Human prenatal progenitors for pediatric cardiovascular tissue engineering

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Human Prenatal Progenitors for Pediatric Cardiovascular Tissue Engineering

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“Movement is the cause of all life.” - *Leonardo da Vinci*

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Summary

Human Prenatal Progenitors for Pediatric Cardiovascular Tissue Engineering

Pediatric cardiovascular tissue engineering is a promising strategy to overcome the lack of autologous, growing replacements for the early repair of congenital malformations in order to prevent secondary damage to the immature heart. Therefore, cells should be harvested during pregnancy as soon as the cardiovascular defect is detected enabling the generation of living autologous implants with the potential of growth, remodeling and regeneration ready to use at or shortly after birth. Furthermore, the ideal cell source should be easily accessible and allow cell harvest without substantial risks for both the mother and the child and without sacrifice of intact infantile donor tissue. In this work, human prenatal progenitor cells obtained from different extra-embryonically situated fetal tissues were investigated with regard to the pediatric cardiovascular tissue engineering concept.

In individual studies prenatal progenitor cells were isolated from different fetal tissues including umbilical cord blood and cord tissue, chorionic villi and amniotic fluid. Cells were expanded and differentiated into cell types that are required for cardiovascular replacements in order to match the characteristics of their native counterparts: a myofibroblast-fibroblast-like cell type producing extracellular matrix and an endothelial cell type forming an antithromobogenic and blood-compatible surface. Thereby, cell phenotypes were analyzed by flowcytometry and immunohistochemistry and genotypes were determined. For the fabrication of cardiovascular tissues, biodegradable cardiovascular scaffolds (PGA/P4HB) were seeded with fibroblast-myofibroblast-like cells derived from either umbilical cord tissue, chorionic villi or amniotic fluid. Constructs were implanted in an in vitro pulse duplicator and exposed to biochemical and/or mechanical stimulation. After, in vitro maturation time, the surfaces of cardiovascular constructs were endothelialized with differentiated umbilical cord blood-derived endothelial progenitor cells or amniotic fluid-derived endothelial progenitor cells and conditioned for an additional 7d. Analysis of the neo-tissues comprised histology, immunohistochemistry (vimentin, α-SMA, desmin, Ki-67), biochemistry (extracellular matrix (ECM) - analysis, DNA), mechanical testing and scanning electron microscopy (SEM). Neo-endothelia were analysed by immunohistochemistry (CD31, vWF, thrombomodulin, tissue factor, eNOS).

After differentiation, cells demonstrated characteristics of fibroblast-myofibroblast-like cells expressing vimentin, desmin and partly α-SMA independent of the cell source. Furthermore, umbilical cord blood-derived endothelial progenitor cells and amniotic fluid-derived cells expressed typical endothelial cell markers such as CD31, vWF, thrombomodulin, tissue factor, and eNOS, respectively. Genotyping confirmed the fetal origin of the cells without contamination with maternal cells. All cardiovascular constructs showed cellular tissue formation with functional endothelia as indicated by the expression of eNOS. Expression of Ki-67 confirmed proliferation of cells in all parts of the neo-tissues. Matrix analysis (collagen and proteoglycans) and DNA content demonstrated constituents typical of native cardiovascular tissues. Mechanical properties revealed native analogous profiles but did not reach native values. SEM showed cell-ingrowth into the polymer and smooth surfaces covered densely with endothelial cells.

Prenatal progenitors from different sources were successfully used for the in vitro fabrication and maturation of living autologous cardiovascular constructs. With regard to clinical application the use of amniotic fluid-derived prenatal progenitor cells represents the most attractive approach as it enables the prenatal fabrication of cardiovascular replacements based on a single cell source ready to use at birth.

Abbreviations and acronyms

α-SMA = α-smooth muscle actin
CD = Cluster of differentiation
ECM = Extracellular matrix
EGF = Epidermal growth factor
eNOS = Endothelial nitric oxide synthase type III
EPCs = Endothelial progenitor cells
FGF = Fibroblast growth factor
GAG = Sulphated glycosaminoglycans
H&E = Hematoxylin-Eosin
HUVEC= Human umbilical cord endothelial cells
IGF-1 = Insulin-like growth factor-1
PGA = Polyglycolic-acid
P4HB = Poly-4-hydroxybutyric acid
WMF = Wharton's Jelly-derived myofibroblasts
UEA-1 = Ulex europeus agglutinin-1
VEGF = Vascular endothelial growth factor
VEGFR-2 = Vascular endothelial growth factor-receptor-2
vWF = von Willebrand factor

