well as enhancing its quality, they are associated with a number of major complications that limit their success (Schoen and Levy, 1994).

Advances in engineering and biomaterials have enabled the design of efficient mechanical heart valves (Korossis et al., 2000). The major advantage of mechanical heart valves is their durability and longevity (life span > 25 years) – making them more suitable than bioprosthetic valves for

paediatric patients. However, the body's natural response to foreign materials can often result in thrombosis, which can lead to mortality. For this reason, patients receiving mechanical heart valves are placed on long-term anticoagulation therapy. Other limitations with mechanical valves are their association with infective endocarditis, risk of failure or separation of valvar components which may become embolised (Vongpatanasin *et al.*, 1996).

Bioprosthetic valves may be constructed from either animal-derived tissue (xenografts) or human-derived tissue (homografts) (Butany *et al.*, 2003b). Xenografts are typically composed of either porcine aortic tissue or bovine pericardial tissue, and are normally mounted on a supporting frame. These valves are generally treated with a chemical cross-linking agent, such as glutaraldehyde, in order to increase their durability. Advantages of xenografts include an unlimited supply of donor tissue and superior hemodynamics to mechanical valves (Butany *et al.*, 2003b). Additionally, recipients generally do not require anticoagulation treatment since the surface of bioprosthetic valves carries a lower thrombogenic risk than mechanical valves. However, progressive tissue deterioration afflicts biological replacements with a significantly short life-span (10-15 years) (Hammermeister *et al.*, 1993), and consequently they are seldom used in younger patients in whom they are more susceptible to calcification (Curcio *et al.*, 1981; al-Khaja *et al.*, 1991).

Cryopreserved cadaveric homografts or healthy valves from heart transplant patients are also used as valve replacements, the natural biological form of the leaflets contributing to the enhanced functional performance of the graft. The tissue contains viable, living cells, making it more biocompatible than either mechanical or xenograft valves. Thromboembolic complications are also quite low (Mitchell *et al.*, 1995). In spite of these advantages, however, the long-term function and durability of homografts is still inadequate (Angell *et al.*, 1989). There is always a risk of damage to the valves through chronic rejection, with the foreign tissue evoking an immune response in the patient. Although there is little evidence of acute rejection, there are reports of rapid failure of homograft valves in infants (Rajani *et al.*, 1998). A further drawback of homografts lies in their limited supply due to a shortage of donor tissue.

The underlying problem with all current methods of heart valve replacement is that the non-living material lacks an intrinsic repair system that can recondition the valve after structural injury. Optimal valve replacement tissue should be biocompatible whilst exhibiting growth and reparative capabilities (Nugent and Edelman, 2003). In recent years investigators have begun to focus their efforts on the development of living, functional replacements for tissue damaged through disease or injury (Langer and Vacanti, 1993). Current strategies in this arena of tissue engineering include transplantation of constructs containing specific cell types to the site of injury following an *in vitro* conditioning period (*in vitro* colonisation approach), or constructs which will recruit endogenous progenitor or differentiated cells from the surrounding tissue (*in vivo* colonisation approach). Either of these constructs, theoretically, could functionally integrate with the host tissue and encourage tissue regen-eration. A thorough knowledge of normal heart valve development and functional tissue composition is an essential prerequisite to establish the desired structural make-up of a tissue-engineered alternative (Rabkin and Schoen, 2002).

Heart Valve Structure – The Blueprint for a Tissue-Engineered Replacement

The four heart valves arise from embryonic mesenchymal outgrowths referred to as the endocardial cushions in a process termed valvuloseptal morphogenesis (Eisenberg and Markwald, 1995); two valves form in the atrioventicular (AV) canal (the tricuspid (trileaflet) and mitral (bileaflet) AV valves), with two valves forming in the ventricular outflow tract (aortic and pulmonary arterial valves – both trileaflet). It has been widely accepted that all heart valves are simple structures whose contribution to the unidirectional flow of blood was merely passive movement in response to pressure gradients across the valve (Cooper *et al.*, 1966). This understanding of 'passive structures' led to the development of the 'passive' mechanical and bioprosthetic valve replacements. It is now becoming apparent, however, that the heart valves have a more complex structure specialised for the microenvironment in which they are placed (Yacoub *et al.*, 1999). A living artificial heart valve alternative (LAHVA) will need to assume an identical structure if it is to replace the current modes of valve treatment; an in-depth knowledge of valvar components and their interaction will be critical to accomplish significant breakthroughs in the field.

Principal heart valve cell types

The principal cell types in the heart valve are the valvar interstitial cells (VICs) and valvar endocardial cells (VECs), with smooth muscle, cardiac muscle and nerve cells also present to varying degrees depending on the specific valve under consideration (Cooper *et al.*, 1966; Fenoglio *et al.*, 1972; Hibbs and Ellison, 1973).

Valvar interstitial cells. VICs, believed to be responsible for maintenance of valvar structure, are numerous, elongated cells with many long, slender processes extending throughout the valve matrix (Filip *et al.*, 1986). They connect to each other to establish a three-dimensional network throughout the entire valve, and are intimately associated with the valve matrix. It has been suggested that there may be two morphologically and structurally distinct populations of VICs – one possessing contractile properties, characterised by prominent stress fibres, and one possessing secretory properties, characterised by prominent rough endoplasmic reticulum (rER) and Golgi apparatus (Filip *et al.*, 1986; Lester *et al.*, 1988; Zacks *et al.*, 1991). Components characteristic of smooth muscle cells suggest VICs have functional capabilities other than fibroblast matrix secretion (Filip *et al.*, 1986; Messier *et al.*, 1994), and it is possible that *in vivo* some VICs are able to contract in order to maintain a limited intrinsic valvar force and withstand hemodynamic pressures (Mulholland and Gotlieb, 1997). Contractile function is further supported by the expression of both cardiac and skeletal contractile

proteins in VICs, which include α- and β-myosin heavy chain and various troponin isoforms (Roy *et al*., 2000). Valve leaflet contraction has been demonstrated in response to a range of vasoactive agents, suggesting a coordinated contribution of biological stimuli to successful valve function (Chester *et* al., 2000; Chester *et* al., 2001; Misfeld *et al*., 2002). The main characteristics of VICs are summarised in Table 1.

VICs are also the essential components of the intrinsic repair system of the valve. The constant motion of the valve leaflets, and connective tissue deformations associated with that movement produce damage to which the VICs respond in order to maintain valvar integrity. This regenerative process appears to be vital to normal valvar function (Schneider and Deck, 1981; Henney *et al*., 1982), and the absence of VICs in current prosthetic heart valve models is a probable factor contributing to structural failure.

Although extensive data exists regarding the heterogeneity and reparative properties of the VIC population, their function is still quite vague. An important avenue of VIC research will be translation of the cross-talk between the cells and their surrounding matrix mediated through focal adhesion molecules. Focal adhesions are specialised cell-matrix attachment sites linking the cell cytoskeleton to the matrix proteins via integrins. They also act as signal transduction sites, transmitting mechanical information from the ECM, which can elicit a number of responses including, but not limited to, cell adhesion, migration, growth and differentiation (Sastry and Burridge, 2000; Weber *et al*., 2002). An understanding of the cell biology of VICs is vital in order to determine the mechanisms by which these cells interact with each other and their surrounding environment, in order that this function can be reproduced in a LAHVA.

Valvar endocardial cells. The other major valve cell type is the VEC, which forms a functional envelope around each of the four heart valves. Whilst there is significant data on the various functions of vascular endothelium (Bachetti and Morbidelli, 2000), the specific role of valve endocardium remains unclear and has not been widely studied. However, it seems reasonable that the VECs act to maintain a non-thrombogenic valve surface, similar to the vascular endothelium (Frater *et al*., 1992). Current valve replacements are devoid of a functional, protective endocardium. This may result in platelet and fibrin deposits on glutaraldehyde-fixed bioprosthetic valves, bacterial infection and tissue calcification (Frater *et al*., 1992; Lehner *et al*., 1997).

