Towards new therapies for calcific aortic valve disease

Improving heart valve tissue engineering protocols and the search for pharmacological targets

Paul W. Riem Vis

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Cover: Characteristic phenotype staining for adult valvular interstitial cells in culture.
Top to bottom: Vimentin, SMemb, Desmin, CD31 and α-SMA.
Produced by J. W. van Rijswijk and P. W. Riem Vis.

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Op weg naar nieuwe therapieën tegen verkalking van aorta kleppen

Verbeteren van protocollen voor hartklep weefsel reproductie en het zoeken naar nieuwe farmacologische doelwitten

(met een samenvatting in het Nederlands)

Proefschrift

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Paul Willem Riem Vis

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Promotores:	Prof. dr. L. A. van Herwerden
	Prof. dr. C. V. C. Bouten
Copromotores:	Dr. J. Kluin
	Dr. J. P. G. Sluijter

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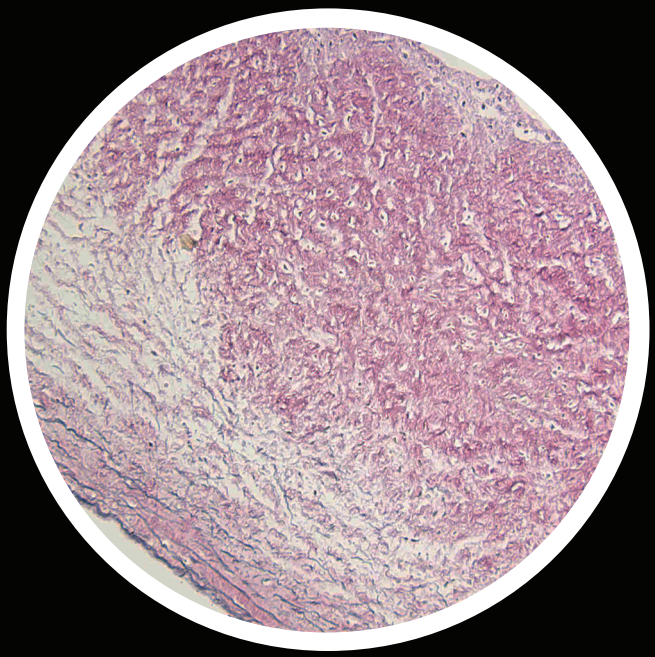
Voor Ilse

Content

Chapter 1



Chapter 1

General Introduction And Outline

Introduction

The aortic valve is located between the left ventricle of the heart and the aorta and has an important function to maintain a unidirectional flow of oxygenated blood from the heart into the systemic circulation. For this purpose, it consists of three pliable but load-bearing cusps that open and close approximately 4 billion times per lifetime, thereby withstanding high transvalvular pressures. These pressures are the net result from low blood pressure in the ventricle after contraction and high blood pressure in the aorta, which results in a net force directed from the aorta to the ventricle. In the adult aortic valve, total stresses in the aortic valve have been estimated to be as high as 500 kPa during diastole.[3] Aortic valve cusps are specialized structures able to absorb these forces and direct them towards the aortic wall. Improper functioning of the valve, caused by the inability to open smoothly or close properly, can ultimately lead to heart failure. Recent studies have identified that moderate aortic valve dysfunction already seriously increases the risk for cardiomyopathy and stroke.[3]

This chapter will serve to explain the burden of heart valve disease and to introduce currently used methods for aortic valve replacement and their shortcomings. Subsequently, the aortic valve structure and functioning will be more thoroughly described to provide a basic overview of this complex structure. This is required background information to understand the slow progression in development of new therapies for aortic valve disease. Two strategies that may lead to new possible treatments, tissue engineering and pharmacological therapies, will be introduced in this chapter as well. In this thesis, our work and our contributions to the development of these treatment strategies will be provided.

Aortic Valve Disease

Aortic valve disease may have a variety of genetic and/or environmental causes, but in general, calcification of the cusps is a common endstage. Deposition of calcific noduli causes stiffening of the aortic valve cusps, leading to obstruction of flow or improper closing. The severity of calcific aortic valve

Severity	Valve Area (cm^2)	Maximum Jet Velocity (m/s)	Mean Pressure Gradient (mmHg)
Sclerosis	-	<2.6	-
Mild	1.5-2.0	2.6-3.0	<25
Moderate	1.0-1.5	3.0-4.0	25-40
Severe	0.6-1.0	>4.0	>40
Critical	<0.6	-	-

Table 1. *Classification of the severity of valve disease in adults, according to the AHA and ACC*

disease (CAVD) can be classified according to the guidelines of the American Heart Association (AHA) and the American College of Cardiology (ACC) (Table 1). The only effective therapy for endstage CAVD, aortic stenosis (AS), is aortic valve replacement surgery and the indications for this invasive procedure are formulated by a working group of the European Society for Cardiology (ESC, Figure 1).[4] According to these guidelines, the patients symptoms are identified first, followed by tests to determine heart function, severity of aortic valve disease and the ability of the patient to exercise.

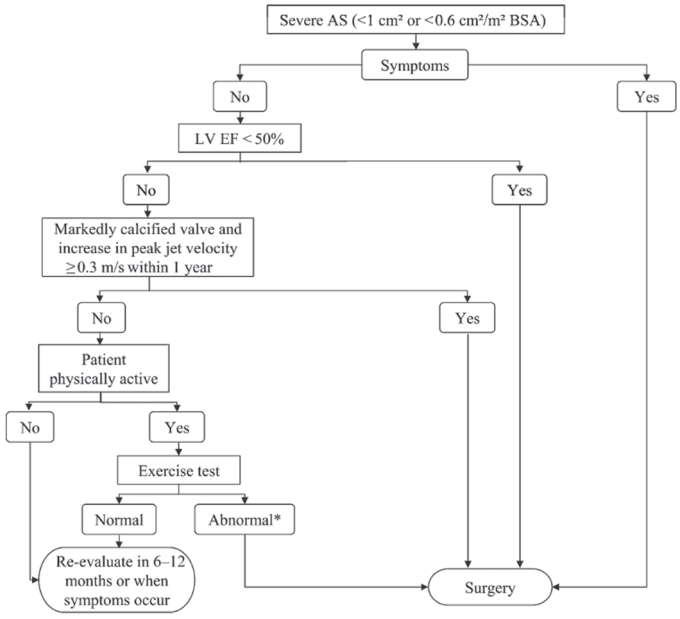


Figure 1. *Guidelines on management on valvular heart disease, according to the ESC: indications for surgery. AS: Aortic stenosis, LV: left ventricle, EF: ejection fraction, BSA: body surface area.*

Approximately 2-3% of adults over 65 years of age are thought to suffer from valve stenosis, requiring aortic valve replacement.[5] This amounts to a total number of approximately 300,000 aortic valve replacements worldwide and this number is thought to triple by 2050.[6] This increase is driven by the aging population and the high estimated prevalence of aortic valve sclerosis in adults.

The prevalence of aortic sclerosis over 65 years of age and over 75 years, is thought to be 26% and 37% respectively.[5] About one third of the patients with aortic valve sclerosis will develop aortic valve stenosis in a follow-up period of approximately four years.[7] To further underline the grim outlook for patients with valve stenosis, it has been shown that asymptomatic patients with a maximum jet velocity of >4 m/s have a 21±18% likelihood to be alive without clinical events which require operative treatment after two years.[8] The prognosis for symptomatic patients is even worse. After onset of severe symptoms, average survival ranges between 2-5 years, depending on the symptom.[9] Alternatively, current mortality risks during surgery for pure aortic valve stenosis are 2-5% for patients younger than 75 years of age and 5-15% in patients over 75 years [10], showing why invasive surgical procedures are preferred over not replacing diseased aortic valves in symptomatic patients.

Currently Used Aortic Valve Prostheses

There are several different aortic valve prostheses available, which all have several advantages and disadvantages, making them more or less suitable for specific groups of patients.

Mechanical valves are usually produced of pyrolytic carbon or titanium covered with pyrolytic carbon (Figure 2a), which makes them very durable. For this reason, they are attractive for use in patients under 60-70 years of age. However, their unnatural form and composition causes high shear stress, leading to induction of thrombus formation. For this purpose, anticoagulation therapy is required for patients that have received these valves, which in turn increases the risk of hemorrhagic events.[11, 12] In addition, the most commonly used anticoagulant is warfarin, which is toxic to fetuses, making mechanical valves undesirable for young women of child bearing age.

Alternatively, biological valve prostheses produced from bovine pericardium, porcine valve leaflets or equine valves may be used (Figure 2b, c and d). These constructs are prepared through a series of processes in which cells are removed, often followed by crosslinking steps to increase durability and specific treatments to inhibit calcification. Biological prostheses are also less thrombogenic as compared to mechanical valves, making anticoagulation therapy unnecessary. Unfortunately, the use of biological valves is hampered by their limited durability. The continuous opening and closing of the valve, the preparation process, lack of living cells to repair tissue damage and eventually calcification, all contribute to structural deterioration of biological prostheses.[11] In general, the durability of biological valves is limited to approximately 15-20 years, but this is strongly age-dependent. Valves in patients under 35 years of age generally fail after 5 years, while in patients over 65 years only 10% fails after 10-20 years.[11, 13] This makes biological tissue valves the current prosthesis of choice in patients over 60-70 years of age, in which the risk for reoperation is significantly lower when compared to younger patients. Recent investigations have pointed out that biological prostheses may even be the preferred over the use of homografts. The durability of the homograft in specific groups of patients is limited because of low-grade immunological responses and is not higher than biological prostheses.[14] Currently, homografts are therefore only the preferred choice after severe destruction of the annulus, following endocarditis.

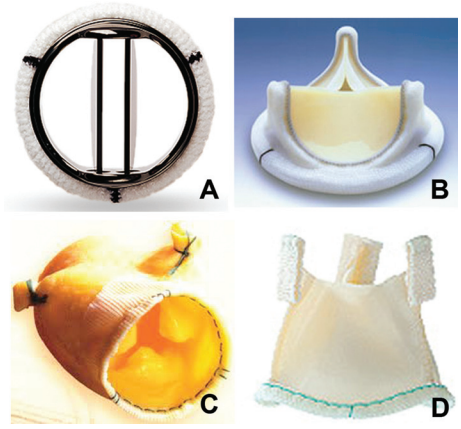


Figure 2. *Several prosthetic aortic valves.* ***2A.*** *St. Jude Medical Regent mechanical valve.* ***2B.*** *Carpentier-Edwards Perimount biological valve, derived from bovine pericardium.* ***2C.*** *Medtronic Freestyle porcine aortic valve.* ***2D.*** *Medtronic 3F equine aortic valve. Pictures have been obtained from manufacturers websites.*

These data clearly show that currently used aortic valve prostheses offer a limited solution for specific groups of adult patients, but they are still not ideal. They are also especially undesirable for use in children, because their lack of growth. Currently, the Ross-procedure and aortic valve repair with autologous pericardium are suggested treatments for these patients. In the Ross procedure, the diseased aortic valve is replaced with the pulmonary valve (Figure 3). The Ross-procedure is reported to result in higher survival rates for patients as compared to replacement with homografts [15], but this procedure requires advanced surgical expertise and still requires implantation of another prosthesis or homograft to replace the pulmonary valve. Moreover, it has been shown that the neo-aorta dilates in 17-34% of the patients within 10-16 years, which requires high risk reoperations.[16]

Because of the invasiveness of conventional surgical aortic valve replacements, they are preferably not performed on older, high risk patients. Recently developed aortic valve implantation techniques decrease the invasiveness of aortic valve replacement, and have proved to be a promising alternative for these patients.[10] These approaches include trans-apical and trans-femoral aortic valve implantations (transcatheter aortic valve implantation, TAVI) (Figure 4), in which the aortic valve is approached through the apex of the heart, the femoral artery or the subclavian artery, followed by deployment of the stented valve over the stenotic valve using a balloon or a self-expanding frame [10]. The first published results of follow-up studies in old, high risk ("inoperable") patients reveal that these less invasive therapies have a lower mortality after 3 years than is to be expected without surgical intervention.[10] Unfortunately these therapies also have several limitations to overcome, like high incidence of stroke, pacemaker implantation, major

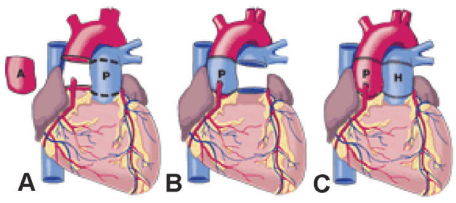


Figure 3. *Ross-operation.* ***3A.*** *The disease Aortic valve (A) is excised and the Pulmonary valve (P) removed.* ***3B.*** *The pulmonary valve is placed in the aortic position.* ***3C.*** *The pulmonary valve is replaced with a homograft (H).*

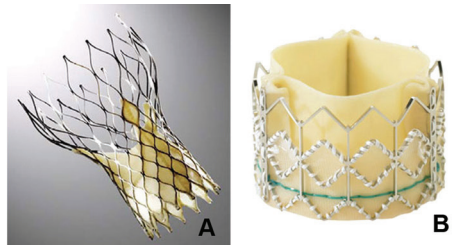


Figure 4. *An example of a self-expanding aortic valve (****4A.*** *Medtronic CoreValve) and a balloon-deployed aortic valve (****4B.*** *Edwards Sapien), suitable for minimally invasive aortic valve replacement therapies.*

vascular complications and residual aortic valve regurgitation.[10, 17, 18] In addition, the durability of these prostheses needs to be determined with long-term follow-up studies. Non-invasive therapies thus remain to be considered as a last resort for otherwise untreatable elderly patients.

Taken together, these data show that patients treated with currently used aortic valve prostheses still suffer from a variety of complications. None of them offers a viable, easily implanted, durable replacement for the diseased valve. Importantly, it has been shown that the life expectancy of patients after aortic valve replacement therapies is significantly lower when compared to age-matched healthy individuals living in the same environment, even if no valve-related events occur. This is especially the case in patients in which the valve is replaced at a relatively young age.[19] There is increasing evidence that this difference is related to the use of sub-optimal valve substitutes.[20] Taken together, this means that suitable therapies are still not available for a large proportion of patients and that there is a need for alternative treatment strategies. In this thesis, our contributions to the development of these alternatives are described.

In the first part of this thesis (chapter 2-5), the concept of tissue engineering is discussed. Researchers have turned to the development of tissue engineered heart valve constructs, with the aim to combine patient-derived cells and synthetic scaffolds in patient-specific tissue constructs, offering the ability to grow and remodel *in vivo*. This search has resulted in several promising constructs that performed well in experimental, *in vitro* settings and even in the pulmonary position in large animals.[21] Currently existing culture protocols have to be adapted for use in humans, to allow for fully autologous production of these tissue constructs. Next, the valves have to become stronger to withstand high pressures in the aortic position. In the second part of this thesis (chapter 6-8), the focus will be on the underlying causes for CAVD and the search for new pharmacological treatment options. Research in this field aims to halt progression of CAVD in early stages, thereby reducing the probability that patients will require surgery.

A better understanding of valve structure and function is essential for successful development of new therapies. Load-bearing capacities, durability, viability and susceptibility of heart valves to disease are all determined by cellular and molecular components of the valve. An overview of several of the most important aortic valve features is therefore presented in the following sections.

Aortic Valve Structure And Function

The cusps are attached to the aortic wall through a fibrous ring, called the annulus. The forces put on the leaflets are redirected through the annulus to the wall of the Sinus of Valsalva, which is the space between the cusp and the aorta.[22] The shape of the sinuses allows formation of vortices in blood flow behind the leaflets during early diastole, which are thought to assist in the closing of the leaflets. Without the vortices, the leaflets would also close, but probably less efficiently.[22] Proper functioning of the aortic valve cusps requires high flexibility to ensure low resistance for blood leaving the ventricles, but also high tensile strength and ability to strain in order to resist transvalvular pressure.[22] It is generally accepted that the trilayered structure of the valve and the valvular interstitial cells (VICs), dispersed throughout the valve tissue, contribute to this high functionality. The three layers in the aortic valve cusps include the collagen-rich fibrosa layer on the aortic side of the cusp, the elastin-rich ventricularis layer on the ventricular side and the hydrated sponiosa layer in the middle (Figure 5). The dense collagen network in the fibrosa provides the strength to resist high strains. It is thought that stretching of crimped collagen fibers is the first step in the bearing of load imposed by aortic blood pressure. During the stretching of collagen fibers, the elastin network in the ventricularis is the primary load-bearing structure, which shifts as soon as collagen fibers are completely unfolded.[11, 23] Circumferentially-oriented collagen fibers are fully stretched at 20 mmHg and modest strain is followed by rapid stiffening (locking).[22, 24] Circumferential strain in the aortic valve is thus limited since collagen fibers in the fibrosa are mainly organized in this direction. Movement of the ventricularis and fibrosa layers of the valve is facilitated by the spongiosa layer between them, which contains glycosaminoglycans in a hydrated matrix.[11] Together, these data show that the leaflet-architecture,

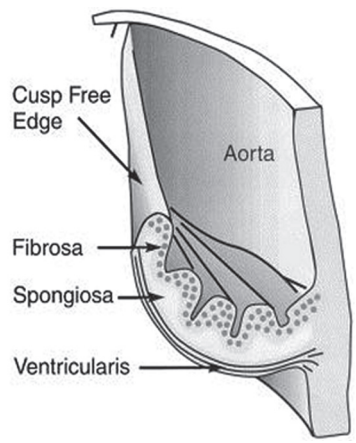


Figure 5. *Representation of the aortic valve cusp with its layered architecture, showing crimped collagen fibers in the fibrosa layer during systole. Elastin fibers are presented as straight lines in the ventricularis layer.*

i.e. the three different layers and the (an)isotropy of collagen and elastin fibers, is important for proper functioning of the valve. Matrix formation and remodeling is therefore an important focus in chapters 3-5, in which new culture protocols for tissue engineered valves are described.

Matrix Remodeling And Regeneration In The Aortic Valve

The cellular component of heart valves consists of the VICs and the valvular endothelial cells (VECs) lining the cusps. These cells are extremely important for the development of the valve, which is reviewed in chapter 2. However, they are also important in physiological maintenance of the matrix in mature heart valve tissue and the onset of aortic valve disease, through active remodeling and regeneration processes. The next section will therefore describe these cells, their role in valve biology and their importance in newly developed therapies.

Continuous application of forces on the valve cusps causes wearing and tearing of the valvular matrix, which necessitates ongoing remodeling and repair of the matrix to prevent structural deterioration. It is generally accepted that matrix maintenance is performed by VICs, possibly controlled by signals derived from the VECs.[25-27] VICs are generally described to be a mesenchymal, myofibroblast-type of cells, which can switch between different levels of activation. In normal healthy valves, quiescent VICs are phenotypically only characterized by expression of the intracellular protein vimentin, which also serves as a marker for mesenchymal cells.[28] However, in embryonic development or during active matrix repair and remodeling, they also express α-Smooth Muscle Actin (αSMA) and non-smooth muscle myosin heavy chain (SMemb) as phenotypic markers for their activated state.[28] In general, they do not express muscle marker desmin, which distinguishes them from vascular smooth muscle cells and thus makes them resemble myofibroblasts more closely (Figure 6). For tissue engineering, these phenotypical markers are important because in general, researchers aim to use cells in their constructs that will eventually resemble VICs, or at least their ability to produce and remodel matrix.

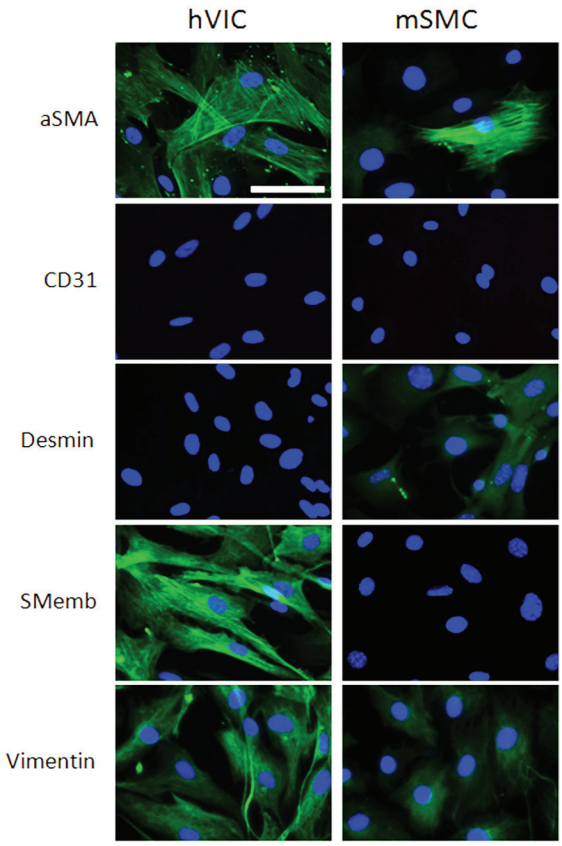


Figure 6. *Phenotype characterization of human valve interstitial cells and murine aortic smooth muscles cells. Both celltypes express aSMA and vimentin (positive green staining) and lack endothelial marker CD31 (showing only blue nuclear staining). VIC express SMemb, whereas SMCs do not. This is vice versa for muscle cell-marker desmin. Original magnification: 40x, scale bar similar in all images: 25 µm*

However, besides switching between quiescent and activated states, VICs have also been described to posses the capacity to differentiate into cells from other mesenchymal lineages, like bone-forming osteoblasts.[29-33] It is assumed that this ability marks later stages of CAVD, as described in chapter 6. Excessive, or pathological, activation and differentiation of VICs are stages of CAVD. Successfully inhibiting these cellular responses could possibly arrest the progression of CAVD before it becomes symptomatic. Therefore, the holy grail in prevention of CAVD progression is to identify pathways involved in these processes and successfully inhibit them. In chapter 6, known processes involved in the most common causes for CAVD

are discussed, followed by studies investigating possible new pathways and signaling networks in CAVD in chapters 7 and 8. In the end, these studies may also prove valuable for application in tissue engineering, to prevent differentiation and calcification in the neo-constructs after implantation.

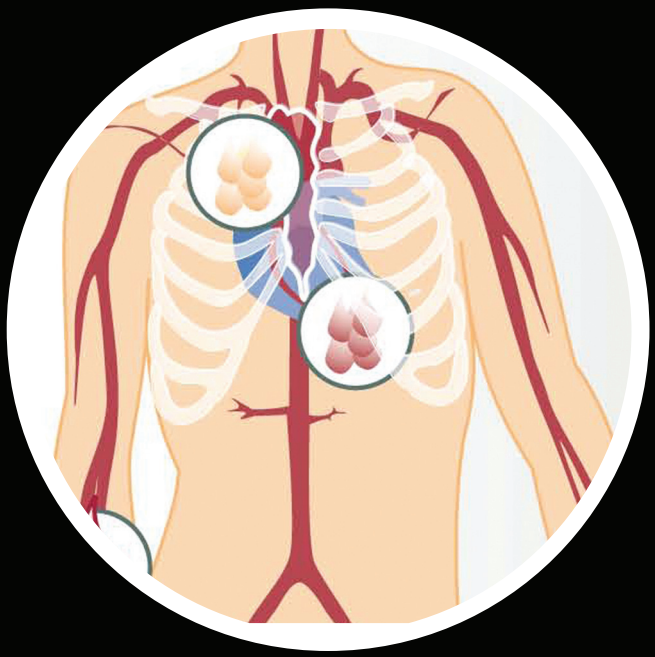
Outline Of This Thesis

The first chapters of this thesis describe our efforts to replace animal serum in existing culture protocols for heart valve tissue engineering with human-derived alternatives. Chapter 2 summarizes embryonic development of the aortic valve and uses information from valvulogenesis to formulate a number of possibilities to improve currently existing culture protocols for heart valve tissue engineering. Available cell sources and culture protocols will also be discussed. In chapter 3, a first step is made towards autologous heart valve tissue engineering, by replacing animal serum with human platelet-lysate in cell culture. The focuses in this chapter are on comparison of cell proliferation, differentiation, matrix production and matrix remodeling as a result of specified culture media. Next, chapter 4 describes the efforts to produce tissue with cells expanded in platelet-lysate. The emphasis of the chapter is on load-bearing tissue production and matrix remodeling. Lastly, chapter 5 shows our efforts to use human serum in tissue culture to produce load-bearing tissue. A culture protocol is proposed in which the benefits of platelet-lysate are combined with those of human serum, to allow production of load-bearing constructs, suitable for heart valve tissue engineering in the future.

The second part of this thesis emphasizes on CAVD. In chapter 6, the most common processes behind CAVD are reviewed, in addition to the pharmacological therapies that have been unsuccessfully applied to halt progression of the disease. Following this summary chapter 7 presents a newly identified pathway mediated by Wnt-signaling. Chapter 8 describes work performed on microRNAs, which have recently emerged as regulators of cellular proliferation, differentiation and other physiological and pathological processes.

Lastly, the general discussion in chapter 9 shows the relationship between these parts of this thesis, followed by a discussion of the presented work and formulates recommendations and directions for future research.

Chapter 2



Chapter 2

Environmental Regulation Of Valvulogenesis: Implications For Tissue Engineering

This chapter has been published in:

Riem Vis PW, Kluin J, Sluijter JP, van Herwerden LA, Bouten CV. Environmental regulation of valvulogenesis: implications for tissue engineering. Eur J Cardiothorac Surg. 2011;39:8-17.[34]

Abstract

Ongoing research efforts aim at improving the creation of tissue engineered heart valves for *in vivo* systemic application. Hence, *in vitro* studies concentrate on optimizing culture protocols incorporating biological as well as biophysical stimuli for tissue development. Important lessons can be drawn from valvulogenesis to mimic natural valve development *in vitro*. Here, we review the up-to-date status of valvulogenesis, focusing on the biomolecular and biophysical regulation of semilunar valve development. In addition, we discuss potential benefits of incorporating concepts derived from valvulogenesis, as well as alternative approaches, in tissue engineering protocols, to improve *in vitro* valve development. The combined efforts from clinicians, cell biologists and engineers are required to implement and evaluate these approaches to achieve optimized protocols for heart valve tissue engineering.

Introduction

To date, prostheses used in valve replacement therapies suffer from limitations, mostly related to the lack of living tissue.[11] Tissue engineering aims to reduce these limitations by creating living heart valve replacements that are able to grow and adapt to changes in physiological environment (Figure 1). In its most frequent strategy, tissue engineering involves the isolation of cells, *in vitro* cell expansion, cell seeding on carriers (scaffolds) and mechanical conditioning in bioreactors, prior to implantation (Figure 2a).

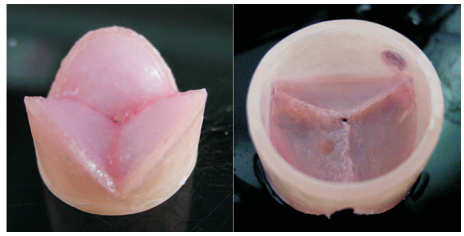


Figure 1. *Tissue Engineered Heart Valve, derived from Mol et al.[1] Reprinted with permission.*

Abbreviations and acronyms

αSMA	α-Smooth muscle actin
AV-valves	Atrio-ventricular valves
BMPs	Bone morphogenetic proteins
ECM	Extra-cellular matrix
EGF	Epidermal growth factor
EMT	Endocardial-to-Mesenchymal Transition
EPCs	Endothelial progenitor cells
EPDCs	Epicardial-derived cells
FGF	Fibroblast growth factor
GAGs	Glycosaminoglycans
GTP	Good tissue practice
HA	Hyaluronan
HB-EGF	Heparin-binding epidermal-like growth factor
HSCs	Hematopoietic stem cells
miRNAs	MicroRNA, or micro ribonucleic acids
OFT-valves	Outflow tract valves
P4HB	Poly-4-hydroxybutrate
PGA	Poly-glycolic acid
TGFβ	Transforming growth factor-β
VEGF	Vascular endothelial growth factor-A
VICs	Valvular interstitial cells

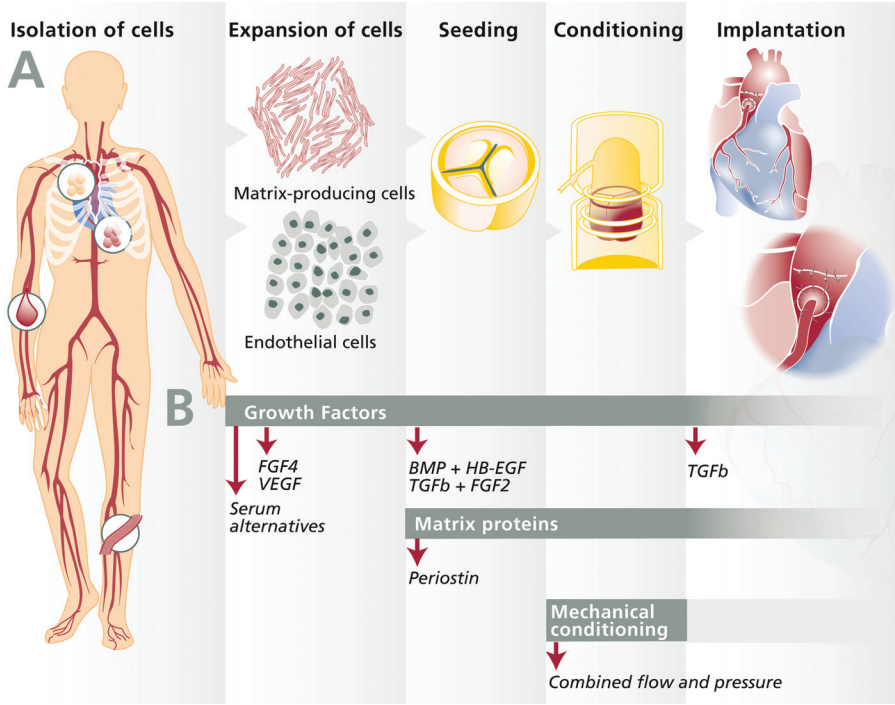


Figure 2. *Concept of tissue engineering and suggestions presented in this paper.*
2A. *Stages of general tissue engineering strategies are: isolation of cells, expansion of cells, seeding on synthetic scaffolds, mechanical conditioning and implantation in the patient. Some sources for autologous cells enable isolation of matrix-producing cells in addition to endothelial-like cells, that can be used to form a monolayer on the construct. After seeding and potentially during conditioning, cells produce extracellular matrix leading to formation of tissue.*
2B. *Suggestions for tissue engineering strategies as described in this paper. These suggestions include looking at alternative cell sources, like cells from the heart (blue in Figure A), next to sources that have already been reported (red). In addition, suggestions for use of growth factors, matrix proteins and mechanical conditioning are presented. Growth cocktails can be designed for specific stages of tissue engineering, to induce proliferation or tissue formation. After implantation, exogenously added growth factors can be released slowly, but their effect will diminish eventually. An example of this is TGFβ, as described in the future perspectives. Matrix-protein coating of scaffolds can lead to improved valve-like niches for cells, which could influence cell behavior. After implantation, exogenous matrix proteins can be replaced by proteins produced by the cells. Lastly, mechanical conditioning should involve flow and pressure combined and crosslink formation can be analyzed as an additional predictor for tissue strength. Mechanical conditioning continues after implantation, albeit uncontrolled.*

Although initial feasibility-studies showed successful application of tissue engineered heart valve constructs in the pulmonary position in sheep [21], the clinically more relevant application in the aortic position has not yet been achieved. This is mainly due to difficulties in the production of strong, flexible and durable tissue that can withstand systemic pressures for prolonged periods of time. These characteristics are strongly related to the microscopic and macroscopic valvular architecture [11], motivating the numerous efforts to induce this architecture *in vitro*.

The aortic valve consists of the aortic root comprising three sinuses and three flexible semilunar cusps attached to a ring of fibrous tissue, called the annulus. The cusps are the main load-bearing parts of the valves and they consist of three layers: the collagen-rich fibrosa on the aortic side, the intermediate spongiosa, mainly containing glycosaminoglycans (GAGs), and the elastin-rich ventricularis on the ventricular side. In mature valves, two main cell types have been identified: valvular interstitial cells (VICs) are dispersed in all three tissue layers, and endothelial cells cover the cusps (Figure 3). VICs are thought to be responsible for maintenance and remodeling of the tissue, in particular of the extracellular matrix and thus for the durability and adaptive capacity of the cusps.

The architecture of the aortic root and cusps is organized such that it reduces the mechanical stresses on the valve. The root withstands continuous deformations, whereas the typical fiber architecture of the cusps transmits applied forces from the cusps to the annulus and the aortic wall. [35] Scientific progress has not yet resulted in tissue engineered prostheses that mimic the adult tri-layered natural aortic valve architecture *in vitro*, but it is likely that this architecture is achieved *in vivo*, as was demonstrated for engineered valves placed in the pulmonary position.[21]

As suggested by Butcher and co-authors in 2007 and 2008, the embryonic development of heart valves, or valvulogenesis, can be used as a template by tissue engineers to optimize reconstruction of native aortic valves.[36, 37] This has resulted in suggestions for new tissue engineering strategies, like the use of growth factors and specific mechanical stimulation protocols. However, there are additional, perhaps more challenging, lessons available from valvulogenesis that can be incorporated. In the present review, we evaluate up-to-date knowledge of human aortic valve development, integrating the roles of the biomolecular and biomechanical environments during pre- and postnatal morphogenesis of the aortic valve. Next, we provide

suggestions for aortic valve tissue engineering protocols derived from our analysis, with specific emphasis on cell sources, growth factor cocktails and serum-alternatives, matrix proteins and mechanical stimulation. Lastly, we discuss alternative strategies that have not yet been studied in relation to valvulogenesis.

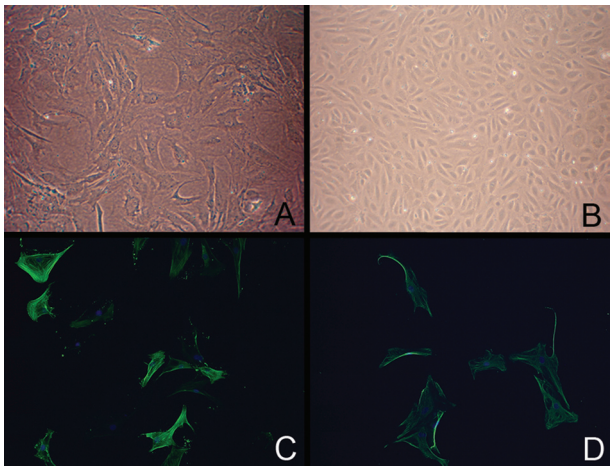


Figure 3. *Valvular interstitial cells (VICs) and endothelial cells (VECs).* ***3A.*** *VICs in culture with light microscopy have a fibroblast-like appearance, while VECs have a "cobblestone-pattern" (****3B.****). Valvular interstitial cells in culture are positive for (myo)fibroblast markers α-Smooth Muscle Actin (****3C.****) and vimentin (****3D.****).*

Natural Valve Development

Initiation of embryonic valve formation

Heart valve development starts with formation of the cardiac jelly at embryonic day 8.5 (E8.5) in mice, and in the middle of the third week after conception in humans. The cardiac jelly is formed by myocardial secretion of GAGs, like hyaluronan (HA).[38] This initial synthesis of extra cellular matrix (ECM) is thought to be stimulated by Bone Morphogenetic Protein-4 (BMP-4) in the outflow tract (OFT) at E8.5 (Figure 4a).[36] This is followed by cardiac cushion formation at E9 in mice and day 20 in humans, through a process known as endocardial–to-mesenchymal-transition (EMT, Figure 4b).[36, 39] During this process, endocardial cells become activated, lose their cell-adhesion molecules, migrate into the cardiac jelly, and transform into mesenchymal cells.[36, 40]. Both cardiac jelly formation and induction of EMT rely on the presence of matrix proteins that stimulate the invasion of mesenchymal cells into matrix and initialize transformation.[41, 42]

The human embryonic heart starts to contract at 65 beats per minute (bpm) at the beginning of the fourth week after gestation and creates unidirectional flow few days later.[43, 44] Thus, EMT starts under (mild) cyclic shear stresses, while transvalvular pressure is believed to be zero.[44] Though cardiac contraction and EMT start at the same time, the relative and combined contribution of hemodynamics and growth factors to valvulogenesis at this stage remain to be elucidated. Shear forces are likely to contribute to cardiac cushion formation and EMT, but they are not indispensible in presence of specific growth factors [45, 46] and might hence be relevant for fine-tuning morphogenesis.

Regulation of Endocardial-to-Mesenchymal Transition

The most frequently described molecular processes involved in the initiation of EMT are down-regulation of vascular endothelial growth factor (VEGF) [39, 47] and upregulation of BMP-2.[36, 48, 49] Interestingly, the role of BMP-2 in EMT was primarily identified in atrio-ventricular (AV)-valves, but not in OFT-valves. At this location, BMP-4 has been suggested as an alternative [50, 51], but the exact mechanism of EMT-initiation in OFT-valves is still not clear.