Chapter 1

General introduction

The contents of this chapter are partly based on

D. Schmidt and S.P. Hoerstrup (2006).
"Heart Valve Tissue Engineering: Choosing the Right Cell Source."
In: Transcatheter Valve Repair. Editors: Hijadzi Z.M., Bonhoeffer P., Feldmann T., Ruiz C.E. Pp. 347-356; Taylor & Francis [ISBN: 1-84184-472-1]

and

D.Schmidt and S.P. Hoerstrup
"Tissue Engineered Heart Valves Based on Human Cells."
Swiss Med Wkly, 136: 618-623 (2006)

1.1 Introduction

1.1.1 Congenital heart disease

Today, congenital heart disease is one of the most frequent congenital disorders affecting almost 1% of all newborns (8 per 1000). Moreover, it remains the most important cause of death in the first year after birth (Centers for Disease Control, 1989). Congenital heart disease, also called congenital heart defect, includes a variety of malformations of the heart or of its major blood vessels that are present at birth. It ranges from minor heart murmurs that require no treatment to fatal complex structural defects such as tetralogy of Fallot, transposition of great arteries and hypoplastic left or right heart.

Causes and risk factors

The majority of congenital heart diseases occur as an isolated defect that is not associated with other extracardiac diseases. In 90% of the cases there is no identifiable cause leading to the heart defect and multifactorial inheritance, the combined effects of one or more alleles at a number of loci interacting with stochastic and/or environmental factors, is considered as reason (Nora, 1993). Maternal infection, e.g. rubella infections during the first trimester of pregnancy, and maternal medication including anticonvulsiva, retinoic acid and alcohol represent risk factors and might contribute to the development of congenital heart defects. Furthermore, maternal illness such as lupus erythematosus, diabetes and phenylketonuria increases the prevalence.

In 8% of affected infants (Nora, 1993) congenital heart disease is associated with genetic and chromosomal syndromes such as trisomies 13, 18, 21, the monosomy X0 (Turner`s syndrome), Marfan syndrome, Noon syndrome, Ellis-van Creveld syndrome and microdeltion 22q11 (CATCH 22 syndrome). Other studies suggest that the frequency of congenital heart defects due to single-gene-defects is even higher (Payne *et al.,* 1995).

Diagnosis

Although no prevention therapy for congenital heart disease exists, early detection of the defect is crucial in order to enable a careful monitoring of pregnancy as well as an optimal perinatal and postnatal management of the affected child. Since most of the cardiovascular defects develop during pregnancy without increased risk fetal ultrasound screening for detection of congenital malformations is recommended and today part of routine prenatal care in most of the western countries. As ultrasound technology and operator skills improved in the last years more fetal malformations now are recognized already prior to birth by ultrasound scanning (Sollie *et al.*, 1988; Sharland and Allan, 1992; Grandjean *et al*., 1999) facilitating the initiation and planning of the most optimal interdisciplinary peri- and postnatal therapeutic strategy.

Current treatment and limitations

Representing a complex anatomical and patho-physiological spectrum, congenital heart disease usually becomes symptomatic at or shortly after birth when the fetal circulation has to adapt to the new extra-maternal environment.

Therefore, the optimal time for therapeutic interventions is shortly after birth to prevent cardiopulmonary decompensation as well as secondary damages to the infantile heart. Over the past 30 years approaches to optimize the outcome of congenital heart disease therapy have mainly been focused on establishing early surgical treatment, improved surgical methods and a more detailed understanding of the underlying patho-physiology. As a result, both short term and long-term survival have improved considerably (Daenen *et al*., 2003). Today, the overall mortality for closed procedures is estimated at 1.7% (range 0 %-3.8%) and 4.8% (range 2.2%-7.4%) for open heart surgery (Stark *et al*., 2000).

However, a major limitation for the repair of congenital cardiovascular defects still persists: the lack of viable replacement materials with the capacity of growth and regeneration. Particularly for heart valve replacements, the commonly used artificial prostheses being either mechanical or bioprosthetic (Mayer, 1995; Schoen and Levy, 1999) are unable to grow with the infant body and cardiovascular system necessitating re-operations associated with increased risks of morbidity and mortality each time. Even in the Ross procedure when the diseased aortic valve is replaced with the autologous pulmonary valve an prosthesis to replace the transplanted pulmonary valve is still required.

Remaining inherently different from the tissue they replace and representing non-living materials, both manufactured mechanical and biological valve replacements do not actively adapt to the physiological environment such as pressure changes and mechanical demands. In addition, all contemporary non-living heart valve replacements lack the capacity for self-repair, remodeling or growth. Furthermore, they are often associated with shortcomings such as material failure, calcifications, obstructive tissue ingrowth, and increased rate of infections, thromboembolism and immunological reactions against the foreign material (Hammermeister *et al*., 1993; Schmidt and Baier, 2000).