Another likely function of VECs is the regulation of the underlying VICs, similar to the regulation of vascular smooth muscle cells by the endothelium (Mulholland and Gotlieb, 1997). A complex interaction exists between endothelium and neighbouring cells, mediated in part by soluble factors secreted by endothelial cells (Guarda *et al*., 1993). A sensory role for VECs has also been proposed by Hill and Folan-Curran (1993); these cells present a large surface area covered with microappendages on their luminal aspect thus increasing exposure to, and possible interaction with, metabolic substances in the circulating blood.

Endothelium often displays morphological and functional differences related to the stress generated by blood flow, and the same is true for VECs, with both elongated and polygonal cells being observed (Hurle *et al*., 1985). The changes in cell structure may be due to the effects of local hemodynamics on either the cytoskeletal components of the cell, or a secondary effect incurred by changes in the underlying extracellular matrix material. Ultrastructurally, VECs have been shown to possess cell junctions, plasmalemmal vesicles and rER (Table 2); although they express von Willebrand factor (vWf) both *in vitro* and *in vivo*, they lack Weibel-Palade bodies {specific storage granules for von Willebrand factor} (Lester *et al*., 1993), which are characteristic organelles of vascular endothelium. *In situ*, abutting VECs are linked together by tight junctions and gap junctions as well as overlapping marginal edges – the so-called marginal folds (Harasaki *et al*., 1975; Manduteanu *et al*., 1988).

Endothelial cells and endocardial cells preserve some of their main metabolic activities *in vitro* (Manduteanu *et al*., 1988), providing suitable cell markers for their identification. The cells express vWf in two-dimensional cell culture (Manduteanu *et al*., 1988) and demonstrate angiotensin converting enzyme (ACE) activity (Manduteanu *et al*., 1988; Bachetti and Morbidelli, 2000). The cells have also been shown to produce prostacyclin (Manduteanu *et al*., 1988) and have demonstrated endothelial nitric oxide synthase (eNOS) expression and activity (Smith *et* al., 1993; Siney and Lewis, 1993). These markers are useful in identifying ideal cell culture isolates in developing a LAHVA, but the immune-stimulating potential of VECs themselves may limit their use in tissue-engineered structures. Simon *et al*. (1993) report the expression of major histocompatability complex (MHC) antigens on cultured VECs, suggesting that an allogeneic valve may provoke immunological reactions in the recipient contributing to rapid valve degeneration. VECs also express the adhesion molecules ICAM-1 and ELAM-1, which are important for the binding of mononuclear cells during an immune response (Simon *et al*., 1993). Batten *et al*. (2001) have demonstrated a similar range of MHC and adhesion/co-stimulatory molecules in VECs and VICs, but only VECs were immunogenic. Although patient-derived autologous VECs present an ideal cell source to construct a LAHVA it may not be clinically feasible to harvest an adequate valve tissue sample. Finding a suitable endocardial replacement cell source is just one of the many hurdles that must be overcome in realising the goal of a LAHVA.

Heart valve extracellular matrix

Histologically, the heart valve leaflet consists of a valve interstitial matrix enveloped by a continuous monolayer of VECs. The principal components of the valve extracellular matrix (ECM) are the fibrous macromolecules collagen and elastin, proteoglycans and glycoproteins, each component conferring unique physical and mechanical properties (Kunzelman *et al*., 1993). Collagen accounts for ~60% dry weight of the valve, elastin for ~10% and proteoglycans for ~20% (Kunzelman *et al*., 1993). The collagen component, which provides most of the mechanical strength of the valve, is predominantly type I and III (74% and 24% respectively) with ~2% of type V collagen (Cole *et al.*, 1984). The collagen

Table 1 Characteristics of valvar interstitial cells (VICs)

Anatomical or Physiological Feature:	Evidenced by:	Reference:
Cytoskeleton	α-smooth muscle actin (α-SMA) staining	Filip *et al*., 1986; Taylor *et al*., 2000
	Vimentin staining	Lester *et al*., 1988; Messier *et al*., 1994; Taylor *et al*., 2000
	Desmin staining	Messier *et al*., 1994; Taylor *et al*., 2000
	Smooth muscle cell myosin staining	Lester *et al*., 1988; Taylor *et al*., 2000
	Troponin T, I, C staining	Roy *et al*., 2000
	α-myosin heavy chain (α-MHC) staining	Roy *et al*., 2000
	β-myosin heavy chain (β-MHC) staining	Roy *et al*., 2000
	Cardiac myosin light chain-2 (MLC2) staining	Roy *et al*., 2000
	α-tubulin staining	Messier *et al*., 1994
	β-tubulin staining	Taylor *et al*., 2000
Contractility	Positive response to epinephrine, angiotensin II	Filip *et al*., 1986, Messier *et al*., 1994
	Positive response to bradykinin, carbachol, potassium chloride, endothelin I	Messier *et al*., 1994
Cell communication	Functional gap junctions (tested by microinjection of carboxyfluorescein)	Filip *et al*., 1986
Matrix secretion	Prolyl-4-hydroxylase / Collagen type I staining	Messier *et al*., 1994; Taylor *et al*., 2000
	Fibronectin staining	Messier *et al*., 1994; Flanagan *et al.*, 2003
	Chondroitin sulphate staining	Messier *et al*., 1994; Flanagan *et al.*, 2003
	Laminin staining	Flanagan *et al.*, 2003
Innervation	Close apposition to motor nerve terminals	Filip *et al*., 1986
	Proximity to nerves displaying:	
	Neuropeptide Y activity	Marron *et al*., 1996; Ahmed *et al*., 1997
	Tyrosine hydroxylase activity	Marron *et al*., 1996; Ahmed *et al*., 1997
	Acetylcholineesterase activity	Marron *et al*., 1996; Ahmed *et al*., 1997
	Vasoactive intestinal polypeptide (VIP) activity	Marron *et al*., 1996
	Substance-P activity	Marron *et al*., 1996; Ahmed *et al*., 1997
	Calcitonin gene-related peptide (CGRP) activity	Ahmed *et al*., 1997
Mitogenic factors	Platelet-derived growth factor (PDGF) – mitogenic effect	Johnson *et al*., 1987
	Basic fibroblast growth factor (bFGF) – mitogenic effect	Messier *et al*., 1994
	Serotonin (5-HT) – mitogenic effect	Hafizi *et al*., 2000
Fibroblast characteristics	Incomplete basal lamina	
	Long, thin cytoplasmic processes	
	Intimate association with matrix	
	Well-developed rough endoplasmic reticulum and Golgi apparatus	Filip *et al*., 1986
	Rich in microfilaments	
	Adherens junction formation	

Table 2 Characteristics of valvar endocardial cells (VECs)

Anatomical or Physiological Feature:	**Evidenced by:**	**Reference:**
Morphology	Cobblestone shape (2-D culture)	Manduteanu *et al*., 1988
	Regional differences *in situ* (cobblestone/ elongated)	Hurle *et al*., 1985
	Surface microappendages	Hill and Folan-Curran, 1993
	Marginal folds	Harasaki *et al*., 1975
Cytoskeleton	Actin staining	Garcia-Martinez and Hurle, 1986
	Vimentin staining	Garcia-Martinez and Hurle, 1986
Metabolic function	Von Willebrand factor (vWf) production	Manduteanu *et al*., 1988
	Angiotensin converting enzyme (ACE) activity	Manduteanu *et al*., 1988
	Prostacyclin biosynthesis	Manduteanu *et al*., 1988
	Endothelial nitric oxide synthase (eNOS) production	Smith *et* al., 1993; Siney and Lewis, 1993
Cell communication	Tight junction formation	Lupu and Simionescu, 1985
	Functional gap junctions (tested by microinjection of carboxyfluorescein)	Lupu and Simionescu, 1985
Ultrastructural features	Lack of Weibel-Palade bodies	Lester *et al*., 1993
	Plasmalemmal vesicles / caveolae	Filip, 1984; Rajamannan *et al*., 2002
	Prominent rough endoplasmic reticulum and Golgi apparatus	Manduteanu *et al*., 1988

Table 3 Principal ECM components of the heart valve layers

Valve layer	**Main ECM component**	**Function**
(Atrialis)	(Elastin)	(Recoil of AV valves during closure)
Fibrosa	Collagen	Tensile strength
Spongiosa	Proteoglycans	Formation of hydrated lattice Resistance against compressive forces
Ventricularis	Elastin	Retention of corrugated collagen structure in aortic valve Recoil of arterial valves during closure

fibre bundles appear to be surrounded by an elastin matrix, which provides interconnections between the collagen fibres (Scott and Vesely, 1995). The glycosaminoglycan (GAG) side-chains of proteoglycan molecules tend to form a gel-like ground substance in which other matrix molecules interact to form permanent cross-links and on which other components are deposited. Human heart valve GAGs consist mainly of hyaluronic acid (HA), followed by dermatan sulphate (DS), chondroitin-4-sulphate (C-4S) and chondroitin-6-sulphate (C-6S), with minimal heparan sulphate (HS), in decreasing concentration (Murata, 1981).