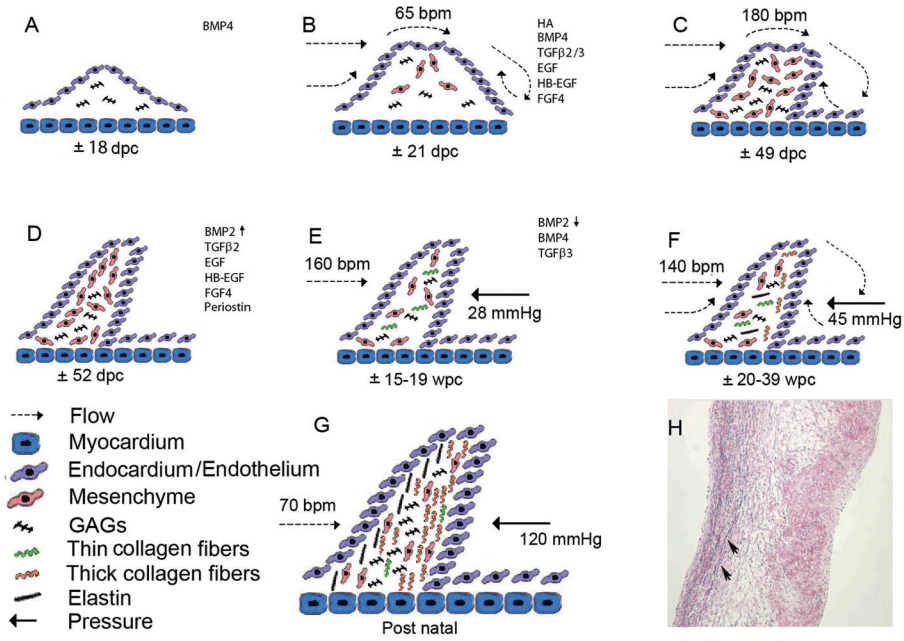


Figure 4. *Overview of aortic valve development.* ***4A.*** *Cardiac jelly is formed by secretion of GAGs.* ***4B.*** *The heart starts to beat, resulting in unidirectional flow few days later. EMT results in mesenchymal cell differentiation and infiltration and formation of cardiac cushions. Flow is not laminar.* ***4C.*** *Flow increases and different flow patterns on each side of the cushion are suggested to lead to initiation of valvular morphogenesis.* ***4D.*** *During the elongation phase, mesenchymal cells on the tip of the valve proliferate, causing elongation of the primitive cusp.* ***4E.*** *During fetal development, the first transvalvular pressure co-occurs with the first thin collagen fibers.* ***4F.*** *At later stages of fetal development, transvalvular pressure increases and the first thicker collagen fibers and elastin fibers can be observed. There is also some preliminary stratification.* ***4G.*** *Post-natally, the valve reaches its final, stratified morphology. Different layers including primarily elastin, GAGs or collagen can be observed.* ***4H.*** *Histological section of a porcine aortic valve, stained with elastin von Giesson, showing the final tri-layered valve architecture. Elastin fibers (arrows) are present at the ventricular side of the cusp, followed by a GAG-layer (white) in the middle and a dense collagen layer (stained red) on the aortic side. Abbreviations next to each image indicate regulatory involvement of specified molecular (growth) factors. These abbreviations are explained in Table 1, elsewhere in this chapter. Other abbreviations: dpc: days past conception, wpc: weeks past conception, bpm: beats per minute.*

The onset of EMT in OFT-valves is followed by (de)activation of complex signaling networks by growth factors, that have been described in more detail by others and which are beyond the scope of this paper. [36, 51, 52] To mention, some of the relevant growth factors involved are Transforming Growth Factor (TGF)-β2 and β3, Fibroblast Growth Factor (FGF)-4 [53-55], BMP-4 [51], Epidermal Growth Factor (EGF) [51] and Heparin-binding Epidermal-like Growth Factor (HB-EGF) [56]. These factors can influence either proliferation, migration into the matrix, or differentiation of endocardial cells into mesenchyme. (Figure 4b)

In chick AV-valves, transition of cardiac jelly into cardiac cushions coincides with tissue stiffening and increased tissue thickness due to higher cell mass and collagen deposition.[46] This leads to preliminary valvular functioning of the cushions in regulation of blood flow. Mathematical models of embryonic hemodynamics suggest that subsequent 'leafing' of the cushion in direction of the flow results from differences in flow patterns (laminar or vortex) up- and down-stream of the cushions (Figure 4c).[57] The primitive cusps remain short and thick upto the 7th week of human development, while the heart rate increases to 180 bpm and arterial blood pressure stays below 1 kPa (10 mmHg).[43, 44]

Together, these findings indicate that biomolecular factors, like growth factors and transcription factors, as well as hemodynamic forces, contribute to the regulation of EMT and early morphogenesis of valvular leaflets.

The elongation phase

Valvular maturation starts in humans at embryonic day 52 (mice E14.5) and occurs in several stages: mesenchymal proliferation first causes a phase of cusp elongation, which is followed by differentiation of the cells and remodeling of the ECM.[58] During the elongation phase, FGF-4 stimulates proliferation of distal mesenchymal cells [36, 55], while TGF-β2, HB-EGF and EGF are thought to act as inhibitors of this process to prevent hyperplasia (Figure 4d).[36, 51, 56, 59]

During the differentiation phase, proliferation stops and signaling cascades involved in EMT are partially reversed.[36, 39] Also, BMP-2 is expressed in the OFT cushions at E12.5, potentially inducing valve maturation through the expression of the matricellular component periostin (Figure 4d).[60] Periostin has been located in both OFT and AV valve cusps where it plays an important role in valve maturation through stimulation of mesenchyme invasion and production and compaction of matrix.[53, 60, 61] The differentiation phase continues into the second trimester of human development and coincides with the expression of growth factors, like FGF-4 and BMP-2, as potential regulators of mesenchymal proliferation and matrix remodeling.

Final stage of valve maturation: pre- and postnatal remodeling

During the last phase of valvular development, additional matrix components are formed and ultimately arranged into the tri-layered architecture as observed in mature valves. These changes are depicted in Figure 4e-g and include:

- A decrease in cell proliferation from 30% in the endocardial cushions, to 5% pre-natally and 1% in the post-natal valve.[62]
- Upregulation of α-Smooth Muscle Actin (α-SMA) by interstitial cells in mouse AV-valves between E15.5 and E18.5, associated with activated myofibroblasts and contractile properties.[28, 63] In mature, post natal valves, αSMA-expression is lost, indicating differentiation towards the final, quiescent fibroblast phenotype.[64]
- In humans, the elastin content increases from 1% during the first part of the second trimester (14-19 weeks after gestation) to 4% at the third trimester (20 to 39 weeks after gestation) and 25% post-natally.[28]
- Where collagen exists only in small, disorganized fibers in earlier stages of valvulogenesis, collagen content reaches a maximum during the maturation-period. After this, only the thickness and orientation of collagen fibers change: the frequency of thin collagen fibers decreases from 72% in the second trimester to 24% in the third, while the frequency of thick fibers increases from 5% to 30%. During post-natal remodeling, mature collagen fibers compose 47% of the total surface area.[28]

This maturation could be driven by changed hemodynamic loads, such as high shear stresses and increased transvalvular pressures during this period.

- The heart rate remains high at 155-160 bpm at 20 weeks after gestation and only gradually decreases to 140 bpm prior to labor [44], followed by a more gradual decrease to 70 bpm at mature age.
- Aortic blood pressure increases from 4 kPa (28 mmHg), to 6 kPa (45 mmHg) at 40 weeks of gestation.[65] Post-natally, the shunts between the pulmonary and systemic circulation close and the aortic pressure increases to 9/5 kPa (70/40 mmHg) after birth, slowly rising to 16/12 kPa (120/90 mmHg) in adults.[28]
- Shear stresses in adults are estimated to be 8.0 Pa on the ventricular side of the cusps in adults, and between -0.8 and 1.0 Pa in the sinuses on the aortic side.[66] These different shear stresses are thought to induce differences in matrix depositions on different sides of the cusp. [52]

The mechanisms underlying the transduction of mechanical stimuli to cellular behavior are still only partly known but may involve the inhibition of BMP-2-signaling.[67] In addition, TGF-β3 is thought to increase collagen production and both BMP-4 and TGF-β3 have been suggested to play a role in semilunar valve maturation.[68, 69] Although mechanical stimuli may become more important for regulation of valvulogenesis in maturing valves, it would again be interesting to determine the most favorable combination of growth factors and mechanical stimuli, to optimize valvular tissue development and maturation in tissue engineering strategies.

Implications of Valvulogenesis for Tissue Engineering

In the following sections, different steps of currently used *in vitro* heart valve tissue engineering protocols will be analysed with respect to potential improvements derived from valvulogenesis.

Available cell sources to mimic valvulogenesis

Valvular cells are predominantly endocardial-derived mesenchymal cells and endothelial cells.[70] Vascular myofibroblasts have been suggested as cells of mesenchymal origin for use in heart valve tissue engineering.[1, 28, 71] They can be easily harvested from vascular grafts and allow concomitant isolation of vascular endothelial cells as a source for valvular endothelial cells.[1, 72, 73] However, relatively invasive procedures are required to obtain venous grafts and the grafts may not be available from all patients.

Alternatively, adipose- or bone marrow-derived mesenchymal cells are used for heart valve tissue engineering. [74-77] These cells can be differentiated towards endothelial cells and thereby allow formation of a valve construct with both mesenchymal cells and endothelial cells from a single biopsy.[78] Limitations of these cells might be the invasive harvest procedure and their susceptibility for osteogenic differentiation, which has been shown repetitively for mesenchymal stem cells as well as for hematopoietic stem cells (HSC).[79] It has even been suggested that the natural ongoing replacement of VICs with HSC *in vivo* can lead to valvular pathologies in time.[79] Thus, future studies should determine whether valvular constructs produced with mesenchymal progenitors are prone to calcification *in vivo*.

Recently, studies have focused on the use of Endothelial Progenitor Cells (EPCs) circulating in the bloodstream. An advantage of these cells is the relatively non-invasive way in which they can be obtained. Moreover, EPCs can differentiate into mature endothelial cells and are able to undergo transdifferentiation into mesenchymal cells [80], which mimics EMT-processes as witnessed during valvulogenesis. EPC-like cells have already been used for reseeding of decellularized allograft pulmonary valves [81] and synthetic poly-glycolic acid (PGA) scaffolds [82], while future applications aim at *in vivo* recellularization of implanted scaffolds.[83]

Although EPC characterization is far from complete, earlier *in vitro* work indicated that the EPC-population is comprised of at least two different cell types referred to as 'early' and 'late' EPCs.[84] These different EPC-populations produce different proinflammatory cytokines that could limit their use for heart valve tissue engineering. Early EPCs produce Tissue Factor for instance, which could increase the risk of thrombosis on the graft surface, whereas late EPCs produce Monocyte Chemoattract Protein-1, a proinflammatory cytokine that may stimulate invasion of inflammatory

cells.[85] It should be further investigated whether the production of these cytokines limits the application of EPCs for tissue engineering and if pharmaceutical treatment is required to reduce their release.[85] Thus, EPCs are clinically accessible in a relatively non-invasive manner, but their true potential for clinical applications remains to be determined.

Another interesting cell source are the cells derived from the proepicardium, known as epicardial-derived cells (EPDCs), which give rise to cardiac fibroblasts and have a speculated role in valve development.[86] EPDCs and cardiac fibroblasts are able to produce periostin and collagen type I, relevant for heart valve tissue formation.[86] Future studies should elucidate if EPDCs or cardiac fibroblasts can be easily isolated from adult patients and if they can be useful for heart valve tissue engineering purposes.

Use of matrix proteins in heart valve tissue engineering

Despite the relevance of matrix proteins in regulation of valvular interstitial cell behaviour, they are generally not used in tissue engineering protocols. The application of decellularized allografts to preserve the highly specialized valvular matrix and architecture may favor this natural cellular environment, or niche, but low availability of donor valves limits the use of this technique. [71, 81, 87, 88] Scaffolds for tissue engineering, are predominantly made out of synthetic polymers, like PGA, or natural polymers such as fibrin.[80, 82, 89] An extracellular matrix niche that resembles the matrix-environment during valvulogenesis may be obtained by coating of synthetic scaffolds with defined matrix proteins, to manipulate cell behavior and ultimately tissue properties.

An interesting candidate protein is periostin, which might be involved in maturation of the valve and modulation of cellular phenotype. [53, 60, 61, 69] Lack of periostin has even been related to calcific aortic valve disease, stressing its relevance for normal valve development.[90] Incorporation of periostin in collagen gels showed beneficial effects on invasion of cells into the gels and enhanced collagen remodeling, as indicated by condensation and compaction.[53] Care should be taken, however, to control matrix compaction due to remodeling, since it may lead to retraction of valvular cusps and impaired function of engineered valves. Keeping the delicate balance between healthy and excessive compaction will be an important problem to overcome in the coming years.[91]

Tailored culture media for specific stages of heart valve tissue engineering

The previous sections have shown that multiple growth factors are expressed in spatio-temporal patterns during valvulogenesis. In the last few years, the first papers on heart valve tissue engineering have been published using different growth factors to improve tissue properties.[75, 92, 93] However, research has focused primarily on the growth factors most commonly used in cell culture, like FGF-2, EGF and TGFβ. Although the use of FGF-2 and EGF can be useful for tissue engineering strategies in general, their family-members FGF-4 and HB-EGF are specifically beneficial for aortic valve development. Moreover, it has been shown that BMPs are indispensible for valvulogenesis, but they are not reported for heart valve tissue engineering purposes. This might be explained by their role in osteogenic differentiation [94] and the development of diseased and stenotic valves *in vivo*.[32, 95, 96] However, HB-EGF-signaling was suggested to interfere with BMP-dependent signaling pathways [97, 98] and to reduce chondrogenic differentiation [59].Thus, using BMPs in combination with other growth factors, as occurs during valvologenesis, could result in protection from osteogenesis during tissue culture. Likewise, FGF-2 can disrupt TGFβ-mediated myofibroblast activation, thereby preventing unbalanced extracellular matrix production. [99] Exploring the addition of less conservative growth factor cocktails to culture media should therefore be considered when optimizing tissue engineering strategies.

Such cocktails can be sequentially added *in vitro,* based on their temporal presence and regulatory roles in valvulogenesis. The first step in general tissue engineering paradigms is *ex vivo* expansion of endothelial and/or mesenchymal cells, followed by cell seeding and tissue culture. Proliferation of endothelial-like cells can be induced *in vitro* using VEGF-containing media. [39, 100] Next, these cells can be seeded as an endothelial surface layer on tissue engineered constructs or allografts. As source of mesenchymal cells, EPCs can be transdifferentiated by TGFβ-mediated induction of EMT, as seen in natural valvular development.[54, 101] If mesenchymal target cells are directly isolated, like venous myofibroblasts or bone marrow-derived cells, proliferation can perhaps be stimulated by addition FGF-4 to the culture media.[55, 102] When sufficient numbers of cells have been acquired, cells can be seeded on scaffolds and growth factors can be added to induce tissue formation. Growth factors that can enhance tissue formation according to valvulogenesis are BMPs and TGFβ.[58, 60, 69, 94] To prevent stiffening

of the tissue constructs by fibrosis or osteogenesis, we hypothesize to add HB-EGF and FGF-2 respectively. Further experiments are required to determine at which timepoints growth factors should be added, at which concentrations, and whether they should be added in bursts or via gradual release. Eventually, this should lead to tailored culture media for different stages in tissue engineering to mimic natural valve development more closely.

Serum-free or autologous culture conditions?

The FDA has formulated Good Tissue Practice-guidelines (GTP) for the use of cell based products in clinical application, in order to prevent introduction of animal components and contamination in patients.[103] Possible complications might be rejection of the implanted construct through an immune response against animal proteins, as has been shown for cells expanded in bovine serum.[104, 105] Though this may not seem relevant for *in vitro* bench-studies, these guidelines may affect tissue properties, *e.g.* when using serum alternatives to prevent cross-species contamination due to non-autologous serum.[73] As an alternative, serum-free culture media are becoming commercially available. Use of specialized serum-free culture media, with growth factor additives optimized for each different step of heart valve tissue engineering, could reduce expansion time, induce tissue formation and allow clinical application. It should be considered, however that cellular behavior in these highly defined culture media most likely does not reflect cellular behavior after implantation. Exposure to blood subjects the cells to completely different concentrations of humoral factors like growth factors, cytokines and hormones, and leads to different cellular responses. A promising alternative was studied by our group through replacement of bovine serum with autologous platelet-lysate [73] or serum as a source for nutrients and growth factors. Supplementing autologous serum-alternatives with growth factors could stimulate proliferation and tissue formation of cells, while mimicking *in vivo* circumstances and valvulogenesis more closely. Future studies need to address whether fully autologous culture of heart valves, hence using the patient's plasma or serum and cells, can be achieved and allows clinical application of heart valve tissue engineering.

Mechanical conditioning

As for most load-bearing tissues, the concept of *in vitro* mechanical conditioning, *e.g.* in bioreactors, to improve functional tissue architecture and properties is also well acknowledged for heart valve tissue engineering and has been confirmed in various *in vitro* studies.[37, 106-108] The most important mechanical stimuli during heart valve development are shear stress and (transvalvular) pressure. Involvement of shear stresses has been suggested in processes directing EMT, valvular morphogenesis and stratification.[52, 57] Further, analysis of collagen content and collagen ultrastructure has shown that the increase in transvalvular pressure and/or shear stresses during valvulogenesis leads to thickening of the lamina fibrosa and to strain-related changes in orientation of fibers.[109] Similar strain-related changes can be observed when comparing adult pulmonary and aortic valve cusps. The collagen fiber architecture of aortic cusps is modestly, but significantly, different to accommodate for the higher transvalvular pressure loads during diastole.[110] For aortic heart valve tissue engineering it is therefore beneficial to mimic native like fiber architecture and mechanical functioning by applying (increased) transvalular pressures during culture.

Since collagen fibers are the most important load-bearing structures in heart valves, collagen content and fiber thickness have been used as a marker for mechanical properties.[11, 23] However, maturation of collagen fibers through crosslink formation is also important. Balguid *et al*. have demonstrated that application of cyclic strains enhanced collagen crosslink formation in engineered tissues, as compared to unloaded controls. Crosslink density was further found to be highly correlated to tissue strength *in vitro*, as well as in native aortic valve leaflets.[23] Crosslinks are essential for tensile strength and mechanical stability of the collagen microfibrils and protect the microfibrils against enzymatic degradation.[111] Mechanical stimulation to improve crosslink density can therefore be considered an important additional regulator of tissue maturation and mechanical properties in engineered valves, next to collagen content and fiber organization.[112]

Lastly, valvulogenesis suggests that physical stimuli are important for the formation of the tri-layered architecture of aortic valve cusps. Mechanisms behind stratification likely involve different flow conditions on each side of the cusp, resulting in differentially expressed genetic profiles by endothelial and matrix-producing cells.[36, 52, 113] Ultimately, these differences might influence expression of proteins involved in matrix deposition or degradation,

leading to specific niches favorable for development of specific matrix proteins.[52, 113] At this moment, there are only few studies available that investigate the synergistic or inhibitory effects of mechanical stimulation on growth factor and protease expression.[106, 107] In the future, however, such studies might shed more light on the formation of specialized collagen and elastin layers and indicate whether growth factors and mechanical stimulation should be applied together or separately.

The studies above show that mechanical conditioning of engineered valves in bioreactors is indispensible prior to implantation and that cyclic, strain-based conditioning is beneficial for the production of strong aortic valves. These protocols, however, are only partially related to the hemodynamic processes during valvulogenesis. Potential mechanical regulation of *e.g.* EMT and tissue stratification, as well as interaction with biomolecular regulation of these processes is hardly studied. This should be subject of future studies and include the use of bioreactors that allow for controlled application of different flow and pressure profiles to opening and closing valves.

Discussion and Future Perspectives

The aim of this overview is to provide an up-to-date analysis of natural aortic valve development, the regulatory roles of biomolecular and biomechanical stimuli during different stages of valvulogenesis, and their relevance for optimizing heart valve tissue engineering strategies. Several concepts from valvulogenesis have already been applied in tissue engineering, but new and perhaps more challenging strategies can be suggested from our analysis. If one desires to mimic valvulogenesis as closely as possible, we suggest the combined or subsequent application of:

1. Progenitor cell sources that may reconstitute both valvular endothelial and matrix-producing interstitial cells. However, alternative sources like cells directly derived from the heart, such as cardiac fibroblasts or EPDCs, should also be considered, because they produce matrix proteins relevant for heart valve and are used to a mechanically dynamic environment.

2. Addition of different growth factors relevant for valvulogenesis to culture media, like BMPs and FGF-4. This can potentially reduce expansion time and increase tissue formation *in vitro*. BMPs for instance are indispensible for heart valve formation, but not used for valve tissue engineering. Specific combinations of growth factors in cocktails might solve undesired side effects of the use of these BMPs.
3. The use of specific matrix proteins like periostin in scaffolds. Natural valve development shows that matrix proteins can influence cellular behavior and using them could lead to tissue formation that resembles heart valves more closely.
4. Mechanical stimulation to enhance collagen formation, organization and crosslink formation to improve tissue strength during valvular maturation. In addition, valvulogenesis suggests that shear stress and transvalvular pressure contribute to other stages of valvular development and should therefore be considered for optimizing tissue engineering strategies.
5. Alternatives for the use of animal-derived products in culture media required to expand cells *in vitro*. Highly specific culture media can be developed to mimic different stages of valvulogenesis. Alternatively, the use of media with autologous serum or plasma combined with growth factor cocktails, or as an alternative source for growth factors can be investigated.

Our suggestions are incorporated in Figure 2b. Complete mimicking of the complex and highly organized valve and copying all natural factors involved, like cells, growth factor combinations and mechanical conditioning, is a highly challenging and unrealistic approach and may likely be unnecessary. To determine the most relevant processes and regulators, we suggest to compare stages in tissue engineering strategies with developmental stages in valvulogenesis and to evaluate the effects of identified regulators of valvulogenesis on tissue engineering outcomes. Obviously, this approach can and should be combined with new emerging strategies that are not well studied or understood in natural valve development.

An example of these new strategies is the recent successful clinical application of a tissue engineered airway.[114] In this study, a layered construct was created using two different autologous cell types and specialized culture media for each individual cell type. This concept can also be considered for heart valve tissue engineering. For instance, the attachment of TGFβ to scaffolds within the construct to induce EMT, can be combined with endothelial growth promoted via VEGF on the outside.

Secondly, several groups have reported the presence of fibroblast progenitor cells in the blood, called fibrocytes.[115] These cells have been suggested to be involved in the repair of damaged tissues, including heart valves, and therefore a potential attractive source of mesenchymal cells for heart valve tissue engineering.[116] However, future studies should point out if these cells can be isolated, expanded and used for heart valve tissue engineering.[116]

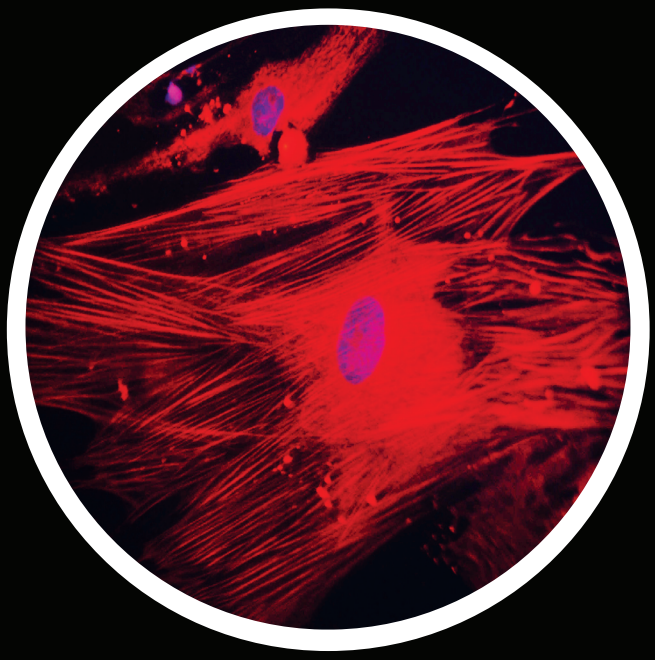
Another example are microRNAs (miRNAs), which have been show to block synthesis of specific target-proteins *in vivo*.[117] MiRNAs are suggested to be important regulators of several physiological processes, including angiogenesis, heart development and osteogenic differentiation.[118-120] Additionally, the therapeutic use of mimicking or inhibitory miRNA-molecules is described after experiments in several animal models and pre-clinical investigations in primates.[119, 121] Therefore, microRNAs might also be interesting targets for future research in development or pathology of heart valves, like calcification.

In conclusion, we have shown that current knowledge of valvulogenesis provides several unexplored but attractive options to improve heart valve tissue engineering and to take another step towards clinical application. Our suggestions are summarized in Table 2. Currently, clinicians are primarily involved at the end-stage of tissue engineering protocols, *i.e.* implantation of constructs. However, their involvement can also be extremely valuable in earlier stages, for instance to optimize scaffold design and to improve cell recruitment. Eventually, it will require the combined efforts of clinicians, biologists and engineers to achieve optimized protocols for heart valve tissue engineering and to and to bring clinical application of tissue engineered aortic valves within reach.

Summary of suggestions and recommendations
Use cell sources that can resemble valvular interstitial cells and search for alternative cell sources, like EPDCs or blood-derived fibrocytes
Investigate if matrix proteins can be used to improve the cellular niche on artificial scaffolds, like periostin
Explore new combinations of growth factors to enhance tissue maturation and reduce compaction. Examples are new combinations with BMPs or TGFβ
Aim for tissue engineering protocols based on serum-free culture media, or autologous serum alternatives
Mimic physiological mechanical stimuli for tissue conditioning and allow opening and closing of cusps
Apply techniques derived from other tissue engineering studies, like combined culture of different cells in specialized media or use of microRNAs

Table 2. *Summary of recommendations and suggestions for future research on heart valve tissue engineering, presented in this paper.*

Chapter 3



Chapter 3

Platelet-lysate As An Autologous Alternative For Fetal Bovine Serum In Cardiovascular Tissue Engineering

This chapter has been published in:

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Abstract

There is an ongoing search for alternative tissue culture sera to engineer autologous tissues, since use of fetal bovine serum (FBS) is limited under Good Tissue Practice (GTP) guidelines. We compared FBS with human platelet-lysate (PL) in media for *in vitro* cell culture. A threefold increase in duplication rate was found when human, saphenous vein-derived myofibroblasts were cultured in PL, while expression of marker proteins (α-smooth muscle actin, vimentin, desmin and non-muscle myosin heavy chain) was similar. Hsp47 mRNA-expression was increased in PL-cells and type III collagen fibers were seen on PL-cell monolayers, but not on cells cultured in FBS. These results imply a more efficient collagen fiber production. We also found higher levels of proteins involved in tissue repair and collagen remodeling, which could explain increased production of proteases and protease inhibitors by PL-cells. Our findings indicate that PL is beneficial because of the increased duplication rate, in addition to the increased matrix production and remodeling. This could lead to production of strong tissue with properly organized collagen fibers, which is important for heart valve tissue engineering.

Introduction

General tissue engineering strategies require the *in vitro* expansion of cells, which usually involves the use of animal-derived products like fetal bovine serum (FBS). However, the use of FBS for expansion of bone marrow-derived mesenchymal stem cells (MSCs) has been related to prion transmission [122] and to immune responses in patients after cell transplantation.[104, 105] The use of animal products is thus not desirable and limited under the guidelines of Good Tissue Practice (GTP) [103], which is a hurdle in the translation of our tissue engineering concept into clinical practice. Human, autologous platelet-lysate (PL) has been suggested as an alternative source to culture cells under autologous conditions.[123-126]

Tissue engineering is considered a promising alternative method for production of viable heart valve constructs to replace diseased valves in patients.[127] Currently used valve replacement therapies suffer from several limitations, mostly related to lack of viable tissue.[11] The proof of principle for heart valve tissue engineering was provided by seeding of autologous vessel-derived myofibroblasts (MFs), expressing vimentin and α-smooth muscle actin (αSMA), on artificial scaffolds.[21, 64, 128] Upon implantation into the pulmonary position in juvenile sheep, the constructs remained viable and intact for up to 20 weeks after implantation. For clinical application of MFs in heart valve tissue engineering, it is important that cells can be rapidly expanded *in vitro* to quantities large enough to seed on the scaffold surface. Subsequently, the valve constructs should be able to produce and remodel abundant amounts of extra cellular matrix (ECM).

Native aortic heart valves have a tri-layered architecture including highly specialized and functionally adapted cells and ECM, to be able to direct high tensile forces generated during systole from the belly to the commisures and aortic wall.[22, 35] Valvular interstial cells (VICs) are considered the most important regulators of specialized ECM synthesis and remodeling and, hence, the maintenance of strength and durability of valve cusps.[129] VICs display a certain degree of plasticity *in vivo*, which can be distinguished by expression of different markers. In developing, diseased, remodeling and tissue engineered heart valves, VICs have been reported to express markers of a contractile, activated myofibroblastic cell type, including vimentin, α-SMA and non-muscle heavy myosin chain (SMemb). The activated, developing or remodeling state is usually followed by normalization of the phenotype

to a secretory, quiescent fibroblast phenotype, in which VICs express only vimentin.[64]

In this study, we investigated the application of PL as a replacement for FBS in the *in vitro* expansion of MFs and the effect on morphology, proliferation, phenotype and the ability to produce collagen and proteins involved in matrix remodeling.

Materials and Methods

Cell isolation

Segments of vena saphena magna (± 3 cm) were obtained from 10 patients undergoing coronary artery bypass surgery using a venous graft. Individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht was obtained and tissue was further treated anonymously, as adapted from Schnell *et al*.[130] Briefly, venous segments were transferred to the laboratory in serum-free medium (DMEM-advanced (Gibco, 12491015), 2 mM GlutaMax (Gibco, 35050-028), 10 µg/mL gentamycin (Lonza, BE02-012E)), where the adventitia was removed from the medial/intimal layer. Subsequently, the vessel was washed in antibiotics solution (PBS, 2.5 µg/mL AmphotericinB (Biochrom, AG A2612) and 200 µg/mL Gentamycin (Gibco, 15710-049)). The lumen of the vessel was incubated in endothelial cell medium with collagenase (EBM2 (Lonza), EGM2 single quots (CC-3162, Lonza), 20% FBS (HyClone, CH30160.03) and 2 mg/mL collagenase A (Roche, 10103578001)), after which endothelial cells were scraped off with a cell scraper. Tissue segments were cut into small squares (2x2 mm) and plated on culture plastic with the lumen faced down, receiving either FBS-medium (serum-free medium + 10% (v/v) FBS) or PL-medium (serum-free medium with 5% v/v PL and 10 U/mL heparin (LEO, 013192-03)). Human thrombocytes in serum were obtained from the hospital blood bank and were pooled from 5 donors with similar blood type and rhesus-factor and buffered with citrate-phosphate dextrose. PL was frozen in aliquots at -80°C and thawed and centrifuged (8 min 900 rcf) prior to addition to the culture medium to obtain platelet-lysate, as described by others.[125, 131] Cells were cultured in humid environment at 37°C and 5% CO_2.

Cell culturing, morphology, growth curves

Cells derived directly from the tissue (passage 0) were cultured up to passage 5 (P5). Passage 1 and 2 (P2) were seeded at a density of approximately 2000 cells/cm^2, while subsequent passages were split in a 1:3 surface ratio. After each passage, the time required to reach 85% confluency was noted per cell isolation and the number of cells was determined. A number of 10^8 cells was considered clinically significant. The time in days and the logarithm of the total number of cells that could have been obtained at each passage were fit into a linear model using SPSS 15 software. Slopes of these models were determined and represent duplication rate. They were used to calculate the mean duplication rate of all patients per culture medium. Viability was determined using tryptan-blue staining and quantification with a Bürker-Türk-hemocytometer.

At P2 and P5, cells were plated for further culturing, for analysis by zymography, for collection in Tripure Isolation Reagent (Roche, 11667165001) and plated for immunocytochemistry (ICC) on coverslips. These passages were selected because P2 was needed to obtain enough cells for all the different analyses, while P5 represents the last stage, prior to seeding for possible tissue studies in the future. Morphology-changes were analyzed using light microscopy and length/width-ratio measurements on cells plated for ICC from all patients in both conditions and both passages. Cell^P-software (Olympus, Japan) was used to determine length and width of the ICC-cells in µm.

Immunocytochemistry

Immunocytochemistry was performed on cells at P2 and P5, seeded on coverslips and cultured for 3 days. Cells were subsequently fixed in 4% paraformaldehyde and permeabilized with 0.1% Triton-X100. Prior to staining, cells were washed and blocked in a 2% BSA (Roche, 10735086001) (w/v), 0.1% saponin (w/v) solution in PBS, and subsequently incubated for 1 hr with primary antibodies in PBS. For extracellular proteins, all detergents were removed from the staining procedure. Antibodies used: αSMA (1:400, Sigma, A2547, stock concentration (sc): 4.5 mg/mL), vimentin (1:400, ab20346, VI-10, Abcam, sc: 1.0 mg/mL), SMemb (1:400, ab684-100, Abcam, sc: ND), desmin (1:50, Clone D33, M0760, DAKO, 0.235 mg/mL) and CD31 (1:200, JC70A, M0823, DAKO, sc: 0.45 mg/mL). Staining

for Heat shock protein 47 (Hsp47) (1:200, ab13510, Abcam, 1.0 mg/mL) and collagen type III (1:200, ab7778, Abcam, sc: 1.0 mg/mL) were used to address collagen synthesis.[109, 132, 133] Expression of CD31 was analyzed to exclude the presence of venous-derived endothelial cells. Control stainings were performed by omitting primary antibody. The used secondary antibodies were: Texas-Red labeled Goat-anti-Mouse (1:400, Vector lab, TI-2000, sc: 1.5 mg/mL), FITC-labeled goat-anti-mouse (1:400, Southern Biotech, 1010-02, sc: 2 mg/mL), or AlexaFluor-555-labeled goat-anti-rabbit (1:400, invitrogen, A21429, sc: 2 mg/mL). Cell nuclei were stained in 1 ng/mL Hoechst dye for 5 minutes. Coverslips were mounted with a 10% mowiol-solution (w/v) (25% glycerol, 50% Tris-HCl, pH 8.5). Positive controls were included for CD31 (human venous-derived endothelial cells) and desmin-staining (human adult cardiomyocytes). Cells were viewed by fluorescence microscopy (Olympus, BX60) and percentage of positively stained cells was analyzed using Cell^{P}-software (Olympus, Japan). For each condition, the average percentage of positively stained cells was determined per patient.

RNA isolation and quantification

RNA was extracted with Tripure Isolation Reagent (Roche) according to the manufacturer's protocol. Per sample, 500ng DNA-free RNA was used to produce cDNA using the iScript cDNA synthesis kit (Bio-Rad, 170-8891). For PCR, we used the MyIO qRT-PCR system and accompanied software (MyIQ, Bio-Rad). Template cDNA was added to a mastermix containing 2x iQ SYBR-Green Supermix (Bio-Rad, 172-5006CUST) and forward and reverse primers (each 0.5 µM). Primers were designed (OligoPerfect, Invitrogen) and optimal PCR conditions determined. Product specificity was confirmed by post-run melting analysis and gel-electrophoresis. PCR conditions: 50 cycles of 95°C (30 sec) and different annealing temperatures (45 sec, details are given in Table 1). Samples were normalized for β-actin ($\Delta Ct = Ct_{sample} - Ct_{\beta\text{-actin}}$).

Zymography

Zymography analysis was performed on conditioned medium, containing low-serum (2.5% for PL and 5% for the FBS-group), corrected for the number of cells. The samples were prepared with LaemmLi buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 40% glycerol and 0,004% Broomphenol blue) and loaded on 10-15% poly-acrylamide gels containing 2 mg/mL gelatin or 4 mg/mL casein, as described before.[133] After running, gels were incubated overnight at 37°C in Brij-solution (50 mM tris-HCL 7.4, 10 mM CaCl2, 0.05% Brij35 (w/v dilute 600x) and stained with Coomassie Blue (25% MeOH, 15% HAc and 0.1% Coomassie Blue (Briljant Blue R, CI42660)). Unconditioned low-serum medium was loaded as a negative control. Analysis and quantification of total (active and inactive) protease secretion was performed using the ChemiDoc XRS system (Bio-Rad) and QuantityOne software (Bio-Rad, USA). Quantification was corrected for presence of proteases in unconditioned medium.

Superarray

To determine relative presence of growth factors and cytokines in both culture media, a biotin label-based human antibody array (RayBiotech, AAH-BLM-1) was performed, according to the manufacturer's protocol. For this, complete, unconditioned culture medium was used, containing 5% PL or 10% FBS-medium. Arrays were normalized based on internal positive controls and background signals were substracted. An exposuretime of 15 seconds was used for the analysis to prevent saturation of the signal. An exposuretime of 137 seconds was additionally used to determine presence of a set of a priori selected growth factors. Homology was determined using the Homologene function at the National Center for Biotechnology Information (NCBI) website.

Statistics

Results are expressed as means ± standard error of the mean (SEM). We used SPSS 15 software for statistical analysis. All results from similar passage were compared using paired samples T-tests (two-tailed) and a p-value < 0.05 was considered significant. We used a regression analysis to create the linear models of the growth curves.

Target	Forward primer	Reverse primer	Annealing temp (°C)
Hsp47	AAGAGTAGAATCGTGTCGCGG	GCCTTGGGGCTCAACTTCT	60
COL1A1	TGCCATCAAATGCTTCTGC	CATACTCGAACTGGAATCCATC	56
COL3A1	CCAGGAGCTAACGGTCTCAG	CAGGGTTTCCATCTCTTCCA	48
MMP1	CGCACAAATCCCTTCTACCC	CTGTCGGCAAATTCGTAAGC	55
MMP2	ATGACAGCTGCACCACTGAG	ATTTGTTGCCCAGGAAAGTG	60
TIMP1	TGACATCCGGTTCGTCTACA	TGCAGTTTTCCAGCAATGAG	49
TIMP2	CACCCGCAACAGGCGTTTT	TTTCCTCCAACGTCCAGCGA	60
β-actin	GATCGGCGGCTCCATCCTG	GACTCGTCATCCTGCTTGC	60

Table 1. *List of primers used for qRT-PCR*

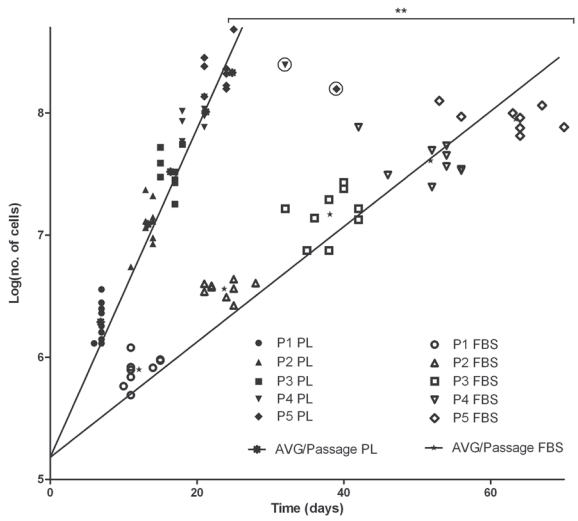


Figure 1. *Duplication rate analysis, starting at 150,000 cells. The time in days and number of cells per cell isolation are shown in this graph. The PL-cells are depicted by the filled symbols and the FBS cells with the clear symbols, including a different symbol per passage. The line with the averaged slope is also shown in the Figure. The slope of the PL-cells was significantly higher than the FBS-group ($p < 0.01$). The circled datapoints indicate outlying data for one PL-patient (P4 and P5).*

Results

Proliferation and morphology

Successful cell isolations for follow-up in both culture media were obtained from 9 patients, including 6 males and 3 females (61 ± 4 years old). Cultures from one patient developed infection at an early passage in both culture media and were therefore excluded. Cell viability was approximately 95%, independent of the culture medium. PL-cells reached 10^8 cells in approximately 20 days, while FBS-cells required approximately 60 days on average (Figure 1). The linear models used to determine duplication rate closely resembled the raw data sets (Figure 1, R2 = 0.985 ± 0.007 and 0.975 ± 0.005 for PL-cells and FBS-cells respectively). Analysis of the slopes of the individual linear models showed significant differences in proliferation of the cells between culture media and showed a three-fold increase when cultured with PL-medium (0.133 ± 0.006 and 0.043 ± 0.002 for PL- and FBS-cells respectively, $p < 0.01$).