1.1.2 Requirements for the "ideal" cardiovascular replacement

Many criteria have to be considered as to the optimal vascular replacement. The main requirements are summarized in Table 1.1.

Table 1.1: *Requirements for the ideal cardiovascular substitute.*

Properties of the ideal cardiovascular replacement
• biocompatiple
• non-thrombogenic
• non-immunogenic
• matching mechanical properties of native counterpart
• capacity to remodel and regenerate
• nature-analogous healing process
• resistant to infection after implantation
• causing no inflammation
• no hyperplasia
• integration into surrounding tissue
• physiological responses as to environmental demands

The ideal cardiovascular tissue replacement would be a copy of their native counterparts comprising adequate mechanical function, durability, adequate haemodynamic performance, as well as the absence of immunogenic, thrombogenic and/or inflammatory reactions. Such goals were first outlined by Dwight F. Harken in 1962 (Harken *et al*., 1962) for artificial heart valve prostheses but do represent commandments for cardiovascular replacements in general.

Furthermore, after implantation in vivo the integration of the cardiovascular replacement to the host tissue is crucial. Thus, the implant must induce an acceptable nature-analogous healing response that does not result in inflammation, hyperplasia, or fibrous capsule formation. Ideally, it would lead to a complete integration of the

substitute into the cardiovascular system becoming indistinguishable from the surrounding and connecting nature tissue.

Additionally, the ideal substitute would exhibit mechanical properties similar to its native counterparts including appropriate physiological compliance and tissue strength in order to withstand hemodynamic pressure changes without failure. Moreover, responses to physiological changes and integration into the complex cardiovascular system by means of adequate vasoconstriction and relaxation pose an additional challenge for the ideal vascular replacement. For pediatric application it would be crucial that the substitute grows with the infant body in order to avoid re-operations today associated with increased mortality and morbidity each time. Therefore, a living autologous cardiovascular prosthesis with growth, remodel and repair capability would be the ideal substitute.

1.1.3 The "gold standard" - Architecture and characteristics of native cardiovascular tissues

General structure

The architecture of cardiovascular structures such as heart valves and blood vessels differs depending on the function of the tissues in the complex cardiovascular system. However, there is a common structural pattern that can be found in all cardiovascular tissues. The surface represents a blood-compatible and anti-thrombogenic layer that is formed by endothelial cells. The tissue beneath the endothelium is composed in a layered fashion and consists of interstitial cells that are responsible for the production of extracellular matrix. The extracellular matrix of cardiovascular tissues comprises mainly collagen, glycosaminoglycans and elastin enabling the mechanical properties of the tissues i.e. elasticity, tissue strength and flexibility.

Heart valves

The four heart valves have microstructural similarities. However, the trileaflet semilunar aortic valve best illustrates the essential features and serves as a paradigm for microstructural and cellular adaption to functional requirements.

The aortic valve leaflets are covered with endothelial cells enabling the smooth blood flow during opening and closing of the valve and regulating immune and inflammatory reactions. The heart valve leaflet tissue itself consists of three layers (i) the ventricularis that is closest to the inflow surface and rich in elastin (ii) the fibrosa that is closest to the outflow surface and comprises densely packed collagen and (iii) the between these two layers located spongiosa that is largely composed of glycosaminoglycans. Figure 1.1 illustrates this layered architecture schematically.

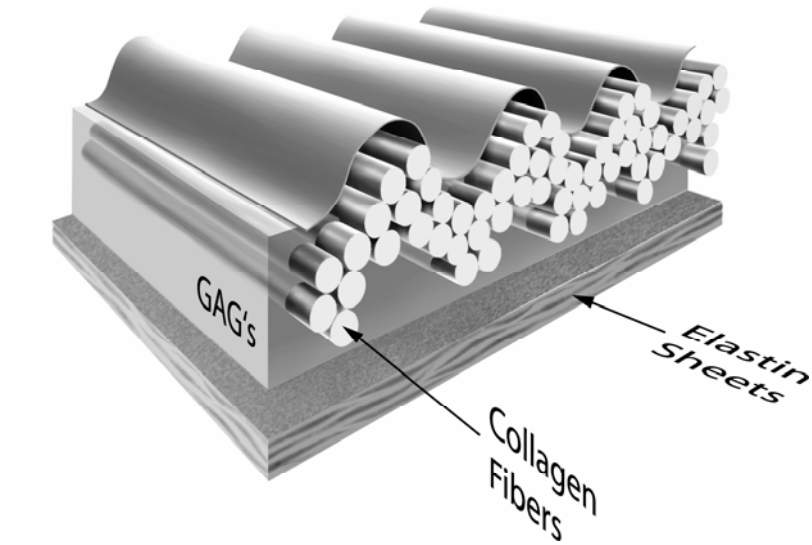

Figure 1.1: *Schematic of the extracellular matrix formation in heart valve leaflets.*