The deposition and subsequent remodelling of ECM components help to determine many of the mechanical characteristics of the heart valve; however, the appropriate bio-logical, chemical and physical cues are not yet completely understood. Both tissue remodelling and renewal observed in the connective tissue of structures such as heart valves are mediated primarily by the matrix metalloproteinases (MMPs) and their tissue inhibitors (TIMPs). These molecules also participate in a broader spectrum of physiological and pathological processes (Nelson *et al*., 2000; McCawley and Matrisian, 2001; Galis and Khatri, 2002). Several MMPs, including interstitial collagenases (MMP-1, MMP-13) and gelatinases (MMP-2, MMP-9) and their TIMPs (TIMP-1, TIMP-2, TIMP-3) have been localised in all four heart valves (Dreger *et al*., 2002). Over-expression of MMPs has also been demonstrated in pathological heart valve conditions (Rabkin *et al*., 2001). Further stud-
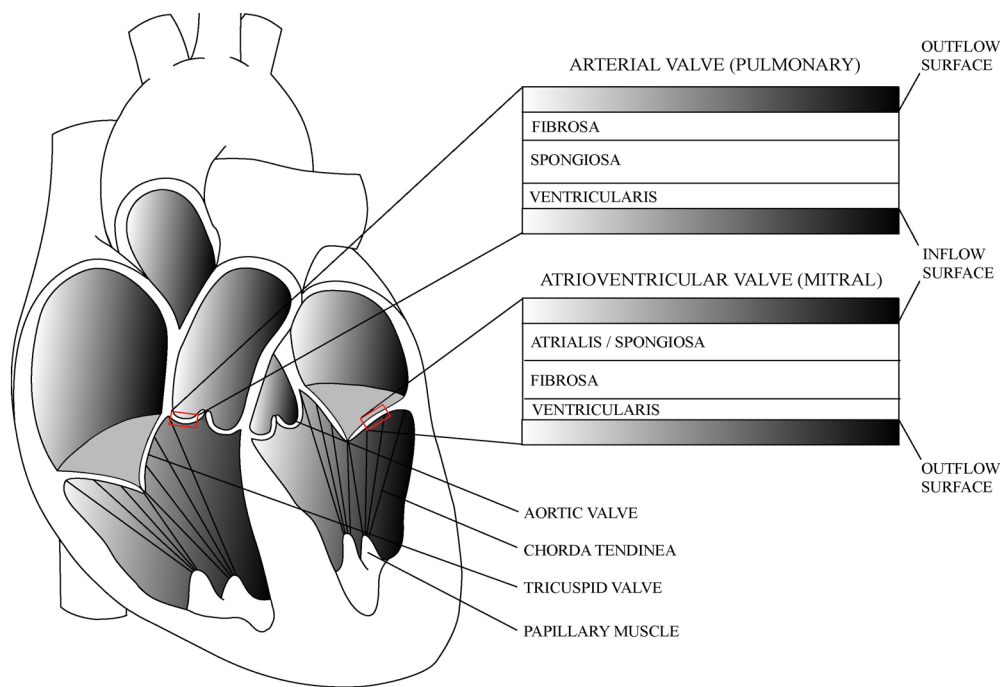
Figure 1 Schematic arrangement of the connective tissue layers through the mid-zone of the heart valve leaflets

ies are necessary to elucidate their mechanism of action and functional implications in valve matrix turnover and tissue morphogenesis.

Each of the three major structural valve components (collagen, elastin and proteoglycans) constitutes a significant proportion of each of the three morphologically distinct and functionally significant layers of the valve leaflet matrix (Table 3). These layers are termed the fibrosa, spongiosa, and ventricularis (Gross and Kugel, 1931), although the arrangement of these layers differs in the AV and arterial valves (Figure 1). The AV valves also possess an atrialis layer, but the atrialis and spongiosa layers will generally be considered together as the spongiosa layer for the purpose of this review.

Fibrosa. The fibrosa layer forms the load-bearing fibrous backbone of the valve leaflet, consisting of sheets of collagen bundles (Kunzelman *et al*., 1993). These bundles preferentially run in the transverse direction of the aortic valve leaflets and are arranged in a corrugated manner to allow expansion during valve closure (Missirlis and Armeniades, 1977). The fibrosa of the AV valves is continuous with the collagen core of the chordae tendineae (Figure 1) – a network of cord-like support structures anchored to the cardiac papillary muscles (Fenoglio *et al*., 1972). The chordae make up an important component of the AV valve complex, as they prevent the leaflets from ballooning into the atria during ventricular contraction. The fibrosa layer of the heart valves generally lies towards the outflow surface, and helps maintains proper function in the arterial valves as they are unsupported by chordae tendineae (Christov *et al*., 1999).

Spongiosa. The spongiosa layer consists of loosely organised connective tissue in a semi-fluid ground substance (Walmsley, 1978). The dominant matrix components of this layer are proteoglycans, with randomly oriented collagen interspersed with multiple fine layers of elastic tissue. GAG side-chains of proteoglycan molecules are highly negatively charged which results in a significant water-binding capacity and thus the formation of a porous gel matrix. Important mechanical functions of the proteoglycans include this matrix hydration, and the ability to resist compressive forces (Culav *et al*., 1999). The spongiosa layer of the heart valve may absorb stresses generated during valve closure (Kunzelman *et al*., 1993) and may sustain flexibility despite continuous valve motion (Murata, 1981). This layer may also enable shearing between the fibrosa and ventricularis layers of the aortic valve during loading and unloading.

Ventricularis. The ventricularis layer is much thinner than the other two layers, and is abundant in elastic fibres (Kunzelman *et al*., 1993). Elastic fibres allow tissues to withstand repeated deformation and reformation (Culav *et al*., 1999). Elastin is believed to be responsible for maintaining the aortic valve collagen fibre architecture in its neutral corrugated state (Scott and Vesely, 1995). It is also evident that the side of the valve which faces *against* the direction of blood flow (ventricularis in arterial valves, spongiosa in AV valves) contains a higher proportion of elastin than the opposite layer, and its flexibility allows the valve leaflets to recoil during valve closure (Christov *et al*., 1999).

In order to meet the extraordinary demands placed upon it, the valve possesses incredible strength, durability and flexibility due to its composite laminated structure. It will be crucial to recreate this layered structure in a LAHVA to maintain proper functionality amidst the turbulent conditions of the cardiac cycle. Any analysis of normal valve function must take into account the cellular organisation of the valve as well as the reciprocal interactions between the cells and their surrounding matrix. Knowledge gained from such

studies is central to the design and development of a living replacement valve employing a tissue-engineered approach.

Tissue Engineering Approach to Heart Valve Replacement

Tissue engineering approaches emerged during the last 15 years in response to limitations associated with tissue and organ transplantation, with the scarcity of available donor tissue being perhaps the most significant limiting factor (Fuchs *et al*., 2001). The primary goal of tissue engineering is the 'restoration of function through the delivery of living elements which become integrated into the patient' (Vacanti and Langer, 1999). Tissue engineering approaches to the construction of a heart valve, or any tissue or organ, typically rely on three essential components (Figure 2): *cells*, which will ultimately form the new tissue; *scaffolds*, designed to maintain the cells in a three-dimensional environment at the implantation site, and *signals* that guide the gene expression and ECM production of the cells during tissue development.