Platelets and small platelet-clots are visible in the platelet-enriched PL-medium (Figure 2 b,d,f). A distinct cell shape was already observed during the first few passages between cells cultured in different media (Figure 2). Cell shape of cells cultured in FBS showed a more stellate morphology, with abundant stress-fibers, while cells cultured in PL were spindle-shaped and very dense. These observations were confirmed by length/width-ratio analysis, which showed a significantly higher ratio of cells cultured in PL at P5, as compared to the FBS-group (6.07 ± 0.57 vs. 2.83 ± 0.24, $p < 0.01$). The difference was not statistically significant at P2 (5.06 ± 0.46 vs. 4.06 ± 0.62) (Figure 3b). Individual length- and width-analysis demonstrated an elongation of the PL cells ($p < 0.01$) and increased width of the FBS cells ($p = 0.06$) (Figure 3a).

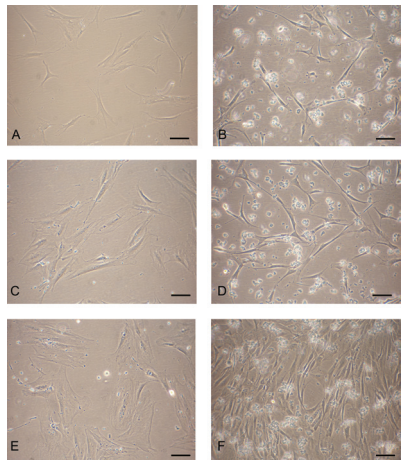


Figure 2. *Human MFs cultured in PL (**2B, D, F.**) have a dense, spindle-shaped morphology, while FBS cells (**2A, C, E.**) have a more stellate morphology with visible stress fibers. Light microscopy pictures show cells at passage 1 (**2A-D.**) and P3 (**2E-F.**) from different patients. The difference in cell-shape between cells of both conditions is more obvious at P3. Original magnification: 10x. Scale: 100 µm.*

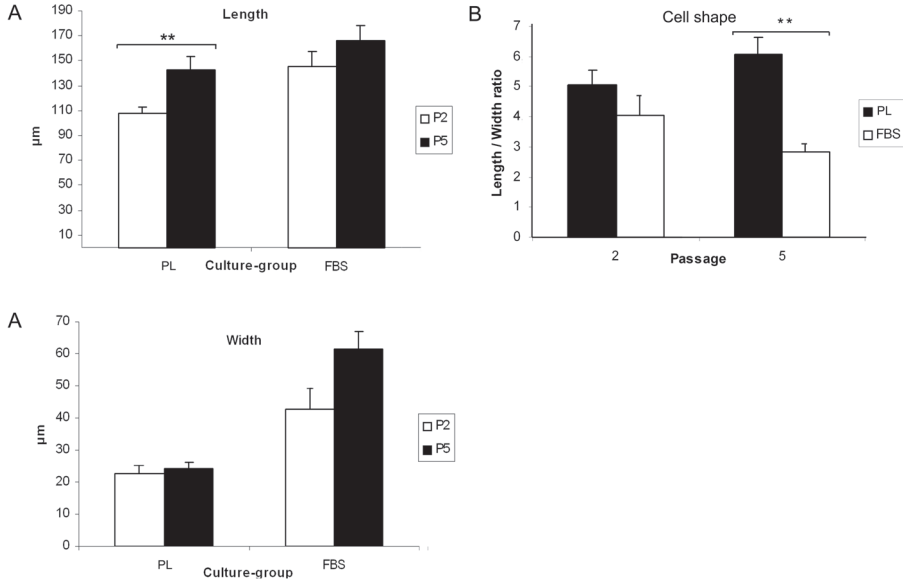


Figure 3A. *Individual analysis of length and width in µm of cells in both culture groups. PL-cells elongate over several passages (**: $p < 0.01$), while FBS-cells do not (mean ± SEM). Width increases in FBS-cultured cells, although not statistically significant ($p = 0.06$). **3B.** The length/ width ratio of human MFs (mean ± SEM) is significant at P5 ($p < 0.01$).*

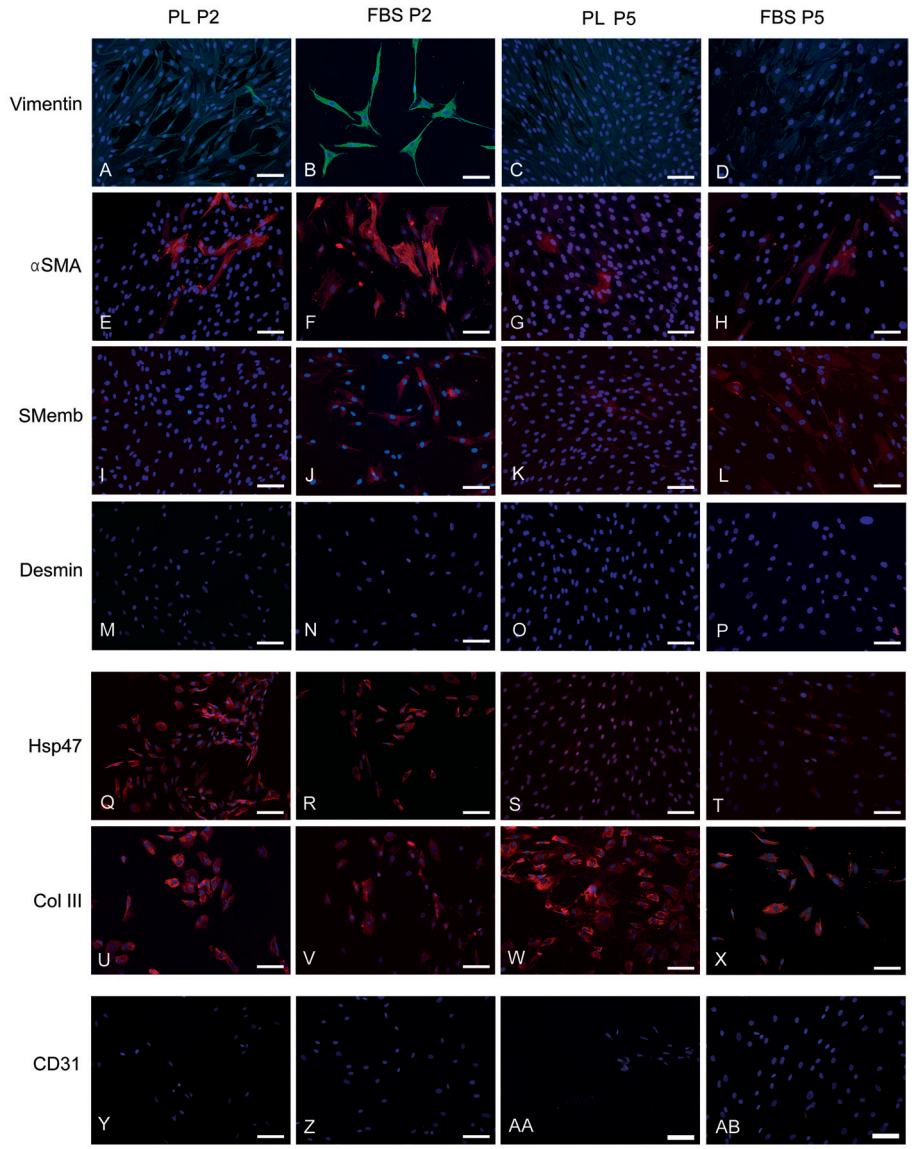


Figure 4. *Immunofluorescent staining of MF cultured in both medium and at different passages. Results for phenotyping of one patient are displayed.* ***4A-D.*** *Vimentin,* ***4E-H.*** *αSMA,* ***4I-L.*** *SMemb,* ***4M-P.*** *desmin,* ***4Q-T.*** *Hsp47,* ***4U-X.*** *Collagen type III and* ***4Y-AB.*** *CD31. Nuclei are stained with Hoechst. Original magnification was 20x. Scale: 100 µm.*

Phenotype

All cells of both media were positively stained for vimentin (Figure 4a-d). For αSMA, the percentage of positive cells was higher in FBS-cells as compared to PL-cells at P2 (83 ± 8% and 31 ± 14% respectively, p< 0.01), but this difference was absent at P5 (69 ± 13% and 76 ± 11%, respectively) (Figure 4e-h and 5). For SMemb, the percentage of positive cells was not significantly different at P2 (PL: 16 ± 9% and FBS: 46 ± 13%), but reached significance at passage 5 (37 ± 16% and 73 ± 14%, respectively, p = 0.047) (Figure 4i-l and 5). The FBS samples displayed prominent intracellular SMemb- and αSMA-fiber structures, while these stainings appeared more diffuse in PL-cells. There was a large variation of the percentage of cells positively stained for αSMA and SMemb between patients (Figure 4e-l). Desmin staining was absent in all cells of all cultures (Figure 4m-p). The positive control demonstrated intracellular fibers (supplementary data, Figure1a). CD31 staining was negative for all cells in both groups and at both passages (Figure 4y-ab), while the positive control showed a positive membrane staining (supplementary data, Figure 1b).

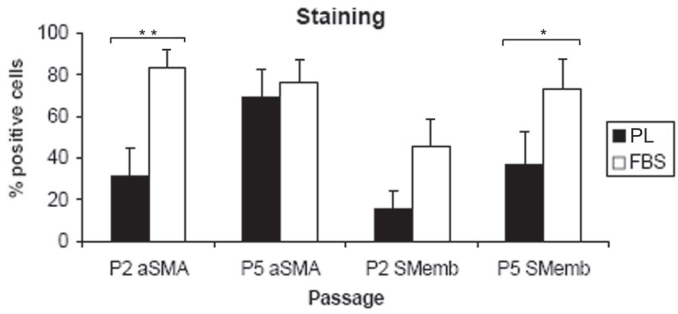


Figure 5. *Quantification of percentage of human MFs positive for expression of αSMA and SMemb (mean ± SEM). For αSMA, there was a significant difference in positively stained cells at P2 (p < 0.01), but not at P5. For SMemb, differences only reached significance at P5 (p = 0,047).*

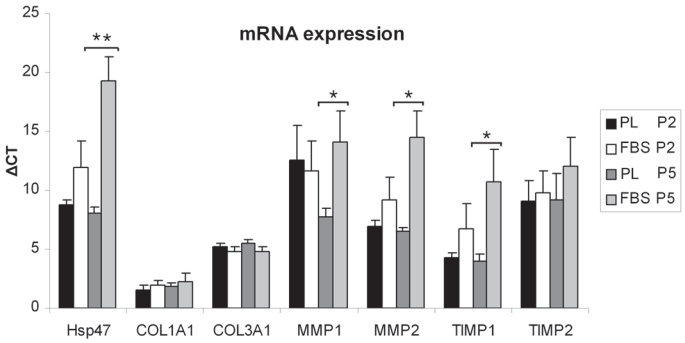


Figure 6. *mRNA expression (mean ± SEM) in human MFs cultured in FBS or PL-supplemented medium at P2 and P5. Significant differences were present at P5, where PL had higher gene expressions for Hsp47 ($p < 0.01$) and MMP1, MMP2 and TIMP1 ($p < 0.05$). PL-cells also had higher gene-expression for proteins involved in matrix remodeling: MMP1, MMP2 and TIMP1 ($p < 0.05$). Error bars represent SEM.*

Collagen synthesis

The ability to produce collagen fibers was investigated by immunostaining for Hsp47 and Collagen type III (Figure 4q-x). All cells of both PL and FBS were positively stained for Hsp47 (Figure 4q-t). On the other hand, collagen staining was also positive in all cells of all conditions, but this appeared more prominent in the PL-group. Cells cultured in both the PL- and FBS-group showed intracellular staining of collagen around the nucleus (Figure 4u-x), but the PL-group showed depositions of extracellular fibers covering the cells in addition to this (supplementary data Figure 1f).

On mRNA levels, lower ΔCt-values indicated that PL-cells showed significantly higher expression of Hsp47 at P5, when compared to FBS-cells (8.10 ± 0.43 vs. 19.33 ± 1.99, $p < 0.01$). There was no difference at passage 2. The amount of mRNA for collagen type 1 (COL1A1) and 3 (COL3A1) was similar at P2 and 5 (Figure 6).

Protease and TIMP production

The amount of mRNA coding for proteins involved in matrix remodeling (MMP1, MMP2, TIMP1 and TIMP2) was not significantly different for any of the tested genes at P2. However, at P5, lower ΔCt-values indicated higher mRNA expression in the PL-group for several genes: MMP1 (7.78 ± 0.71 vs. 14.10 ± 2.67), MMP2 (6.52 ± 0.28 vs 14.47 ± 2.25) and TIMP 1 (4.00 ±

0.63 vs. 10.77 ± 2.66) were all significantly increased when compared to the FBS-group ($p < 0.05$) (Figure 6).

Zymographic analysis of gelatinolytic activity displayed high expression of active and inactive forms of MMP2 (72 and 64 kDa) in both PL- and FBS-conditioned medium at P2 and P5 (Figure 7a). Levels of MMP9 and MMP9/NGAL-complexes were not different when compared to unconditioned medium. Quantification of total gelatinase activity indicated that the increase was more pronounced in PL-cells at P2 ($11.6.10^4 \pm 1.3.10^4$ vs. $7.4.10^4 \pm 1.1.10^4$, $p < 0.01$) and P5 ($11.1.10^4 \pm 1.2.10^4$ vs. $5.0.10^4 \pm 1.4.10^4$, $p < 0.01$) (Figure 7b).

On the casein-gels, the PL-conditioned medium showed some additional expression when compared to the unconditioned medium, suggesting MMP8 and MMP1 expression. This is also an increase when compared to FBS-conditioned medium, which did not show any increase of presence of proteases when compared to unconditioned medium (P2: $1.0.10^3 \pm 0.2.10^3$ vs $0.3.10^3 \pm 0.2.10^3$ respectively, $p = 0.011$ and P5: $1.6.10^3 \pm 0.4.10^3$ vs. $0.1.10^3 \pm 0.1.10^3$ respectively, $p < 0.01$)(Figure 7c).

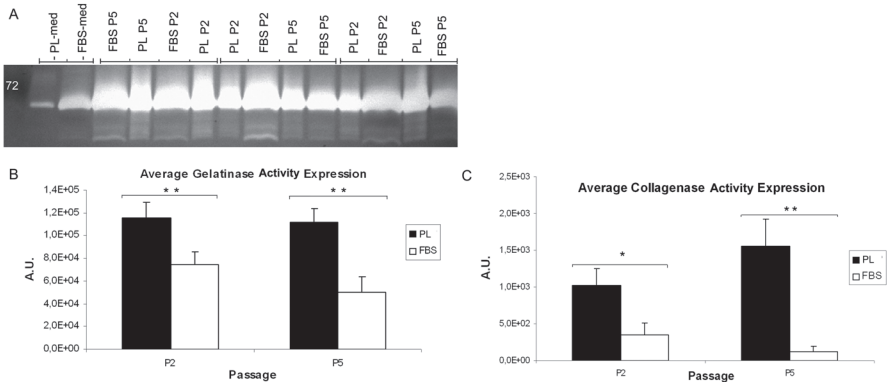


Figure 7A. *Zymography results for total MMP2-expression by human MFs cultured in PL or FBS-supplemented medium. Molecular weight is in kD. Results show negative control (first two lanes) and 3 out of 9 patients (3 x 4 lanes).* ***7B.*** *Quantification of total gelatinase expression. Cells culture in both media produce gelatinases (primarily MMP2), but total gelatinase expression was significantly higher at both passages in the cells cultured with PL ($p < 0.01$). A.U. = Arbitrary units as a measure for number of counted pixels in a predetermined surface.* ***7C.*** *Quantification of total collagenase expression. Collagenase expression is only minimally increased in FBS-cells, when compared to unconditioned medium. PL-cells produce significantly more collagenases. Error bars represent SEM. (*: $p = 0.011$, **: $p < 0.01$).*

Superarray and Western blots

Images of the superarrays are depicted in supplementary Figure 2. The top 50 of proteins with the highest expression for each culture medium are displayed in supplementary Tables 1a and 1b. Results of the growth factor expression at 15 and 137 exposure time are presented in supplementary Tables 1c and 1d. Generally, the results show higher expression of proteins in PL. An analysis of the most abundantly expressed proteins indicated that high concentrations of HB-EGF were present in PL, but not FBS (Table 2). Also, high levels of specific interleukins, involved in tissue repair and matrix remodeling, were detected (Table 2). Higher exposure time analysis revealed higher concentrations of VEGF and bFGF in PL when compared to FBS.

Possible regulators of matrix remodeling and proliferation

Protein	Value PL	Value FBS	Homology	Possibly relevant function
IL-23	**1480**	23	82.5% (IL-23A)	Stimulation of MMP9
Urokinase	**1281**	42	75.4%	Plasmin activator: matrix/fibrin degradation
HB-EGF	**1020**	11	88.0%	Strong inductor of smooth muscle cell proliferation
MIP-1a	**673**	20	NA	Indirectly stimulates wound repair
IL-17F	**608**	19	56.1%	Induces MMP1, MMP3 and MMP13 expression
IL-1 alpha	45	**146**	73.3%	Induces MMP1, MMP3, MMP9 expression
IL-1 F6	57	**108**	70.8%	Similar to IL-1
LRP-1	0	**101**	NA	Inhibits matrix-remodeling
IL-1 F7	36	**39**	62%	Similar to IL-1

Table 2. *Selection of proteins observed using the superarray that might explain differences observed between PL and FBS. Values indicate relative densities of spots in the array. NA: Not Available. Complete top 50 of proteins with highest expression at different exposure times are shown in Supplementary Tables 1a-b, while the pre-selected growth factor results are displayed in Supplementary Tables 1c-d.*

Discussion

The aim of this study was to find an alternative, autologous source of growth factors for future clinical application of cardiovascular tissue engineering. Our results suggest a threefold increase in proliferation rate of MFs cultured in PL, potentially explained by higher concentrations of HB-EGF and bFGF in PL-medium when compared to FBS. The pattern of marker-proteins expressed by the cells resembles the pattern that has been previously reported for activated valvular interstitial cells [64, 134, 135] and not smooth muscle cells [136, 137]. The change from FBS to PL thus caused a major improvement of culture time.

To meet the high mechanical demands of the systemic circulation, required for use of tissue engineered heart valves in patients, active matrix production and remodeling is desired during the conditioning of the cells in the bioreactor prior, and after, implantation. We found higher RNA expression of Hsp47 in the PL-cells. Hsp47 binds procollagen fibers, facilitates triple-helix formation and is known to be an excellent marker protein for collagen synthesis.[109, 132, 133] Combined with the extracellular staining for collagen type III on PL-cells, this suggests that culturing in PL stimulates collagen expression, which could be beneficial for heart valve tissue engineering.

Our data were consistent in finding increased expression of MMPs and TIMPs by PL-cultured cells when compared to FBS, indicating an increased ability to remodel matrix. However, there should be a balance between production and degradation of matrix proteins and a net degradation of matrix reduces the strength of the tissue in which remodeling takes place. Other groups have previously used collagen gels to analyze matrix remodeling in a 3D-model.[138-140] However, since it is difficult to simultaneously quantify newly produced collagen in this model, we have decided to investigate the balance of production and remodeling of collagen in a separate tissue model. The results of this study will show if cells cultured in PL are capable of production of load-bearing tissue. Glycosaminoglycan and elastin production will also be addressed in these studies.

To start to elucidate some of the mechanisms behind our observations, we investigated a wide spectrum of cytokines and growth factors in our culture media, instead of *a priori* selected proteins, using a super array. We found higher amounts of HB-EGF and bFGF in PL when compared to FBS, which can explain the higher proliferation in PL.[141, 142] Moreover, our results suggest that the levels of several interleukins (IL17, IL23) and other proteins involved in tissue repair and matrix remodeling (MIP1a and uPA), are higher in PL than FBS, which could be an explanation for the higher increase of MMPs in PL.[143-145] Interleukins are also among the proteins with highest expression in FBS-medium, but expression is believed to be generally lower when compared to PL. FBS also has relatively high expression of LRP-1, which has been shown to cause uptake of MMP2 by cells [146, 147] and inhibition of matrix-remodeling.[148]

The superarray did not find several growth factors that have been previously shown to be abundantly present in platelets, like EGF, PDGF and TGF-beta.[131, 149] Since TGF-beta has previously been shown to be identical in bovine and human [150], this was the most suitable growth factor to test platelet activation using ELISA (R&D systems, DB100B) and allow comparison between PL and FBS. Our results showed higher concentrations of total TGF-beta in PL, confirming the release of growth factors from the platelets (supplementary data, Table 2). The fact that several growth factors were not detected with the super array, can be explained by differences between the activated and non-activated forms of these growth factors. In our case, TGF-beta was activated prior to the ELISA, but not the superarray. TGF-beta is a known stimulator of collagen production in wound repair and the high levels in PL might thus contribute to the increased fiber production by the PL-cells.[151]

The aim of this study was to investigate the effect of replacing current culture protocols using animal serum with an autologous protocol for cells most often used for heart valve tissue engineering. For practical reasons, some steps were not performed completely autologously during this investigation, like the use of pooled PL, use of FBS in the collagenase solution to collect endothelial cells and the use of antibiotics during cells culture. We expect that these small modifications will not have affected the outcome of this study or the general outcome, that PL is suitable for use in cardiovascular tissue engineering. For clinical application, we aim to obtain plasma from the patient together with cells for expansion, to ensure production of a completely autologous engineered valve.

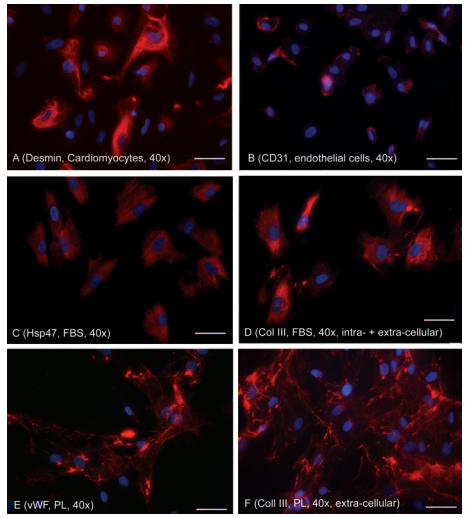
Conclusion

Our data suggest that the expression of marker proteins of cells cultured in PL and FBS are similar. We also found indications for increased matrix production and ability to remodel matrix in PL-cells, which will be more thoroughly investigated in future tissue studies. The major increase in proliferation of cells cultured with PL is an important, beneficial effect, which reduces waiting time when applied in clinical practice. Thus, our data suggest that PL can be an alternative for FBS in cell culture for heart valve tissue engineering. This is an important step forwards in translating this technique from the laboratory bench to clinical application.

Acknowledgements

The authors would like to thank Henk-Jan Prins, Anton Martens and Ineke Slaper for sharing their experience with PL, and Marie-Jose Goumans for the quantification of TGF-beta in our samples.

Supplementary Figures



Supplementary Figure 1. *Additional results from immunocytochemistry assays.* ***1A.*** *Positive results for desmin staining in adult cardiomyocytes.* ***1B.*** *Positive control for CD31 staining on endothelial cells. Figures* ***1C-F.*** *give an overview of the formation of collagen fibers and the differences between FBS and PL-cells.* ***1C.*** *Hsp47 staining, which is intracellularly situated around the nucleus. Hsp47 binds to procollagen during collagen formation, which causes a similar staining pattern for intracellular collagen type III in picture D.* ***1D.*** *collagen III staining in the same patient. Extracellular collagen could also be stained, but this is present in minimal amounts. Cells from the same patients, cultured in PL show more abundant extracellular collagen fibers, as shown in Figure F.* ***1F.*** *to reduce background from intracellular staining and allow better visualization of stained fibers, these cells were not treated with any detergents during the staining procedure, allowing only extracellular staining.* ***1E.*** *In vivo, collagen is a potent activator of von Willebrand Factor (vWF), which binds to the collagen fibers. E shows a vWF-staining that has a similar staining pattern as the collagen staining. The staining is only extracellularly present on cells conditioned with PL, which proves that the bound vWF is derived from the PL in the culture medium and not intracellularly produced. Original magnification is 40x in all pictures.*

A

15s		PL	FBS
no	name	value	value
1	Thrombospondin	1997	2925
2	SIGIRR	1896	20
3	CXCL14	1704	57
4	Thrombospondin-2	1564	16
5	IL-23	1480	23
6	APRIL	1373	38
7	uPA	1281	42
8	Leptin-R	1245	25
9	Angiopoietin-like 1	1165	44
10	Siglec-9	1134	26
11	TMEFF1	1096	36
12	IL-1 RA	1051	13
13	Angiostatin	1031	90
14	HB-EGF	1020	11
15	FGF R4	954	22
16	IL-4 R	876	37
17	CCR7	824	21
18	CCR9	811	0
19	Osteoprotegrin	800	154
20	Thrombospondin-1	727	1317
21	I-TAC / CXCL11	696	33
22	CXCR2	693	9
23	Erythropoietin	680	28
24	MIP-1a	673	20
25	MDC	664	10
26	BMPR-IB	662	43
27	Lipocalin-1	653	18
28	Angiopoietin-2	651	31
29	IL-29	645	37
30	CXCR1	638	5
31	Follistatin	634	18
32	IL-17F	608	19
33	FGF 9	603	8
34	M-CSF	582	21
35	Follistatin-like 3	581	176
36	TRADD	569	0
37	BDNF	521	7
38	CTACK	537	14
39	Gasp-1	488	30
40	Glypican 5	487	15
41	Glut-2	474	17
42	Glucagon	465	11
43	Luciferase	448	118
44	CTNF	385	0
45	Adiponectin	337	31
46	FGF-13 IB	334	5
47	ICAM-5	290	11
48	Osteoactivin	285	0
49	CCR8	235	24
50	ROBO4	225	0

B

15s		FBS	PL
no	name	value	value
1	Thrombospondin	2925	1997
2	Thrombospondin-1	1317	727
3	sFRP-4	856	0
4	IGFBP-7	630	14
5	Glypican 3	610	42
6	ETL	560	138
7	LRP-6	260	5
8	MMP19	225	148
9	MMP20	217	164
10	Follistatin-like 3	178	581
11	Osteoprotegrin	154	800
12	GDF 3	149	103
13	IL-1 alpha	146	45
14	FAM3B	142	0
15	IL-12 R beta2	136	0
16	Insulin	135	24
17	Luciferase	118	448
18	IL-17 RC	117	19
19	GREMLIN	113	118
20	IL-1 F6	108	57
21	GDF 5	105	86
22	IL-1 R4	105	88
23	LRP-1	101	0
24	SCF	93	0
25	GDF-9	92	84
26	Angiostatin	90	1031
27	Endocan	76	0
28	ICAM-2	73	69
29	TNF RII	72	0
30	Endostatin	67	10
31	NT-4	63	0
32	CXCL14	57	1704
33	Csk	56	8
34	IL-9	56	129
35	MMP16	55	0
36	TGF beta 5	55	21
37	Lymphotactin	47	9
38	GITR Ligand	46	6
39	NCAM-1	46	0
40	VE-Cadherin	46	10
41	Angiopoietin-like 1	44	1165
42	BMP R IB	43	662
43	uPA	42	1281
44	Angiopoietin-like 2	41	115
45	RANTES	39	13
46	IL-1 F7	39	36
47	APRIL	38	1373
48	FGF-5	38	0
49	IL-29	37	645
50	IL-4 R	37	876

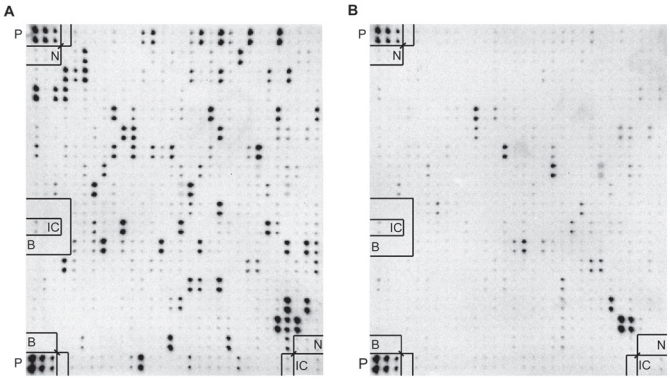
C

Growth Factors	PL	FBS
EGF	0	2
HB-EGF	1020	11
bFGF	45	10
IGF-I	2	16
IGF-II	1	7
PDGF-AA	0	11
PDGF-AB	1	5
PDGF-BB	10	24
TGFb1	0	4
TGFb2	4	0
TGFb3	3	7
VEGF	175	28

D

Growth Factors	PL	FBS
EGF	0	0
HB-EGF	oe	175
bFGF	622	8
IGF-I	9	0
IGF-II	0	82
PDGF-AA	0	95
PDGF-AB	0	46
PDGF-BB	18	117
TGFb1	0	0
TGFb2	0	83
TGFb3	0	77
VEGF	2228	216

Supplementary Table 1. *Top 50 of proteins with highest expression. Results after 15 seconds exposure time.* ***1A.*** *Expression of proteins in PL.* ***1B.*** *expression of proteins in FBS.* ***1C.*** *Expression of pre-selected growth factors at 15s exposure time.* ***1D.*** *Pre-selected growth factors at 137 seconds exposure time. oe = over expressed.*



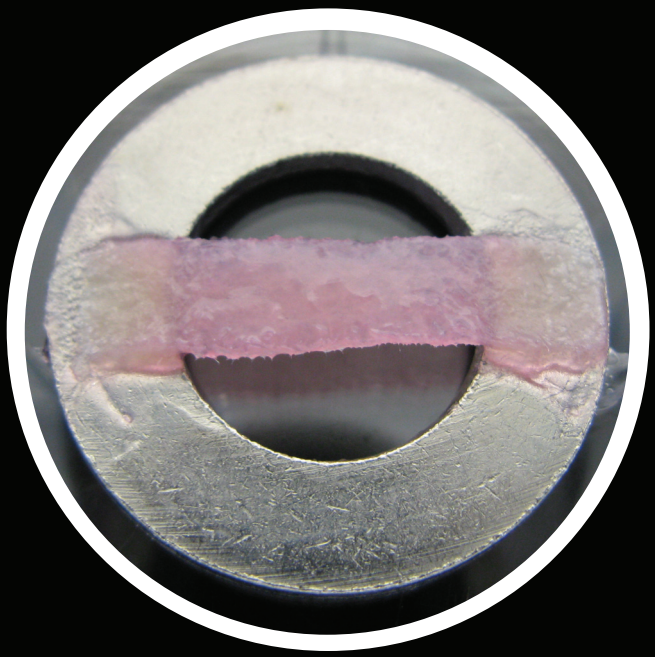
Supplementary Figure 2. *Superarray images obtained after 15s exposure time. The contrast in these images was enhanced to allow better presentation.* ***2A.*** *Image after incubation with PL-medium.* ***2B.*** *Image after incubation with FBS-medium. P: Positive control, N: Negative control, B: Blanks, IC: Internal control.*

Total TGF-beta 1 concentrations

	PL	FBS	
Unconditioned medium	965	334	
Conditioned medium	4359	556	
Normal human serum (a)			36.1

Supplementary Table 2. *Total TGF-beta 1 content (pg/mL) in unconditioned medium used for the superarray and conditioned medium (3 days) from a randomly selected selected patient. (a) Concentration in normal human serum derived from Torre et al.[2]*

Chapter 4



Chapter 4

Decreased Mechanical Properties Of Heart Valve Tissue Constructs Cultured In Platelet-lysate As Compared To Fetal Bovine Serum

This chapter has been published in:

*Riem Vis PW *, van Geemen D *, Soekhradj-Soechit RS, Sluijter JP, de Liefde - van Beest M, Kluin J, Bouten CVC. Decreased mechanical properties of heart valve tissue constructs cultured in platelet lysate as compared to fetal bovine serum. Tissue Eng Part C Methods. 2011;17:607-17.[152]*

Abstract

In autologous heart valve tissue engineering, there is an ongoing search for alternatives of fetal bovine serum (FBS). Human platelet-lysate (PL) might be a promising substitute. In the present paper we aimed to examine the tissue formation, functionality, and mechanical properties of engineered 3D tissue constructs cultured in PL as a substitute for FBS. Our results show that tissue constructs that were cultured in PL and FBS produce similar amounts of collagen, GAGs and collagen crosslinks, and that the cellular phenotype remains unchanged. Nevertheless, mechanical testing showed that the ultimate tensile strength in PL constructs was on average approximately three times lower as compared to FBS (0.25 MPa vs. 0.74 MPa respectively, $p < 0.01$), and also the elastic modulus was almost three times lower (1.33 MPa of PL constructs vs. 3.94 MPa of FBS constructs, $p < 0.01$). Additional tests indicated that this difference might be explained by different collagen fiber architecture possibly due to increased production of matrix-degrading proteases by cells cultured in PL. In summary, our results indicate that PL is not preferred for the culture of strong heart valve tissue constructs.

Introduction

Autologous heart valve tissue engineering (TE) is an emerging strategy for future heart valve replacements.[11, 127] Currently, heart valve replacements enhance survival and the quality of life, but have several limitations.[153] Most importantly, these valves do not consist of living tissue and, therefore, will not grow. Heart valve TE seeks to overcome these limitations by creating a heart valve that has the ability to grow, repair and remodel.

In general, the heart valve TE strategy involves isolation of autologous cells, followed by *ex vivo* expansion and seeding of these cells on biodegradable scaffolds of synthetic or natural origin. The cell-scaffold constructs are then subjected to mechanical triggers in a bioreactor to stimulate extracellular matrix formation until a strong and functional load-bearing heart halve is grown that can be used for implantation. *Ex vivo* expansion of cells and the culture of the TE valve require a source of nutrients and growth factors in basal culture media. Fetal Bovine Serum (FBS) is usually selected for this purpose. However, several studies have shown that cells are able to take up animal-derived proteins from FBS and present these antigens after implantation.[154] In some studies, this has led to immune responses against the implanted cells and thus failure of the treatment.[104, 105, 155, 156] Therefore, there is an ongoing search for alternative and preferable autologous sources of nutrients and growth factors.

One of the alternatives for FBS is human platelet-lysate (PL). Platelets contain granules that are rich in several interleukins and growth factors, including EGF, TGF-β and bFGF, which, *in vivo*, are released at sites of injury when proliferation of cells and remodeling of matrix is required.[131, 149, 157] PL is formed by forced release of growth factors in human serum and is believed to induce proliferation and extracellular matrix (ECM) remodeling in tissue regeneration and repair. In addition, it can be obtained autologously. Furthermore, unlike platelet-rich plasma, platelet numbers are largely reduced in PL due to centrifugation prior to addition to the medium, and it does not need to be activated by thrombin. However, to prevent clotting, heparin should be added to PL-enriched medium. Nevertheless, PL has been shown to be a promising substitute for FBS when applied for autologous culture of adult stem cells. PL is able to promote mesenchymal stem cell expansion[123, 158], while multilineage differentiation is maintained.[123,

125, 158] Furthermore, at the tissue level, PL shows promising results regarding bone TE applications.[124, 159]

For autologous valve TE, it is desired that optimal tissue properties can be achieved in the shortest time possible. Hence, cultured cells should have a high duplication rate to reduce culture time, produce high amounts of ECM proteins and are able to remodel produced matrix to optimize fiber arrangements. In a previous study, we have shown that venous-derived mesenchymal cells (myofibroblasts), frequently used for heart valve TE, meet these primary criteria when cultured in PL.[73]

However, for the culture of load-bearing heart valves, not only the amount of cells and matrix are important to ensure strong tissue. The formation of strong tissue depends on a delicate balance between formation of ECM and degradation of newly formed fibers. As collagen is the main load-bearing component of functional heart valves, the collagen in engineered valves should be good enough to withstand hemodynamic forces upon implantation. Therefore, the aim of this study was to examine tissue formation, functionality, and mechanical properties of engineered heart valve constructs cultured in PL as an alternative for FBS.

Materials and Methods

An established 3D tissue model, consisting of human myofibroblasts seeded onto a biodegradable scaffold was used to study mechanical properties and tissue composition. For this purpose we harvested human myofibroblasts (MFs) of 9 patients. These cells were expanded up to passage 7 and for each patient 10 constructs were engineered per condition, *e.g.* PL or FBS. These tissue constructs were cultured for 4 weeks and, hereafter, mechanical properties and tissue composition of the constructs were analyzed, quantitatively and qualitatively.

Cell isolation

Segments of vena saphena magna (±3 cm) were obtained from 9 patients undergoing coronary artery bypass surgery using a venous graft. Individual permission using standard informed consent procedures and prior approval of the ethics committee of the University Medical Center Utrecht was obtained, and tissue was further treated anonymously, as described previously.[73, 130] Briefly, venous segments were transferred to the laboratory in serum-free medium (Dulbecco's modified Eagle's medium-advanced [Gibco; Invitrogen, Breda, NL], 2mM GlutaMax [Gibco], and 10 µg/mL gentamycin [Lonza, Verviers, Belgium]), wherein the adventitia was removed from the medial/intimal layer. Subsequently, the vessel was washed in antibiotics solution (phosphate-buffered saline [PBS; Sigma, Venlo, NL], 2.5 µg/mL AmphotericinB [Biochrom; VWR, Amsterdam, NL], and 200 µg/mL Gentamycin [Gibco]).

The lumen of the vessel was incubated in endothelial cell medium with collagenase (EBM2, EGM2 single quots [Lonza], 20% FBS [HyClone, Etten -Leur, NL], and 2 mg/mL collagenase A [Roche, Almere, NL]), after which endothelial cells were scraped off with a cell scraper.

Tissue segments were cut into small squares (2x2 mm) and plated on culture plates with the lumen faced down, receiving either FBS medium (serum-free medium + 10% FBS) or PL medium (serum-free medium with 5% PL and 10 U/mL heparin [LEO Pharma, Breda, NL]). Human platelets in serum were obtained from the hospital blood bank, pooled from five donors with similar blood type and rhesusfactor, and buffered with citrate-phosphate dextrose. The pooled plasma bags were estimated to contain a platelet count of 10^6 platelets per mm^3. Moreover, the presence of several growth factors and cytokines were screened in our previous study.[73] PL was frozen in aliquots at -80°C, thawed, and centrifuged (8 min 900 rcf) prior to addition to the culture medium, as described by others.[125, 131] Cells were expanded using standard culture methods as previously described.[1, 130] The expansion medium (2D medium) for MFs consisted of DMEM Advanced [Invitrogen, Breda, NL] supplemented with 1% Penicillin/Streptomycin (P/S; Lonza) and 1% GlutaMax (Gibco). The medium was further supplemented with 10% FBS (Greiner Bio-one, Alphen a/d Rijn, NL) or 5% PL and 0.2% heparin (LEO Pharma) for the FBS and PL groups respectively.

Scaffold preparation and sterilization

Rectangular scaffolds (30x6x1 mm) composed of rapidly degrading non-woven polyglycolic acid (PGA; thickness, 1.0 mm; specific gravity, 70 mg/cm^3; Cellon, Bereldange, Luxembourg), were coated with poly-4-hydroxybutyrate (P4HB; Symetis Inc., Zürich, Switzerland) to provide structural integrity to the mesh. After drying overnight, the two outer 5 mm of the long axis of each construct were glued to stainless steel rings using a 20% solution of polyurethane (PU; DSM, Geleen, NL) in tetrahydrofuran, leaving a 20×6 mm area for TE. The solvent was allowed to evaporate overnight.