The collagen fibers in the fibrosa represent the strongest part of the valve leaflets and are mainly responsible for bearing diastolic stress. In contrast, the elastin in the ventricularis restores the contracted configuration of the leaflets during systole. During valve opening elastin stretches during extension of collagen crimp and corrugations. When the valve is closing, the elastin gets unfolded and the load shifts from elastin to collagen. Thereby, glyocosaminoglycans in the spongiosa may serve as a shock and shear absorber.

Valvular interstitial cells are responsible for the production of this well defined microstructure by synthesizing the mentioned extracellular matrix components and expressing matrix degrading enzymes and their inhibitors. Their phenotype is dynamic ranging from fibroblast-like to myofibroblast-like depending on the environmental conditions.

Blood vessels

Native blood vessels demonstrate a three-layered tissue architecture comprising (i) an endothelium lined intima, (ii) a media containing smooth muscle cells, and (iii) an adventitia with connective tissue as demonstrated in Figure 1.2.

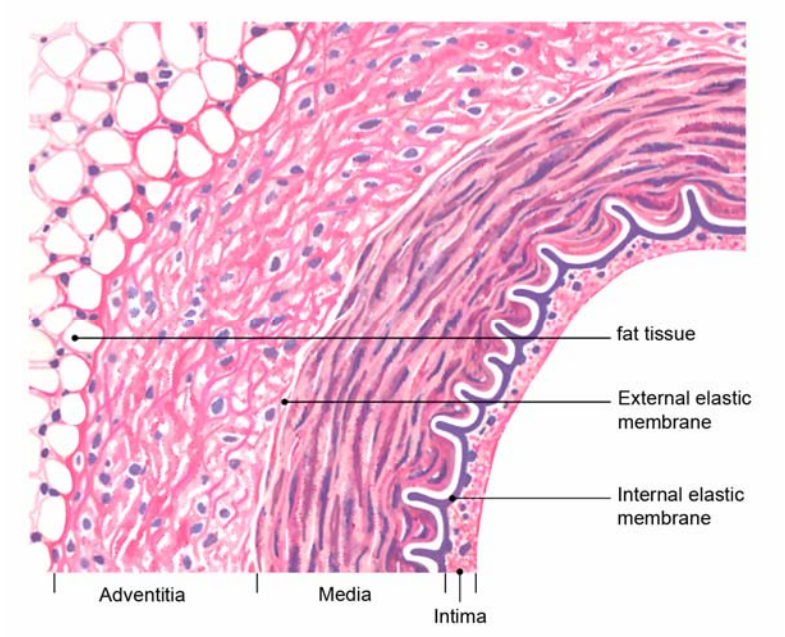

Figure 1.2: *Architecture of a blood vessel.*

(i) As the inner layer the intima delimits the vessel wall towards the lumen. It is formed by a functional endothelium having anti-thrombogenic properties. Because of tight intercellular connections it works as a barrier. Beneath the endothelium an associated connective tissue is present followed by an internal elastic membrane that separates the intima from the media.

(ii) As the middle layer the media consists of one or multiple layers of smooth muscle cells (SMC) and elastic lamellas with variable amounts of connective tissue. Each layer of SMC and elastic lamella form a lamellar unit. The external elastic membrane is located beneath the outmost lamellar unit and separates the media from the adventitia.

(iii) As the outer layer the adventitia consists of blends of connective and fat tissue anchoring the vessel with the surrounding neighbour-tissue.

Two major types of blood vessels, namely arteries and veins, differing in their functionality and therewith in the thickness of the different layers can be distinguished.

Arteries can be classified into three different types: (i) elastic arteries such as the large diameter vessels originating from the pulmonary trunk, the pulmonary trunk and the aorta, (ii) muscular arteries that are medium seized arteries such as the carotic arteries, and (iii) the arterioles representing the small arteries with a diameter less than

2 mm. The main histological characteristic of differently sized arteries represents the thickness of the different layers. The intima of elastic arteries is much thicker compared to that of arteries from the muscular type. In contrast, the media of muscular arteries is dominated by numerous concentric layers of smooth muscle cells with fine elastic fibers, whereas the media of arteries from the elastic type comprises about 50 elastic lamellae.