Cells

Establishing a reliable cell source is a key to effective tissue regeneration (Langer and Vacanti, 1999). A variety of cell sources is applicable to tissue-engineered structures, each with associated advantages and disadvantages (Heath, 2000). One possibility is autogeneic cells, harvested from patients themselves, which would not elicit an immune reaction following re-implantation. However, in diseased states or with geriatric patients, autogeneic cells may not be an appropriate transplantation source (Heath, 2000). They would also be unavailable 'off-the-shelf' for immediate use clinically (Nerem and Seliktar, 2001).

Recent developments in stem cell research have had a significant impact on the progress of tissue engineering (Chapekar, 2000). This research has focussed particularly on multipotent (capable of forming a number of cell types) adult stem cells and totipotent (capable of forming all cell types) embryonic stem cells. However, a clearer understanding of the factors involved in their differentiation, as well as preservation of gene expression and phenotypic function *in vivo* is critical to maintain normal structure and function in a LAHVA.

The remaining possibilities for a LAHVA cell source include xenogeneic or allogeneic cells, although xenogeneic cells carry a risk of transferring animal viruses to humans (Nerem and Seliktar, 2001). There may be a possibility, however, to alter allogeneic cells genetically to reduce antigenicity or prevent host rejection (Heath, 2000). Regardless of cell source, the cells composing a tissue-engineered structure must express the appropriate genes and maintain the appropriate phenotype in order to preserve the specific function of the tissue (Kim and Mooney, 1998).

Scaffolds

Exogenous ECM scaffolds are designed to immobilise the appropriate cell populations at the implantation site of a tissue-engineered structure, and also to provide mechanical support until newly-formed tissue has been laid down,
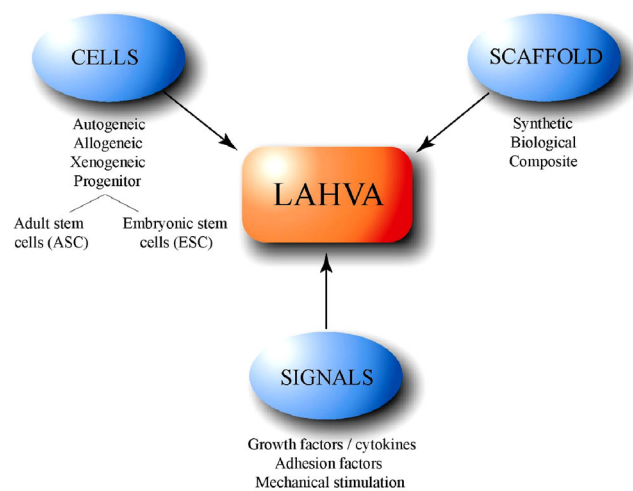
Figure 2 The principal building blocks for LAHVA fabrication

organised and become structurally stable (Putnam and Mooney, 1996). The traditional concept of tissue engineering involves the use of three-dimensional polymeric scaffolds as cell transplant devices. Once the cells are seeded on the scaffolds *in vitro*, the resulting three-dimensional construct can be implanted *in vivo* at the site of injury or disease. Implanted cells can then use the body's natural structural cues to promote organisation, growth and development of the replacement 'tissue'. The polymeric scaffolds are designed to degrade or hydrolyse *in vivo* as the implanted cells produce and organise their own extracellular matrix network, such that eventually only newly synthesised, functionally integrated tissue remains at the transplantation site. The two principal choices of scaffold currently under investigation are synthetic polymeric scaffolds or biological polymeric scaffolds.

Signals

Successful tissue regeneration may not always be achieved 'simply' by combining cells and scaffolds. The gene expression of cells and tissue formation can be regulated or promoted by the addition of growth factors, cytokines or hormones. A drug delivery feedback system can exploit this method of biochemical signalling, by controlling growth factor release at the site of tissue regeneration (Pandit *et al*., 2000; Tabata, 2000).

Cell adhesion molecules also impart a level of signalling that controls the regulation of gene expression and cellular organisation. Bioactivity of a synthetic polymer scaffold can be modified with covalently-attached adhesion peptide sequences, such as arginine-glycine-aspartic acid (RGD) or tyrosine-isoleucine-glycine-serine-arginine (YIGSR) (Hubbell, 1995). Such sequences are present on a number of ECM molecules, forming a physical link between the ECM and the cells via integrin cell surface receptors (Van der Flier and Sonnenberg, 2001). Incorporated sequences function to improve cell adhesion, spreading and higher functions, whilst also enabling integrin triggering of a number of cell signalling pathways critical to tissue development (Kim and Mooney, 1998).

In vitro methods of mechanical stimulation or physical signalling have also been shown to improve cell and tissue growth. This has led to the design and development of bioreactor systems for the *in vitro* pre-conditioning of tissue-engineered structures (Barron *et al.*, 2003); tissue formation thus takes place in an environment closely mimicking that of the transplant site.

Current progress towards a LAHVA

The design of LAHVAs incorporating cells, scaffolds and signals presents a unique challenge to researchers in the field. The optimum design will possess the following characteristics: an anatomical structure comparable to native valve; physiological function; biomechanical adaptability; reparative ability and growth potential. The valves must be able to open and close synchronously at a frequency of approximately 1Hz, resulting in bending deformations, and be exposed to the turbulent hemodynamics experienced within the heart (Mann and West, 2001).

Significant progress has been made to date in the tissue engineering of heart valve structures, with researchers concentrating their efforts on the ideal source of cell populations, the use of cell-seeded scaffolds and the promotion of tissue growth using various signals, in particular *in vitro* mechanical stimulation. The results of selected studies are summarised in Table 4 (to be found in supplemental file 'v006a04s.pdf').

Populations investigated as cell sources for a LAHVA

The concept of a LAHVA was first presented by Shinoka *et al.* (1995), who constructed valve leaflets using both allogeneic and autogeneic cells seeded on a biodegradable polymeric scaffold. A mixed population of smooth muscle cells and fibroblasts representing VICs was isolated from ovine femoral artery and subsequently seeded into scaffolds composed of polyglactin (PGLA) and polyglycolic acid (PGA). The constructs were then seeded with a monolayer of femoral artery endothelial cells representing VECs. The single leaflet structures were transplanted in the low-pressure pulmonary position in ovine models. The autogeneic structures performed better than allogeneic, the latter evoking inflammatory responses and infectious complications in the animal hosts. The study did however demonstrate the feasibility of LAHVA leaflet construction.

Subsequent studies also utilised ovine femoral artery-derived cells to fabricate valve leaflet constructs (Breuer *et al.*, 1996; Shinoka *et al.*, 1996). However, the use of the femoral artery is not ideal as an autogeneic cell source, due to the added complications of limb ischaemia from disruption of the lower limb arterial blood supply. To address this problem, Shinoka *et al.* (1997) designed a study comparing dermal fibroblasts to arterial myofibroblasts[*] as cells of origin for a LAHVA. Leaflets derived from dermal fibroblast were much thicker, more contracted and less organised than those derived from arterial myofibroblasts. The authors suggest that cells of mesodermal origin (such as arterial myofibroblasts) may provide more specialised phenotypic properties than ectodermally-derived skin fibroblasts, making mesodermal cell populations more suitable for the construction of a LAHVA.

Schnell and co-workers (2001) evaluated myofibroblasts derived from human saphenous vein as an alternative cell source for the tissue engineering of cardiovascular structures. The choice of peripheral veins represented a more clinically realistic cell source than the arterial structures previously used, and can be harvested with minimally invasive surgery. The myofibroblasts were cultured on polyurethane scaffolds for 6 weeks, following which they were shown to be viable and confluent. Collagen production was increased in saphenous 'neo-tissue' compared to that constructed using human aortic myofibroblasts, while mechanical testing also demonstrated superior stability in the saphenous structures. However, as with most previous studies using arterial cells, the production of other important structural and regulatory molecules remains to be elucidated.