The rings with the scaffold strips were placed in 6-well plates and sterilized in 70% ethanol for 30 minutes and, subsequently, washed twice in PBS (Sigma, Venlo, NL). Hereafter, the strips were placed in TE medium (2D medium, supplemented with L-ascorbic acid 2-phosphate [0.25 mg/mL; Sigma]) overnight.

Cell seeding

MFs were seeded in the constructs (n = 10 per group) using fibrin as a cell carrier, as previously described by Mol *et al*.[160] In short, MFs were suspended in TE medium, without FBS or PL, containing thrombin (10 IU/mL; Sigma). Subsequently, this cell suspension was mixed with an equal volume of TE medium, without FBS or PL, containing fibrinogen (10 mg/mL; Sigma). This fibrin/cell suspension (20.10^6 cells/mL) was mixed until onset of polymerization of the gel after which 180 µl was dripped onto the strip. To allow further firming of the fibrin gel, 6-well plates were placed in an incubator at 37 °C and 5% CO_2 for 30 minutes. Hereafter, the wells were filled with 6 mL TE medium, with PL or FBS, and placed back into the incubator. The constructs were cultured for 4 weeks and TE medium was changed every 2-3 days.

Mechanical testing

Mechanical properties were determined after 4 weeks of culture by uniaxial tensile testing in longitudinal direction of the engineered constructs (n = 4 per patient and culture condition) using a tensile stage equipped with a 20N load cell (Kammrath-Weiss, Dortmund, Germany). Measurements were averaged per culture condition.

A Digimatic Micrometer (Mitutoyo America Corporation, Aurora, USA) was used to measure the thickness of the strips prior to tensile testing. Stress–strain curves were obtained at a strain rate equal to the initial sample length (20 mm) per minute. The Cauchy stress was defined as the force divided by the cross-sectional area. The ultimate tensile strength (UTS; *e.g.* the maximal Cauchy stress) was determined from the curves while the slope of the linear part of the curve represented the elasticity modulus (E-modulus) of the tissue.

Histology

Tissue formation was analyzed qualitatively by histology. Tissue constructs were fixed in 3.7% formaldehyde in PBS and subsequently embedded in paraffin. Samples were sectioned at 10 µm and stained with hematoxylin and eosin (H&E) for general tissue development and Masson Trichrome (MTC kit, Sigma) for collagen visualization. The stainings were analyzed using a Zeiss light microscopy (Carl Zeiss, Sliedrecht, NL). To distinguish between juvenile and mature collagen fibers, Picrosirius Red Staining (after Puchtler *et al*.[161] and Junqueira *et al*. [162]) was performed and examined by means of crossed polar microscopy (Carl Zeiss).

Immunofluorescence

Immunofluorescence was performed on paraffin sections. The used antibodies and their corresponding secondary antibodies are depicted in Table 1. Sections were deparaffinized and antigen was retrieved by incubation in either boiled TRIS-EDTA buffer for 20 minutes or in 0.04% pepsin buffer for 8 minutes. Afterwards, sections were incubated in 1% BSA (Roche) in PBS to block non-specific binding. Prior to overnight incubation (4 °C) with the primary antibodies (Table 1), the sections were permeabilized with 1% Triton-X-100 (Merck, Amsterdam, NL) in PBS. The specific stainings were visualized with fluorescent secondary antibodies (Table 1). After an additional staining with DAPI to stain cell nuclei, sections were mounted with mowiol (Calbiochem, San Diego, USA). Stained sections were analyzed and pictures were taken by means of fluorescent microscopy (Axiovert 200, Carl Zeiss).

Biochemical assays

The total content of DNA (as an indication of cell number), sulfated glycosoaminoglycans (GAGs) and hydroxyproline (HYP) was determined on constructs previously used for the tensile tests. Lyophilized samples were digested in papain solution (100 mM phosphate buffer (pH=6.5), 5 mM L-cystein, 5 mM EDTA and 125-140 µg papain per mL) 60°C for 16 hours. The Hoechst dye method [163] with a reference curve prepared of calf thymus DNA (Sigma) was used to determine the amount of DNA. The content of sulfated GAGs was determined on the basis of the protocol described by Farndale *et al*. [164] and shark cartilage chondroitin sulfate was used as a reference (Sigma). In short, 40 µl of diluted sample, without addition of chondroitin AC lyase, chondroitin ABC lyase and keratanase, was pipetted into a 96-wells plate in duplicate. Hereafter, 150 µl dimethylmethylene blue was added and absorbance was measured at 540 nm. To determine the HYP quantity, an assay according to Huszar *et al*. [165] and a reference of trans-4-hydroxyproline (Sigma) was used. The number of mature collagen hydroxylysylpyridinoline (HP) cross-links, as a measure for tissue maturity, was measured in the same hydrolyzed samples using high-performance liquid chromatography as described previously.[166-168] The number of HP cross-links was expressed per collagen triple helix (TH).

	Primary antibody		Corresponding secondary antibody		
collagen type I	IgG1	Sigma	Alexa 488	IgG1	Invitrogen
collagen type III	IgG1	Sigma	Alexa 488	IgG1	Invitrogen
αSMA	IgG2a	Sigma	Alexa 488	IgG2a	Invitrogen
vimentin	IgM	Abcam (Cambridge, UK)	Alexa 555	IgM	Invitrogen
Hsp47	IgG2b	Stressgen (Michigan, USA)	Alexa 488	IgG2b	Invitrogen
Desmin	IgG1	DAKO (Heverlee, Belgium)	Alexa 555	IgG1	Invitrogen
SMemb	IgG2b	Abcam	Alexa 488	IgG2b	Invitrogen

Table 1. *The antibodies used in this study are listed in this table. All primary antibodies were monoclonal mouse anti-human antibodies. All secondary antibodies were goat anti-mouse antibodies. Only in case of monoclonal IgG1 mouse anti-human antibody against collagen type III, the antigen was retrieved by incubation in pepsin buffer. For all other antibodies, the antigen was retrieved by incubation in TRIS-EDTA buffer.*

Medium analysis

Concentrations of the remodeling enzymes matrix metalloproteinases (MMP)-1, MMP-2, and procollagen type I C-peptide (PIP; a marker for collagen I synthesis) were determined on medium samples after 4 weeks of culturing. ELISAs were performed according to the recommendations from the supplier. MMP-1 and MMP-2 concentrations were quantified by immunoassays for human MMP-1 and MMP-2 protein (RayBiotech; Tebu-bio, Heerhugowaard, NL). PIP was determined using a procollagen type I C-peptide ELISA kit (Takara Bio, Otsu Shiga, Japan).

Zymography

Zymography analysis was performed on medium conditioned by cells in tissue constructs, corrected for the amount of DNA. Similar amounts of medium from patients that produced tissue in both PL and FBS was pooled, to give an averaged overview of protease expression. To reduce aspecific signals from abundant serum proteins, albumin and IgG-fractions were removed using the Aurum Serum Protein Mini Kit (Bio-Rad, Veenendaal, NL), according to the manufacturer's instructions. The samples were prepared with LaemmLi buffer (0.25 M Tris-HCl pH 6.8, 8% SDS, 40% glycerol and 0.004% Bromophenol blue) and loaded on 10-15% poly-acrylamide gels containing 2 mg/mL gelatin or 4 mg/mL casein, as described previously. [133] After running, gels were incubated overnight at 37°C in Brij-solution (50 mM tris-HCL 7.4, 10 mM $CaCl_2$, 0.05% Brij35 (w/v dilute 600x) and stained with Coomassie Blue (25% MeOH, 15% HAc and 0.1% Coomassie Blue [Polysciences, Inc., Eppelheim, Germany]). Analysis and quantification of total (active and inactive) protease secretion was performed using the ChemiDoc XRS system (Bio-Rad) and QuantityOne software (Bio-Rad).

Statistics

Data on quantitative analysis of tissue composition, mechanical properties and collagen remodeling are provided as mean ± standard deviation. We used SPSS 17 software for statistical analysis. Paired t-tests were used to test differences in tissue outcome for tissues cultured in FBS or PL. A p-value < 0.05 was considered significant.

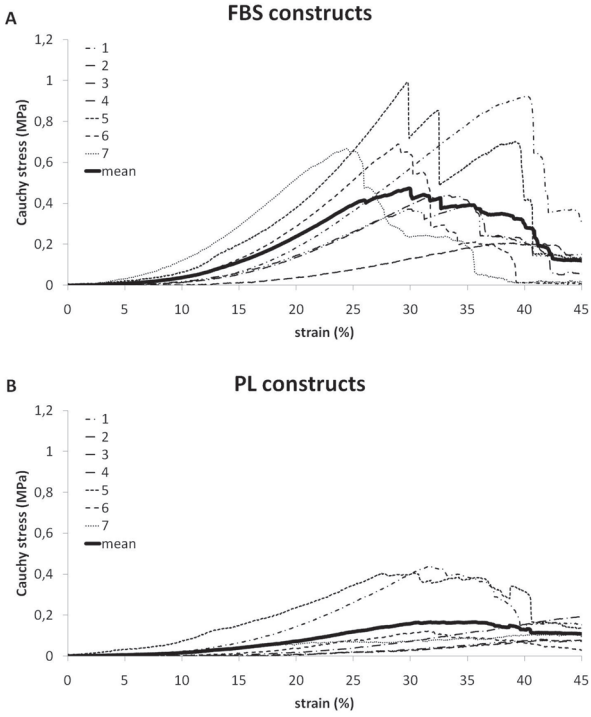


Figure 1. *Stress-strain curves of the tissue engineered constructs cultured in media supplemented with FBS (**1A.**) or PL (**1B.**). The stress-strain curves of the constructs of the different patients are indicated with the different lines. The dark black line indicates the averaged stress-strain curve of all constructs cultured in FBS (**1A.**) or PL (**1B.**).*

Results

Biomechanical properties

Tissue constructs were engineered from myofibroblasts of 9 different patients. In the tissue constructs of two patients cultured with FBS a bacterial infection was found. The constructs cultured with the cells of these patients were, therefore, excluded from this study. The results of the constructs of the remaining seven patients are described below.

The averaged stress-strain curves are shown in Figure 1, and tissue stiffness (E-modulus) and strength (UTS) are shown in Figure 2 and Table 2. The E-modulus ranges from 1.10 ± 0.17 MPa to 7.46 ± 0.82 MPa in the tissue constructs cultured in FBS medium, while the constructs cultured in

PL medium have an E-modulus that ranges from 0.46 ± 0.26 MPa to 2.83 ± 0.36 MPa (Figure 2a). The Ultimate Tensile Strength ranges from 0.25 ± 0.07 MPa to 1.37 ± 0.19 MPa in the constructs cultured in FBS medium and from 0.12 ± 0.09 MPa till 0.52 ± 0.09 MPa in the constructs cultured in PL medium (Figure 2b). There are differences in E-modulus and UTS between constructs of different patients. However, the constructs of all patients were significantly stiffer (higher E-modulus) and stronger (higher UTS) when cultured in medium supplemented with FBS ($p < 0.01$).

Qualitative tissue analysis

Pictures of general tissue development (H&E), collagen deposition (MTC), and collagen fiber thickness (picrosirius red) are shown in Figure 3. Although there is a slight variation between the constructs of the different patients, the general tissue development, as seen in the H&E staining, between cardiovascular constructs cultured in FBS or PL is similar. Collagen is seen throughout the tissue; however it is more abundant at the surface layers. This is observed for all tissue constructs.

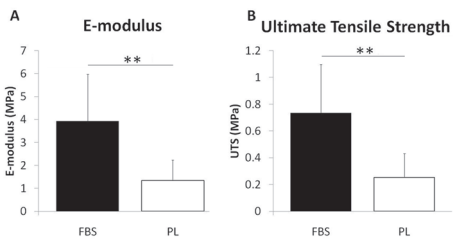


Figure 2. *Mechanical properties including E-modulus (**2A.**) and UTS (**2B.**). ** ($p<0.01$) represents significant difference between constructs cultured in FBS- and PL-medium. Tissue constructs engineered in FBS-medium are stiffer and stronger than constructs cultured in PL-medium.*

Tissue properties	FBS constructs	PL constructs
E-modulus (MPa)	3.94 ± 2.02	1.33 ± 0.90 **
UTS (MPa)	0.74 ± 0.36	0.25 ± 0.18 **
Thickness (mm)	0.74 ± 0.07	0.79 ± 0.07
Width (mm)	4.08 ± 0.42	4.33 ± 0.51 *
DNA (µg/strip)	15.78 ± 2.63	19.27 ± 3.09
GAG (µg/strip)	126.63 ± 16.90	121.69 ± 22.45
HYP (µg/strip)	89.84 ± 34.83	88.42 ± 23.35
HP/TH (-)	0.17 ± 0.05	0.17 ± 0.06
PIP (ng/ml)	36032 ± 8844	40767 ± 14257
MMP-1 (ng/ml)	49 ± 44	155 ± 107 *
MMP-2 (ng/ml)	3.4 ± 0.5	10.1 ± 1.9 **

Table 2. *Mechanical properties and tissue composition of engineered constructs cultured in media supplemented with FBS or PL. * indicates a difference compared to the FBS group. Single and double symbols indicate $p < 0.05$ and $p < 0.01$, respectively.*

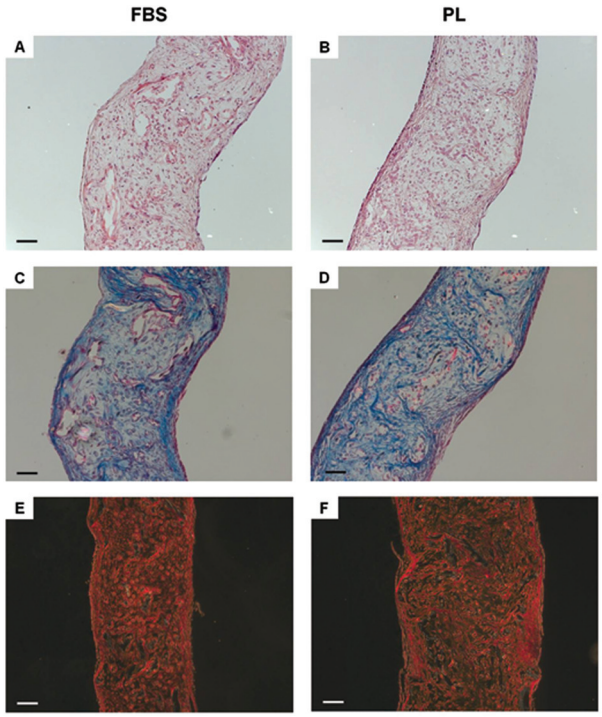


Figure 3. *Histology of tissue engineered constructs cultured in FBS (**3A, C, E.**) and PL (**3B, D, F.**) medium. Representative pictures for tissue composition are displayed. (**3A-B.**) H&E staining, (**3C-D.**) Masson Trichrome Staining, and (**3E-F.**) picrosirius red staining. Original magnification was 10x. Scale bars indicate 100 µm.*

The phenotype of the myofibroblasts in the cardiovascular constructs was analyzed with immunofluorescence (Figure 4). In all engineered constructs, cells stain positively for α-smooth muscle actin (αSMA), vimentin, heat shock protein 47 (hsp47), and non-muscle myosin heavy chain (SMemb), but not for desmin. No consistent difference between FBS and PL is observed, though, there are differences between the constructs of different patients.

To distinguish between collagen type I and collagen type III, the engineered constructs were analyzed by immunofluorescent staining (Figure 5). Within PL and FBS groups large inter-subject variations were observed. These were not related to collagen type. Typically, if collagen type I was abundant, collagen type III was also and vice versa. Between FBS and PL groups no consistent differences in collagen production could be recognized.

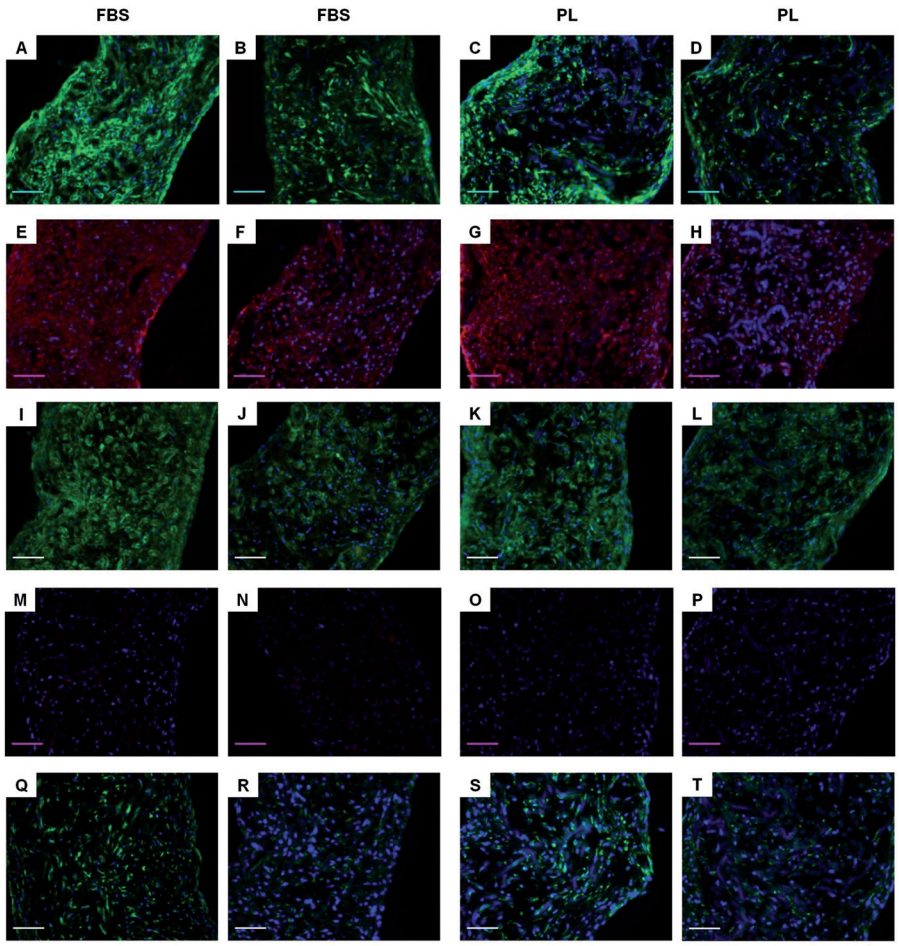


Figure 4. *Immunofluorescent staining of tissue engineered constructs cultured in both media. Results for phenotyping of two patients are displayed to indicate differences between patients. (**4A-D.**) α-smooth muscle actin, (**4E-H.**) vimentin, (**4I-L.**) nonmuscle myosin heavy chain (SMemb), (**4M-P.**) desmin, and (**4Q-T.**) heat shock protein 47. Nuclei are stained with DAPI. Original magnification was 20x. Scale bars indicate 100 μm.*

Some scaffold remnants, which were still present in all tissue engineered constructs, were also stained with the DAPI staining (Figure 4 and 5). In Figure 5 such remnants are indicated with an arrow to illustrate the distinction between the remnants and the nuclei.

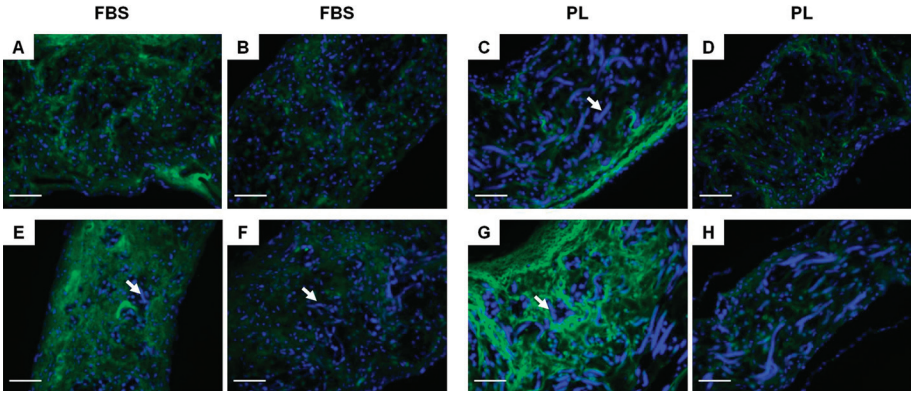


Figure 5. *Immunofluorescence of tissue engineered constructs cultured in FBS (**5A, B, E, F.**) or PL (**5C, D, G, H.**) medium. Results of collagen type I and collagen type III immunofluorescent staining of two patients are displayed. (**5A-D.**) collagen type I, and (**5E-H.**) collagen type III. Nuclei are stained with DAPI. Original magnification was 20x. Scale bars indicate 100 µm. The white arrows indicate scaffold remnants.*

Quantitative tissue analysis

Tissue composition of the engineered constructs was quantified by DNA, GAG, and hydroxyproline analysis, and the amount of collagen crosslinks (Table 2). The DNA per tissue construct was comparable for the constructs of all patients in the PL and FBS groups, and did not differ between the groups. Averaged DNA content was 15.78 ± 2.63 µg/tissue strip for the FBS group, and 19.27 ± 3.09 µg/tissue strip for the PL group. This difference is not significant. Also no differences were observed in the GAG content, HYP concentration, and the number of HP cross-links per triple helix. Although there were no differences in HYP content between the PL and FBS groups, there was a slight variation between the constructs of the different patients. These results correspond with the Masson Trichrome, picrosirius red staining, and immunofluorescent stainings.

Collagen remodeling capacity

The effects of tissue culture in either FBS or PL on remodeling markers are shown in Figure 6 and Table 2. Collagen synthesis, indicated by PIP concentration measurements, was similar in tissue cultured in FBS or PL. The PIP concentration ranges from 18,133 to 45,100 ng/mL in the culture medium with FBS, and from 19,712 to 55,985 ng/mL in the culture medium

supplemented with PL (Figure 6a). For collagen degradation/remodeling, the concentration of matrix metalloproteinases was measured. As indicated by ELISA analysis, MMP-1 and MMP-2 were elevated in tissue culture supplemented with PL (MMP-1: 155 ± 107 ng/mL; MMP-2: 10.1 ± 1.9 ng/mL) when compared to culture medium with FBS (MMP-1: 49 ± 44 ng/mL; MMP-2: 3.4 ± 0.5 ng/mL). These variations were also observed in the zymography analysis, that indicate the contribution of activated MMPs. Figure 6d shows a higher activated MMP-2 and MMP-9 in tissue culture medium supplemented with PL.

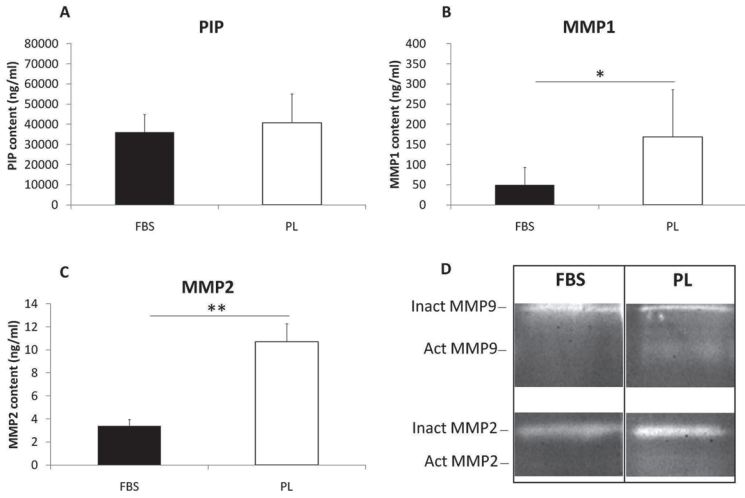


Figure 6. *Concentrations of the remodeling markers PIP (**6A.**), MMP-1 (**6B.**), and MMP-2 (**6C.**) in culture medium. * ($p<0.05$) and ** ($p<0.01$) indicate significant differences between tissue engineered constructs cultured in FBS- and PL-medium. Higher levels of MMP-1 and MMP-2 are observed in PL-medium. (**6D.**) Zymography results for total MMP-2 and MMP-9 expression. More active and inactive MMP-2 and MMP-9 are present in the culture medium of constructs cultured with PL.*

Discussion

In our previous study, published in April 2010 [73], we studied in 2D whether PL could serve as an alternative for FBS in human heart valve TE. This study showed promising results with respect to the expansion and matrix production and remodeling potential of cells cultured in PL as compared to FBS. In the present study, we therefore aimed to verify whether PL can serve as an alternative for FBS in 3D tissue engineered heart valve construcs. Engineered constructs, consisting of PGA/P4HB scaffolds seeded with myofibroblasts of 7 different patients, were cultured in TE-medium supplemented with either PL or FBS and mainly tested for tissue composition and mechanical properties. Contrary to the 2D studies, however, this 3D study indicates that, despite similarities in matrix production of tissues cultured in PL and FBS, tissue mechanical properties are drastically reduced after culture in PL. In addition, E-moduli and ultimate tensile stress of PL-constructs reached values of about 2 times lower than those found for native heart valve leaflets in the radial direction [23], whereas tissue mechanical properties of the constructs cultured in FBS-rich medium more closely resembled those of native valve leaflets.

Tissue mechanical properties strongly depend on ECM formation and maturation. Haut *et al*. described that the tensile modulus was positively correlated with the content of insoluble collagen in the canine tendon. [169] Elbjeirami and co-workers found that the tensile strength and elastic modulus was increased in TE constructs, in which the ECM was enzymatically cross-linked.[170] Moreover, Balguid and colleagues showed that there is a significant correlation of collagen cross-linking with tissue stiffness in circumferential direction in native heart valves.[23] Thus, in determining the mechanical properties of tissue constructs, not only collagen formation is of interest, but especially collagen cross-links appear important. Nevertheless, despite similar collagen content and a similar number of HP cross-links, which are the main type of collagen cross-links present in cardiovascular tissue, the tensile properties of constructs cultured in FBS were higher than those cultured in PL. Therefore, these structural properties alone do not explain the difference in mechanical properties found in this study.

Another factor that specifies tissue biomechanical properties is the collagen architecture.[171] Lindeman *et al*. described that mechanical properties of vessels are strongly influenced by collagen microarchitecture and that perturbations in the collagen network may lead to mechanical failure.[172] In addition, Guidry and Grinnell reported that heparin modulates the organization of hydrated collagen gels.[173] Control collagen gels were composed of a uniform network of interlocking fibrils, while this network was disrupted in heparin-containing gels.[173] Heparin must be added to PL medium, to prevent coagulation of the platelets in the medium. Heparin, in combination with the increased matrix remodeling abilities, can alter the collagen architecture of the engineered constructs. To verify the collagen architecture of the constructs cultured in the present study, additional tissue constructs were engineered in PL or FBS medium and collagen fiber organization was visualized by whole mount collagen type I immunofluorescent staining. An inverted Zeiss Axiovert 200 microscope (Carl Zeiss) coupled to an LSM 510 Meta (Carl Zeiss) laser scanning microscope was used to visualize collagen type I organization. The collagen architecture in the TE constructs cultured in PL was observed to be different from that of the constructs cultured in FBS (Figure 7) in a blinded procedure using 3 independent observers. It was concluded that the collagen architecture was less dense in the PL group as compared to the FBS group. Furthermore, in the PL-constructs the collagen fibers appeared to be shorter. This difference of the collagen network in PL, is probably caused by enhanced collagen degradation by MMPs in the PL constructs and might indeed explain the decreased mechanical properties of the PL constructs as compared to the FBS constructs. Additional studies, incorporating a more quantitative approach including image analysis, are required to verify this relation between tissue architecture, MMP activity, and tissue mechanical properties in PL constructs

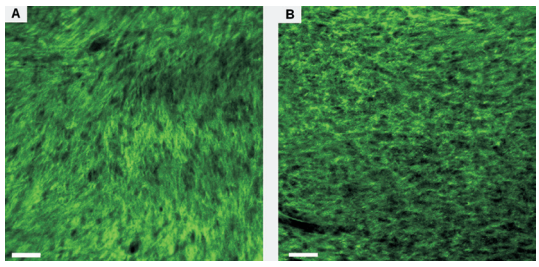


Figure 7. *Whole mount collagen type I immunofluorescent staining of engineered constructs cultured in FBS (**7A.**) or PL (**7B.**). The collagen network in the PL group seems less dense with shorter fibers. Original magnification was 20x. Scale bars indicate 50 µm.*

A last factor that might have affected the difference in the mechanical properties of the engineered constructs is the contribution of the scaffold material. Due to degradation of the material used in our studies, the mechanical properties of the cell-scaffold constructs will rapidly change with time. We have previously tested these properties for tissue constructs and bare scaffolds kept in FBS.[174, 175] Initially, tissue mechanical behavior is linear in nature and resembles that of the bare scaffold, indicating a large contribution of scaffold material to total mechanical properties. However, with time the scaffold degrades and tissue is being formed, leading to a rapid increase in mechanical properties (E-modulus, UTS) and the typical non-linear stress-strain behavior of native cardiovascular tissues after 3 to 4 weeks of culture. Although scaffold degradation in PL in the present study may have been different from that in FBS, it is unlikely that this affected ultimate tissue mechanical properties after 4 weeks. For both culture conditions only scaffold remnants are present at this point in time, unlikely to significantly contribute to total tensile mechanical properties. Furthermore, by this time, both PL and FBS constructs have reached the non-linear stress-strain behavior typical of 'native' tissue without artificial material.

The composition and quality of the tissue are subject to change during tissue remodeling due to mechanical or biochemical environmental stimuli. In this study, the biochemical triggers were different for the PL and FBS groups. Superarray and western blot analyses, performed in our previous study [73], showed generally higher expression of proteins involved in tissue repair and remodeling in PL-medium. PL medium contains higher concentrations of heparin-binding epidermal growth factor (HB-EGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and total transforming growth factor-beta (TGF-β) when compared to FBS. Furthermore, the levels of interleukins, IL17 and IL23, and proteins involved in tissue repair and matrix remodeling, MIP1a and uPA, are higher in PL than FBS. This suggests that the matrix remodeling capacities of tissues cultured in PL might be higher than of tissue cultured in FBS. Therefore, the level of collagen remodeling markers (MMP-1, MMP-2, and PIP) secreted in these tissues was also investigated in this study. MMP levels were higher in the PL group, which might be explained by the high levels of IL17, IL23, MIP1a and uPA in PL-medium.[143, 144, 176] These proteins are also involved in the wound healing process.[177, 178] Moreover, MMPs are crucial in the inflammatory and remodeling phases of wound healing. [179] This suggests that the tissue formed in PL has similar properties as

the scar tissue formed during wound healing. Characteristic of scar tissue is a disorganized collagen network and high remodeling properties, both resulting in initial weaker tissue. A hypothesis could be that, with time, tissue cultured in PL can remodel to stronger and more organized tissue.

Our main reason to test the use of PL was to define an autologous culture medium for future clinical application of heart valve tissue engineering. PL can be obtained autologously, offering the potential to culture fully autologous tissue engineered valves in the future. Though, we used pooled PL from several human subjects instead of autologous PL in the present study, we believe to have provided proof of principle for the use of PL in heart valve tissue engineering and sufficient data to conclude that culture of heart valve tissue constructs in PL medium results in tissue with poor mechanical properties.

In conclusion, at the cellular (2D) level PL might be a promising substitute for FBS; however, at the 3D tissue level, FBS induces a more stable and organized ECM, relevant for improved mechanical properties. Hence, our results indicate that serum is required for mechanically functionally heart valve tissue engineering, though this might preferably be obtained autologously. Future studies should point out if autologous serum can be used as an appropriate substitute for FBS in 3D tissue culture.

Acknowledgements

The authors would like to thank Leonie Grootzwagers for her help during cell seeding and the tensile tests, and Jessica Snabel (TNO Leiden, department Tissue Repair) for performing the crosslink assays. This research is supported by the Dutch Technology Foundation (STW), applied science division of NWO and the Technology Program of the Dutch Ministry of Economic Affairs.

Chapter 5

Chapter 5

Sequential Use Of Human-derived Medium Supplements Favors Cardiovascular Tissue Engineering

This chapter has been published in:

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Abstract

For clinical application of tissue engineering strategies, the use of animal-derived serum in culture medium is not recommended, because it can evoke immune responses in patients. We previously observed that human platelet-lysate (PL) is favorable for cell expansion, but generates weaker tissue as compared to culture in fetal bovine serum (FBS). We investigated if human serum (HS) is a better human supplement to increase tissue strength. **Methods:** Cells were isolated from venous grafts of 10 patients and expanded in media supplemented with PL or HS, to determine proliferation rates and expression of genes related to collagen production and maturation. Zymography was used to assess protease expression. Collagen contraction assays were used as a 2D-model for matrix contraction. As a prove of principle, 3D tissue culture and tensile testing was performed for two patients, to determine tissue strength. **Results:** Cell proliferation was lower in HS-supplemented medium than in PL-medium. The HS-cells produced less active MMP2 and showed increased matrix contraction as indicated by gel contraction assays and 3D-tissue culture. Tensile testing showed increased strength for tissues cultured in HS when compared to PL. This effect was more pronounced if cells were sequentially cultured in PL, followed by tissue culture in HS. **Conclusion:** These data suggest that sequential use of PL and HS as substitutes for FBS in culture medium for cardiovascular tissue engineering results in improved cell proliferation and tissue mechanical properties, as compared to use of PL or HS apart.

Introduction

The use of engineered cardiovascular tissues to replace diseased tissues in patients has been proposed as an alternative for current replacement therapies, because of their ability to grow and adapt *in vivo*.[11] Although this has been demonstrated in long-term animal studies [181], studies in humans have yet to be performed to evaluate the efficacy of this approach. Recent studies by our group have demonstrated differences in cardiovascular tissues engineered from human and ovine cells (van Geemen *et al*., submitted). This further indicates the relevance of specific, deviating culture protocols for either ovine or human engineered tissues and emphasizes that results obtained with either species cannot be easily compared. In order to prepare for future clinical studies and to circumvent risks associated with the use of animal-derived culture additives, it is relevant to investigate the outcomes of autologous culture of human engineered tissues.

Engineered (cardiovascular) tissues are generally produced by isolation of cells from patients, expansion of those cells in culture media and seeding on scaffolds of synthetic or biological origin, followed by culture and conditioning in bioreactors to promote tissue formation. Culture media used for cell expansion and tissue culture generally contain fetal bovine serum (FBS) as an essential source of proteins and growth factors. Ideally, this animal-derived serum is replaced for preparation of constructs for clinical application, since it may lead to rejection of grafted cells *in vivo*.[104, 156] Cells are able to take up proteins from culture media and present them on their membranes, thereby activating the immune system and leading to failure of the implanted construct.[104, 155, 156, 182] Therefore, our research concentrates on identification of human alternatives for FBS that can be used for cell and tissue culture in (cardiovascular) tissue engineering, as a step in the translation to clinical application.

In previous studies on cardiovascular (heart valve) tissue engineering, we investigated the use of culture media supplemented with human platelet-lysate (PL), because platelets contain many granules with growth factors and because good results had been reported with PL in other areas of tissue engineering, like clinical scale expansion of bone marrow cells and bone tissue engineering.[123, 158, 159] Several of the identified growth factors are involved in the process of wound repair *in vivo*, which includes

deposition and remodeling of collagen [131, 149, 157], or in heart valve development and maturation.[152] The production of collagen and a balance in remodeling of the collagen matrix is also desirable in tissue engineered heart valves, because a strong and well-organized collagen fiber network is extremely important to support the valves' load-bearing function. We established at first that PL was indeed a suitable human alternative for FBS to stimulate cell proliferation and expression of proteins involved in matrix remodeling, as a result of these differences in growth factor content. [34] However, we subsequently also observed that 3D tissue culture in PL strongly reduced tissue mechanical properties as compared to the culture in serum, supposedly caused by increased cellular expression and activation of matrix metallo-proteinases (MMPs).[73, 152] In our search for clinically favorable medium supplements, the present study investigates if human serum (HS) can improve tissue mechanical properties, while maintaining high cell proliferation rates.

In cultured cells obtained from 10 patients, we investigated the individual effects of PL and HS on cell proliferation, expression of genes related to collagen production and maturation, and MMP expression and activity. Gel contraction assays with cells from all patients were performed to investigate the potential of the cells to contract matrix. As a proof of principle, tensile testing on engineered 3D tissue constructs with cells from 2 patients was included, to illustrate potential effects in engineered cardiovascular tissue.

Materials and Methods

Culture media

PL was produced by obtaining pooled human thrombocytes in serum from 5 donors with similar blood type and rhesus-factor from the hospital bloodbank, buffered with citrate-phosphate dextrose. These thrombocytes were frozen in aliquots at -80°C and thawed and centrifuged (8 min 900 rcf) to produce PL prior to addition to the culture medium.[131, 159] HS was obtained by collecting blood from 5-6 healthy controls in coagulation tubes and centrifuging for 15 minutes at 1800 g. The serum was pooled and frozen in aliquots before use as well. Culture was performed in DMEM-advanced (Gibco: Invitrogen, CA, USA, 12491015) supplemented with 2 mM GlutaMax

(Gibco, 35050-028), 1% penicillin/streptomycin and either 5% HS or 5% PL with 10 U/mL heparin (LEO-pharma, Breda, The Netherlands, 013192-03).

Cell isolation and expansion

Segments of great saphenous vein (± 3 cm) were obtained from venous grafts from 10 patients undergoing coronary artery bypass surgery. Individual permission using standard informed consent procedures and prior approval of the medical ethics committee of the University Medical Center Utrecht, according to the world Medical Association Declaration of Helsinki was obtained and tissue was further treated anonymously.[73] Tissue pieces were plated on culture plastic and culture medium supplemented with either PL or HS was added to stimulate outgrowth of myofibroblasts.

Cells were cultured in a humid environment at 37°C and 5% CO_2 up to passage 5, which in our hands has proved to be a suitable passage to obtain abundant cells for seeding on scaffolds in future tissue engineering strategies. A number of cells were frozen at each passage for use in subsequent experiments. Passage 1 (P1) was seeded at a density of 3000 cells/cm^2, while subsequent passages were split in a 1:3 surface ratio. After each passage, the time required to reach 85% confluency was determined per cell isolation and the number of cells was determined using the Countess Automated Cell Counter (Invitrogen, California, USA). The time in days and the logarithm of the total number of cells that would have been obtained at each passage were fit with a linear model using regression analysis with SPSS 16 software (IBM, Chicago, Illinois). Slopes of these models were used to compare duplication rates of cells derived from individual patients in each condition, followed by determination of the average slope per condition.

Immunocytochemistry

Immunocytochemistry was performed to identify myofibroblasts on cultured cells at P5, seeded on coverslips and cultured for 3 days. Staining procedures were previously described [73] and included α-Smooth Muscle Actin (α-SMA, Sigma, St. Louis, MO, USA; A2547, 11.25 µg/mL) and vimentin (Abcam, Cambridge, UK; ab20346, VI-10, 2.5 µg/mL) as positive markers, and desmin (DAKO, Glostrup, Denmark; M0760, Clone D33, 4.7 µg/mL) as a negative marker. This combination of markers is specific for

both venous myofibroblasts and valvular interstitial cells [28], indicating why venous myofibroblasts may serve as a cell-source for heart valve tissue engineering [1, 71, 72]. Alexa Fluor-labeled goat-anti-mouse (Invitrogen, A21428, 5.0 µg/mL) was used as the secondary antibody. Stainings were visualized by fluorescence microscopy (Olympus, BX60, Tokyo, Japan) and Cell^{P}-software (Olympus).