In contrast to artery walls, those of veins are thinner while their diameter is larger. Furthermore, the stratified architecture of vein walls is not very distinct. The intima is thin, internal and external elastic membranes are absent or tenuous. The media appears thinner than the adventitia and the two layers tend to blend into each other. Moreover, valves, essentially endothelial folds, are found in many veins, particularly in veins of the extremities.

1.1.4 Pediatric cardiovascular tissue engineering – A strategy to overcome the lack of living graft materials for the repair of congenital cardiovascular defects

The above mentioned limitations of the currently available cardiovascular prostheses have motivated the exploration of novel approaches towards living autologous cardiovascular replacements. A series of studies has been undertaken to determine if tissue engineering principles can be used to develop viable, cardiovascular substitutes with a thromboresistant surface and a viable interstitium with repair, remodelling and growth capabilities. Several groups demonstrated the feasibility of creating living cardiovascular structures by autologous cell seeding on synthetic polymers, biological matrices, or xenogeneic scaffolds (Shinoka *et al*., 1995; Bader *et al*., 1998; Hoerstrup *et al*., 2000a; Elkins *et al*., 2001; Taylor *et al*., 2002; Sutherland *et al*., 2005). As to heart valve tissue engineering the first milestone was the successful replacement of a single pulmonary valve leaflet by a tissue engineered autologous leaflet (Shin`oka *et al.*, 1995) followed by the implantation of a living, autologous tissue engineered trileaflet heart valve in an animal model (Hoerstrup *et al.,* 2000a).

Regarding the requirements for an ideal living autologous replacement with growth, remodelling and regeneration potential for the repair of congenital heart defects, optimally, the tissue engineered construct would be available when the first corrective intervention is performed in order to avoid re-operations. This might be at or shortly after birth in order to prevent secondary damage to the immature heart. Thus, the tissue engineering process has to be initiated prior to birth providing sufficient time for the fabrication of a living autologous cardiovascular replacement. Consequently, the prerequisites have to be provided and prepared prenatally including the fetus` own cells enabling the successful engineering of the tailor-made living autologous cardiovascular tissue replacement. Ideally, autologous cells are harvested in a minimally invasive low-risk procedure as soon as the cardiovascular defect has been detected by ultrasound and processed for the fabrication of the living cardiovascular replacement.

1.1.5 Strategies in autologous cardiovascular tissue engineering

Two strategies have been used to generate such living autologous cardiovascular replacements. One requires an in vitro phase generating the replacement ex vivo (Langer and Vacanti, 1993), the other bypasses the in vitro tissue culture phase by direct implantation of e.g. natural tissue-derived heart valve matrices for potential cell ingrowth and remodelling in vivo (Matheny *et al*., 2000). Matrices used for the latter approach included decellularized tissues derived from pericardium or valves, cell free porcine small intestine submucosa (Matheny *et al*., 2000), synthetic biodegradable polymeric scaffold (Hoerstrup *et al*., 2000a) or biological matrices such as collagen (Taylor *et al.*, 2006) or fibrin gel (Jockenhövel *et al*., 2001). Hitherto, decellularized scaffolds implanted in humans demonstrated ingrowth of host cells, no calcification but a strong inflammatory response (Simon *et al*., 2003). The structural failure of these materials inhibited further use.

1.1.6 Concept of in vitro cardiovascular tissue engineering

Following the approach of in vitro tissue engineering, the successful fabrication of autologous living cardiovascular replacements similar to their native counterparts is based on three main components: (1) autologous cells that resemble their native counterparts in phenotype and functionality; (2) a temporary supporter matrix (biodegradable scaffold) which promotes tissue strength until the extracellular matrix produced by the autologous cells guarantees functionality on its own; and (3) culture conditions enabling tissue formation and maturation by in vitro conditions similar to a physiological environment.

In cardiovascular tissue engineering, these three components have to be chosen and controlled in a highly orchestrated manner to meet the mechanical requirements of the neo-tissue at the time of implantation

Figure 1.3 summarizes the interplay of these components for in vitro tissue engineering.