Carotid artery-derived myofibroblasts have also been harvested for the construction of a LAHVA (Stock *et al.*, 2000; Sodian *et al.*, 2000a; Hoerstrup 2000a); however, as with all other previous cell types isolated for the same purpose, this requires the sacrifice of an intact tissue structure and requires invasive surgery. In the search for an alternative cell source, one group demonstrated the possibility of using autogeneic umbilical cord cells (Kadner *et al.*, 2002a). The isolated cells represent a mixed population from umbilical cord artery, vein and the surrounding Wharton's jelly. Absence of endothelial cells was confirmed *in vitro*; cells demonstrated features of myofibroblast-like differentiation, such as expression of alpha smooth muscle actin, vimentin, and deposition of collagen types I and III. The cells were shown to attach well to biodegradable scaffolds, and formed a layered tissue-like structure comparable to scaffolds seeded with vascular cells (Hoerstrup *et al.*, 2000a). Deposition of collagen types I and III was demonstrated, although there were low levels of GAGs produced, and no report of elastin production. There are also a number of other limitations to this study; although Wharton's jelly myofibroblasts, the umbilical cord artery or vein-derived cells on their own may represent a promising cell source, the authors present a study with a 'mixed' population of cells from all three structures. There is also limited immunohistochemical characterisation of this 'mixed' population, and further characterisation studies of pure cell

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[*]Many papers have misleadingly referred to cells isolated from veins and arteries as 'myofibroblasts', with minimal characterisation. It has been proposed that most examples of cells cited in the literature as being myofibroblasts and seen in normal, non-traumatised, non-pathological tissues may be pericytic or smooth-muscle in nature (Eyden *et al.*, 1994). Indeed, the vascular-derived cells are more likely to be smooth muscle cells, which are generally either synthetic or contractile. This again highlights the importance of extensive cell characterisation, and a standardisation of the cell terminology is of paramount necessity. Solely for the purpose of this review, the authors will cite the terminology used by the original authors.

lines from each source will be necessary to significantly evolve this approach.

Mesenchymal stem cells (MSCs) are present in adult bone marrow, and have been demonstrated to develop into a variety of connective tissues, including bone, cartilage, muscle and fat (Pittenger *et al*., 1999; Caplan and Bruder, 2001). With this in mind, Kadner and colleagues investigated the feasibility of creating functional LAHVAs using human bone marrow stromal cells (Kadner *et al*., 2002b; Hoerstrup *et al.*, 2002). Cell populations are easily harvested from a patient using simple bone marrow puncture, avoiding the sacrifice of an intact blood vessel. Isolated cells were partially characterised using a number of myofibroblast markers, and, like umbilical cord cells, were shown to express alpha smooth muscle actin and vimentin, and produce collagen types I and III. Biodegradable polymeric scaffolds cultured with MSCs *in vitro* demonstrated an organised internal structure and mature tissue development. Despite the encouraging results, it is essential that the bone marrow stromal cells differentiate into the appropriate cell type in the scaffold, and that they remain differentiated *in vivo*, in order to ensure long-term function and durability of the implanted LAHVA (Perry *et al*., 2003). Further investigation into the mechanisms and signals that direct bone marrow stromal cell differentiation are warranted before introduction in the clinical setting.

Although some promising results have been obtained using the aforementioned cell types, the ideal cell replacements for a LAHVA would be VICs and VECs themselves. A recent report discusses the use of heart valve leaflet biopsy samples from an ovine model as a tissue source for the cultivation of VICs for use in LAHVA fabrication (Maish *et al*., 2003). In most cases, the biopsy procedure did not appear to compromise leaflet function, with the majority of animals showing intact valves with normal leaflet anatomy post-mortem (9 out of 13). Long-term follow-up studies are required (the maximum investigation period was 17 weeks); however, it is unlikely that enough cells could be isolated and cultured from a small biopsy to be of clinical use. It is also known that cell numbers decrease with age, making this technique even less feasible in the older patient. The risks involved in valve biopsy may also be too high to justify human trials.

The search for an ideal replacement for both cell types is ongoing, with other potential sources of interest including circulating endothelial and smooth muscle progenitor cells (Rafii, 2000; Simper *et al*., 2002). However, all current techniques assume that transplanted cell populations achieve the same distribution and differentiation pattern of cells in the native valve (Bertipaglia *et al*., 2003). In previous studies, there is limited data regarding the phenotypic profile of these cells once they are implanted into the scaffolds, and future studies will need to provide extensive characterisation profiles of tissues both prior to implantation and during long-term follow-up studies. It is highly probable that both embryonic and adult stem cells will become valuable resources for construction of a number of tissues *ex vivo*, including heart valves.

Scaffolds investigated as ECM templates for a LAHVA

Synthetic biodegradable polymeric scaffolds. The work by Mayer *et al.* at the Children's Hospital, Boston in the mid-to-late 1990s involved the application of the biodegradable polymers PGA and polylactic acid (PLA), and composites of these polymers (PGLA), in the development of a LAHVA (Shinoka *et al*., 1995; Breuer *et al*., 1996; Shinoka *et al*., 1996; Zund *et al*., 1997). The scaffolds were composed of a PGLA woven mesh sandwiched between two non-woven PGA mesh sheets. The constructs were first seeded with arterial myofibroblasts, secondly with arterial endothelial cells, and were subsequently transplanted as a single leaflet into the pulmonary position in an ovine model. Seeded cells were shown to persist in the structure after 6 weeks *in vivo* (Shinoka *et al*., 1996). Histological examination of the leaflet structures post-mortem revealed a cellular architecture and tissue structure similar to that of native valve, although inflammatory responses and complications due to infection in the animal study were evident (Shinoka *et al*., 1995). The scaffold also persisted for at least 6 weeks after implantation. There was evidence for elastin and collagen production in the leaflets, which had mechanical properties similar to native leaflets (Shinoka *et al*., 1996). The success of these studies was limited, however, as the PGA-PLA polymers proved to be too immalleable to function as flexible valve leaflet structures. Kim and associates (2001) attempted to conduct a study to compare the biological responses of autogeneic cell-seeded PGA-PLA scaffolds and an acellular PGA-PLA scaffold by implanting both structures in the pulmonary position of the same animal. However, they failed to achieve long-term survival in animals with double valve leaflet replacement. They attributed this to heart failure caused by high-grade valvar insufficiency.

The high initial stiffness and thickness using PGA-PLA scaffolds warranted the investigation of new polymers. Stock and colleagues (2000) evaluated the use of polyhydroxyoctanoate (PHO) as part of a composite scaffold for the creation of a LAHVA. The fabricated valved-conduit consisted of four different biomaterials: the conduit wall was composed of a layer of non-porous PHO film (240µm thick) sandwiched between two layers of non-woven PGA felt (each 1mm thick). The leaflets consisted of a monolayer of porous PHO (120µm thick), and were sutured to the wall of the conduit using polydioxanone sutures. Microscopical examination after explantation revealed a uniformly organised, fibrous tissue with significant amounts of stainable collagen and proteoglycans, but without stainable elastin. However, the PHO scaffold was still evident in the conduit and leaflets after 24 weeks, suggesting a much longer degradation profile than PGA or PLA, and this extended period could have the potential to augment host-tissue reactions. The study was also limited to implantations in the low-pressure pulmonary position.

An additional study involved moulding a porous PHO scaffold into a trileaflet valved-conduit through a thermal processing technique, with subsequent seeding of ovine carotid artery myofibroblasts and jugular vein endothelial cells (Sodian *et al*., 2000b). The constructs, devoid of

polydioxanone sutures, were implanted into the pulmonary position in ovine models, and were harvested after 1 - 17 weeks. All valve constructs opened and closed synchronously, and showed an increase in inner diameter and length. This could not conclusively be attributed to actual tissue growth, and may simply have been dilatation of the construct. Scanning electron microscopical studies demonstrated a smooth flow surface on the leaflets, although they did not demonstrate a confluent endothelium using histological techniques. This limitation could affect the long-term durability of the structures. There was evidence for collagen and GAG deposition, but the constructs were devoid of elastin. All valve constructs demonstrated mild stenosis and regurgitation, and there was evidence for inflammatory reaction. The scaffold material remained after 17 weeks *in vivo*, and further long-term studies are required to monitor complete scaffold degradation.