RNA isolation and quantification

RNA of cells cultured in HS or PL was extracted with Tripure Isolation Reagent (Roche, Basel, Switzerland) according to the manufacturer's protocol. Production of cDNA, qRT-PCR and post-run product verification confirmation were performed as previously described.[73] Primers were designed (OligoPerfect, Invitrogen) for Collagens type I and III and Collagen crosslinking enzymes lysyl-oxidase (LOX) and lysyl-hydroxylase (PLOD2) and annealing temperatures were determined (Table 1). Samples were normalized for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, $\Delta Ct = Ct_{Target} - Ct_{GAPDH}$). Fold-difference of cells cultured in HS-medium per patient were compared to cells cultured in PL-medium ($2^{-\Delta\Delta Ct}$ per patient, with $\Delta\Delta Ct$ per patient = $\Delta Ct_{PL} - \Delta Ct_{HS}$). The fold-differences were averaged per medium. We were unable to obtain cDNA from one of the HS-samples, thus all calculations were n=9 at most. Also, LOX was not detected in one PL patient and PLOD2 in one HS patient.

Target	*Forward primer*	*Reverse primer*	*Annealing temp (°C)*
COL1A1	TGCCATCAAATGCTTCTGC	CATACTCGAACTGGAATCCATC	56
COL3A1	CCAGGAGCTAACGGTCTCAG	CAGGGTTTCCATCTCTTCCA	48
LOX	CCACTATGACCTGCTTGATG	TGTGGTAGCCATAGTCACAG	60
PLOD2	AGCTGTGGTCCAATTTCTGG	CTAGCATTTCGGCAAAGAGC	55
GAPDH	ACAGTCAGCCGCATCTTC	GCCCAATACGACCAAATCC	56

Table 1. *qRT-PCR-primers and annealing temperatures. COL: Collagen type 1 or 3, LOX: lysyl-oxidase, PLOD2: lysyl-hydroxylase, GAPDH: glyceraldehyde 3-phosphate dehydrogenase*

Zymography

To assess MMP expression and activity, zymography was performed as previously described.[73] Conditioned medium was collected and phenol red was removed through dialysis for 2 x 24 hrs using Slide-a-lyzer Mini Dialysis units (Pierce, Thermo Fisher Scientific, Waltham, MA, USA) in 5L of phosphate-buffered saline. After dialysis, protein quantification was performed using a BCA-assay (Sigma) according to the manufacturer's protocol. An equal amount of total protein was loaded on the gels. Analysis and quantification of protease secretion was performed using the ChemiDoc XRS system (Bio-Rad) and QuantityOne software (Bio-Rad). Quantification was corrected for presence of proteases in non-conditioned medium, *i.e.* complete medium that was not exposed to cells.

Collagen gel contraction assay

To evaluate the potential of cells cultured in different media to contract the extracellular matrix, we used a collagen gel contraction assay as described by Merryman *et al*. [139], with small modifications. In brief, the collagen gels (PureCol, Advanced BioMatrix, San Diego, CA, USA) were made and seeded as has been described according to their protocol and the instructions of the collagen manufacturer. The gels were cast in teflon rings with a 13 mm inner diameter and a 2 mm thickness. After polymerization of the gel, cells were seeded on the gel in 200 µL medium at a concentration of $1.5x10^5$ cells per mL and left to attach for 30 min, before additional medium was added and the Teflon ring was removed. Cells were seeded in the same medium as was used for expansion and the gels were subsequently kept in this medium as well. Pictures were taken 2, 16 and 40 hours after removal of the ring. The area covered by the gel was measured using Cell^P-software (Olympus) and noted as the percentage of the initial area of the gel, *i.e.* the inner surface area of the teflon rings. The difference was calculated by subtracting the surface are of HS-gels from the surface area of the PL-gels per patient.

Preparation and testing of engineered tissue constructs

As a proof of principle for tissue forming capacities in both media, we set-up a small experiment with 3D tissue constructs (n = 2 patients, and 2-3 strips per patient). For this purpose, cells were transferred to the Eindhoven University of Technology, where rectangular constructs (20 x 6 mm strips) were cultured, tested and stained according to previously published protocols. [152] Briefly, $1.88.10^6$ cells were seeded onto rapidly degrading scaffolds using fibrin as a cell carrier, as previously described by Mol *et al*. [160]. The constructs were cultured under constrained conditions (*i.e.* attached at both ends) for 4 weeks and medium was changed every 2-3 days. During the tissue culture experiments, medium was supplemented with L-ascorbic acid 2-phosphate (0.25 mg/mL, Sigma).[183]

Mechanical properties were determined after 4 weeks of culture by uniaxial tensile testing in longitudinal direction of the engineered constructs.[152] The Ultimate Tensile Strength (UTS in MPa) was defined as the maximum force at break divided by the cross-sectional area.

Collagen organization in the tissue constructs was visualized with the CNA35-probe directly labeled with Oregon-Green, as designed by Krahn *et al*. [184] and used as described for cardiovascular constructs by Boerboom *et al*. [185]. The probe has been shown to bind to several types of collagen (types I-VI), but with the highest affinities for collagen types I and III.[184] It has previously been used in combination with other vital stains like cell tracker blue [185-188] and compared with other histological stainings like picrosirius red and masson trichrome.[189-191]

Statistics

All results were compared between PL and HS groups, using paired samples T-tests (two-tailed) and a p-value < 0.05 was considered significant. Results are expressed as means ± standard deviation (SD). SPSS 16 software (Microsoft corporation, Redmond, WA, USA) was used for all statistical analyses.

Results

Proliferation rates

Successful cell outgrowth and isolations were obtained from all 10 patients in both culture media, including 7 males and 3 females (71 ± 6 years old). Overall, cell viability was similar for both medium groups and was on average 94% ± 8 in PL-medium and 93% ± 7 in HS-medium. Analysis of proliferation rates showed an approximate two-fold increase when cells were cultured in PL-medium (Figure 1, slopes: 0.173 ± 0.054 and 0.092 ± 0.034 for PL- and HS-cells respectively, $p < 0.01$). Thus, when starting with $2.25.10^5$ cells, PL-cells reached 10^8 cells in 17 ± 5 days, while HS-cells on average required 32 ± 12 days. This number of cells is considered sufficient for seeding onto scaffolds for heart valve tissue engineering purposes.[192]

Immunohistochemistry

At passage 5, all cells in both media were positively stained for αSMA (Figure 2a-b) and vimentin (Figure 2c-d), while being negative for desmin (Figure 2e-f). This indicates that after repetitive divisions, cells in both media display phenotypes that resemble myofibroblasts.

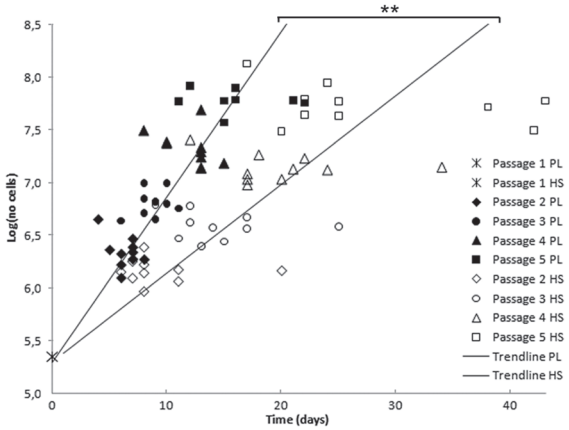


Figure 1. *Duplication rates, starting at 225,000 cells, expressed as log(number of cells) for cells cultured in PL and HS for 10 patients. The time in days and number of cells per cell isolation are indicated. The PL-cells are depicted by the filled symbols and the HS cells with the clear symbols, including a different symbol per passage. The regression lines with the averaged slope are also shown in the Figure. The averaged slope of PL-cells was significantly steeper than for the HS-cells ($p < 0.01$).*

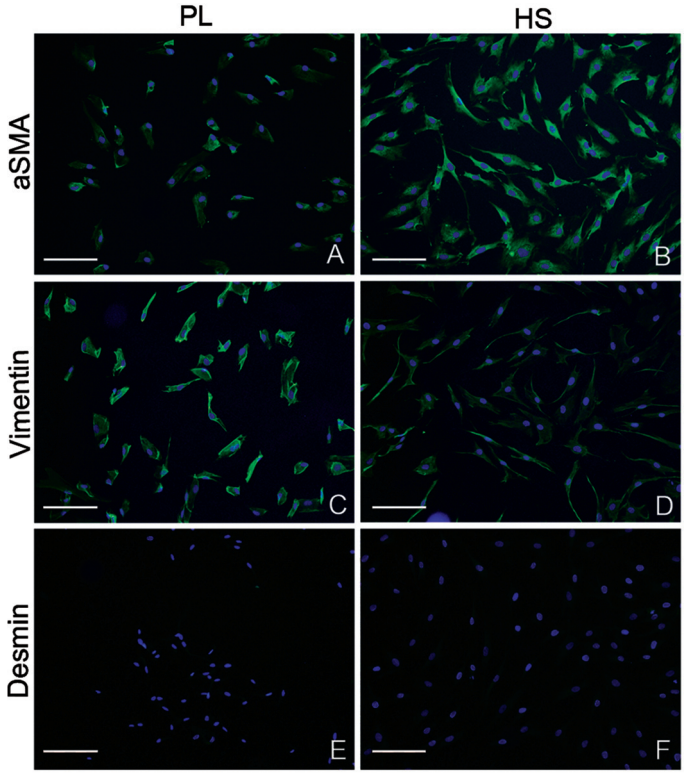


Figure 2. *Immunofluorescent staining of MF cultured in PL and HS. Results for phenotyping of one patient are displayed, but results were comparable for all patients.* ***2A-B.*** *αSMA.* ***2C-D.*** *Vimentin.* ***2E-F.*** *Desmin. Nuclei are stained with Hoechst. Original magnification was 10x. Bars represent 200 μm.*

mRNA quantification

We studied mRNA expression of collagen types I and III, which are considered most relevant for heart valve tissue engineering, and collagen cross-linking enzymes LOX and PLOD2. Significant differences in expression of these mRNAs could be an indication for a difference in the ability to form strong and mature collagen fibers. Although we did not observe a statistically significant difference between the two groups, we did see a two-fold increase in average production of collagens type I and III and PLOD2 by HS-cells as compared to PL (Figure 3: 2.30, 3.16 and 1.96-fold increase, respectively). LOX-expression was comparable in both media (1.10 ± 0.81 fold increase in HS relative to PL).

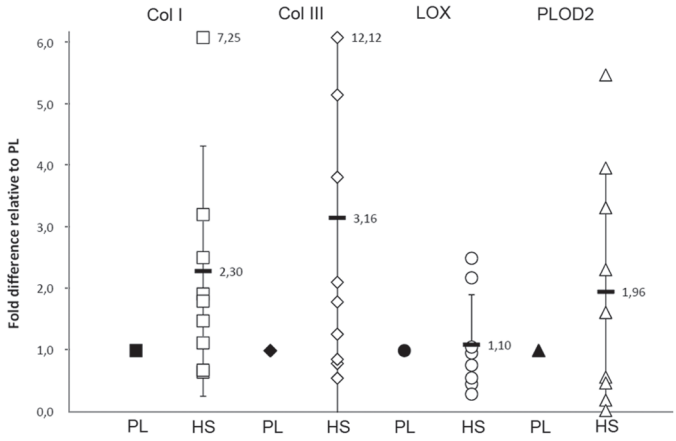


Figure 3. *Fold-change of target genes in cells cultured in presence of HS (mean ± SD), when normalized for cells cultured in presence of PL. LOX-expression is comparable between groups, but Collagens type 1 and 3 and PLOD2 expression tends to be higher in cells cultured in HS, though differences are not significant due to large interpatient variations.*

Zymography

Figure 4a shows zymographic analysis of gelatinolytic activity corrected for their presence in non-conditioned media, and displays release of active and inactive forms of MMP2 (72 and 64 kDa) and active MMP9 (82 kDa) by the cells in both PL- and HS-conditioned cells, but not of inactive MMP9 (92 kDa). When expression in PL was compared to HS, no significant differences were found for inactive forms of MMP2 and MMP9 and active MMP9 (Averages PL: 27,718 ± 6,803, 0 ± 4,479 and 4,306 ± 1,701 and HS: 23,299 ± 5,299, 91 ± 3,151 and 6,105 ± 1,640 arbitrary units (a.u.) respectively), but quantification of active MMP2 revealed a significant two-fold increase in PL-cultured cells (PL: 6,215 ± 1,278 vs HS 3,608 ± 1,375 a.u.). On the collagen-substituted gels (Figure 4b), we did not find significant differences in MMP-activity between cells cultured in PL or HS. Taken together, zymography analysis revealed a reduced matrix-degradation capacity through a significant decrease of active MMP2-levels when cells are cultured in HS-supplemented medium.

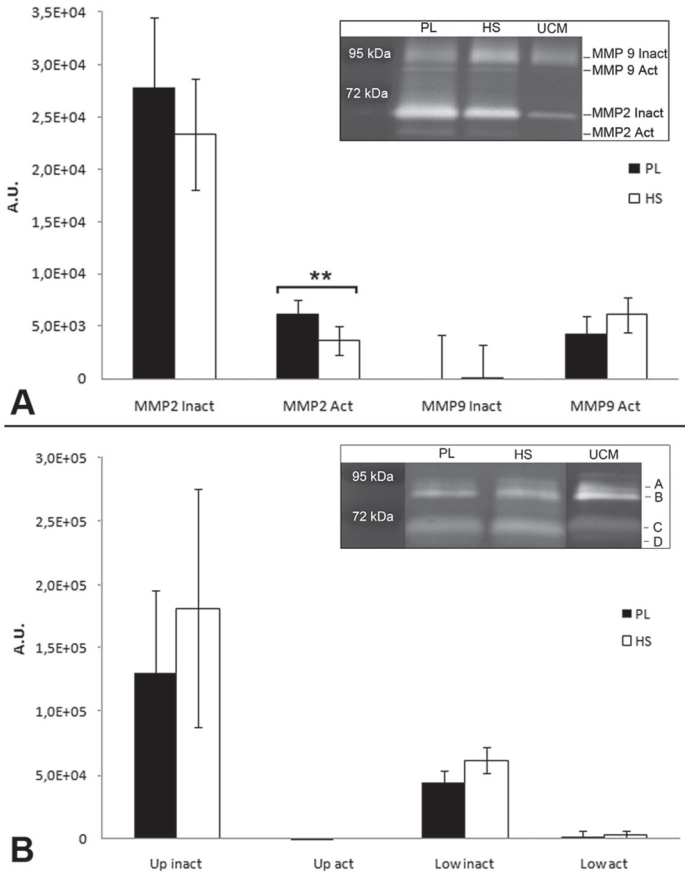


Figure 4A. *Quantification of MMP2 and 9 expression by cells in culture (mean ± SD), corrected for unconditioned medium (UCM). Cells cultured in both media produce and activate MMP2, while MMP9 did not show a difference compared to the unconditioned control. Activated MMP2 expression was significantly lower in the cells cultured with HS (**: p<0.01). The inlay shows an example of expression patterns by one patient and the size of the signals that were quantified. A.U. = Arbitrary units as a measure for number of counted pixels in a predetermined surface.*

Figure 4B. *Quantification of collagenases by cells in culture (mean ± SD). Expression patterns are presented in the inlay. We did not find significant differences in collagenase expression between cells cultured in PL or HS. Up = MMPs expressed at mark A and B, Low = MMPs expressed at mark C and D. Active forms (Act, B and D), are slightly smaller in size when compared to inactive forms (Inact, A and C) of these MMPs.*

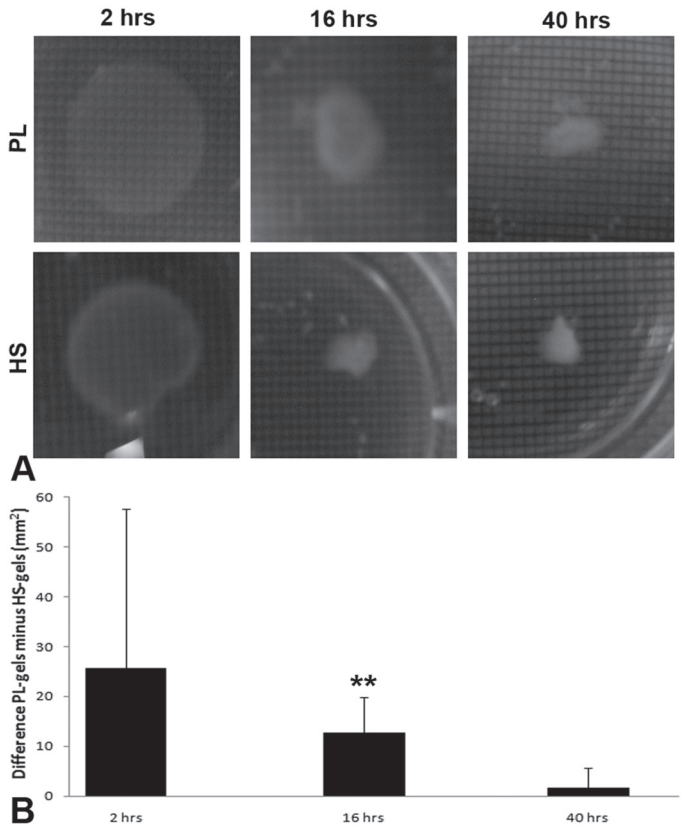


Figure 5. *Results of the collagen gel contraction assay (mean ± SD). The picture series (**5A.**) show shrinking of the gels over time and clearly show the difference after 16 hrs between PL and HS-conditions. The 1 mm² grids that were used to determine the actual size of the gels are also visible. After quantification the difference of the gel areas between PL and HS over time is depicted in the graph (**5B.**) and shows significant differences after 16 hrs (**: p<0.01).*

Collagen Gel Contraction Assay

Collagen gel contraction assays were performed to assess cell potential to contract matrix. Collagen gels seeded with cells cultured in PL and HS were shown to contract over time (Figure 5a). After 16 hours, HS gels were significantly smaller than PL gels (Figure 5b, $p<0.01$). After 40 hours, a maximal contraction was observed in all gels. This suggests that cells in HS-medium are more active in contracting collagen matrices.

Tissue studies: contraction, matrix and tensile testing

Similar to the collagen gels, the tissue constructs revealed increased contraction when cultured in HS as compared to PL (Figures 6a and 6c). This was accompanied by enhanced alignment of collagen structures in HS (Figures 6b and 6d). Lastly, tensile testing was performed to test the load-bearing capacities of the constructs cultured in either PL- or HS-supplemented medium. The first two columns in the bar plot in Figure 6 show a difference in load-bearing capacity between tissue cultured in PL (0.27 MPa ± 0.21) and HS (0.81 MPa ± 0.16), though obviously significance cannot be tested.

Discussion

In the present study, we aimed to test if HS is a better human replacement for FBS than PL in culture media for (cardiovascular) tissue engineering strategies and future clinical applications.[104, 156] We have shown here and in previous studies that the use of human PL resulted in an increased cell proliferation rate, but weaker tissue.[73, 152] The use of HS resulted in lower MMP2 activity levels, enhanced tissue remodeling capacity, and higher load-bearing properties.

We found that the proliferation rate of cells expanded in HS was lower than when PL was used, requiring 28 days to reach 100 million cells instead of 15 days. However, earlier studies showed that cells cultured in FBS required 60 days to obtain a similar number of cells, meaning that the use of HS would still reduce time by 50% as compared to the use of FBS.[73] These results with PL and HS were consistent with the results others obtained when used for expansion of other human cells.[193, 194] The duplication rate of cells is of considerable importance to obtain high a number of cells for clinical use of tissue engineering strategies, as it will reduce the time patients have to wait for autologous constructs to be generated. In this perspective, PL would be preferred as medium supplement for cell expansion over HS and FBS.

In contrast to other groups, who have primarily investigated the use of PL and HS in combination with mesenchymal stem cells and bone tissue engineering [122, 123, 125, 193-200], we focus on soft tissue engineering. One of the most important determinants for cardiovascular, and in particular heart valve, tissue engineering is the load-bearing capacity of the tissue,

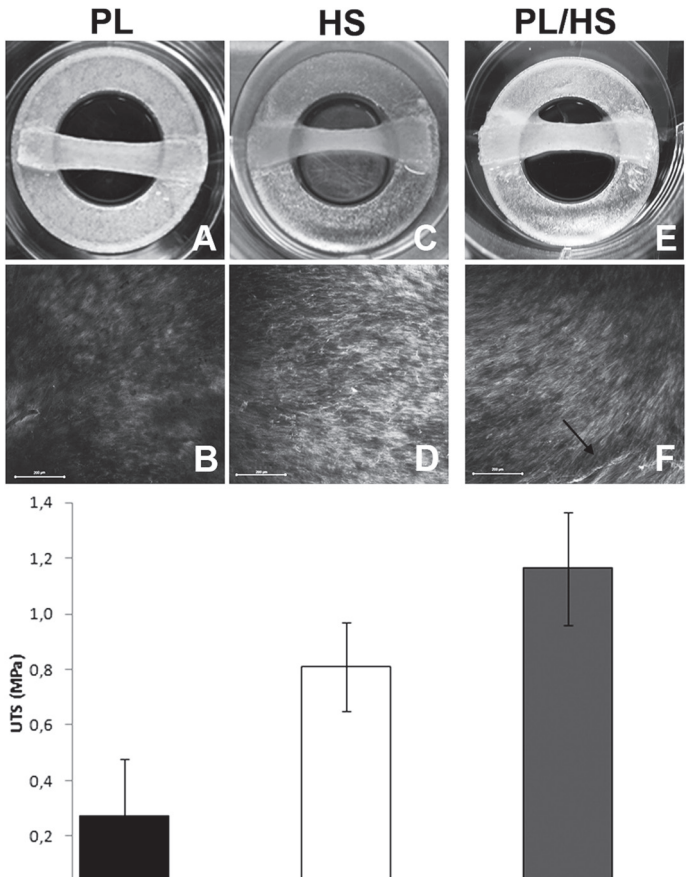


Figure 6. *Results of the tissue-formation experiments (mean ± SD). The pictures in* ***6A.*** *and* ***6C****. show tissue contraction 4 weeks of tissue culture in both PL-supplemented medium and HS-medium respectively. Figures* ***6B.*** *and* ***6D.*** *shows fluorescent images obtained with a collagen-staining probe showed differences in matrix structure in PL and HS. The pictures (10x magnification), taken in the middle of the constructs at an approximate depth of 7-11 µm, reveal differences in fiber density. Collagen fibers in HS-medium also seem thicker and more aligned. Scale bars represent 200 µm. Tensile testing (****Figure 6*** *first two bars of bar plot) suggests differences in Ultimate Tensile Strength (UTS) between constructs cultured in PL and HS medium. Figure* ***6E.*** *shows tissue contraction when cells expanded in PL-medium are transferred to HS-medium for tissue culture (PL/HS). The collagen-fiber staining reveals abundant aligned collagen bundles in this condition, emphasized with an arrow (Figure* ***6F.****). The third column of the bar plot shows an additional increase in UTS in PL/HS as compared to the conditions in which cells were solely cultured in PL or HS.*

which is highly related to the quality and architecture of the extracellular matrix, in which collagens type I and III predominate.[127] Indicative for the importance of matrix maturation and architecture are our previously reported results, in which a significantly reduced tissue strength was presented in tissue constructs prepared in PL as compared to FBS, although no differences in collagen content were found.[152] Similarly, our current study does not show significant differences in expression of collagen mRNA when cells are cultured in HS or PL, but indicates that tissue strength is higher when HS is used. We have previously reasoned that matrix remodeling and formation regulated by proteins present in PL can negatively influence the load-bearing capacity of tissue engineered constructs.[73, 152] In the present study, we did observe that the activation of MMP2 was reduced when cells are cultured in HS as compared to PL. Interestingly, the load-bearing capacities of tissue constructs cultured in HS-supplemented medium, was slightly higher than the ultimate tensile strength of tissue constructs previously produced in FBS-supplemented medium (0.81 and 0.73 MPa, respectively). Constructs cultured in PL did not reach this strength in both the present and previous study (0.27 and 0.25 MPa, respectively).[152] The strength of tissue constructs cultured in HS is also closer to the UTS of 2.0 MPa previously measured in circumferential direction of native adult aortic valve leaflets.[152] This emphasizes our earlier reasoning that tissue culture in serum is preferable over tissue culture in PL because it generates stronger tissue as a result of lower protease activity levels.

In order to combine the benefits of both PL and HS, we performed additional experiments to test if cell proliferation with PL-supplemented medium can be followed by tissue formation in HS-supplemented medium. For this purpose, the 2D collagen gel assays and culture of 3D tissue constructs were also performed in HS-containing medium after cell expansion in PL (a condition named: "PL/HS"). The collagen gel assays revealed that average gel areas became smaller than PL gels and larger than HS gels after 2 hours and slightly larger than PL gels after 16 and 40 hours (13, -1 and -1 mm2 respectively when PL/HS was subtracted from PL, similar to Figure 5b), but not significantly different when compared to either of the other groups. The 3D-tissue constructs also showed moderate contraction, an organized collagen architecture, and an increased ultimate tensile strength (1.16 MPa ± 0.20, Figure 6e, 6f and bar plot respectively) as compared to strips culture in only PL or HS. This UTS is higher than the values previously reported for constructs exclusively cultured in FBS, PL or HS (0.73, 0.27 and 0.81

MPa respectively).[152] Thus, these results indicate that high proliferation caused by PL-supplemented medium can be followed by sequential tissue formation in HS-supplemented medium, resulting in an improved protocol for tissue engineering with human serum substitutes.

It also underlines the relevance of designing specialized or 'chemically defined media', optimized for every step of tissue engineering strategies. [34, 201] Our studies with FBS, PL and HS show that growth factors and cytokines in the medium supplement have a distinct and large influence on cellular proliferation and formation and remodeling of a collagen network, which is difficult to predict from early expression markers, such as obtained from cell studies alone. Future studies should systematically investigate the effects of the different growth factors abundantly present, or absent, in HS and PL, to determine which growth factors could be specifically responsible for either proliferation, increased MMP levels or formation and maturation of collagen fibers.

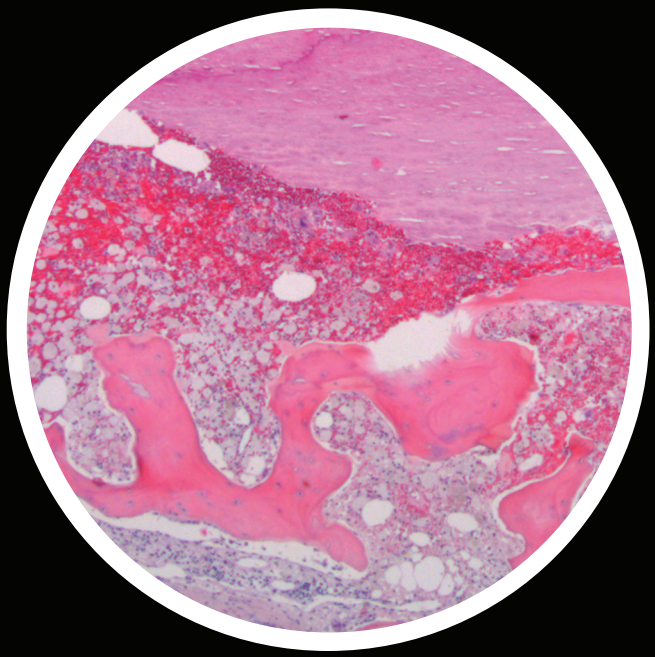
We included a 2D-collagen contraction assay to how the cells would contract the matrix under different culture conditions, to see if this assay has predictive value for tissue formation in 3D constructs. The assay predicted the ability of cells cultured in different media to cause tissue contraction in the 3D-tissue constructs. Recent reports pointed out there was ongoing compaction of heart valve constructs after implantation leading to failure of the valve constructs *in vivo*, making it more important to test contractive activity of cells *in vitro*.[91] Methods to stop excessive tissue compaction *in vivo* will be important in heart valve tissue engineering and the collagen gels can be a fast and simple *in vitro* model for future experiments.

In conclusion, we have shown that the use of HS during tissue culture results in stronger tissue, while PL is a more potent stimulator of cellular proliferation during expansion. More importantly, sequential use of human platelet-lysate and human serum can be used as a substitute protocol for the use of FBS in tissue engineering strategies. This means another step towards optimal autologous tissue engineering protocols and future clinical applications of cardiovascular tissue engineering.

Acknowledgements

The authors would like to thank P. K. Wagemakers, BSc for his technical contributions.

Chapter 6



Chapter 6

The Pathophysiological Basis For Pharmacological Interventions In CAVD

The content of this chapter has been accepted for publication in the Netherlands Heart Journal.

Riem Vis PW, van Rijswijk JW, Chamuleau SAJ, Vink A, van Herwerden LA, Kluin J.

Abstract

Calcific aortic valve disease (CAVD) results in aortic valve stenosis and is one of the most common cardiac diseases in both Western and developing countries. The burden of this disease is expected to increase rapidly in the future, but there are still no effective pharmacological therapies available and aortic valve replacement remains the sole definite therapy. This review presents an overview of the most common causes of CAVD, followed by current debates and trials related to the onset and progression of this disease. Several differences and similarities between the different causes of CAVD are presented. Additionally, stages of CAVD are compared with stages in atherosclerosis. At the end, future directions for research on CAVD will be discussed.

Introduction

Calcific Aortic Valve Disease (CAVD) is the most frequent native valve disease in Europe (43.1%) followed by mitral regurgitation (31.5%), aortic regurgitation (13.3%) and mitral stenosis (12.1%).[202] Aortic valve stenosis (AS) is thought to represent the late stage of the pathological process of CAVD, following aortic valve sclerosis, *i.e.* thickening of the aortic valve cusp without obstruction of the left ventricular outflow. (Figure 1) Sclerotic and stenotic aortic valves are characterized by a chronic inflammatory cell infiltrate, which consists mostly of macrophages and T-lymphocytes, accumulation of lipids, thickening and fibrosis and eventual mineralization.[203] The prevalence of aortic valve sclerosis is 29% in the overall population, and up to 37% in those older than 75 years [5]. Estimates of patients in which sclerosis develops into AS ranges from 15-30% within 6 to 8 years.[7] Approximately 2-3% of the population of 65 years and older have been estimated to have AS.[5] Life expectancy in patients with AS is severely reduced, as indicated by Otto *et al*., who found that the probability to be alive after two years for asymptomatic patients with a peak jet velocity of > 4m/s and without aortic valve replacement, was only 21±18%.[8]

Grossly, the most common etiologies for CAVD are degenerative, rheumatic and congenital (81.9%, 11.2% and 5.4% of the patients respectively).[202] Despite several prospective clinical trials, there are no effective pharmacological therapies available for CAVD and the only effective treatment is valve replacement. Several procedures are available for aortic valve replacement, which include conventional replacement surgery with biological or synthetic prostheses and less invasive trans-apical or trans-femoral therapies. Surgical treatment options for end-stage aortic stenosis will not be discussed any further in this review.

In this review, we will provide an overview of the three most common etiologies and pathogeneses of CAVD and present some of the latest concepts and results in clinical trials aiming to prevent CAVD.

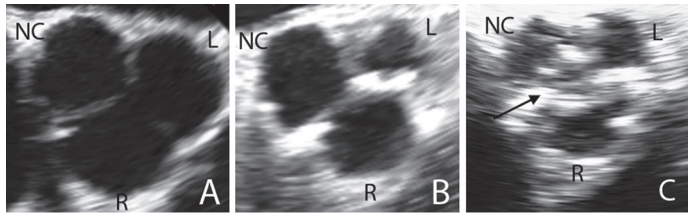


Figure 1. *Echographic images showing the aortic valve in different stages of the disease: normal (**1A.**), sclerotic (**1B.** early stage, low transvalvular gradient) and stenotic (**1C.** end stage). All images have been obtained using short axis TEE in the same angle. NC: Non-coronary cusp, L: Left coronary cusp, R: Right coronary cusp. Arrow marker improper closing of the severely stenotic valve causing a valvular leak.*

Degenerative Aortic Valve Disease

The most frequent cause for CAVD is degenerative valve disease and several risk factors have been correlated to the progression of this condition. The prospective Cardiovascular Health Study correlated age, male gender, hypertension, elevated levels of lipoprotein (a) and low-density lipoprotein cholesterol (LDL) and smoking with the presence of aortic valve sclerosis and stenosis.[5] Others also identified these risk factors, in addition to diabetes and elevated body mass index [204], the metabolic syndrome [205] and end-stage renal disease [206], amongst others [3]. Risk factors for degenerative CAVD are thus suggested to be similar to the traditional risk factors for atherosclerosis, which also include increasing age, male gender, hypertension, diabetes, triglycerides, and smoking [5, 204] and it has been hypothesized that acquired valve disease is a manifestation of atherosclerosis. [207] However, an inconsistency has been found in coexisting prevalence between CAVD and coronary artery disease (CAD) as only 50% of patients with severe CAVD have significant CAD, and the majority of patients with CAD do not have CAVD.[208] This shows that risk factors may be similar, but that there are also noteable differences between atherosclerosis and CAVD.

Pathogenesis of Degenerative CAVD

Injury and activation of the valve endothelium by mechanical forces, like shear stress and transvalvular pressure, is thought to be causative for the onset of CAVD.[209] Similar to atherosclerosis, endothelial damage might initiate a number of events like accumulation of lipoproteins and inflammation. [203, 210] Several adhesion molecules which are normally not expressed by the valvular endothelium, are found in non-rheumatic aortic valve disease. Monocytes can adhere to these adhesion molecules and migrate into the subendothelial space [210], where they release cytokines, chemokines, growth factors and proteolytic enzymes. In addition, Apo(a) and apoB have been found to accumulate in developing lesions of CAVD. Oxidative modifications make these lipoproteins highly cytotoxic for most cells and the products generated by lipid oxidation have shown pro-inflammatory properties. It is likely that inflammation and lipid oxidation cause activation and differentiation of the valve interstitial cells (VICs), which are responsible for the maintenance and repair of the valve matrix structure.

In pathological processes, inflammatory cells are the main source of matrix-metallo proteinases (MMPs).[211] Tenascin-C (TN-C) is an extra-cellular matrix glycoprotein, which is often co-expressed with MMPs, and is overexpressed by interstitial myofibroblasts in aortic valve calcification. TN-C actively participates in physiologic bone formation and stimulates osteoblastic differentiation [212]. During progression of aortic valve sclerosis to CAVD, positive feedback mechanisms between MMP-2 and TN-C lead to increased alkaline phosphatase expression, which is a crucial enzyme for bone formation and bone cell differentiation, and deposition of calcium phosphates.[213] MMP activity has also been identified as an activator of transforming growth factor-beta 1 (TGF-β1) [214], which is overexpressed in stenotic valves [215] and has been suggested to play an active role in progression of the disease.[216]

Macrophages have also been found to express angiotensin-converting enzyme (ACE) in atherosclerosis. In valvular tissue, local production of ACE has been demonstrated.[217] Enzymatically active ACE was also found to be present in aortic sclerosis and CAVD lesions, where it might participate in disease progress with local production of angiotensin II.[218] Angiotensin II is an important mediator of inflammation and fibrosis and therefore a potential initiator of processes involved in CAVD progression.[219]

Lastly, it has been suggested that VIC differentiation towards osteoblasts in valvular calcification is enhanced by the osteoprotegerin (OPG) / receptor activator of nuclear factor-κB (RANK)/RANK ligand (RANKL) system. RANKL expression was increased, while OPG was decreased in human calcified aortic valves.[220] In cultured VICs, RANKL stimulation leads to the induction of an osteogenic phenotype, a significant rise in matrix calcification, alkaline phosphatase activity, expression of osteocalcin, and increased functioning of the transcription factor Runx2, which is essential for onset of genes involved in osteogenic differentiation.

Together, these data provide a brief overview of possible processes involved in risk factors, onset, progression and end-stage differentiation in degenerative CAVD, while additional pathways have been reviewed by others [3]. Risk factors similar to atherosclerosis, combined with high continuous mechanical stress, are thus most likely important for onset of CAVD, followed by inflammatory processes and gradual osteogenic differentiation with AS as the end-stage (Figures 2a and b).

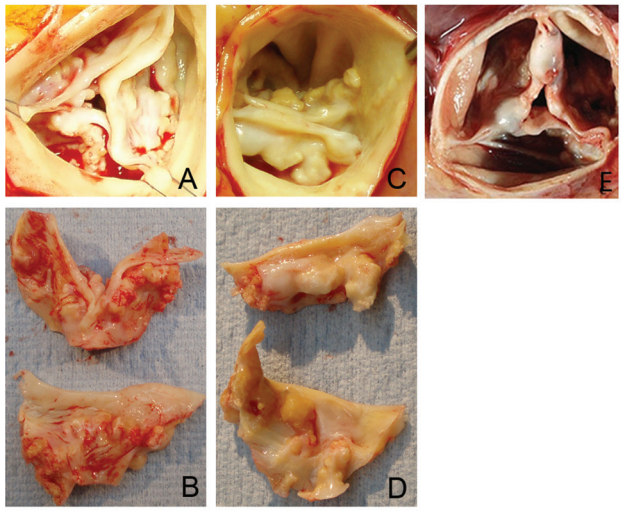


Figure 2. 2A. *Stenotic tricuspid valve, prior to replacement.* ***2B.*** *Excised cusps of stenotic tricuspid valve, showing large calcific nodules in the tissue, primarily located at the belly region of the valve.* ***2C.*** *Stenotic congenital bicuspid valve, prior to replacement.* ***2D.*** *Excised cusps of stenotic congenital bicsupid valve, also showing large calcified deposition in the belly region of the cusp.* ***2E.*** *Explanted aortic valve after rheumatic aortic valve disease, obtained from Robins and Cotran, Pathologic basis of Disease, 7th edition, Elsevier Health Sciences. This shows thickening of the cusps and fusion of the commissures following healing and scarring after the acute phase of the disease.*

Congenital Aortic Valve Disease

Congenital aortic valve disease include unicuspid, bicuspid, quadricuspid and even pentacuspid valves. Bicuspid aortic valve (BAV) is the most common congenital cardiac abnormality, occurring in about 1-2% of the population, with a higher prevalence in males.[221] Unicuspid aortic valves, which is the second most common congenital valve disease, occurs in 0.02% of the population.[222] In this review, the focus will be on BAVs.