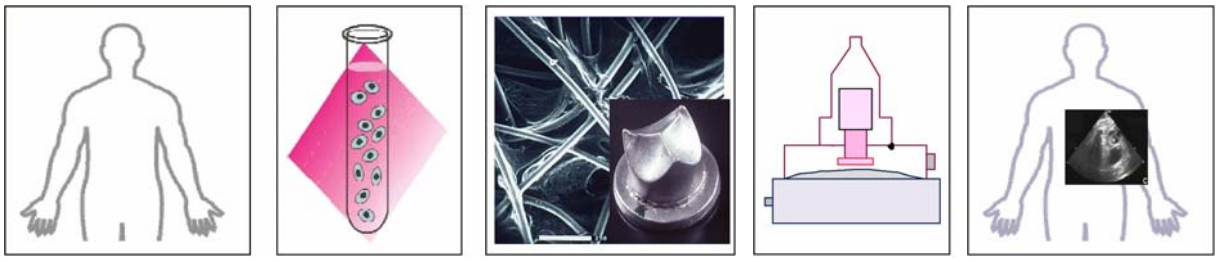

Figure 1.3: *Concept of cardiovascular in vitro tissue engineering: Autologous cells are harvested from the patient. After expansion, cells are seeded onto a biodegradable scaffold material (here: PGA/P4HB) and preconditioned in a bioreactor. After tissue development, the autologous replacement is ready for implantation.*

As depicted schematically in Figure 1.3, cells first are harvested from an autologous donor structure. After in vitro expansion, cells are seeded onto a biodegradable cardiovascular scaffold, preferably with a porosity of at least 90% (Agrawal and Ray, 2001). Scaffolds could be fabricated either from synthetic materials or biological structures. Table 1.2 provides an overview of the most common scaffold types applied in cardiovascular tissue engineering.

Table 1.2: *Most commonly applied scaffold materials for cardiovascular tissue engineering.*

Scaffold	**Source**	**Examples**	**Reference**
synthetic	biocompatible and biodegradable polymers	• polyglycolic acid (PGA) • polylactic acid (PLA) • polyhydroxyalkanoates (P3HB) • PGA and PLA (PGLA) • PGA and P4HB	• Shin`oka *et al.,* 1996 • Shin`oka *et al.,* 1998 • Sodian *et al.,* 2000 • Zund *et al.,* 1997 • Hoerstrup *et al.,* 2000
biological	xenogenic or allogenic	• decellularized porcine pulmonary heart valves • pulmonary heart valves on allogenic acellular matrix conduits	• Schenke-Layland *et al.,* 2003 • Steinhoff *et al.,* 2000
biological	biodegradable collagen	• cardiovascular scaffold sponge • rapid prototyped collagen scaffolds	• Taylor *et al*., 2002 • Taylor *et al*., 2006
biological	fibrin-gel	• heart valves based on fibrin-myofibroblast cell suspension	• Jockenhövel *et al.,* 2001
hybrid	decellularized heart valves coated with synthetic polymer	• porcine aortic heart valves dip coated with biodegradable poly(hydroxybutyrate)	• Stamm *et al.,* 2004

After seeding, the constructs are cultured in nutrient media in a pulse duplicator in vitro system (bioreactor) mimicking the in vivo environment. In order to improve cell migration, proliferation and extracellular matrix production mechanical load has been applied to the seeded cardiovascular constructs in bioreactors (Niklason *et al*., 1999; Hoerstrup *et al.*, 2000a; Mol *et al*., 2003). In the bioreactor, tissue formation takes place and after several days the constructs are ready for implantation.

This concept for in vitro cardiovascular tissue engineering has already been proven by investigating completely autologous tissue engineered heart valves in an animal model (Hoerstrup *et al.,* 2000a). In this proof of principle study, trileaflet heart valve scaffolds based on bioabsorbable polyglycolic acid coated with poly-4-

hydroxybutyrate (PGA/P4HB) were subsequently seeded with autologous ovine vascular-derived myofibroblasts and endothelial cells. The constructs were grown for 14 days in a pulse duplicator in vitro system under gradually increasing flow and pressure conditions and implanted into a growing sheep model (n=6 lambs; mean weight at cell harvest 9±2.8kg). Echocardiography demonstrated mobile, functioning leaflets without stenosis, thrombus or aneurysm. These autologous tissue engineered valves functioned up to 5 months in vivo and resembled normal heart valves as to microstructure, mechanical properties and extracellular matrix formation.

1.1.7 Selection of the appropriate cell source

Regarding the three main components for the tissue engineering concept, the related interdisciplinary technologies have reached already an advanced state. Synthetic scaffolds with adjustable biodegradation properties are available and can be fabricated into three-dimensional matrices. The technology of in vitro cell culturing has made significant progress and allows controlling tissue growth conditions. The cell source is the least controlled factor, yet most important for the quality of the living part of the replacement. The quality of cells varies from patient to patient depending on the individual tissue characteristics and co-morbidities. Thus, the choice of the right cell source is of major importance for the success of cardiovascular tissue engineering.