PHO scaffold was evaluated as an alternative to PGA-PLA, but demonstrated prolonged degradation time *in vivo*, and was not sufficiently replaced by neotissue after 24 weeks (Stock *et al.*, 2000). Hoerstrup and colleagues (2000a) developed a novel composite scaffold material consisting of PGA coated with a thin layer of poly-4-hydroxybutyrate (P4HB) – a flexible, thermoplastic polymer with a more rapid degradation time than PHO (Martin and Williams, 2003). Trileaflet heart valve scaffolds were fabricated from the composite material using a heat-application welding technique. Myofibroblasts and endothelial cells from ovine carotid artery were seeded onto the scaffolds, which were then cultured in a bioreactor for 14 days (Hoerstrup *et al.*, 2000a). The conditioned constructs were subsequently implanted into the same lambs from which the cells were harvested, where they remained for up to 20 weeks. After *in vitro* conditioning, constructs showed increased ECM synthesis, more organised internal structure and improved mechanical properties over static controls. The leaflets demonstrated a layered architecture after 16 and 20 weeks, with a loose, spongy layer containing elastin and GAGs on the inflow (ventricular) surface, and a more fibrous layer containing primarily collagen on the outflow (arterial) surface. However, the valves showed moderate regurgitation at 20 weeks, and only partial endothelial cell coverage of the leaflet surface.

Decellularised tissue scaffolds. Decellularisation approaches may reduce the immune response of the host to bioprosthetic valves and generate natural materials for use in tissue engineering applications (Schmidt and Baier, 2000). The acellular biological matrix that remains offers a physiological template, which holds the potential for endothelialisation by surrounding host VECs and repopulation by local VICs. Theoretically, VECs will form a non-thrombogenic surface layer, while VICs will induce matrix turnover in the valve. This process would ultimately transform the allogeneic or xenogeneic acellular matrix into a primarily autogeneic, living valve tissue, with concurrent reduction in immune response. There are various methodologies available for decellularisation of heart valve tissue, including detergent treatments, enzymatic digestion, hypo-/hypertonic immersion and sonication.

Wilson and colleagues (1995) developed a cell extraction process for decellularising heart valves using both detergent (Triton X-100) and enzymes (DNAse, RNAse). The process removes cell membranes, nucleic acids, lipids, cytoplasmic structures, and soluble matrix molecules, while retaining the collagen and elastin ECM suitable for recellularisation (Zeltinger *et al.*, 2001). After one month of follow-up using decellularised allografts in a canine model (Wilson *et al.*, 1995), explant histology demonstrated no inflammatory process, partial VIC infiltration at the valvar base, and partial endothelialisation. Long-term calcification studies were not performed.

Bader and colleagues (1998), employing the decellularisation strategy of Wilson *et al.* (1995), demonstrated reseeding of acellularised porcine aortic valves with human endothelial cells, resulting in a confluent surface monolayer of viable cells. Endothelialisation of LAHVAs is an important preconditioning factor, in order to reduce thrombogenic risk. These authors demonstrated a largely acellular structure across the thickness of the leaflet. The collagen network appeared wavelike, as in normal valve tissue; however, there were large interfibrillar spaces that may have affected the mechanical properties of the tissue. It was also impossible to exclude the presence of cellular remnants in the tissue structure. This may be an important factor, as cellular remnants have been associated with calcification in microscopic investigations (Valente *et al.* 1985, Schoen *et al.* 1985). The extraction of cellular remnants should also minimise the immune response (Schmidt and Baier, 2000).

Bader and colleagues extended their studies to the *in vivo* application of an acellular matrix valve scaffold conditioned by tissue engineering *in vitro* using autogeneic cells (Steinhoff *et al.*, 2000). Carotid artery myofibroblasts and endothelial cells were added sequentially to acellularised pulmonary valve conduits and were implanted into ovine models. The valve leaflets were completely endothelialised at 4 weeks, with the endothelium remaining confluent at 12 weeks after implantation, and had been infiltrated by a dense population of myofibroblasts. However, there was evidence for subvalvular calcification and inflammatory reaction. There was also an increased thickening of the valve leaflets; long-term studies were not undertaken.

The decellularisation approach is the basis for the commercially available SynerGraft™ valve manufactured by CryoLife Inc., USA (O'Brien *et al.*, 1999). The SynerGraft™ decellularisation process involves cell lysis in sterile water, enzymatic digestion of nucleic acids (RNase and DNase enzymes in an unspecified buffer), followed by a multi-day isotonic washout period. The process was developed to replace the use of glutaraldehyde cross-linking to limit xenograft antigenicity. Histological examination after 150 day implantation in porcine models revealed intact leaflets with ingrowth of host myofibroblasts and no evidence for calcification. However, early failure of the valve has been reported in human trials (Simon *et al.*, 2003). The SynerGraft™ matrix elicited a strong inflammatory response which was non-specific early on and was followed by a lymphocyte response. Structural failure or rapid degeneration of the valves occurred within one year. There was no host cell repopulation of the valve matrix, and evidence for calcific deposits and cellular remnants pre-implantation may indicate manufacturing problems.

The ideal heart valve decellularising agent remains unknown. In a recent effort to determine the most efficient decellularising agent from a panel of candidates (Triton X-100, sodium dodecyl sulphate (SDS), sodium deoxycholate, MEGA 10, TnBP, CHAPS and Tween 20), Booth and colleagues (2002) demonstrated that only SDS (0.03 - 1%) or sodium deoxycholate (0.5 - 2%) resulted in total decellularisation after 24 hours. However, the possible toxicity of decellularisation reagents remains a factor for investigation; more detailed characterisation studies of the matrix scaffold are crucial to determine appropriate matrix composition and complete elimination of cellular remnants. Biomechanical properties of decellularised valve tissue matrices also need to be analysed.

Natural biodegradable polymeric scaffolds. A number of naturally occurring biodegradable polymers have been investigated as potential LAHVA matrix structures, as they offer a more native environment than synthetic structures to the cells. Acellular small intestinal submucosal (SIS) matrix has been isolated to construct tissue substitutes (Badylak *et al.*, 1989; Badylak *et al.*, 1998). Matheny and colleagues (2000) have applied porcine SIS matrix as a resorbable scaffold for pulmonary valve leaflet replacement in porcine models. Analysis of explanted constructs revealed resorption of the submucosal matrix, progressive replacement with fibrous connective tissue and vascularisation similar to host leaflets. This approach appeared to yield complete resorption of the implanted scaffold in comparison to decellularised valve matrix applications.

The use of fibrin gel as a natural, autogeneic scaffold in heart valve tissue engineering has been demonstrated by Ye and associates (2000a), and has been the subject of investigation for the development of cardiovascular 'tissue equivalents' by Tranquillo and colleagues (Grassl *et al.*, 2002; Neidert *et al.*, 2002). Fibrin gel is a biodegradable polymer which can be produced from the patient's own blood. In the study by Ye and colleagues (2000a), cell-fibrin gel structures were constructed using human aortic myofibroblasts suspended in a solution of fibrinogen, thrombin and calcium chloride ($CaCl_2$) which was allowed to polymerise at 37°C. The growth media was supplemented with L-ascorbic acid 2-phosphate – a potent promoter of collagen synthesis (Grinnell *et al.*, 1989), and varying concentrations of aprotinin, a protease inhibitor, to adjust and control the rate of degradation. Microscopical studies demonstrated homogenous cell distribution and collagen synthesis; degradation was controlled by altering the aprotinin concentration. A moulding technique has also been developed for the application of this compound to trileaflet heart valve fabrication (Jockenhoevel *et al.*, 2001a). An adjustable mould consisting of 'aortic' and 'ventricular' stamps was constructed from aluminium, and used to cast a trileaflet fibrin gel structure seeded with myofibroblasts within 1 hour. The tissue could withstand suturing, although its mechanical integrity was insufficient for direct implantation. Shrinkage of the gel structure also presents a complication. However, the fibrin gel model has a number of advantages: the scaffold itself can be produced directly from a sample of patient's blood eliminating the risk of rejection; the rate of degradation can be controlled and a ho-

mogenous cell distribution can be achieved upon polymerisation.