BAV is the second most common cause of CAVD and is the result of abnormal aortic cusp formation during a complex developmental process.[223] From a recently suggested classification system, it can be extracted that there are several types of BAVs, with different incidences.[224] It showed that only few patients congenitally have two commissures and no raphe (type 0: 7%) and that the majority has conjoined or fused cusps resulting in one (type 1: 88%) or more (type 2: 5%) raphes. Moreover, there seems to be a genetic predisposition for the location of the raphe in the type 1 BAVs, as suggested by the high incidence of fusion of the left and right coronary cusp (71%) and the low incidence of fusion of the left and non-coronary cusp (3%).[224] Although the information on the effect of these different types of BAVs on calficication is not yet known, there are indications that CAVD tends to progress more rapidly if the cusps are asymmetrical and in antero-posterior position.[221] Patients with BAV are generally 10 years younger than those with tricuspid CAVD when requiring aortic valve replacement. It is likely that the progression of CAVD in these patients is similar as in patients with tricuspid valve degenerative calcification [225], but that the process starts earlier in life due to higher mechanical stresses on the cusps and more turbulent blood flow due to abnormal configuration of the cusps.[226]

Pathogenesis of BAV

It has been suggested that onset of CAVD in BAVs is the result from less natural folding, incomplete opening and wrinkling of the biscuspid valve cusps during the cardiac cycle, making it more prone to tissue damage as compared to tricuspid valves.[227] The increased turbulence in the blood flow, causes trembling and whipping of the cusps, predisposing them to fibrosis and calcification.[227] In addition, recent modeling-studies noted that the orifice area of BAVs, functioning echocardiographically normal, may

already resemble valves with some degree of stenosis.[228] The overloaded stresses revealed by the model may accelerate degeneration as described for degenerative valve disease.[228] The models do not explain why aortic valves become bicuspid in the first place, but this has been correlated with mutations in Notch-signaling. Notch is a receptor which has been attributed several important roles in embryonic development, but that is also linked to endothelial functioning and suppression of osteogenic transcription factor Runx2. Hence, mutations in Notch could be causative for accelerated progression of valve calcification as well.[3] Lastly, recent discoveries have identified altered expression of microRNAs (miRs) 26a, 30b and 195 in calcific and non-calcific regurgitant BAVS.[229] MiRs are small sequences of nucleotides that can influence protein expression and they may provide targets for future therapies as has been shown in several *in vivo* studies.[230]

In summary, progression of calcification in BAV appears to have many similarities with degenerative CAVD in tricuspid valves, although it most likely progresses faster due to higher mechanical stresses on the cusp. A severely stenotic congenital bicuspid valve is presented in Figures 2c and d.

Rheumatic Aortic Valve Disease

Although the incidence of rheumatic aortic valve disease has declined in industrialized countries, it is estimated that worldwide nearly 20 million people are affected by acute rheumatic fever and rheumatic heart disease, of which 95% in developing countries.[231] The first step in the pathogenesis of rheumatic valve calcification is Pharyngitis due to Streptococcus Pyogenes, or group A streptococci (GAS), but only 0.3-3% of individuals with acute GAS pharyngitis go on to develop rheumatic fever.[231] Despite a significant decline in the incidence of acute rheumatic fever in developed countries there has been no apparent decline in GAS pharyngitis.[231] This may be explained by the rapid and appropriate diagnosis, and antibiotic management of even uncomplicated GAS infections, or the occurrence of less virulent serotypes becoming more dominant with time.[232]

Each year there are 470,000 incident cases of rheumatic fever worldwide and 233,000 deaths due to acute rheumatic fever and rheumatic heart disease.[233] The mean annual incidence rates are lowest in American and Western European countries and relatively a bit higher in Eastern Europe, Asia, Australasia, and the Middle East. The highest reported mean annular incidences were found in India and in a Maori community in New Zealand.[234] However, the prevalence in African countries like Kenya and Congo has been estimated to be a lot higher.[231] Even these estimates have been suggested to be underestimations, since more systematic screening with echocardiography resulted in even higher numbers in other developing countries, like Cambodia and Mozambique.[235] (Figure 3)

Pathogenesis of Rheumatic valve disease

The cytoplasmic membrane of GAS contains several proteins and groups that are highly similar to proteins found in the human host. This results in molecular mimicry, or the sharing of epitopes between antigens of the host and the bacteria, and may stimulate existing B and T cells to respond to self proteins.[236] Rheumatic heart disease can be a serious autoimmune consequence of acute rheumatic fever. For example, the sites where anti-streptococcal/anti-myosin antibodies bind to valvular endothelium may serve as infiltration sites for activated T cells and macrophages.[236, 237] Inside the inflammatory infiltrate, cells produce valve inflammation, scarring and destruction of valvular tissue. The valve turns into a local microenvironment for continuous cytokine production, lymphocyte infiltration, inflammation, and scarring.[237] Infiltrated lymphocytes and macrophages allow for the formation of so called Aschoff bodies in the valve. Aschoff bodies are characteristic lesions of rheumatic heart disease, consisting of granulomatous structures with fibrinoid change and chronic inflammation of the valve interstitium. Scarring and eventual neovascularization of the previously avascular valve tissue will lead to irreversible deformation of the valve (Figure 2e).[236, 237] Concomitant identification of osteopontin, osteocalcin, periostin and increased bone matrix protein synthesis, suggests that a similar osteoblast-like phenotype is involved in rheumatic valves as well as in degenerative CAVD.[238]

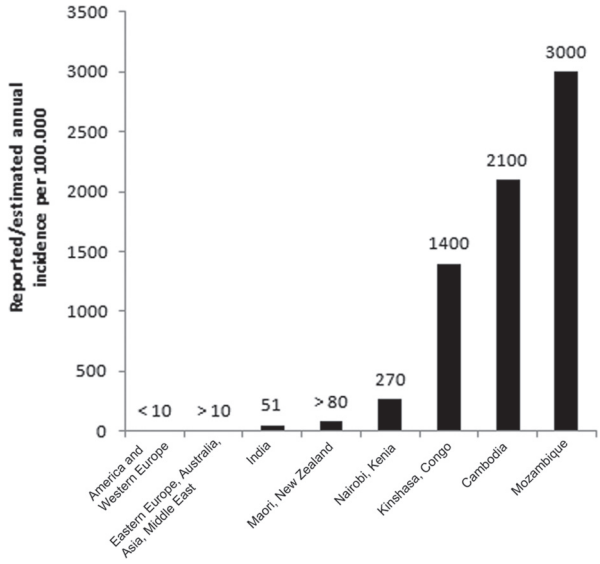


Figure 3. *Graphic presentation of measured or estimated incidence of rheumatic aortic valve disease in western and developing countries.*

Pharmacological Treatment

In search for effective pharmacological treatment of CAVD, the discussion has often focused at similarities between CAVD and atherosclerosis [239], although contradictory studies have also been published.[3, 240] Currently, the debate seems to shift back to focusing on the differences between the two conditions. Atherosclerotic lesions generally consist of foam cells that fuse to form lipid accumulations surrounded by fibrous caps, while early valvular lesions have been suggested to lack foam cells [241] and progress to mineralized matrix and in some cases bone structures.[3] However, lipid accumulations are also observed in minimally sclerotic valves derived from explanted hearts for transplantation [203] and mature lamellar bone formation has also occasionally been described in atherosclerotic lesions [242]. (Figure 4) These data show that both atherosclerosis and CAVD may occur in several manifestations, but that most manifestations of atherosclerotic lesions also occur in valves and vice versa. Thus, in many cases, risk factors and causes for atherosclerotic and valvular lesions may

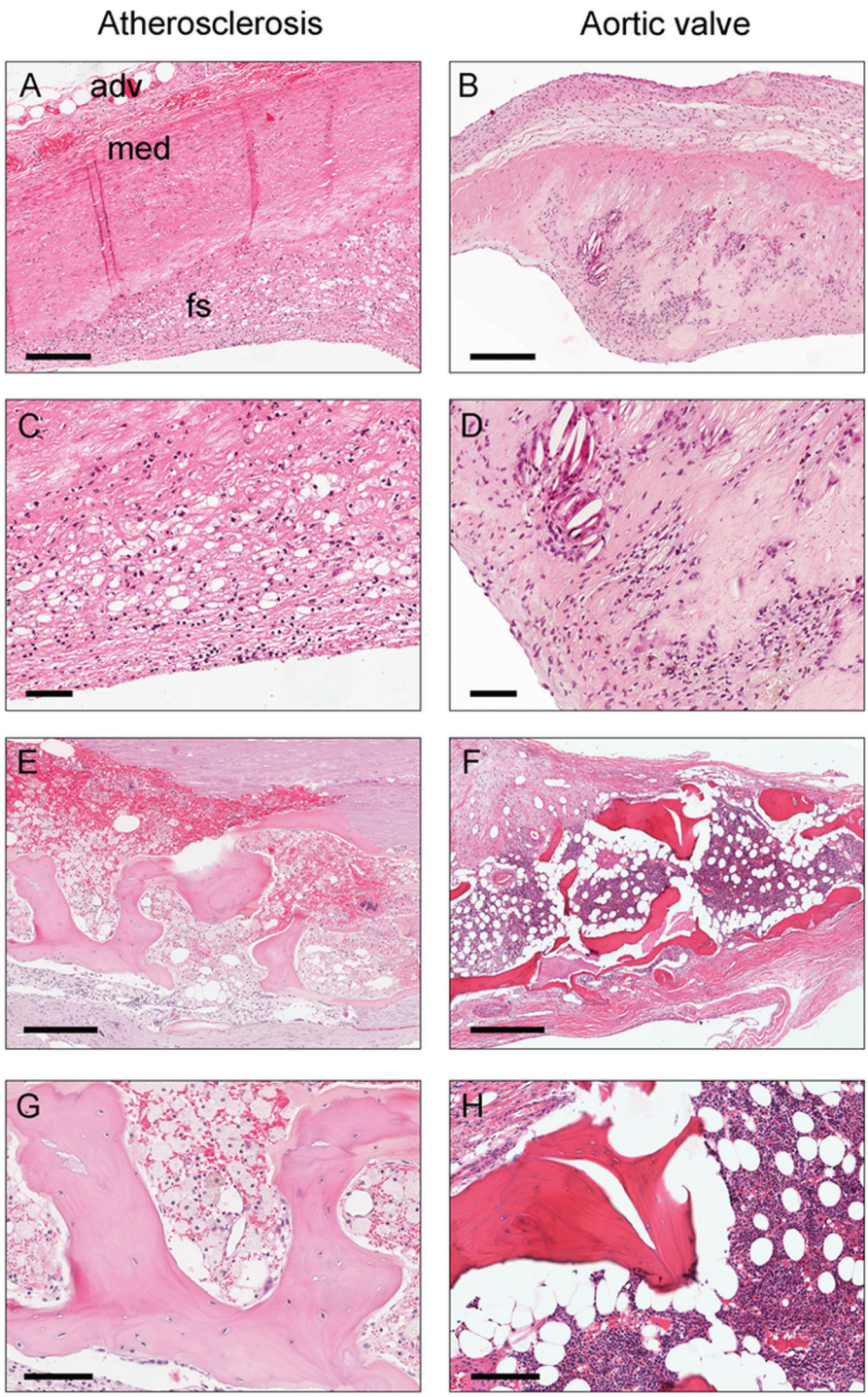

Atherosclerosis
Aortic valve
A
adv
med
fs
B
C
D
E
F
G
H

Figure 4a. *Atherosclerosis versus degenerative aortic valve disease.* ***4A****,* ***C****,* ***E*** *and* ***G.*** *Atherosclerosis.* ***4A.*** *Coronary artery vessel wall with an early atherosclerotic lesion (fatty streak). Adv = adventitia; Med = media and Fs = fatty streak. Bar = 150µm.* ***4C.*** *Higher magnification of A showing foamy macrophages in the intima. Bar = 50µm.* ***4E.*** *Advanced atherosclerotic lesion in the aorta. Bar = 300µm.* ***4G.*** *higher magnification of E showing bone formation in the atherosclerotic lesion. Foamy macrophages are present in between the bone fragments. Bar = 100µm.*
4B*,* ***D****,* ***F****,* ***H****. Degenerative aortic valve disease.* ***4B.*** *Cross section through aortic valve leaflet showing the three layer structure with early lesions. Bar = 250µm.* ***4D.*** *higher magnification of B showing foamy macrophages and cholesterol clefts in the lamina fibrosa of the valve leaflet. Bar = 100µm.* ***4F.*** *Advanced degenerative lesion with bone formation in aortic valve. Bar = 400µm.* ***4H.*** *Higher magnification of F showing blood-forming bone marrow next to the bone fragments in the aortic valve leaflet. Bar = 125µm.*

be similar, but different mechanic environments and cells involved may cause slightly different progressions and end-stages of the diseases. These small differences might explain why therapies used to slow progression of atherosclerosis are not always successful for inhibition of CAVD progression.

One of the key factors in the pathogenesis of CAVD appears to be lipoproteins. The lipid lowering agents HMG-CoA reductase inhibitors (statins) are used to lower cholesterol levels, exert significant anti-inflammatory effects and also preserve endothelial function in atherosclerotic disease. [243] Several studies have been published in which serum cholesterol levels were lowered and calcium accumulation or inflammation in the valve were reduced, resulting in inhibited progression of CAVD.[244] However, these studies were predominantly retrospective or had small sample sizes. Three recent prospective randomized double blind studies revealed that statin treatment did not have any effect on the progression of CAVD. According to the Scottish Aortic Stenosis and Lipid Lowering Trial, Impact on Regression (SALTIRE) study [245] high-dose statin therapy does not halt the progression of CAVD over a 2 year follow-up period, despite reducing the LDL cholesterol by more than 50%. Similar results were found in the Simvastatin and Ezetimibe in Aortic Stenosis (SEAS) trial [246], during a median follow up of at least 4 year. An average reduction in LDL cholesterol of at least 50% was found in addition to reduced incidence of ischemic cardiovascular events, but no changes in CAVD progression. The third prospective trial, Aortic Stenosis Progression Observation: Measuring Effect of Rosuvastatin (ASTRONOMER) [247], included patients that were nearly 10 years younger than in the SALTIRE and SEAS trials, to better represent population suffering from CAVD (58 years on average). There was a 54,5% reduction in LDL concentration,

but no reduction of progression of CAVD. The result of studies are consistent in demonstrating that lipid lowering does not affect CAVD progression in patients with no clinical indications for lipid lowering.

Preliminary observational studies with ACE-inhibitors (ACEi) have produced conflicting results. Rosenhek *et al*. [248] found that progression of CAVD was not significantly delayed in patients using ACEi. In contrast, a small retreospective study found an association between ACEi use and lower rate of aortic valve calcium accumulation.[249] More recent investigations showed that angiontensin receptor 1-blockers inhibited atherosclerotic changes and endothelial disruption in aortic valves.[250] More research may reveal new targets for therapies in this pathway.

Recent observational studies described that patients receiving treatment for osteoporosis, especially bisphosphonates, had significantly less calcium deposition in the aortic valve and slowed the progression of degenerative CAVD.[247, 251] In addition, members from our group have shown that valvular calcification correlates strongly to osteoporotic remodeling. [252] Lastly, a recent trial has shown that bisphosphonates can reduce cardiovascular calcification in patients over 65 years old, but that it had no effect in younger patients.[253] This may indicate that there are different stages in the progression of CAVD, in which recruitment of calcium is the last stage, and that bisphosphonates may be beneficial in inhibiting the progression of this last stage only.

For all therapies described, both successful and unsuccessful results on progression of CAVD have been reported, but better matching of different stages of CAVD with specific applied therapies may shift the balance towards more effective treatment.

Future Directions

The previous sections show that there are no therapies available directed specifically against pathological processes in aortic valve disease. Instead, several experiments have been conducted with pharmacological agents that were successful against other cardiovascular disease that resemble aspects of CAVD and were therefore thought to be promising for inhibition of CAVD as well. Limitations that prevent successful development of pharmacological

therapies for CAVD, are the lack of suitable animal models, insufficient understanding of processes behind progression of the disease and most important: lack of knowledge on normal valve regeneration. VICs are able to actively maintain and repair the matrix structure of the valve, but it remains unknown which triggers are responsible for the activation of these regenerative capacities and more importantly, what causes it to get out of control and thereby initiates fibrosis and sclerosis in the cusps. For this purpose, it is important that *in vitro* and *in vivo* models are developed in which the processes that are specific for CAVD can be identified and targeted with newly developed therapeutic agents.

An example of these therapeutic agents could be modified small nucleotide chains called antagomirs, which can target specific miRs *in vivo*.[117] MiRs are known to be involved in processes like angiogenesis and fibrosis and the first pre-clinical trials indicate that specific miRs can be successfully targeted to prevent or stimulate these events.[119, 121] The first investigations in BAVs reveal that there is also altered miR expression in calcified and non-calcified valves [224] and further investigations may reveal additional miRs, proteins and pathways involved in CAVD.

However, it should be noticed that successful development of pharmacological therapies that slow down progression of CAVD will not suffice to prevent surgery in most patients. Currently, most patients are diagnosed with CAVD when they have become symptomatic and at this stage, it may be too late for pharmacological intervention. Therefore, it is important that the search for medication is combined with additional genetic research in humans in order to identify patients at risk before CAVD is initiated. As described above, researchers have been able to identify several potentially interesting genes that may cause BAVs in mice, but these models cannot be translated to humans yet [3] and there is not much known about genetic predisposition for CAVD.

Summary

The pathogenesis of non-rheumatic aortic valve calcification is probably initiated by damage to the endothelial layer overlying the valve, followed by accumulation of low density lipoprotein and infiltration of inflammatory cells. Oxidative stress, release of inflammatory cytokines and growth factors, and extracellular matrix remodeling create an environment that facilitate the proliferation and osteoblastic differentiation of VICs, which ultimately leads to calcification and occasionally ossification of the aortic valve. In BAV, calcification is likely to occur earlier than in patients with a tricuspid aortic valve, as altered mechanical stress accelerates valvular trauma. The processes following valvular trauma are probably similar in tricuspid and bicuspid valves. Rheumatic valve disease is initiated by inflammation, caused by pharyngitis due to GAS followed by release of cytokines, activation of VICs, matrix remodeling and eventually osteogenic differentiation of VICs. These processes and discovery of osteopontin, osteocalcin, and increased bone matrix protein synthesis in rheumatic aortic valves suggest that a similar osteoblast-like phenotype is involved in rheumatic valve disease as is in non-rheumatic CAVD. The three forms of CAVD, including currently debated pharmacological treatments, are depicted in Figure 5. In conclusion, current evidence suggests that there are differences in the onset of several types of CAVD, but that there are similarities in the progression of the disease, which is interesting for development of pharmacological treatments in the future.

Acknowledgements

The authors gratefully acknowledge L.M. de Heer for providing the pictures a,b,c and d used in Figure 2.

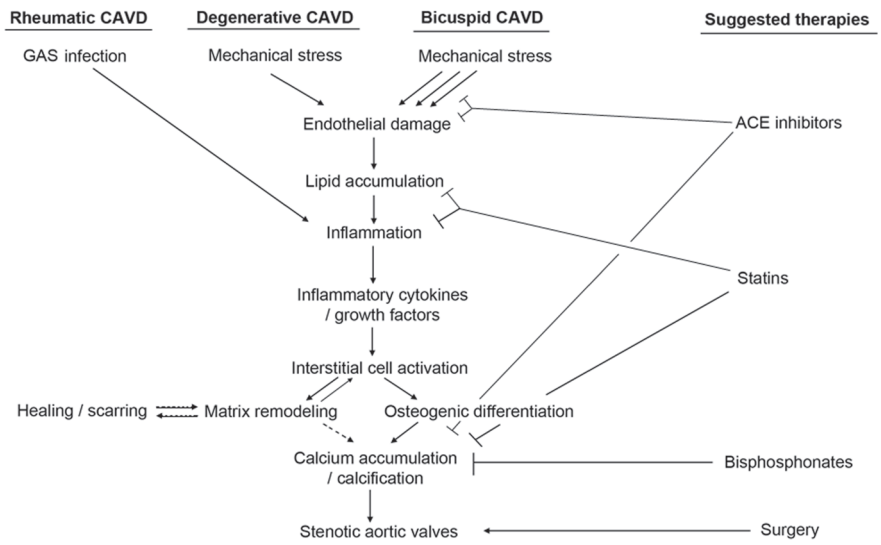
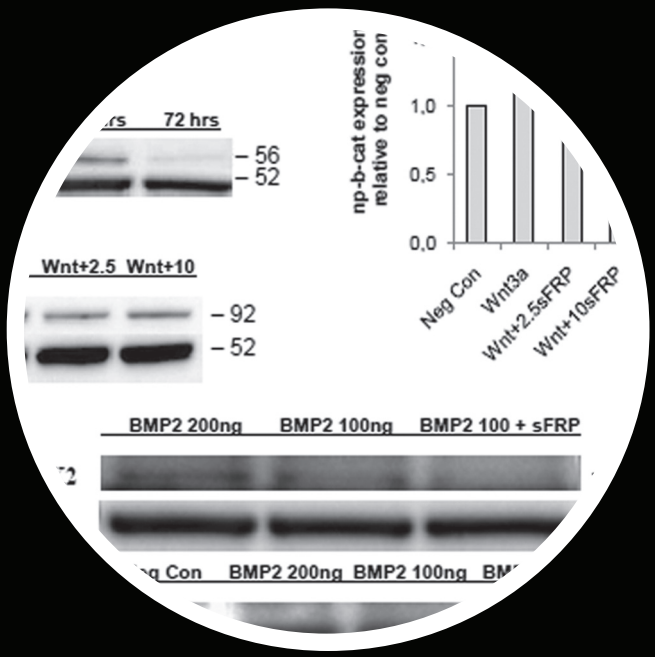


Figure 5. *Overview of possible disease pathways as discussed and presented in this review. Degenerative CAVD and Bicuspid CAVD are likely to follow a similar pathway, but the higher mechanical stress may induce the process early on. Genetical causes for Bicuspid CAVD have not yet been identified, but cannot be ruled out yet. Rheumatic CAVD is caused by specific bacterial infections, but following healing and scarring (dashed/solid arrows), these valves also calcify and interstitial cells have been found to undergo osteogenic differentiation. The dashed arrow between matrix remodeling and calcification shows that this process may occur, but for severely stenotic valves, the path through osteogenic differentiation is probably more likely. The suggested therapies so far have not been successful in stopping progression of CAVD. Statins and inhibitors of ACE-related processes have been suggested to be primarily involved in prevention of onset of CAVD, while bisphosphonates were reported to be beneficial at later stages of the disease. For all therapies, both successful and unsuccessful results on progression of CAVD have been reported. Better matching of different stages of CAVD with specific applied therapies may shift the balance towards more effective treatment.*

Chapter 7


72 hrs
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Wnt+2.5
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BMP2 200ng
BMP2 100ng
BMP2 100 + sFRP
Neg Con
BMP2 200ng
BMP2 100ng

Chapter 7

The Role Of BMP/Wnt Signaling In Human Heart Valve Calcification

The content of this chapter has been submitted.

*Riem Vis PW *, Chester AH *, Osman L, Latif N, Sarathchandra P, Kluin J, Taylor PM, Yacoub MH*

Abstract

Aortic valve calcification is a complex process that is characterised by the expression of bone markers in the valve leaflet. The Wnt pathway and bone morphogenetic proteins (BMPs) have been implicated to play a role in vascular and heart valve calcification. This study examines the regulation of Wnt3a by BMPs and the functional effects mediated by Wnt3a in human valve interstitial cells (VICs). **Methods and results:** Analysis of human pathological specimens by immunohistochemistry showed an increased level of expression of Wnt3a, Msx2 and β-catenin was localised to peri-calcific regions of the tissue. Treatment of VICs by BMPs up-regulated the expression of Wnt3a, ALP, Runx2, and β-catenin. The concentration-dependent proliferation or differentiation of VICs in response to Wnt3a was measured by the incorporation of [3H]-thymidine and activity of alkaline phosphatase (ALP) respectively. Wnt3a treated VICs proliferated in a concentration-dependent manner. At higher concentrations, Wnt3a caused a significant increase in ALP activity and expression of Runx2. Lastly, Western blot experiments showed that BMP-2 induced Runx2 upregulation was mediated through β-catenin/Wnt-signaling and not Smad-signaling. **Conclusions:** This study demonstrates the important role of Wnt-signaling in valve calcification in response to BMPs. Wnt3a may induce valve calcification by regulating the proliferation and osteogenic differentiation of cells within the valve. These findings suggest a key role of Wnt3a, in the calcification process and possibly identify new therapeutic targets.

Introduction

Wnts are a family of secreted glycoproteins that play an essential role in valve development and in skeletal bone formation through paracrine and autocrine mechanisms.[254] They bind to the frizzled seven-transmembrane receptors (FZDs) and the low density lipoprotein-receptor related proteins (LRP) 5 or 6, which function as co-receptors. This leads to activation of β-catenin and subsequently its translocation into the nucleus, where it binds to Tcf/Lef transcription factors and regulates the expression of a number of target genes to induce cell differentiation, proliferation, maturation and activity.[255]

Recent findings show that in adult heart valves, Wnts have been correlated with degenerative calcific aortic valve disease (CAVD). We have previously shown that human valve cusp tissue and VICs express FZD2, to which the LRP5/6 binds, and have a basal expression of Wnts, FZDs and several Wnt inhibitors such as secreted Frizzled Related Protein (sFRP)-1, 3, 4 and Dickkopf (DKK)-1, -2, -3 and -4.[256, 257] Rajamannan *et al*. reported that inhibition of hypercholesterolemia-induced calcification with atorvastatin is mediated by LRP5 [258], and that LRP5 upregulation occurs in human calcified aortic valves.[259] These effects may be caused by Bone Morphogenetic Proteins (BMPs), which have been shown to be expressed in calcified valve tissue [30]. BMPs have also been shown to induce expression of the homeobox gene Msx2, which in turn induces paracrine Wnt signals. [260, 261] Other studies proved that Wnts are able to stimulate osteogenic differentiation by directly stimulating the essential transcription factor for osteogenic differentiation, Runx2.[262-264] Calcified aortic valves were found to have bone-like lesions expressing markers normally found in osteoblasts.[31] However, despite these studies, the functional response of VICs to Wnts has not been determined.

In the present study, expression of Msx2 and Wnt-related proteins are localized in human valve tissue, and the functional response of human adult aortic VICs to BMPs and Wnt is investigated. We hypothesize that a network exists in valve cusp tissue whereby BMPs stimulate expression of Wnts and that Wnt3a plays an important role in regulating the calcification process by promoting the fate of the adult valve cells to proliferate and/or differentiate into an osteoblast-like cell phenotype. The action of Wnt3a in human valves may represent a key mechanism for the development of heart valve calcification.

Methods

Tissue Collection and Processing

The study was approved by the Royal Brompton and Harefield NHS Trust Ethics Committee and patient consent was obtained for the use of heart valves for research that would otherwise be disposed of. Human aortic valve leaflets were collected from explanted valves either at the time of cardiac transplantation or during aortic valve replacement surgery. Eight aortic valves were used, 3 from children (non-calcified) (mean age 13.7 +/- 4.2 years) and 5 from adults (2 non-calcified and 3 calcified) (mean age 62.7 +/- 7.5 years). The valve tissue was put into a freshly prepared Krebs solution and taken immediately to the laboratory. The average time between removal of the tissue and processing for staining or cell culture was 3-6 hours. Microscopic examination of calcified aortic valves showed clear regions of calcification. Sections of these valves stained positive for von Kossa (data not shown). VICs were isolated from non-calcified valve leaflets by enzymatic digestion and were phenotypically characterised as previously described.[265] Cultured VICs were used between passages 4-6. Other calcified and non-calcified leaflets were either snap frozen in liquid nitrogen or fixed in 10% formal saline. The diseased valves were decalcified by neutral EDTA (12.5%) before processing.

Protein Expression of β-catenin, Runx2, ALP and Wnt3a Following BMP stimulation

Cultured human VICs were treated with either 100 ng/mL of BMP-2, BMP-4 or BMP-7, 40 ng/mL of Wnt3a or osteogenic media (which contained ascorbate-2-phosphate (50 μg/mL); dexamethasone (10 nmol/L) and β-glycerol phosphate (10 mmol/L) (all purchased from Sigma, UK)) for 21 days. After treatment, cells were solubilised and homogenised in 1% SDS containing protease inhibitors (Roche, UK). Protein content was determined using the BCA protein assay kit (Sigma, UK) using bovine albumin as a standard. Total protein homogenates (15 μg) were denatured, separated on 10% Bis-Tris gels (Invitrogen, UK) and transferred to nitrocellulose Hybond C (Amersham, UK). Nitrocellulose membranes were blocked (3% w/v non-fat dried milk in PBS containing 0.05% Tween-20) and then probed using primary antibodies against bone ALP (Abcam, UK), Runx2 (Abcam, UK), and

β-catenin (R&D Systems, UK). Membranes were washed and then incubated with horseradish-peroxidase-conjugated secondary antibody (Dako, UK) for 1 hour. Visualization of the protein bands was accomplished using enhanced chemiluminescence (Amersham, UK) and captured on Hyperfilm (Amersham, UK).

Localisation of Signaling Proteins and Bone Markers in Human Aortic Valve Leaflets

Prior to immunoperoxidase staining, 5 μm thick paraffin sections of decalcified aortic valves were dewaxed and rehydrated, washed in phosphate buffered saline (PBS) for 5 minutes before blocking for endogenous peroxidases using 0.3% hydrogen peroxide in PBS for 15 minutes. Sections were washed twice in PBS and blocked with 3% bovine serum albumin (BSA) (w/v) in PBS for 30 minutes. Sections were incubated separately for 1 hour with antibodies against Wnt3a, Msx2, β-catenin, osteocalcin, osteopontin (Abcam, UK), smooth muscle α-actin (SMA) (Sigma, UK), calponin, and CD45 (Dako, UK). Negative control consisted of 3% BSA in PBS and an irrelevant isotype antibody. Primary antibodies were then removed by washing the sections 3 times in PBS followed by a second layer of biotinylated immunoglobulins for 1 hour. Sections were then washed 3 times in PBS before 1 hour incubation with Avidin-Biotin Complex ABC (Vector laboratories, UK). Reactivity was detected using diaminobenzidine tetrahydrochloride (DAB tablets) (Sigma, UK) (25 mg/mL) and hydrogen peroxide (0.01%) (w/v). Sections were then counter stained with haematoxylin and viewed on Zeiss Axioskop microscope. Photomicrographs were taken using Nikon DMX1200 camera.

Growth Stimulation of Human Aortic VICs by Wnt3a

Human VICs were seeded onto a 96-well plate and treated with Wnt3a (2, 4, 10, 20, 40 ng/mL) for 24 hrs at 37°C. [3H]-Thymidine was then added for another 18 hours, cells were then lysed by freezing at -80°C, after which the cell fragments and DNA were passed through a filter membrane, that retained the intact DNA. The filter membrane was then dried and amount of radioactivity was counted in a scintillation counter. Number of cells was expressed as cpm (counts per minute).

Osteogenic Differentiation of Human Aortic VICs by Wnt 3a

Primary cultures of human aortic VICs were seeded onto coverslips at 5 x 10^3 cells (for immunocytochemistry) or into a 24-well plate (for alkaline phosphatase activity assay) and were grown in normal growth medium containing DMEM supplemented with 4 mM L-glutamine, 100 U/mL penicillin, 100 µmol/L streptomycin and 10% FCS (acting as the control). Cells were also treated with osteogenic medium for 21 days containing ascorbate-2-phosphate (50 g/mL); dexamethasone (10 nmol/L) and glycerol phosphate (10 mmol/L) (all purchased from Sigma), in addition to the growth medium. The effect of osteogenic medium was compared to that of Wnt3a.

Immunocyotchemistry

Primary cultures of human VICs were maintained in DMEM, osteogenic medium or Wnt3a (10, 20 and 40 ng/mL) for 21 days. Prior to immunostaining, cells were washed with PBS, then fixed and permeabilised with ice-cold acetone for 5 minutes. Cells were blocked in 2% BSA and incubated with anti-human osteocalcin antibody (Abcam, UK) for 1 hour at room temperature. Cells were then washed with PBS and incubated for 1 hour with the secondary goat anti-mouse antibody FITC-conjugate (Sigma) and DAPI (nuclear stain, Sigma, UK). Cells were washed in PBS and mounted on glass slides in Permafluor aqueous mounting fluid (Coulter, UK). Stained cells were visualised using a Zeiss Axioskop microscope.

Alkaline Phosphatase Enzyme Assay

Cells that had been maintained in DMEM, osteogenic medium or Wnt3a (40 ng/mL) for 21 days were lysed and centrifuged at 10,000 rpm for 5 mins, and enzyme activity was assayed in the supernatant by adding 10 mmol/L of ρ-nitrophenyl phosphate as a substrate in 0.1 mol/L glycine buffer, pH 10.4, containing 1 mmol/L $ZnCl_2$ and 1 mmol/L $MgCl_2$. The quantity of ρ-nitrophenol formed was read immediately using a spectrophotometer at 405 nm and then monitored every 30 min. The ALP activity was calculated from a standard curve. In the supernatant, protein content was also determined using the BCA protein assay kit (Sigma, UK), using bovine albumin as a standard. The specific activity of ALP was calculated as nmol/min/mg protein.

BMP-2/Wnt/Runx2 signaling

Cultured VICs obtained from healthy adult valves were cultured in normal medium and switched to DMEM containing only 0.5% horse serum, penicillin/streptomycin and glutamine 24 hrs prior to the experiment. As a control for normal expression of Runx2, cells were treated for 2, 24 or 72 hours with 100 ng/mL BMP-2. In the experimental conditions, cells were treated with 200 ng/mL BMP-2 or 100 ng/mL BMP-2 albeit after 1 hr pre-treatment with 8 μg/mL rh-sFRP1 (R&D systems). After stimulation, cells were scraped, collected and resuspended in RIPA-buffer with protease inhibitors to collect the total protein content. Western blotting was performed as described above. Protein extracts (10 μg) were probed with antibodies against Runx2, b-tubulin and pSMAD 1/5/8 (Cell Signaling, USA) and followed by incubation with HRP-conjugated secondary antibodies. Visualization was digitally performed using the ChemiDoc system (BioRad, USA). For Smad-signaling, proteins from the sFRP-experiment were used, in addition to an extra control condition, with unstimulated cells in low-serum medium to show basal expression of phosphorylated Smads. Quantification was performed using ImageLab Software (BioRad, USA).

Statistical Analysis

Results are presented as mean ± SEM. Statistical analyses were performed using one way ANOVA. All statistical analyses were performed using SigmaStat software (version 2.03). A p-value of <0.05 was considered as statistically significant.

Results

Effect of BMPs on Protein Expression of Wnt3a and Osteogenic Markers

Treatment of human aortic VICs with BMP-2, BMP-4 and BMP-7 for 21 days resulted in an upregulation in the expression of Wnt3a, β-catenin, ALP and Runx2, compared to control (Figure 1). While treatment of VICs with BMP-2 and BMP-7 gave a marked upregulation in the expression of ALP, Wnt3a, β-catenin and Runx2, the effects seen with BMP-4 on Runx2 expression were variable (Figure 1).

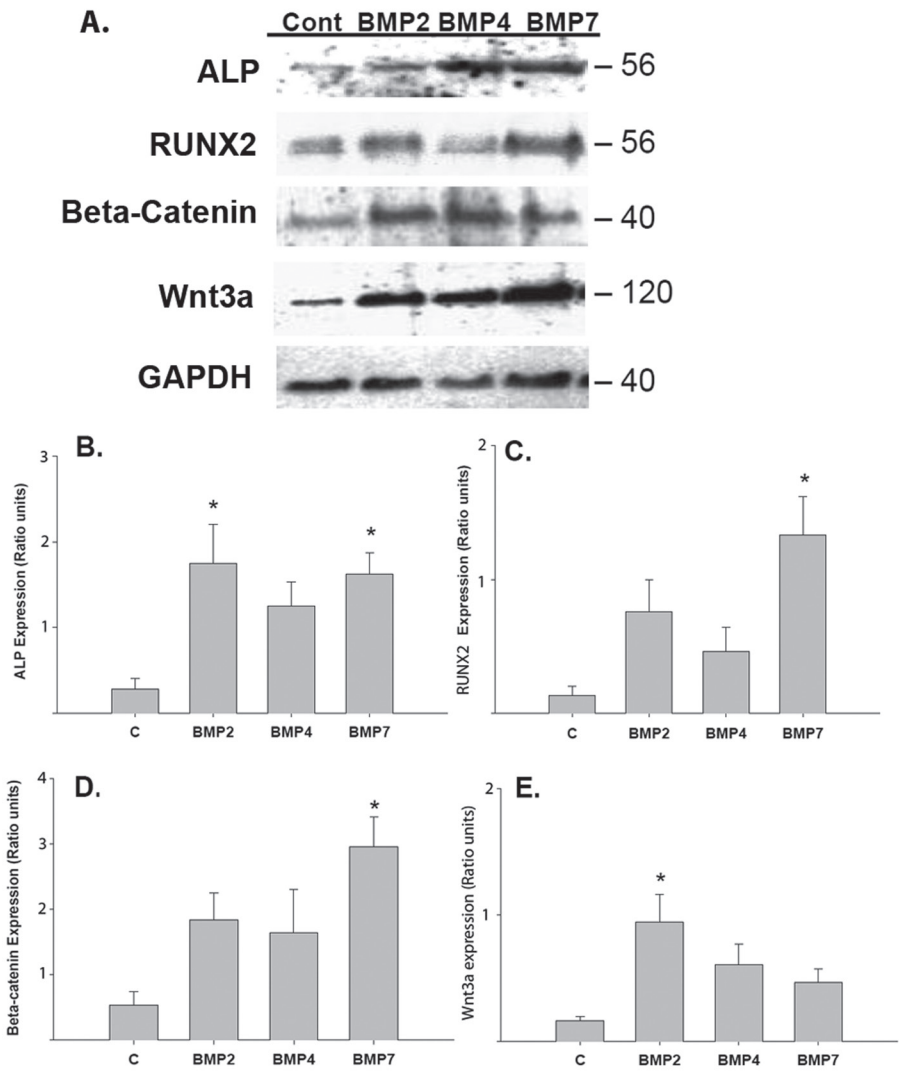


Figure 1A. *Protein expression of alkaline phosphatase (ALP), Runx2, Wnt3a, and β-catenin in VICs treated with BMP-2, BMP-4 and BMP-7 by Western blot analysis. All samples were treated for 21 days. (**1B-E.**) Quantitative analysis of Western blots showing the effects of BMPs on the expression of ALP (**1B.**), Runx2 (**1C.**), Wnt3a (**1D.**), and β-catenin (**1E.**). (* $p < 0.05$ vs. Control, n=3).*

Expression and Localisation of Wnt3a, Msx2 and β-catenin in Human Aortic Valves

Non-calcified human aortic valve leaflets demonstrated a weak expression of Wnt3a, Msx2 and β-catenin (less than 5%) (Figure 2). A similar level of Wnt3a expression was seen in the non-calcified regions of calcified valves, however there was marked expression of Wnt3a in cells adjacent to the calcified region. Similarly in the calcified valves, only cells adjacent to the calcified regions expressed Msx2 and β-catenin. Those cells that were associated with expression of Wnt3a, Msx2 and β-catenin in peri-calcified region of diseased valves, also expressed the bone markers osteocalcin and osteopontin. In addition these regions were also associated with cells that stained positively for the smooth muscle markers, smooth muscle α-actin and calponin. The expression of CD45 was negative in these regions. No staining was observed when the primary antibody was omitted, as shown in the negative control.