Besides cell growth and expansion capacity, an important issue is the possibility to develop a cell phenotype that matches the native counterpart. This is expected to have a major impact on the long-term functionality of the replacements (Butcher and Nerem, 2004). Using cells originating from the tissue to be replaced would be the safest approach. In the case of heart valve tissue engineering, the usage of valvular interstitial cells obtained by biopsy has been shown feasible (Maish *et al*., 2003). However, with respect to clinical applications, these cells are difficult to obtain and the approach bears substantial risks. Therefore, several alternative human cell sources have been investigated for their use in cardiovascular tissue engineering. In order to create functional cardiovascular replacements with the mechanical properties of the native counterparts rapid development of the extracellular matrix is crucial.

Therefore, the choice of cells which are responsible for production of extracellular matrix is an important factor. Two cell types are routinely used for the fabrication of cardiovascular tissues: cells with the capacity to synthezize extracellular matrix elements, commonly myofibroblast/fibroblast-like cells, and endothelial cells forming a monolayer endothelium with antithrombogenic characteristics. The seeding procedure onto three-dimensional scaffolds is mostly performed sequentially: first by seeding of the myofibroblast/fibroblast-like cells, followed by the endothelial cells (Zund *et al.*, 1998). Among the most promising cell sources investigated in the last years are vascular-derived cells, bone marrow-derived cells, blood-derived cells and umbilical cord-derived cells, particularly for pediatric applications.

Vascular-derived cells

In most cardiovascular tissue engineering approaches cells are harvested from vascular structures. With regard to clinical application, several human vascular cell sources have been investigated including mammary, radial artery, and saphenous vein (Schnell *et al.*, 2001). The obtained mixed cell populations consisting of myofibroblasts and endothelial cells were sorted by fluorescence-activated cell sorting and the pure cell populations were cultivated and used for the fabrication of the replacements (Hoerstrup *et al*., 1998). Alternatively, in order to detach the endothelium from the luminal layer of the vessel biopsies were digested with collagenase and the endothelial cells were harvested. Afterwards, the tissue was minced into small pieces and out-growing myofibroblasts were cultured. Unfortunately, harvesting cells from a vascular cell source necessitates the sacrifice of intact vascular donor tissue. Particularly in children, obtaining cells in an invasive procedure can result in dysfunction and disturbance of growth and development of the harmed structures. Additionally, cardiovascular risk factors and co-morbidities such as atherosclerosis or diabetes can influence the quality of vessel tissue. In order to avoid damages of the tissue and ensure high cell quality alternative cell sources have been investigated.

Bone marrow-derived cells

With regard to future clinical application the bone marrow represents an attractive alternative heterogeneous cell source. Apart from progenitor cells for red blood cells, platelets, monocytes, granulocytes and lymphocytes (Hay, 1966) non-hematopoetic stem cells are also present. These adult stem cells have the ability to differentiate into cells with features of mesenchymal or marrow stromal cells because they arise from the complex array of supporting structures found in the marrow (Prockop, 1997). Recently, human marrow stromal cells were successfully used for the generation of living trileaflet heart valves (Hoerstrup *et al.,* 2002a). Flowcytometric characterisation of the marrow–derived cells prior seeding confirmed a myofibroblast-like phenotype. The cells showed the expression of α-SMA and vimentin while the uptake of LDL and the expression of desmin, CD31 and CD14 receptors were negative. Upon seeding on the biodegradable scaffold and preconditioning in a biomimetic environment the cells showed adequate production of extracellular matrix proteins. Additionally, mechanical properties comparable to native tissue were demonstrated. In order to create an anti-thrombogenic surface progenitors for endothelial cells were found in the bone marrow representing a promising cell source (Reyes *et al*., 2002).

Blood-derived cells

Endothelial progenitor cells (EPCs) can be isolated from the peripheral and umbilical cord blood (Ashara *et al.*, 1997; Dimmeler *et al.*, 2001; Vasa *et al*., 2001). They represent a rare heterogeneous population of mononuclear blood cells. Their origin and phenotype is still not fully understood and has been discussed controversially. Several possible sources for EPCs exist: haematopoetic stem cells, myeloid cells, circulating progenitor cells and circulating mature endothelial cells shed off vessel walls (Ubrich and Dimmeler, 2001). EPCs have the potential to differentiate into mature endothelial

cells and show regenerative features. They have been successfully used for the repair of injured vessels, neo-vascularization or regeneration of ischemic tissue (Kawamoto *et al.*, 2001; Kocher *et al*., 2001; Assmus *et al*., 2002; Pesce *et al*., 2003) as well as for coating of synthetic vascular grafts (Shirota *et al.,* 2003a). Recently, animal-derived EPCs have been used for the endothelialization of decellularized grafts in animal models (Kaushal *et al*., 2001) and for seeding of hybrid graft (Shirota *et al.,* 2003b).