As fibrous long-spacing collagen provides most of mechanical and tensile strength of the heart valve, a valve construct fabricated using a natural collagen scaffold is quite a logical choice. Collagens are weakly immunogenic compared to other structural proteins, due to a large degree of homology across species, and are biodegradable on account of their proteinaceous nature (Chevallay and Herbage, 2000). Collagen scaffolds have been investigated for the production of heart valve-like tissue using human and porcine-derived cells (Rothenburger *et al.*, 2001; Flanagan *et al.*, 2002; Taylor *et al.*, 2002). Rothenburger and colleagues used a cryogenically-engineered collagen type I matrix derived from bovine skin tissue, with a homogenous pore size of 40μm and a porosity of 98%. The matrix was cut into disc structures and sequentially seeded with either human or porcine aortic smooth muscle cells. The structures were then seeded with porcine aortic endothelial cells and maintained in culture for 28 days. A tissue-like morphology was observed in all samples, characterised by several layers of cells with newly synthesised ECM components. Proteoglycans and the ECM proteins fibronectin and thrombospondin were also localised.

Ye and colleagues (2000b) took another approach to the construction of tissue using 'natural' materials by producing completely human autogeneic tissue without the use of a supporting scaffold. Myofibroblasts were cultured in large culture dishes in medium supplemented with L-ascorbic acid 2-phospate to promote extracellular matrix production. The cell sheets subsequently formed were folded into quadrilaminar sheets and mounted on customised culture frames. They were then cultured for a further 4 weeks and demonstrated a multilayer tissue pattern, with active viable cells surrounded by extracellular matrix.

Adequate, uniform and reproducible cell seeding of both natural and synthetic polymeric scaffolds remains a difficulty in the field. In an effort to improve scaffold cell-seeding methods, Ye and colleagues (2000c) have demonstrated the possibility of coating scaffolds with an autologous immuno-compatible ECM material; Zund and colleagues (1999) aimed to improve cell-seeding methods by optimising the cell seeding interval (i.e. the length of time between subsequent seeding procedures). Their results suggest that longer seeding intervals of 24- to 36-hours result in higher cell attachment and cell growth on PGA scaffolds, this may have been due to 'pre-conditioning' of the seeded scaffolds by cells previously laid down. Optimisation of rapid seeding techniques will be important in LAHVA fabrication, as it maximises the use of donor cells, hastens the proliferation and subsequent differentiation of cells, decreases the time in culture, and provides a uniform distribution of cells (Vunjak-Novakovic *et al.*, 1998). Advances in the modification of scaffold surfaces to enhance cell adhesion and subsequent function will be necessary for short-term *ex vivo* culture of tissues prior to implantation.

It is not clear whether natural or synthetic scaffolds will ultimately prove to be more appropriate for LAHVA development, as there are still certain limitations associated with both. The optimum heart valve scaffold should display an

accurate, anatomical spatial reconstruction of the valve to be replaced. The design of a replacement aortic valved-conduit, for example, will require the inclusion of three dilated pouches alongside the 'cusp-like' leaflets to approximate the sinuses of Valsalva; AV valve designs may require the addition of other components of the AV valve apparatus, including chordae tendineae and papillary muscles (Bursac *et al*., 1999; Zimmermann *et al*., 2002). Using stereolithography, Sodian and colleagues (2002) fabricated plastic models with an exact spatial representation of human aortic and pulmonary valve. These models were then used to fabricate heart valve cell scaffolds using poly-3-hydroxyoctanoate-co-3-hydroxyhexanoate (PHOH) and P4HB, which were shown to function well in a pulsatile flow bioreactor under both normal and supranormal flow and pressure conditions. The choice and design of scaffolds will be integral to the successful clinical outcome of a LAHVA. As well as conforming to a suitable anatomical shape, heart valve scaffolds will be required to possess both tensile and elastic properties. The scaffolds should be biocompatible, bioabsorbable or remodelable, and should provide a suitable template for facilitating development of new tissue. Scaffold permeability is also vital for the control of cell nutrition and removal of waste products.

Signalling factors investigated in LAHVA development

There is a dearth of research into the application of growth factors, mitogenic factors or adhesion factors in scaffolds and matrices used to develop a LAHVA. However, significant progress has been made in the development of *in vitro* methods of mechanical stimulation or physical signalling to improve cell and tissue growth prior to implantation (Hoerstrup *et al*., 1999; Weston *et al*., 1999; Hoerstrup *et al*., 2000b; Sodian *et al*., 2001; Weston and Yoganathan, 2001; Zeltinger *et al*., 2001; Jockenhoevel *et al*., 2002; Dumont *et al*., 2002; Perry *et al*., 2003; Engelmayr *et al*., 2003).

One method of mechanically stimulating cells to induce tissue morphogenesis is maintaining them in culture under isometric strain (Stopak and Harris, 1982). A number of groups report increased collagen synthesis and tissue organisation in LAHVA scaffolds mounted in cultures frames that provide isometric strain (Hoerstrup *et al*., 1999), border-fixed fibrin gel structures (Jockenhoevel *et al*., 2001b) and cyclically-strained scaffolds (Mol *et al*., 2003).

The mechanical properties obtained by Niklason *et al*. with tissue-engineered vascular constructs cultured under pulsatile flow conditions (Niklason *et al*., 1999) prompted a number of other investigators to develop bioreactor systems to improve the mechanical structure of LAHVAs. Jockenhoevel and colleagues designed and constructed a flow system to investigate the effects of laminar flow conditions on ECM formation and tissue development in cell-seeded synthetic biodegradable polymer scaffolds (Jockenhoevel *et al*., 2002). The system was efficient at maintaining sterile culture conditions, and extracellular matrix production was significantly higher than static controls. Current experiments in the design and development of a system specific for dynamic culture of fibrin and col-

lagen gel LAHVA structures are ongoing (Flanagan and Jockenhoevel, unpublished results).

Pulsatile flow systems have also been engineered to condition both myocardial patch tissue and trileaflet heart valve tissue *in vitro* (Hoerstrup *et al*., 2000b; Sodian *et al*., 2001). The pulse duplicator system of Hoerstrup and colleagues was designed to grow LAHVAs under gradually increasing flow and pressure conditions in order to mirror the physical stimulation encountered *in vivo* (Hoerstrup *et al*., 2000b). The conditioned structures demonstrated a much higher deposition of ECM, improved tissue organisation and better mechanical properties than those grown in static conditions. Zeltinger *et al*. (2001) demonstrated efficient colonisation of decellularised porcine valve scaffolds with human dermal fibroblasts using a pneumatic flow bioreactor apparatus. The application of pulsatile fluid flow was shown to stimulate ECM production and cell proliferation, improving the structural integrity of the LAHVA.

A major drawback with all of these pulsatile flow systems, however, is the limit to the number of test samples (i.e. only one LAHVA sample can be conditioned at a time), which does not offer statistically significant results. Results are also limited to a combination of mechanical stimuli, rather than observing the effects of single stimuli, such as laminar shear and isometric tension studies previously undertaken (Hoerstrup *et al*., 1999; Jockenhoevel *et al*., 2002). A recent bioreactor design allowed studies of the effects of cyclic three-point flexure on LAHVA structures, and incorporated a large test sample size to demonstrate significant results (Engelmayr *et al*., 2003). The investigators report fatigue and fracture in acellular biodegradable polymer scaffolds cultured under dynamic flexure conditions; this may prove to have important consequences in the design and selection of potential LAHVA scaffold candidates.