Effect of Wnt3a on proliferation

Wnt3a increased the proliferation of VICs in a concentration-dependent manner with a significant 3-fold increase in proliferation in response to 20 ng/mL of Wnt3a. At higher concentrations of Wnt3a (40 ng/mL) there was no further increase in the amount of proliferation (Figure 3). Human aortic VICs grown in normal growth medium supplemented with 10% serum demonstrated a significant 5-fold increase in the level of proliferation in comparison to cells treated with serum-free medium ($p < 0.05$).

Effect of Wnt3a on ALP Activity of Human VICs

Immunocytochemical staining of human VICs treated with osteogenic medium for 21 days, showed a high expression of osteocalcin in comparison to control cells. VICs treated with Wnt3a at different concentrations (10, 20, 40 ng/mL) for 21 days showed a gradual, concentration-dependent increase in the expression of osteocalcin (Figure 4). Treatment of VICs with Wnt3a (40 ng/mL) for 21 days, caused a significant increase in ALP activity to 6.8 ± 1.2 nmol/min/mg protein in comparison to 1.5 ± 0.7 nmol/min/mg protein in untreated cells ($p < 0.05$). In comparison, VICs treated with osteogenic medium significantly increased ALP activity to 9.4 ± 1.0 nmol/min/mg protein ($p < 0.05$) (Figure 5).

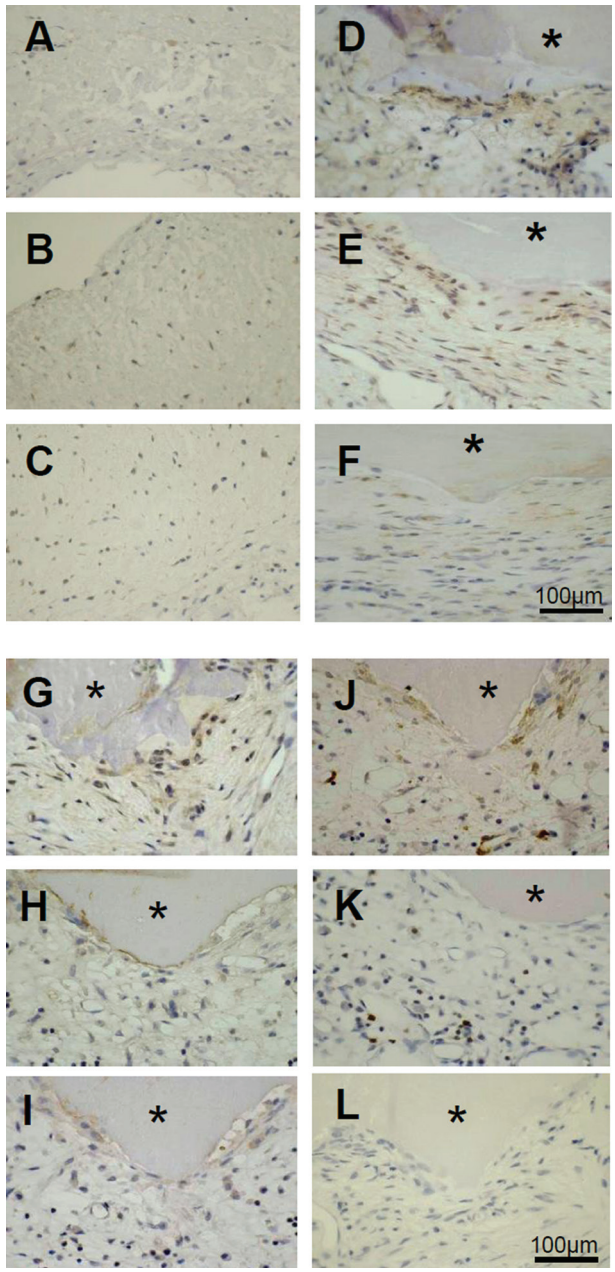


Figure 2. *Immunohistochemical staining of non-calcified (**2A-C.**) and calcified (**2D-F.**) human aortic valves for Wnt3a (**2A,D.**), Msx2 (**2B,E.**) and β-catenin (**2C,F.**). Expression of osteocalcin (**2G.**), osteopontin (**2H.**), calponin (**2I.**), smooth muscle alpha actin (**2J.**), CD45 (**2K.**) and negative control (**2L.**) in sections of calcified human aortic valves. Positive staining is indicated by brown colour. * demonstrates calcified acellular regions (magnification × 40) (n=3).*

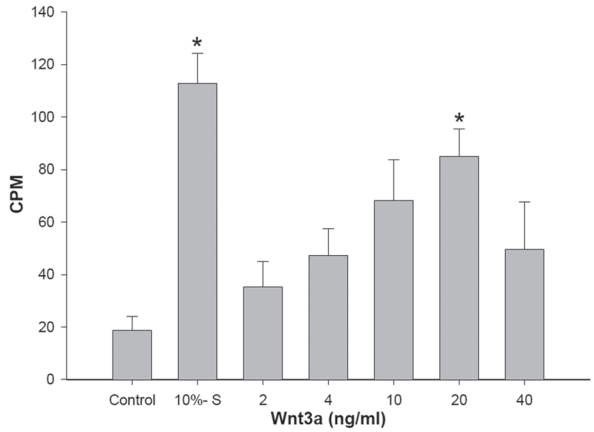


Figure 3. *The proliferation of human aortic VICs grown in serum-free medium (Control), growth medium containing 10% serum (10%-S) or serum-free medium with different concentrations of Wnt3a (2, 4, 10, 20 and 40 ng/mL). Cells were starved for 72 hrs and were then treated with the above conditions for 24 hrs. Values are expressed as counts per minute (cpm). (* p < 0.05 vs Control, n=3).*

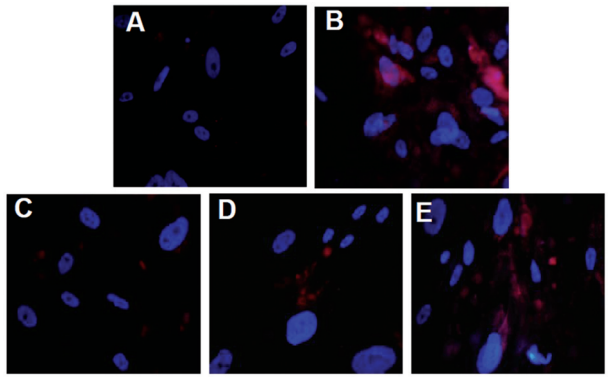


Figure 4. *The expression of osteocalcin in human aortic VICs treated with normal growth medium (**4A.**), osteogenic medium (**4B.**) and with Wnt3a in normal medium at 10 ng/mL (**4C.**), 20 ng/mL (**4D.**), and 40 ng/mL (**4E.**) (n=3). (* p < 0.05 vs. Control, n=3).*

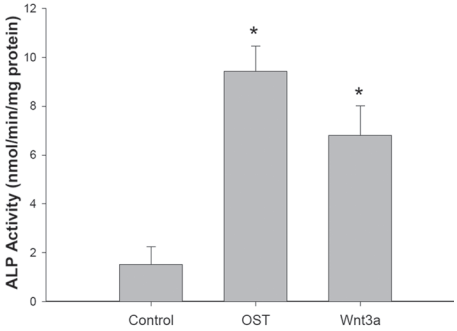


Figure 5. *The activity of ALP in normal human aortic VICs (Control), VICs treated with osteogenic medium (OST), VICs treated with Wnt3a (40 ng/mL) (Wnt3a) for 21 days. Values are presented as nmol/minute/mg of protein ± SEM (* $p < 0.05$ vs. Control, n=3).*

Effect of Wnt3a on Protein Expression of Runx2

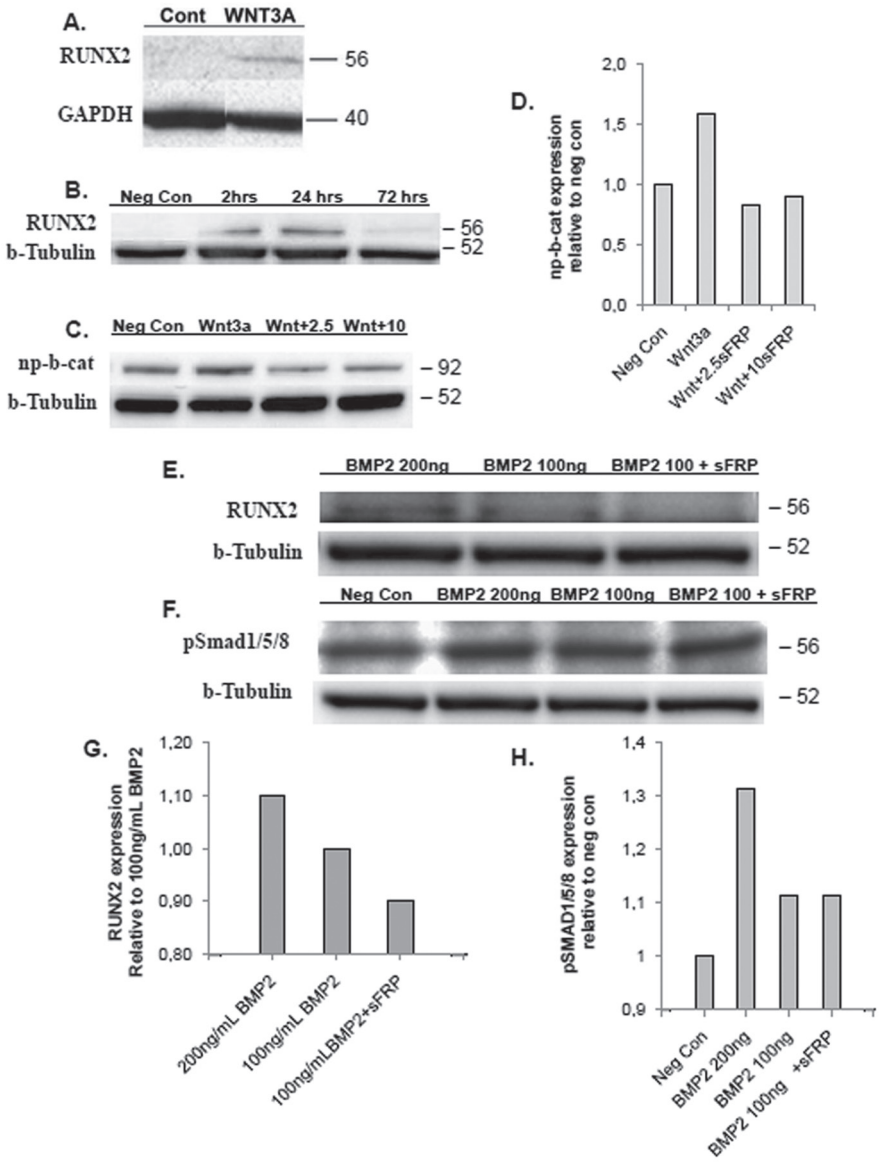
Human aortic VICs treated with 40 ng/mL Wnt3a revealed an upregulation in the expression Runx2, after 6 days of treatment in comparison to control (Figure 6a).

Effect of Wnt-inhibition on Runx2 expression after stimulation with BMP-2

Treatment of VICs with 100 ng/mL and 200 ng/mL BMP-2 increased Runx2 expression compared to unstimulated cells after 2 and 24 hours, however, this effect was lost after three days incubation (Figure 6b). This showed that experiments using 2 hr stimulations can be used to detect differences in Runx2 expression.

sFRP1 pre-treatment effectively inhibited Wnt3a induced upregulation of non-phosphorylated β-catenin, independent of the concentration (2.5 or 10 μg/mL), however the higher concentrations was used in subsequent experiments so to guarantee the inhibitory action of sFRP. (Figure 6c and d) Pre-treatment of the cells with sFRP1 also resulted in a reduction in Runx2 expression when stimulated by BMP-2 (Figure 6e and g).

To check if this effect was mediated by an inhibitory effect of sFRP1 on BMP-2/Smad signaling, we also quantified pSmad1/5/8 in these samples and compared it to a negative control. Stimulation of the cells with 100 ng/mL or 200 ng/mL BMP-2 gave a progressive increase in the expression of pSmad1/5/8, while pre-treatment of the cells with sFRP1 did not significantly affect the activation of pSmad1/5/8 by BMP-2 (Figure 6f and h). These results show that BMP-2 induced Smad1/5/8 signaling was not blocked with the sFRP1 treatment.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BMP2 100ng
BMP2 100ng +sFRP

Figure 6. 6A. *Protein expression of Runx2 in response to incubation with Wnt3a by VICs.* ***6B.*** *Timeframe of Runx2 expression after 2 hr, 24 hr or 72 hr of stimulation with 100 ng/mL BMP-2, compared to unstimulated control, which shows no expression.* ***6C.*** *Expression of non-phosphorylated beta-catenin (np-β-cat) in unstimulated cells, cells stimulated with Wnt3a and stimulated cells that were pre-treated with 2.5 or 10 µg/mL sFRP1.* ***6D.*** *Quantification of np-β-cat, corrected for β-tubulin and relative to the unstimulated cells.* ***6E.*** *Expression of Runx2 after 2 hr stimulation with BMP-2 with 200 ng/mL, 100 ng/mL and 100 ng/mL pre-treated with 10 µg/mL sFRP1.* ***6F.*** *Expression of phosphorylated Smad1/5/8 by cells with the same stimulations as for Runx2, including the basal level of pSMAD1/5/8 in unstimulated cells.* ***6G.*** *Graphical presentation of Runx2 expression after stimulation with BMP-2 and sFRP, corrected for β-tubulin and relative to stimulation with 100 ng/mL.* ***6H.*** *Quantification of pSmad1/5/8 expression after stimulation with BMP-2 and sFRP, corrected for β-tubulin and relative to unstimulated cells.*

Discussion

In this study we have demonstrated that a signaling network exists in adult human valve where BMPs increase the expression of Wnt3a in human VICs and that Wnt3a has the capacity to stimulate the proliferation of valve cells and their differentiation towards an osteoblast phenotype via upregulation of Runx2. These data provide the missing link between several studies that showed increased expression of BMP-2 and/or Wnt3a in calcified aortic valve leaflets.[30, 32, 266] In addition, our results showed that in addition to Wnt3a, VICs in adult calcified aortic valves expressed the signaling markers Msx2 and β-catenin in peri-calcification regions of human aortic valve leaflets, suggesting that the BMP-2/Msx2/Wnt-axis previously described for vascular tissue [260], may also be involved in calcification of aortic valve tissue.

In calcified human aortic valve leaflets, BMP-2 and BMP-4 are expressed by VICs and pre-osteoblasts in areas adjacent to B-cell and T-cell lymphocytic infiltration.[30] Msx2 can determine osteogenic cell fate of vascular myofibroblasts by upregulating Osx (Osterix-osteoblast factor), ALP (alkaline phosphatase) and mineralised nodule formation, as has been shown by others.[267] It has also been previously determined that the Wnt autocrine/paracrine loop mediates the induction of ALP and mineralisation by BMP-2 in pre-osteoblastic cells.[261] According to our data, the increased expression of the osteoblast transcription factor Runx2 can also be mediated via the Wnt pathway. We were able to show an increase in expression of Runx2 by

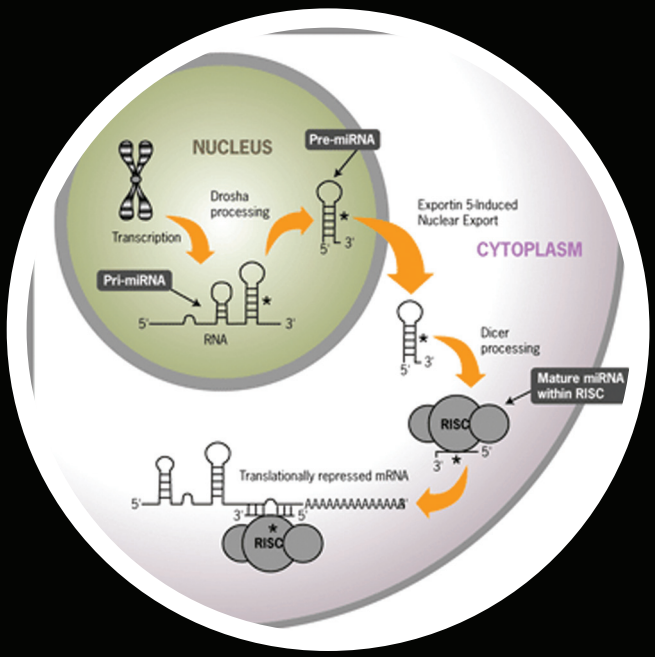
Wnt3a in adult VICs. The link between BMPs, Wnt3a and Runx2 expression is further confirmed by the ability of the Wnt antagonist sFRP1 to inhibit BMP-2-induced expression of Runx2. In addition, we are able to ensure that Runx2-expression was not reduced through sFRP1-mediated inhibition of Smad1/5/8 signaling, as has been previously reported for sFRP2.[268] Our studies showed identical expression of pSmad after stimulation with BMP-2, with or without the presence of sFRP1. However, although our results clearly show that a significant part of BMP-2-mediated Runx2 expression is mediated by Wnts, it should be noted that BMP-2-induced Runx2 seemed slightly more persistent in our hands when compared to expression induced by Wnt-3a alone. This may be explained by a longer half-life of BMP-2 proteins when compared to Wnt3a, but we cannot exclude the possibility that it is caused by alternative BMP-2 signaling paths such as Smad1/5/8.

Wnt signaling has been previously reported to play an important role in embryonic development of aortic valves, in which osteogenic differentiation is undesirable. Studies in zebrafish have previously shown that canonical Wnt-signaling is likely to induce expression of genes involved in proliferation and trans-differentiation of endocardial cells that are required in order to form the endocardial cushions.[34, 254] Alfieri *et al*. demonstrated that Wnt-genes are expressed by valvular endothelial cells, but not valvular mesenchymal cells in embryonic development, while FZDs were expressed similarly in embryonic endothelial and mesenchymal valve cells.[263] Stimulation of embryonic VICs did not result in expression of genes required for differentiation of these cells towards osteoblasts, like Runx2.[263] These results may seem conflicting with our findings of enhanced osteogenic differentiation by Wnt-signaling, but this can be explained by the source of VICs being obtained from valves at different stages of development. It is known that the phenotype of cells changes post-natally as the valve valve adapts to systemic pressures after birth.[28] It has been proposed that cell-responsiveness is partially determined by the level of lineage determination of the cells. Early progenitor cells cannot be pushed towards an osteogenic lineage with Wnts, while Wnt-mediated differentiation may be induced in cells in a more advanced stage of differentiation.[269] In addition, Wnts are known to be secreted proteins that act as morphogens and exert a concentration-dependent effect on cellular fate. It has been shown that Wnts may have differential responses in the same cell.[270, 271] This is also illustrated by our results that show proliferation of VICs at low concentrations of Wnt3a, while higher concentrations induced differentiation.

Future studies will further investigate the role of Wnt signaling in valvular pathogenesis. Recent work has shown that nitric oxide is capable of inhibition the formation of calcific nodules *in vitro*.[272] Observation from our group has identified a role for the valvular endothelial cells in the protection of valve tissue against calcification, through secretion of nitric oxide (NO) and an increase in the levels of cGMP (unpublished observation). Future studies are required to investigate if the BMP-Wnt-Runx2 network is regulated by agents such as nitric oxide, or c-type natriuretic peptide, that are both released by the endothelium and increase levels of cGMP. Such effects could be mediated by protein kinase G on up-stream or down-stream mediators in the BMP-Wnt-Runx2 network, as has been shown for the non-canonical Wnt pathway, or via the ability of the endothelium to regulate the mechanical properties of the valve.[273, 274]

In conclusion, this study has shown that Wnt signaling appears to play an important role in valve calcification by regulating the proliferation and osteogenic differentiation of VICs. The current and future studies could help in understanding further the cellular mechanisms of heart valve calcification and should provide potential therapeutic targets.

Chapter 8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Translationally repressed mRNA
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RISC

Chapter 8

Differential Expression Of MicroRNAs In Human Calcific Aortic Valve Disease

This chapter describes ongoing work:

Riem Vis PW, Joziassen IC, Kluin J, Vink A, van Herwerden LA, Doevendans PAFM, Sluijter JPG.

Abstract

Calcific aortic valve disease (CAVD) is a serious illness that may lead to heart failure and death. There are no known pharmacological treatments available to inhibit progression of this disease. **Aim:** The aim of this study is to determine microRNA (miRNA) profiles from healthy and calcified valves and use these profiles to identify new miRNAs or proteins that may regulate onset and progression of CAVD in humans. **Methods:** RNA was isolated from valve tissue obtained from human CAVD patients. Healthy valve tissue was obtained from patients with dilated cardiomyopathy, who had no signs of valve stenosis or regurgitation. Diseased valves were obtained from patients who underwent aortic valve replacement as treatment for severe aortic valve stenosis. Histological analysis was used to determine the pathological condition of the tissue specimen. Total RNA was isolated and microarray analysis was used to obtain miRNA profiles. Quantitative RT-PCR (qRT-PCR) was used to examine target mRNA expression patterns. **Results:** The miRNA profiles revealed that miR-29a/c, miR-2861 and miR-30b/c/d were differentially expressed in CAVD valves. qRT-PCR analysis showed that potential targets of these miRNAs tended to increase in the tissue samples. **Conclusion:** Our results have identified several miRNAs possibly involved in the onset and progression of CAVD. Future studies are needed to determine their functional contribution in valve cells.

INTRODUCTION

Calcific aortic valve disease (CAVD) is a disease that causes gradual calcification and mineralization of aortic valve tissue over time. The pathological process starts with mild, asymptomatic fibrosis and thickening of the cusp (sclerosis) and ends with aortic valve stenosis, which severely disrupts opening and closing of the valve. This final stage of CAVD is thought to be caused by differentiation of valvular interstitial cells (VICs) towards osteoblasts and subsequent formation of mineralized, bone like structures in valve tissue. Approximately 2-3% of patients over 65 years old suffer from aortic valve stenosis [5] and, due to aging of the population, the number of patients is expected to grow rapidly in the future. Once the disease has become symptomatic, life expectancy is dramatically reduced. [8] Despite these threatening numbers, there are still no pharmacological therapies available to slow down progression of CAVD and there are currently no pathways known that can be successfully targeted in the near future to accomplish this.

MicroRNAs (miRNAs) have emerged as new possible targets for intervention in different cell processes, since they play an important role in cell proliferation, differentiation [275] and several other physiological processes, including angiogenesis and fibrosis in heart failure.[118, 119, 230] MiRNAs are small, 18-22 nucleotides long, endogenous present RNA molecules that can bind to the 3' untranslated regions (UTR) of target mRNA sequences in the cell, thereby prohibiting translation into executing proteins. Using small, modified, complementary oligonucleotides [117], miRNA function can be attenuated *in vivo*, resulting in induction or inhibition of physiological processes.[119, 121, 230] These findings have boosted the search for differential expression of miRNAs in all fields of research, including osteogenic differentiation and valve biology. For instance, miR-204 has been found to bind to Runx2.[120] Runx2 is a transcription factor that is well known for its stimulatory role in osteogenic differentiation. An increase in miR-204 resulted in a decreased protein expression of Runx2 and thereby inhibited osteoblast differentiation. In addition, miR-2861 was discovered to affect Runx2 activity in mouse osteoblasts by targeting an inhibitor of the Runx2 protein, HDAC5.[276, 277] These studies show that miRNAs are indeed involved in regulation of cell differentiation towards osteoblasts. Interestingly, the concept that miRNAs might influence valvular cells in

diseased tissues was recently confirmed by the identification of differentially expressed miRNAs between non-calcified, insufficient bicuspid valves, and calcified bicuspid valves.[229] This was the first study that shows the differentially expression of miRNAs in bicuspid aortic valve stenosis.

In the present study, we compared the miRNA-expression profiles from aortic valves, derived from explanted human hearts from patients suffering from dilated cardiomyopathy (DCM), with profiles derived from severely stenotic valves. The valves that are obtained from DCM-patients are usually healthy, or only mildly sclerotic, and are therefore the ideal control to investigate which miRNAs are differentially expressed once CAVD enters later stages. Investigation of miRNAs involved in progression of CAVD may result in identification of miRNAs, proteins and signaling pathways that were previously not linked to osteogenic differentiation in valves and aortic stenosis in particular and may therefore result in new targets for pharmacological therapies in the future.

MATERIALS AND METHODS

Patient material

All healthy aortic valve tissue specimen are derived from hearts explanted at the University Medical Centre in Utrecht at the time of heart transplantation. They have been cleared for use in research projects by a general hospital waver, approved by the authorized Dutch Ethical Committees and the Dutch Law. As such, no individual informed consent from the patient was necessary. For collection of all diseased valve tissue specimen, separate permission was requested and approved by the Medical Ethics Committee of the University Medical Center Utrecht. Informed consent was obtained according to the world Medical Association Declaration of Helsinki and tissue was further treated anonymously.[73] Tissue samples have been stored in a tissue bank that has been created and previously used by Joziasse *et al.* to study bicuspid aortic valves.[278] Patient characteristics at time of explantation are presented in Table 1. Initial analysis is performed on three groups consisting of healthy valve leaflets, calcified tricuspid valve leaflets and calcified bicuspid valve leaflets in which a raphe is visible (HAV, TRI and Bi + R respectively). For further additional analyses, data of TRI and Bi + R are pooled as diseased valves.

Group	Sample	Gender	Age	Gradient	DM	HC	HT	Obese	Smoking	FH
HAV	1	m	58	10	Y	N	N	N	N	N
HAV	2	f	34	2	N	Y	N	N	N	Y
HAV	3	m	46	4	N	Y	N	N	N	N
HAV	4	f	52	2	N	N	Y	N	N	Y
TRI	1	m	72	ND	N	N	N	N	N	N
TRI	2	f	81	60	N	N	Y	N	N	N
TRI	3	m	50	83	N	N	N	N	N	N
Bi+ R	1	m	76	82	N	N	N	N	P	ND
Bi+ R	2	m	64	72	N	N	Y	N	N	N
Bi+ R	3	m	60	70	N	N	N	N	N	N
Bi+ R	4	f	41	100	N	N	N	N	N	Y
Bi+ R	5	f	54	100	N	N	N	N	N	Y

Table 1. *Patient characteristics. HAV: Healthy Aortic Valve, from patients with DCM, no assist device and no aortic valve insufficiency. TRI: Explanted stenotic tricuspid valves. Bi + R: Explanted stenotic bicuspid valves in which a raphe was visible. Gender: Male or Female. Gradient: peak transvalvular pressure (mmHg), ND: not determined. DM: Diabetes Mellitus type II: Yes or No. Hypercholesterolaemia (HC): Yes or No cholesterol above 5 mmol/L. Hypertension (HT): Yes or No systolic pressure above 140 mmHg. Smoking: Yes, Never or in the past (quit more than 5 years ago). Family History (FH): Valve-related; Yes, No or Not Determined. Pos. his.: HAV: First degree relative with DCM, Bi+R4: Bicuspid valve, aortic dilatation, Bi+R5: Several sudden cardiac deaths on mothers side of family. All patients took blood pressure lowering medication.*

Histology

Histology was used to detect calcific noduli in healthy or diseased valves. Hematoxilin and Eosin (H&E) staining was performed on tissue sections, embedded in paraffin. Elastin von Gieson staining was performed on HAV samples, to demonstrate the clear three-layered matrix architecture of the healthy aortic valves.

Target	*Forward primer*	*Reverse primer*	*Annealing temp (°C)*
RUNX2	GTGGACGAGGCAAGAGTTTC	TTCCCGAGGTCCATCTACTG	56
COL1A1	TGCCATCAAATGCTTCTGC	CATACTCGAACTGGAATCCATC	56
COL3A1	CCAGGAGCTAACGGTCTCAG	CAGGGTTTCCATCTCTTCCA	48
αSMA	ACCCACAATGTCCCCATCTA	GAAGGAATAGCCACGCTCAG	50
GAPDH	ACAGTCAGCCGCATCTTC	GCCCAATACGACCAAATCC	56

Table 2. *Primers used for qRT-PCR*

RNA isolation and mRNA analysis

Total RNA was isolated from tissue samples using Tripure isolation reagent (Roche, Basel, Switzerland) according to the manufacturer's instructions. Production of cDNA, qRT-PCR and post-run product verification were performed as previously described.[73] Primers were designed (OligoPerfect, Invitrogen) for α-Smooth Muscle Actin (αSMA), Collagens type I and III and Runx2 and annealing temperatures were determined (Table 2). Samples were normalized for glyceraldehyde 3-phosphate dehydrogenase (GAPDH, $\Delta Ct = Ct_{Target} - Ct_{GAPDH}$). Fold-difference of diseased valves compared to healthy valves was determined per group ($2^{-\Delta\Delta Ct \text{ per group}}$, with $\Delta\Delta Ct = \Delta Ct_{Healthy} - \Delta Ct_{Diseased}$).

MiRNA-profiling

Total RNA (samples 1-3 from each group) was shipped to a service provider (LC Sciences, Houston, TX, USA) according to instructions, to obtain microRNA profiles and to determine significant differentially expressed miRNAs in the three groups. Detection was performed using the μParaflo™ MicroRNA microarray assay. To find miRNAs related to disease processes *in vivo*, the focus in subsequent analyses was on miRNAs that were similarly reduced or upregulated in the two diseased valve groups as compared to the healthy valves. In addition, the raw data obtained from the profiling were used to combine the data from tri- and bicuspid calcified valves in a "diseased" group, which were compared to the healthy valve data. Log2-scores were calculated to determine the number of duplications required to get from the value in one group to the value in the other group. Potentially important miRNAs were selected by using the miR-base.org, targetscan.org and microrna.org target prediction websites. miRNAs with predicted targets for osteogenic differentiation were selected for follow-up.

Statistics

One-way ANOVA was used to determine significant differences between miRNA expression profiles the three seperate groups. To compare the healthy and the diseased group, Mann Whitney U tests were applied. Results are expressed as means ± Standard Error of the Mean (SEM). A $p<0.05$ is considered significant and marked with *, while $p<0.01$ is marked with **.

RESULTS

Histology

Histological analysis was applied to determine the state of calcification of the tissue specimen used. None of the healthy valves showed signs of calcification following H&E staining, moreover, EvG-staining indicated that the tri-layered architecture of these valves was nicely intact (Figures 1a and b respectively). The diseased valves showed large calcific noduli on the cusps (Figure 1c) and H&E staining confirmed calcification in the valvular tissue (Figure 1d).

MiRNA-profiling

ANOVA analysis of the miRNA profiles obtained from three groups resulted in a list of differentially expressed miRNAs (supplementary Table 1). Analysis of the raw data separated in a "healthy" and "diseased" group revealed additional potentially interesting miRNAs with statistically significant different expression between groups (supplementary Table 2 and Figure 1). Upon further analysis, miR-30b, miR-30c, miR-30d, miR-29a, miR-29c and miR-2861 were selected as microRNAs that are potentially involved in the onset and progression of CAVD. MiR-30b/c/d are predicted to target Runx2 directly, miR-29a/c are known to target collagen production and miR-2861 has been shown to target an inhibitor of Runx2 [276, 277]. Expression of these miRNAs was also significant if healthy tissue samples are compared with the diseased samples combined ($p<0.05$, Figure 2a, c and e respectively).

mRNA-analysis

To determine if the effects of these miRNAs are also reflected on target gene levels in the valves, qRT-PCR analysis was performed on the predicted targets. Expression of Runx2 and Collagen type 1 and 3 tend to increase between healthy and diseased valves (Figure 2b and d).

Expression of αSMA was determined as additional marker for the condition of the healthy and diseased valves. Expression tended to increase in diseased valves, but was not significantly different when compared to healthy valves (Figure 2f).

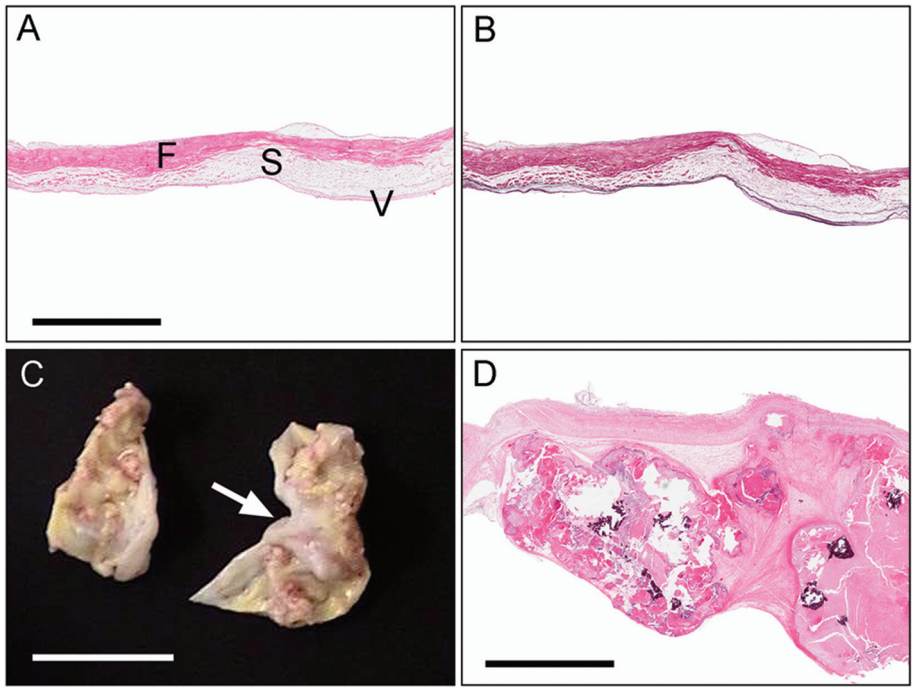


Figure 1. 1A. *and* ***1B.****: representative image of normal leaflets of aortic valves, showing the three layered architecture. F, fibrosa; S, spongiosa; V, ventricularis. Bar = 1.5 mm.* ***1A.*** *haematoxylin and eosin staining.* ***1B.*** *elastin – van Giesson staining.* ***1C.*** *representative image of diseased valve: resected tricuspid aortic valve showing nodular calcifications. The arrow indicates fusion of commissures. Bar = 20 mm.* ***1D.*** *The diseased leaflets show calcification (purple) and degeneration. Haematoxylin and eosin staining. Bar = 1.5 mm.*

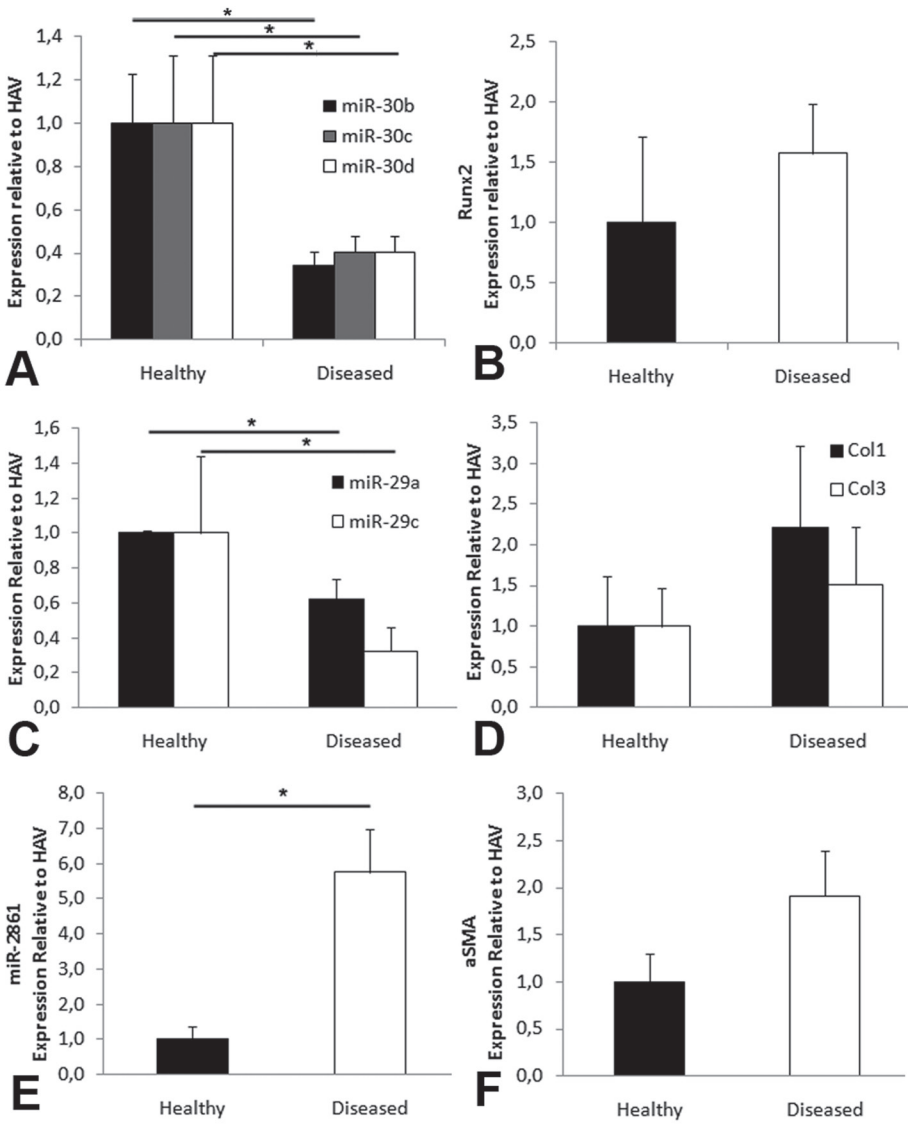


Figure 2. *On the left: comparison of microRNA expression in healthy or diseased valves. MiR-30b/c/d (**2A.**), miR-29a/c (**2C.**) and miR-2861 (**2E.**) all showed a significant differently expression between groups ($p < 0.05$). On the right: comparison of mRNA expression. Runx2 (**2B.**), Collagen type 1 and 3 (**2D.**) and αSMA (**2F.**) all tend to increase between groups.*

Discussion

As described in this paper, we successfully obtained total RNA from non-calcified valves ("healthy") and calcified valves ("diseased"), followed by determination of microRNA (miRNA) profiles. In addition to previous investigations on differentially expressed miRNAs in heart valves, we included healthy and diseased valves. Nigam *et al*. compared calcified bicuspid with insufficient bicuspid valves [229], while El-Hamamsy *et al.* compared calcified bi- and tricuspid valves [279]. Comparison of our profiles has led specifically to identification of miRNAs that may be involved in the onset and progression of calcific aortic valve disease (CAVD). Further analysis and functional follow-up of these miRNAs may result in discovery of novel targets or pathways that may be targeted in the future, to reduce progression of CAVD.

Using miRNA-profiling, we have previously identified miRNAs and proteins involved in regulation of cardiomyocyte-progenitor cell behavior [275] and angiogenesis ([280] and van Mil *et al.*, submitted). We obtained successful modulation of a functional vasculature *in vivo* by a miRNA-inhibition approach. Similarly, our profiles may reveal involvement of proteins and miRNAs that have not been previously identified to be active in CAVD. Successful *in vivo* modulation of these novel targets may be an important step forward in finding new pharmacological treatment options for CAVD ([3] and Riem Vis *et al.*, submitted).

The presented data are a first step to the identification of functionally important miRNAs, but additional experiments are required. First, we have planned *in vitro* experiments in which the functional response of aortic valve interstitial cells to the selected miRNAs is determined, following transfection with potentially important miRNAs. After identifying these miRNAs, we need to confirm that predicted targets are truly targeted and bind to the 3'-UTR. Furthermore, in addition to the presented suggestive changes on gene level, we need to confirm effects on a protein level. Moreover, comparison of miRNA expression in healthy and diseased tissue showed significantly different expression of miRNAs that have no described function yet. Future cell studies will be performed to determine their importance in calcification processes.

Nonetheless, the presented data consistently showed that Runx2 and collagen-targeted miRNAs are differentially regulated to favor osteogenic differentiation and collagen formation in diseased valves. MiR-29a/c have been previously shown to be involved in several fibrotic processes [281] and is known to target collagen. Mir-2861 regulates expression of HDAC5 and thereby influences activity of Runx2, a key transcription factor required for osteogenic differentiation.[276, 277] These observations are supported by a tendency of Runx2 and collagen mRNA expression to increased, as determined by mRNA analysis. In addition, expression of αSMA was enhanced in diseased valves and this protein has been previously shown to be a marker for activation of the valve interstitial cells and related to enhanced matrix remodeling, *i.e.* collagen production and degradation.[28, 134] Therefore, these data indicate that miRNAs may contribute to formation of a favorable climate in the heart valve tissue for disease processes, like osteogenic differentiation of the valvular interstitial cells and fibrosis, which are both processes that have repeatedly been associated with CAVD.[3, 30, 31, 95, 282-284]

In conclusion, we have presented a first comparison of healthy with diseased valves tissue. Our data suggest potential roles for miRs 29a/c, 2861 and 30b/c/d. Future studies are required to determine functional effects of these miRNAs on aortic interstitial valve cells.

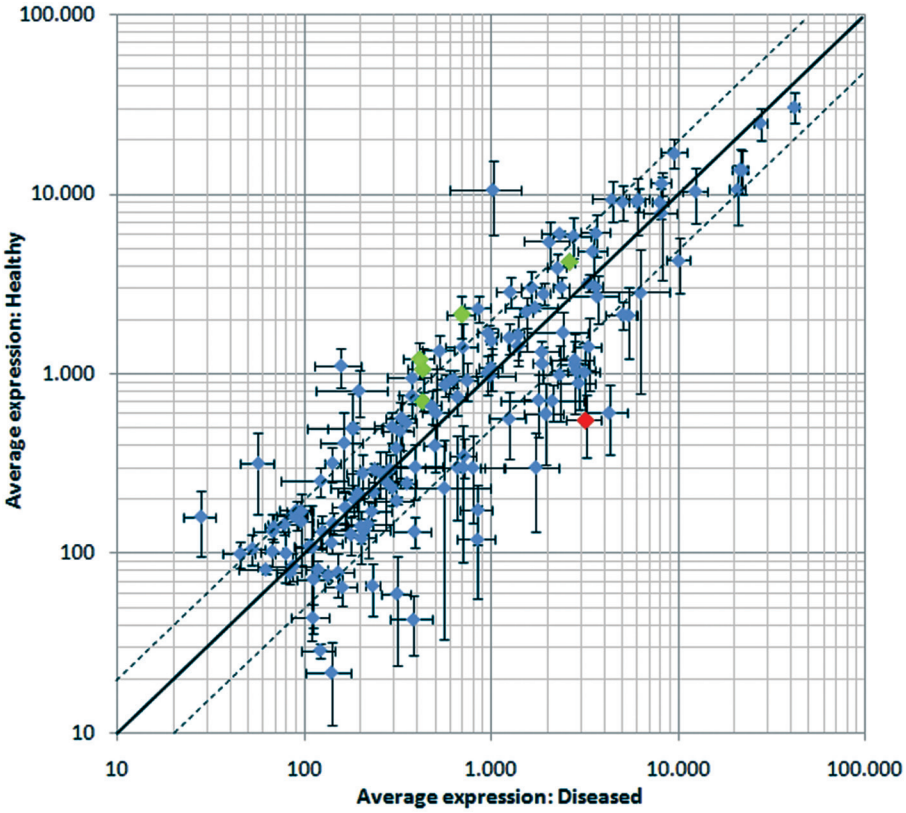
Supplementary Figures

			Group 1	Group 2	Group 3
			HAV	TRI	Bi + R
No.	Reporter Name	p-value	Mean	Mean	Mean
99	hsa-miR-126	4.97E-03	1,115	155	165
1023	hsa-miR-574-5p	5.03E-03	2,357	2,761	566
1132	hsa-miR-671-5p	6.77E-03	175	665	1,181
* 396	hsa-miR-29a	7.45E-03	4,195	2,468	2,896
1020	hsa-miR-572	7.68E-03	43	378	459
60	hsa-miR-1224-5p	8.74E-03	60	256	414
469	hsa-miR-3149	1.19E-02	981	1,655	248
10	hsa-let-7e	1.27E-02	953	530	193
850	hsa-miR-466	1.46E-02	710	478	4,012
* 388	hsa-miR-2861	1.55E-02	555	3,017	3,525
197	hsa-miR-1469	1.59E-02	609	4,616	4,272
1209	hsa-miR-99a	1.84E-02	2,318	914	686
55	hsa-miR-1207-5p	1.98E-02	120	760	1,009
401	hsa-miR-29c	2.04E-02	2,143	563	818
* 426	hsa-miR-30d	2.19E-02	705	467	363
* 421	hsa-miR-30b	2.47E-02	1,209	441	326
1186	hsa-miR-92a	2.99E-02	757	458	286
852	hsa-miR-483-5p	3.06E-02	718	1,482	2,451
118	hsa-miR-1273e	3.15E-02	122	133	363
24	hsa-miR-103	3.17E-02	913	689	450
96	hsa-miR-125b	3.35E-02	17,058	11,550	6,816
215	hsa-miR-151-5p	3.42E-02	666	590	329
33	hsa-miR-107	3.45E-02	872	649	422
524	hsa-miR-32*	3.63E-02	304	661	93
20	hsa-miR-100	3.67E-02	2,806	2,128	1,514
17	hsa-let-7i	3.69E-02	1,699	1,118	684
764	hsa-miR-4267	3.80E-02	302	85	1,139
1124	hsa-miR-663	3.83E-02	2,134	5,069	6,096
194	hsa-miR-145	3.87E-02	2,148	6,310	3,433
843	hsa-miR-451	4.65E-02	10,660	774	1,353
594	hsa-miR-3621	4.69E-02	301	544	1,061
229	hsa-miR-16	4.95E-02	2,878	1,222	1,124

Supplementary Table 1. *Results of ANOVA analysis of miRNA expression profiles of three groups. The asteriks marks miRNA which have a similar expression profile in both diseased groups when compared to healthy tissue and have predicted targets possibly involved in CAVD. p<0.01 is red, while p<0.05 is orange.*

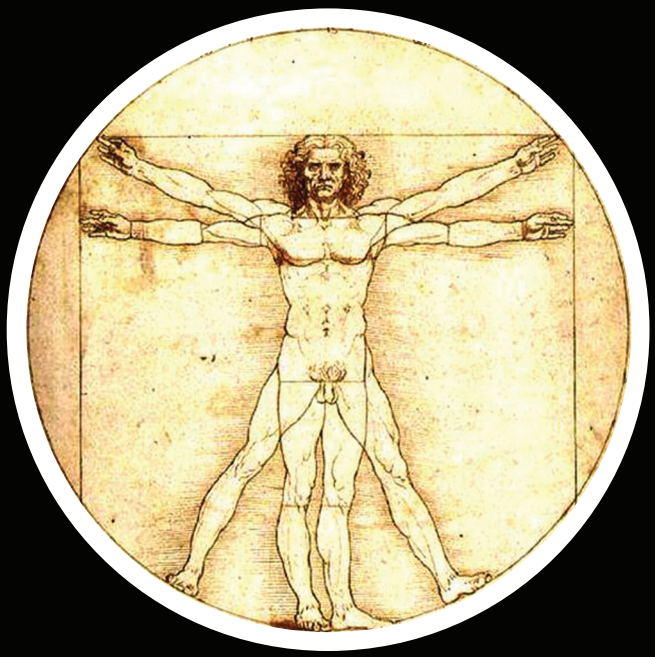
Overexpressed in Diseased						Overexpressed in Healthy					
NAME	AVG H	SD	AVG D	SD	Log2(D/H)	NAME	AVG H	SD	AVG D	SD	Log2(D/H)
hsa-miR-572	43	27	382	238	3,16	hsa-miR-451	10660	8107	1018	1024	-3,39
hsa-miR-1207-5p	120	111	842	475	2,81	hsa-miR-126	1115	489	156	107	-2,83
hsa-miR-1469	609	445	4264	2525	2,81	hsa-miR-486-5p	161	111	28	13	-2,53
hsa-miR-1231	22	18	139	93	2,68	hsa-miR-26b	809	399	195	194	-2,05
hsa-miR-2861	555	369	3198	1676	2,53	hsa-miR-29c	2143	953	694	300	-1,63
hsa-miR-1224-5p	60	63	312	139	2,39	hsa-miR-30b	1209	477	413	183	-1,55
hsa-miR-671-5p	175	109	846	406	2,27	hsa-miR-99a	2318	662	848	321	-1,45
hsa-miR-1307	29	5	121	60	2,07	hsa-miR-101	500	59	183	122	-1,45
hsa-miR-4270	66	37	231	49	1,80	hsa-let-7f	5462	2725	2039	1350	-1,42
hsa-miR-1275	893	444	2928	1630	1,71	hsa-miR-4324	6070	269	2321	1175	-1,39
hsa-miR-149*	1027	810	3152	1510	1,62	hsa-let-7g	1349	499	528	252	-1,35
hsa-miR-143	1044	253	2843	1849	1,45	hsa-miR-30c	1064	580	430	189	-1,31
hsa-miR-663	2134	1602	5416	1340	1,34	hsa-miR-16	2878	966	1258	467	-1,19
hsa-miR-602	44	14	110	62	1,33	hsa-miR-25	320	126	141	34	-1,19
hsa-miR-483-5p	718	475	1791	704	1,32	hsa-miR-140-5p	100	29	45	22	-1,14
hsa-miR-762	4295	2517	10056	3373	1,23	hsa-miR-106b	140	44	68	19	-1,04
hsa-miR-198	75	7	133	51	0,82	hsa-miR-30e	172	23	96	38	-0,84
hsa-miR-638	13758	6558	22118	2214	0,68	hsa-let-7i	1699	313	961	305	-0,82
hsa-miR-3665	30843	10477	42003	5466	0,45	hsa-miR-199a-3p	3906	1346	2257	657	-0,79
						hsa-miR-30d	705	146	431	99	-0,71
						hsa-miR-29a	4195	63	2616	481	-0,68
						hsa-miR-107	872	222	564	133	-0,63
						hsa-miR-100	2806	666	1903	413	-0,56

Supplementary Table 2. *Results of Mann Whitney U analysis of miRNA expression profiles separated in two groups. Only miRNA with p<0.05 are displayed, sorted by the Log2 number which represents the difference between the healthy and diseased groups. Marked in red are the miRNAs selected for further analysis. AVG H: average expression in Healthy tissue, AVG D: average expression in diseased tissue.*



Supplementary Figure 1. *Graphical representation of expression profile: dots represent averaged expression of individual miRNAs in healthy or diseased tissue and their corresponding SEM values. Solid line represents no difference in expression between groups. Dotted lines are arbitrarily drawn to enhance visualization of which miRNAs are overexpressed in each of the two groups. Green dots represent the selected miRNAs overexpressed in healthy tissue, red dot indicates selected miRNA overexpressed in diseased tissue.*

Chapter 9



Chapter 9

General Discussion And Future Perspectives

Overview

The experiments described in this thesis aim to contribute to the search for new treatment options for patients suffering from calcific aortic valve disease (CAVD). The first part of this thesis focuses on the improvement of currently used protocols for heart valve tissue engineering, a technique that may eventually provide an ideal, viable graft for aortic valve replacement. **Chapter 2** summarizes several important issues that need to be addressed to successfully engineer heart valve constructs, like the use of human-derived substitutes for animal serum in culture media. **Chapters 3, 4 and 5** subsequently describe the first steps towards autologous culture protocols and show that platelet-lysate is a potent inducer of cell proliferation, but is ineffective for tissue culture as indicated by the weak tissue strips produced by cells cultured in platelet-lysate. Our results in cell and tissue studies indicate that the weakness of the tissue could be correlated to high production and activation of matrix metalloproteinases (MMPs). Human serum showed less induction of cell proliferation, but the tissue strength was enhanced, possibly due to reduced activity of MMPs. Together, these studies resulted in a culture protocol that combined platelet-lysate-induced cell proliferation, while retaining load-bearing capacities of the tissue constructs by culture in human serum. The remaining chapters of this thesis concentrate on earlier events in CAVD. Processes identified in these studies may be targeted in future therapies to halt progression of the disease before it reaches its end-stage. However, they may also be valuable in future heart valve tissue engineering strategies, to prevent calcification in tissue engineered constructs. **Chapter 6** briefly summarizes early processes in the most common causes of CAVD, compares them with atherosclerosis, shows the ineffectiveness of currently used pharmacological treatments and stresses that alternative solutions have to be found. In **Chapter 7** it is shown that Wnt signaling may be a target for future treatments. In **chapter 8**, microRNA-research is introduced as another method to identify promising new targets for future therapies against valve disease. Especially miR-2861, inhibitor of HDAC5 and therefore inducer of osteogenic transcription factor Runx2, may provide new opportunities for further research. Several important questions and future perspectives in relation to this work will be discussed in the following sections.

Translation Of Heart Valve Tissue Engineering To Clinical Practice

In general, translation of experimental procedures to clinical application starts with the development of therapeutic products in cell studies, followed by experiments in animals, before advancing towards different experimental stages in humans. This is also the path for heart valve tissue engineering which has passed the stage of *in vitro* studies with cells and bioreactors and has been applied in a number of studies in sheep, which is the preferred animal model for heart valve research.[81, 91, 181, 285] Sheep are considered an optimal model because their circulatory system is fairly similar to humans, they are relatively large therefore allowing implantation of nearly human-sized prostheses, they allow implantation in juvenile animals to observe functioning of the prosthesis while the animal grows and last but not least, they are considered a worst-case scenario for heart valve calcification.[181, 286] Unfortunately, like all large animal models, sheep cannot be effectively used to study the functioning of constructs with human cells, because immune responses could compromise the results. Therefore, cells used in these animal models are generally obtained from sheep to have a fully autologous pre-clinical model to mimick future studies in humans. However, a study by members from our group has shown that there are differences in constructs produced with cells from sheep or from humans. (van Geemen, Mol, Grootzwagers, Soekhradj-Soechit, Riem Vis, Baaijens, Bouten, Regenerative Medicine, submitted) Specifically, differences can be observed in proliferation of cells, ultimate tensile strength of tissue constructs and in collagen maturation, if results with human cells are compared with ovine cells. This shows that the autologous animal model might not be a valid pre-clinical model for autologous human tissue engineering and it raises concerns for future steps to bring heart valve tissue engineering to clinical practice: excellent results in sheep with specific protocols might not automatically translate into excellent results with humans and protocols for humans are difficult to test in sheep. Future experiments need to be performed to describe differences between human and sheep cells more closely and to identify which experiments give similar interspecies results, making them more suitable preclinical predictors. An example of this might be the collagen gel contraction assay described in chapter 5, which might be a valid tool to study ongoing tissue remodeling and contraction, witnessed

in vivo [91], with an *in vitro* model. Once it has been fully established how human and sheep cells and tissue constructs respond to mechanical and biological stimuli, we may be better able to predict the outcome in future clinical trials.

From Semi-Autologous To Fully Autologous Tissue Engineering Strategies

Our results are the first to show that human alternatives for fetal bovine serum can be used for heart valve tissue engineering. However, all studies presented in this thesis were performed with badges of platelet-lysate or human serum consisting of pooled products from 5-6 healthy subjects. This approach makes it possible to effectively study cellular responses under similar circumstances, but does not represent future clinical application. Especially if heart valve tissue engineering is to be used in patients with systemic diseases, like diabetes or metabolic syndrome, it is likely that their serum protein profiles are different as compared to healthy humans. Consequently, it is possible that matching of autologous cells with autologous serum elicits different responses as have been observed in our investigations. Considering the significant differences we observed between patients in various experiments, this is concerning. Ideally, experiments should be performed in which serum protein profiles are compared with receptor expression patterns on cells and mechanical properties of tissue produced with these materials, in order to establish a functional relationship between growth factors, cell responsiveness and tissue quality. This would result in data that can be used to prescreen patients and test their eligibility for heart valve tissue engineering. Unfortunately, these experiments would be extremely costly, time consuming and would require a very high sample size in order to identify any significant correlations. It is therefore questionable if thorough experiments like these will ever be performed for a tissue engineering strategy that has not established itself yet.

Cell Choice For Heart Valve Tissue Engineering And Calcification studies

There are currently a number of different cell sources used for heart valve tissue engineering, including mesenchymal stem cells [74-76, 78], venous-derived myofibroblasts [1, 71, 72] and endothelial progenitors from the blood [81-83]. These cell sources all have certain advantages and disadvantages as described in chapter 2, but not many papers have been published in which the potential of these cells to undergo osteogenic differentiation is compared. As described in chapter 6, osteogenic differentiation is an important factor in progression of CAVD and mesenchymal stem cells, for instance, are well known for their ability to undergo osteogenic differentiation.[79, 287] Since tissue engineered heart valve constructs are primarily suggested to be beneficial for pediatric patients and thus have to last a lifetime, it is important to understand processes leading to aortic valve calcification (hence the last three chapters of this thesis), and to examine which processes drive osteogenic differentiation of these cells. For instance, preliminary results from our group, not presented in this thesis, suggest different responses of valvular interstitial cells (VICs) to standard osteogenic culture media when compared to mesenchymal stem cells. While VICs responded with a significant upregulation of transcription factor Runx2, essential for differentiation towards osteoblasts, we found that mesenchymal cells primarily upregulated alkaline phosphatase, which is thought to be important for matrix mineralization [288]. This further complicates interpretation of *in vivo* results from animal models, because it will be difficult to extrapolate successful long term follow-up results in animals to humans. Stimuli that usually cause valvular calcification in sheep may not the same that trigger osteogenic differentiation after implantation in human patients. It would therefore be interesting to know which processes drive calcification in sheep and to compare this to studies that investigate the response of cells to osteogenic stimuli that naturally occur in humans. More insight in osteogenic differentiation may prevent early *in vivo* calcification of tissue engineered heart valve constructs in humans.

Towards New Treatments To Inhibit Progression Of CAVD

The final chapters of this thesis have focused on the search for new pathways in CAVD that may be targeted in order to halt progression of the disease. This may be useful for inhibition of calcification in tissue engineered heart valves, but may naturally also serve as therapy against calcification of native heart valves. We have shown that Wnt signaling possibly plays in important role in valve calcification and ongoing work in the microRNA-project described in chapter 8 may reveal even more new signaling pathways. Unfortunately, there are limited options available to study these signaling networks and possible inhibitors more closely. There are no suitable animal models available to investigate onset of CAVD let alone the effectiveness of experimental therapeutics. For this purpose, our group is currently involved in a collaboration with research partners at Harvard, Boston, which aims to develop hydrogels as 3D models for aortic heart valves. These gels are composed of hyaluronic acid, which is also one of the most abundant glycosaminoglycans in native aortic heart valves. Future experiments will point out if interstitial cells loaded into these hydrogels sufficiently behave like VICs in aortic valves. This would significantly facilitate ongoing efforts to unravel processes that initiate CAVD and allow for proper testing of potentially therapeutic agents.

Where Do We Stand In Identification Of Pathways Leading To CAVD?

In chapters 6-8 of this thesis, we have briefly described what is currently known about pathways leading to CAVD and our contributions to this knowledge. For practical reasons, we have not thoroughly described all pathways that have been previously identified to be involved, but several reviews are available.[3, 95, 239, 282, 289] Popular proteins suggested to be involved in progression of CAVD are Bone Morphogenetic Proteins (BMPs), Transforming Growth Factor (TGF-) β, Tumor Necrosis Factor (TNF-) α and Receptor Activator of Nuclear Factor-κb (RANK) [282], which are supposedly secreted by macrophages following influx of Low-Density Lipoproteins and

monocytes. Although this may present a likely possibility, it does not answer the question on how these processes are initiated and more importantly, which of these numerous options could be a best suitable target for future therapies.

Initiation of CAVD has been suggested to show parallels with atherosclerosis, but pharmacological therapies which can be successfully applied to limit atherosclerosis, do not have the same effect on CAVD (chapter 6). The processes behind onset of CAVD are still unknown. In addition, there is no notable knowledge on production and repair of matrix structures during normal physiological regeneration of the valve, let alone how these processes may become overactive to cause fibrosis and sclerosis. This would be a logical first step in order to study onset of CAVD and to search for possible new treatment options. We have plans for a number of studies to investigate these processes in the near future.

Subsequently, influx of LDL particles and monocytes can be valid explanations for presence of high concentrations of growth factors and cytokines which contribute to progression of CAVD. Unfortunately, it is difficult to determine local concentrations of these growth factors in living tissue and even more difficult to determine their activity. This has led to numerous studies in which the presence of growth factors in calcifying valves are nicely shown, followed by *in vitro* studies in which cells are stimulated with high doses of growth factors, even though it is uncertain if these high concentrations are present in the valve and if they represent similar growth factor activities. The results of these studies therefore show that all these growth factors may contribute to progression of valvular heart disease, given that they are available in the valve at certain concentrations, which are not known exactly. Moreover, most of these studies do not consider crosstalk between several pathways that could be involved, or do not consider the mechanical challenging environment in which the VICs reside, which may also alter their response to stimuli. Taken together, it can be concluded that all the proposed pathways are probably capable of inducing CAVD, but it remains unknown if they play an important role *in vivo*. Using the previously described hydrogels we work on in collaboration with the group at the Harvard Medical School, we aim to investigate the role of growth factors and cytokines in the onset of CAVD more closely. Better animal models are also required to study the importance of these factors *in vivo*.

Personal Perspective On Future Therapies For CAVD

In the introduction of this thesis, I have described the advantages and shortcomings of current heart valve prostheses. I have shown that bio-prostheses can be used in many patients, albeit with a high risk of reoperation in younger age-groups. Subsequently, I have introduced tissue engineering and pharmacological treatment as new options for the future. In the following section I will present my personal perspective on how patients with (severe) CAVD can or should be treated in the future.

The development of tissue engineered heart valves, according to the classical paradigm with *in vitro* cell expansion and seeding onto biodegradable scaffolds, will need more time to allow clinical application. Although promising results have been obtained in the pulmonary position in sheep, it has also been shown that unexpected events can further delay successful use in patients. Fibrosis, stiffness, retraction and calcification of tissue engineered cusps are still frequent observations and have not been properly resolved. In addition, resolving these issue in constructs produced with conventional, animal serum-containing culture media may temporarily be a step forward, but subsequent experiments with human serum-additives may give different results. This brings the tissue engineered valve back to the laboratory bench. Therefore, I personally believe that the preliminary results of our group with autologous human serum-supplements, presented in this thesis, should be applied in tissue engineering strategies rather sooner than later. Existing problems with tissue engineered heart valves should be addressed in constructs produced under protocols suitable for clinical application and the results should be more closely compared to constructs with autologous cells from the applied pre-clinical animal model.

Meanwhile, a number of different "tissue engineering" strategies are being developed. Other groups are experimenting with de- and recellularization of homografts [81, 290] and animal valves [78, 87]. We and others experiment with *in vivo* recellularization of implanted synthetic scaffolds.[83] These approaches are also subjected to limitations. The use of homografts is limited by availability and degeneration, the use of xenograft valves induces immune responses and implantation of synthetic scaffolds is dependent on the development of load-bearing constructs that should not attract cells from the blood other than the cells required for successful reseeding of

the construct. It will therefore be interesting to observe which of these approaches is first to reach good long term results in animal models, which are also considered sufficiently predictive for use in humans to allow clinical application. Although the conventional tissue engineered heart valve is ideally the best possible replacement for a diseased aortic valve, I am anxious to see if funding organizations have enough patience to invest in the concept until it is clinically successful.

The other aim to prevent CAVD would be development of a therapeutic agent that successfully arrests progress of the condition. However, as described in chapter 6, this would raise the question to whom this therapeutic agent would be prescribed. This would primarily be determined by which process is directly targeted by the neo-drug. Patients suffering from congenital valve diseases are known to have a predisposition for CAVD, but it is questionable if therapeutic agents can be applied to protect cells from mechanical cues, which are supposedly causing early onset of CAVD in these patients. Alternatively, it could be proposed to focus primarily on late calcification processes in diseased valves. Sclerotic valves are usually not stenotic or regurgitant until they calcify so it is tempting to think that prevention of calcification significantly postpones onset of symptoms. The problem with this approach, however, would be that patients with sclerotic valves are usually not seen by physicians until they have become symptomatic. My personal opinion would be that development of a therapy that stops calcification would have the highest chance to succeed. It might be an option to explore if this therapy can be included in future "polypills", aiming to reduce risk factors associated with cardiovascular conditions, and to be administered to all patients above a certain age or with certain risk factors. Our work on Wnt-signaling and the comparison of microRNAs in healthy and diseased valves is a small preliminary step in this direction. Possibly, echocardiography could be performed on all patients above a certain age to screen for patients with from aortic valve sclerosis. This group of patients includes 26% of the adults over 65 years old. Identification of patients with aortic valve sclerosis would ensure more directed treatment of CAVD in the future.

In summary, I think that aortic valve replacements in the near future, will still be performed with conventional valve prostheses. The development of alternative prostheses will also have to compete with ongoing improvements of these conventional prostheses by companies, which will also delay the use of the new strategies. If tissue engineering strategies eventually emerge,

they are most needed in children with congenital valve diseases, which are the most vulnerable group with current prostheses. Interestingly, a survey among cardiologists, cardiothoracic surgeons and patients revealed that the first patients in which tissue engineered heart valves may be tested are the old and high risk patients who currently undergo transcathether aortic valve replacement (personal observation). Because pharmacological therapies will not be able to successfully prevents calcification in all patients, there will always be a place for aortic valve replacement surgery.

Conclusion

The work presented in this thesis can be considered as an important, but small step forward in the search for new therapies to cure patients suffering from CAVD. However, we are still far from effective treatment of this disease and obvious alternatives for currently existing valve prostheses have not emerged yet. Therefore, extensive additional research on tissue engineering strategies and pathways involved in aortic valve calcification is warranted.

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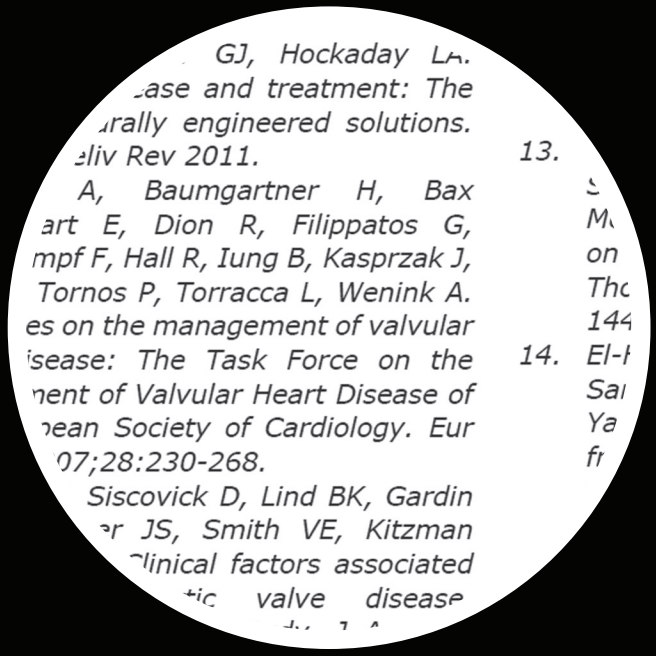

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Nederlandse

Samenvatting

De aortaklep is de klep tussen de linker kamer van het hart en de lichaamsslagader (aorta), die ervoor zorgt dat het bloed slechts in één enkele richting kan stromen. De klep opent en sluit 3-4 miljard keer in een mensenleven en moet bij elke keer dat hij sluit het bloed opvangen dat door drukverschillen terug richting het hart wil stromen. Hartklep verkalking is een aandoening die het functioneren van de aorta klep ernstig kan belemmeren. Kalk afzettingen op de klep maken het openen en sluiten van de klep moeilijk, wat kan resulteren in een vernauwde en/of lekkende klep. Hierdoor moet het hart harder werken, wat uiteindelijk kan resulteren in hart falen en overlijden.

Aortaklep verkalking is voornamelijk een aandoening die leeftijds-gerelateerd is, maar kent ook een genetische component. Van de ouderen boven de 65 jaar heeft 2-3% last van ernstige aortaklep vernauwing en de verwachting is dat het aantal patiënten in de toekomst flink toe zal nemen. Ten eerste omdat mensen steeds ouder worden en de populatie patiënten boven de 65 aanzienlijk zal toenemen. Daarnaast is het bekend dat een veel groter percentage van de mensen lijdt aan een nog milde vorm van aortaklep verkalking (sclerose), waarvan bekend is dat deze uiteindelijk kan overgaan in ernstiger, symptomatische vormen van aortaklep lijden.

Helaas is er op dit moment nog geen geneesmiddel op de markt dat progressie van aortaklep verkalking kan remmen en is de enige behandeling een klepvervanging via een gesloten hartoperatie of een meer uitgebreide open hart operatie op het moment dat de aandoening het leven in gevaar brengt. De huidige hartklep protheses hebben ieder hun specifieke nadelen: er is een beperkte levensduur van de biologische klep of de patient moet levenslang bloedverdunners gebruiken ter voorkoming van bloedstolselvorming door kunststofkleppen. Daarnaast groeien de kleppen niet mee, wat noodzakelijk is voor gebruik bij kinderen.

Tissue engineering, of kunstmatige weefsel reproductie, heeft de potentie om een ideale klepprothese te maken met lichaamseigen cellen van patiënten. In theorie wordt een “tissue engineerde klep” niet afgestoten door het lichaam, groeit mee en kan zichzelf beschermen tegen kleine beschadigingen ten gevolge van het vele openen en sluiten van de klep. Deze techniek is in het laboratorium al meerdere malen getest en de eerste dierproeven, noodzakelijk om de situatie in het menselijk lichaam zo dicht mogelijk te benaderen, zijn ook uitgevoerd. Echter, de manier waarop op dit moment deze weefselkleppen worden gemaakt, zijn niet wenselijk voor

het gebruik in patiënten. Sommige componenten die gebruikt worden om de weefselkleppen te maken zijn van dierlijke oorsprong en het gebruik van deze producten kan tot afstoting van de klep leiden. Het is noodzakelijk om zoveel mogelijk, zo niet alle dierlijke componenten uit te bannen.

In hoofdstuk 2 wordt het proces van embryonale vorming van menselijke kleppen beschreven, gevolgd door een analyse van de mogelijkheden om bepaalde onderdelen van dat proces ook in te zetten bij het perfectioneren van tissue engineering strategieën. In hoofdstuk 3 wordt met dat perfectioneren een begin gemaakt. Dierlijk serum in het medium voor de kweek van klepcellen werd vervangen voor menselijk serum verrijkt met eiwitten uit bloedplaatjes, ook wel plaatjes-lysaat genoemd. Bloedplaatjes helpen bij wondgenezing door vorming van stevige collageen vezels te stimuleren en nabij gelegen cellen te laten vermeerderen. Deze eigenschappen van bloedplaatjes hopen wij ook in te zetten voor tissue engineering. De resultaten in hoofdstuk 3 laten zien dat bij cel studies het beoogde effect aanwezig is. Hoofdstuk 4 beschrijft vervolgens de productie van kleine stripjes weefsel die model staan voor toekomstig klepweefsel. Dit onderzoek laat zien dat het weefsel dat geproduceerd wordt op deze wijze te slap is om de druk van het bloed op te vangen. Vermoedelijk komt dit doordat het collageen dat gevormd wordt ook te snel weer wordt afgebroken. Daarom hebben we in hoofdstuk 5 de stelling getest dat het gebruik van humaan serum zonder plaatjes-lysaat wellicht voor steviger weefsel zorgt. Dit vermoeden blijkt juist en bovendien blijkt dat een combinatie mogelijk is van snel opkweken van cellen met behulp van plaatjes-lysaat en het vormen van sterk weefsel door daarna over te gaan op humaan serum. In toekomstige experimenten zou dit het ideale protocol moeten zijn voor productie van weefselkleppen voor gebruik bij mensen.

Een alternatief voor het vinden van de ideale klepprothese, zou het voorkomen van klepverkalking zijn door de ontwikkeling van nieuwe medicijnen. Om vroege verkalking van kleppen gemaakt met behulp van tissue engineering te voorkomen, is begrip nodig van de processen die aortaklep verkalking veroorzaken. Voor zover bekend worden deze processen in hoofdstuk 6 beschreven en met elkaar vergeleken. Tevens wordt beschreven welke therapieën tot op heden al getest zijn, allen overigens zonder positief resultaat. In hoofdstuk 8 wordt vervolgens een begin gemaakt met het vinden van aangrijpingspunten voor nieuwe doelwitten voor medicijnen. Er wordt beschreven hoe het eiwit BMP een signaleringsmechanisme in de cel activeert dat tot op heden nog niet beschreven was met betrekking tot

aortaklepverkalking. In samenwerking met een onderzoeksgroep uit Londen werd een nieuw signaleringsmechanisme gevonden, aangestuurd door een ander eiwit (Wnt). Mogelijk kan dit mechanisme als aangrijpingspunt gebruikt worden voor toekomstige medicamenten.

Tenslotte wordt er in hoofdstuk 8 nog aandacht besteed aan microRNAs. Dit zijn kleine moleculen waarvan recentelijk is gebleken dat ze een belangrijke rol spelen bij regulering van eiwit productie in de cel. Daardoor kunnen ze vele cellulaire eigenschappen aan sturen. Wij hebben het profiel van microRNAs in gezonde aortakleppen vergeleken met het profiel in verkalkte kleppen en daarin verschillen aangetroffen. Een aantal van de verschillen zouden kunnen wijzen op betrokkenheid van specifieke microRNAs bij klepverkalking en derhalve nieuwe mogelijkheden openen voor onderzoek naar klepverkalking en therapien om dit te voorkomen. Dit proefschrift eindigt met een beschouwing van de door ons behaalde resultaten (hoofdstuk 9), een aantal voorstellen voor toekomstig werk en mijn persoonlijke perspectief op de toekomst van behandeling van aortaklep verkalking.

Dankwoord


THE LAB
THAT TIME
FORGOT!
MARVEL! AT THE LACK OF RESOORCES AND OOT DATED EQOIPMENT
AT THE LETHARGIC PACE AND MIND NOMBING REPETITIVENESS
DISBELIEF AS OOR DESPERATE HEROES STROGGL

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List of Publications

List of publications

1. Platelet-lysate as an autologous alternative for fetal bovine serum in cardiovascular tissue engineering. **Riem Vis PW**, Bouten CV, Sluijter JP, Pasterkamp G, van Herwerden LA, Kluin J. Tissue Eng Part A. 2010 Apr;16(4):1317-27.
2. Environmental regulation of valvulogenesis: implications for tissue engineering. **Riem Vis PW**, Kluin J, Sluijter JP, van Herwerden LA, Bouten CV. Eur J Cardiothorac Surg. 2011 Jan;39(1):8-17.
3. Lack of fibronectin-EDA promotes survival and prevents adverse remodeling and heart function deterioration after myocardial infarction. Arslan F, Smeets MB, **Riem Vis PW**, Karper JC, Quax PH, Bongartz LG, Peters JH, Hoefer IE, Doevendans PA, Pasterkamp G, de Kleijn DP. Circ Res. 2011 Mar 4;108(5):582-92.
4. Decreased mechanical properties of heart valve tissue constructs cultured in platelet lysate as compared to fetal bovine serum. **Riem Vis PW ***, van Geemen D *, Soekhradj-Soechit S, Sluijter JP, de Liefde-van Beest M, Kluin J, Bouten CV. Tissue Eng Part C Methods. 2011 May;17(5):607-17.
5. Sequential use of human-derived medium supplements favors cardiovascular tissue engineering. **Riem Vis PW**, Sluijter JP, Soekhradj-Soechit RS, van Herwerden LA, Kluin J * , Bouten CVC *. J Cell Mol Med. 2011 Jun 7.
6. Variation in ovine and human engineered cardiovascular constructs to predict the outcome of heart valve tissue engineering. van Geemen D, Driessen-Mol A, Grootzwagers LGM, Soekhradj-Soechit RS, **Riem Vis PW**, Baaijens FPT, and Bouten CVC. Submitted.
7. The pathophysiological basis for pharmacological interventions in CAVD. **Riem Vis PW**, van Rijswijk JW, Chamuleau SAJ, Vink A, van Herwerden LA, Kluin J. Accepted for publication. Neth Heart J.
8. The role of BMP/Wnt signaling in human heart valve calcification. **Riem Vis PW ***, Chester AH *, Osman L, Latif N, Sarathchandra P, Kluin J, Taylor PM, Yacoub MH. Submitted.

Curriculum Vitae

Curriculum Vitae

Paul Riem Vis was born on february 24th, 1982 in The Hague, The Netherlands. After graduating from secondary school in 2000 (Dalton Scholen Gemeenschap, Den Haag), he spent one year in the United States to obtain his High School diploma (Riddle High, Riddle, OR, USA). Several attempts to get into Medical School failed and through one year Biology, he entered Bio-Medical Sciences in 2002 and graduated in 2006. After another failed attempt to enter SUMMA, he started his PhD at the department of Cardio-thoracic Surgery at the UMC Utrecht in September 2007, supervised by Prof. dr. L. A. van Herwerden, Prof. dr. C.V.C. Bouten, dr. J. Kluin and dr. J. Sluijter. During this PhD, he also performed a four month internship at the research institute of Prof. Sir Magdi Yacoub, FRS in London, supervised by dr. A. Chester. To finance this, he has been awarded the van Wijck - Stam - Caspers beurs and the EMBO Short term scholarship. As of January 1st 2012, he will continue his work as a postdoctoral research fellow at the Cardio-thoracic Surgery department.