Although significant progress has been made, all LAHVAs constructed to date lack the mechanical strength required for functional performance in the anatomical position. All previous studies have been directed towards the design of LAHVAs for transplantation at the low-pressure pulmonary valve site. A clearer understanding of the mechanical environment of all four heart valves, in particular those of the systemic circulation, will lead to the development and production of improved biomimetic environments for the *in vitro* pre-conditioning of LAHVAs. Cell response mechanisms to mechanical stimuli are unclear (MacKenna *et al*., 2000) and a better understanding will permit the design of more efficient biomimetic protocols. Cell death caused by dynamic bioreactor culture conditions will also need to be investigated, and conditions optimised to minimise cell death. Gene therapy studies have the potential to be exploited in order to promote the expression of suitable mitogenic, angiogenic or neurogenic factors in a LAHVA (Yla-Herttuala and Martin, 2000; Musgrave *et al*., 2002) to enhance development of a more 'native' heart valve tissue in concert with mechanical stimulation studies.

3a   3b
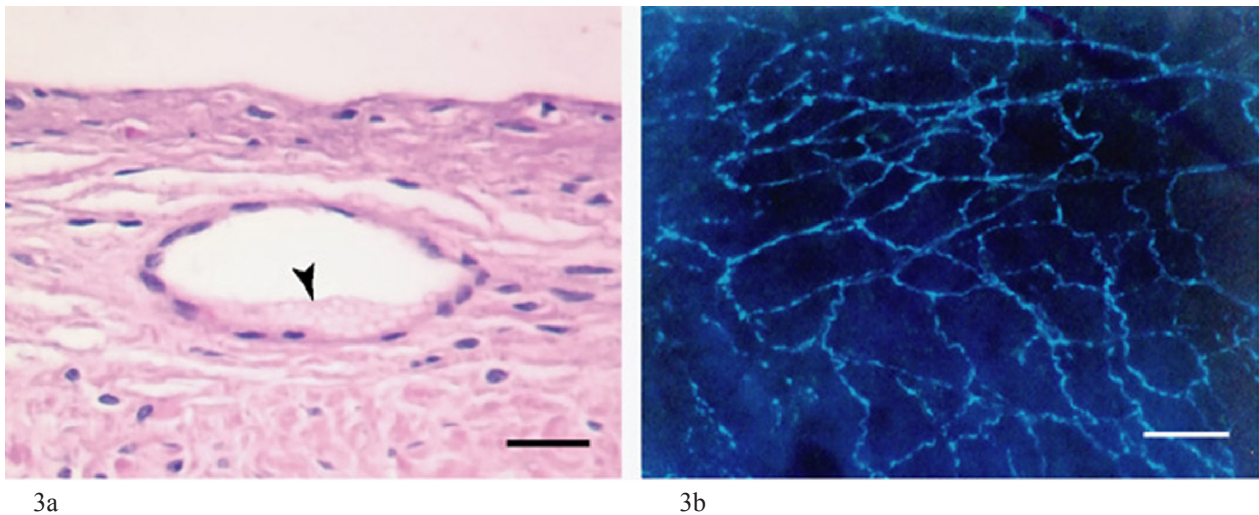
Figure 3 Both vascular elements **(a)** and neural elements **(b)** have been demonstrated in the heart valve interstitial matrix: **(a)** blood vessel embedded in the atrialis/spongiosa layer of the porcine mitral valve containing red blood cells (arrowhead) (haematoxylin & eosin staining). Scale bar 50µm; **(b)** dense adrenergic nerve plexus situated in the anterior leaflet of the rat mitral valve (sucrose-potassium phosphate-glyoxylic acid staining). Scale bar 30µm.

Conclusion

Heart valve tissue engineering holds great promise for improved treatment of valve disease. Significant advances have been made since its inception in the early 1990s, however, the field remains in its infancy and many issues remain to be addressed and resolved. Primary amongst these is our limited understanding of the normal heart valve, in particular normal valvar cell biology (including gene expression and regulation) and normal valvar ECM composition. A blueprint for LAHVA fabrication can only be achieved through substantial advances in our understanding of basic heart valve biology. Identification of the appropriate transplantation cell types and scaffolds remain the key strategies. Data concerning VECs remains limited, thus characterisation of these cells may take precedence over that of VICs. Furthermore, the presence of vascular (Figure 3a) and in particular neural elements (Figure 3b) in the AV valves requires further research to determine their role in normal valve function. It may be possible to control vascularisation and neurogenesis in a LAHVA by investigating, characterising and incorporating angiogenic and neurogenic factors. A thorough understanding of embryonic and fetal heart valve development may permit the control of heart valve tissue morphogenesis both *in vitro* and *in vivo*. As developmental studies improve, the field should acquire an improved understanding of the mechanisms that may allow for reproducible construction of clinically acceptable heart valves *ex vivo*. It would also allow accurate predictions about valvar morphogenic events under specified conditions, allowing advances to be made in the design and development of suitable bioreactor systems. Other key technological advances are required to enable the transition from experimental model to clinical reality, and will require the cooperation of researchers from a broad spectrum of disciplines. Consistent manufacture of LAHVAs and adequate assessment standards will pose equally challenging factors in this exciting area of research.

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Discussion with Reviewers

P. Taylor: The pressures during valve development are not the same as the maintenance pressures during life. What are the authors views on the flow/pressure conditions required to tissue engineer an aortic valve in a bioreactor? Should increasing pressures be used?
Authors: Fetal blood pressure increases gradually with ongoing maturation, and although there is limited data available, it has been suggested that complete development of all four heart valves takes place under pressure values below 10mmHg, with further remodeling of the structures up to 20 weeks gestation under increasing heart rate (Stock and Vacanti, 2001). A number of previous heart valve bioreactor studies have used pressure ranges increasing above late gestation fetal aortic pressure, with upper values of 55-70mmHg (Hoerstrup *et al*., 2000a; Hoerstrup *et al*., 2002). Although some promising results have been obtained, it would be inter-

esting to determine whether or not pressure values closer to those during valve development would improve tissue structure further. Inappropriately high values may inhibit cell proliferation, increase cell-scaffold detachment and may even increase cell death. More gradual increases in pressure over longer time periods, similar to those encountered *in utero*, may also prove beneficial and may establish a more positive feedback mechanism for tissue development. However, different cell sources and scaffold polymers are likely to be influenced in different manners by varying pressure ranges, even if cultured in identical bioreactor systems; therefore, the problems associated with inadequate valve tissue development can only be corrected with all factors taken together.

S. Jockenhövel: A general point of discussion is the expected market for tissue engineered heart valve prostheses. In my opinion the number of potentially needed implants is significantly over-rated. The high production costs will lead to strict indications e.g. for paediatric heart surgery or for the treatment of acute endocarditis.
Authors: The number of necessary implants may not strictly be over-rated, however, the phenomenal costs that will be involved in quality control processes, and in packaging, sterilization and storage of tissue-engineered heart valves, may certainly limit the scale-up of manufacturing processes early on. The preliminary target market for tissue-engineered heart valves is the paediatric population, where these valves would theoretically grow and remodel with the patient and would eliminate the need for successive reoperations. Once a proof of application and efficacy has been established in this market, manufacturing processes can be scaled up to supply <60years market. As the market size increases, production costs will decrease; this may particularly benefit patients in developing countries where rheumatic fever and rheumatic heart disease continue to have a high prevalence rate.

S. Jockenhövel: The current investigations in that field are encouraging – but we have to take care not to implant a tissue engineered heart valve too early. Animal models like sheep are not ideal models concerning endothelialization and hemostasis and might lead to an early failure of the prostheses in human clinical use like we have seen in tissue engineered small calibre vascular grafts.
Authors: Certainly the SynerGraft™ valves have shown that animal models have failed to predict the failures in humans (Simon *et al*., 2003). Animal experimental models can normally be encouraging but it is very difficult to translate the same success rate to humans. With regard to the poor success rates in animal models of heart valve tissue engineering, we strongly feel that it is the fundamental heart valve biological issues that need to be addressed, and significantly more *in vitro* research must be carried out. Also, the same test models have been used in the heart valve industry for years; more appropriate test models must be developed to be able to further develop living, tissue-engineered alternatives for introduction in the human clinical setting.

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