Furthermore, the feasibility of using human umbilical cord blood-derived EPCs for tissue engineering of cardiovascular replacements for pediatric application has recently been demonstrated (Schmidt *et al.,* 2004 and 2005). When differentiated EPCs were co-cultured with non-endothelial cells as well as when exposed to mechanical stimuli they showed stabile phenotypes. The extracellular matrix production of undifferentiated EPCs was demonstrated to be insufficient whereas the differentiation into endothelial cells on biodegradable scaffolds was observed (Dvorin *et al.*, 2003). In the overall tissue engineering concept, EPCs represent a promising cell source for the endothelialisation of cardiovascular replacements. Since EPCs are easily accessible current research aims at their transdifferentation into myofibroblast-like cells in order to enable blood as a sole cell source, particularly for pediatric applications (Sales *et al*., 2006).

Umbillical cord-derived cells

With regard to pediatric cardiovascular tissue engineering the umbilical cord may serve as an optimal perinatal cell source. It is composed of two arteries and one vein transporting the blood of the embryonic circulation. The vessels are imbedded in the Wharton's jelly, an embryonic connective tissue. Due to its anatomical structure the umbilical cord is rich in different cell types: myofibroblasts, endothelial cells, and progenitor cells, harvested from the different parts of tissue (Schoenberg and Moore, 1958). Furthermore, the presence of mesenchymal progenitor cells in the Wharton's jelly of human umbilical cords with multilineage potential has been demonstrated (Wang *et al.*, 2004; Sarugaser *et al.*, 2005).

Currently, only rare experience exists with umbilical cord-derived cells for pediatric cardiovascular tissue engineering. In 1996 Sipehia et al. (Sipehia *et al*., 1996) described the use of human umbilical cord vein-derived endothelial cells. In 2002, human umbilical cord-derived myofibroblasts were established as a new cell source for pediatric cardiovascular tissue engineering and used for the fabrication of pulmonary conduits (Hoerstrup *et al.*, 2002b; Kadner *et al*., 2002).

In 2004, Koike et al. (Koike *et al*., 2004) created long lasting blood vessels with endothelial cells from umbilical cord vein in a three-dimensional fibronectin-type-I-collagen-gel connected to the mouse circulatory system. Recently, the fabrication of cardiovascular replacements based on myofibroblasts derived from Wharton's jelly and endothelial cells derived from umbilical cord blood EPCs has been demonstrated (Schmidt *et al.*, 2005). Despite these achievements the engineering of autologous pediatric cardiovascular tissue from umbilical cord is still in an early stage of development and a number of issues remain to be investigated.

1.1.8 The search for an autologous fetal cell source enabling the fabrication of cardiovascular constructs prior to birth

An important aspect for the realization of the pediatric cardiovascular tissue engineering concept concerns the selection of the appropriate autologous cell source. Of particular significance is cell-harvesting at early prenatal stage, for example by ultrasound-guided tissue sampling that allows having the tissue engineered replacement ready for implantation at or shortly after the birth of the patient. Furthermore, besides easy cell harvest in a low risk-procedure substantial requirements for applicable cells comprise a high growth potential and constant phenotypic quality. Progenitor cells that can be isolated from many tissues are an interesting source as due to their immaturity they represent a pool for many types of cells. Moreover, their immaturity might lead to more native-analogous tissues.

Bone marrow-derived progenitor cells may not be the optimal choice as prenatal harvest is difficult and bears high risks. In contrast, the umbilical cord appears to be a more promising cell source as prenatal cell harvesting could be realized by ultrasound guided cordocentesis without harming intact fetal structures.

However, although many interesting cell sources have been explored and have been successfully used for the fabrication of autologous cardiovascular constructs, the adequate cell source for pediatric application has still not been identified.

1.2 Objective and outline of the thesis

In this thesis, human prenatal progenitor cells obtained from different extra-embryonically situated fetal tissues were investigated with regard to their usability for the realization of the pediatric cardiovascular tissue engineering concept, introduced in this chapter. As first step towards an antithrombogenic surface that is crucial for functional tissue engineered cardiovascular replacements umbilical cord blood-derived endothelial progenitor cells were investigated, described in **Chapter 2.** These cells represent an attractive source for endothelial cells due to the fact that prenatal sampling of umbilical cord blood by ultrasound guided percutaneous cordocentesis is already a routine and well established low-risk procedure. It was explored whether blood derived-endothelial progenitor cells would form a functional endothelium also on a living matrix that was fabricated from Wharton`s Jelly-derived cells. This feasibility study is presented in **Chapter 3.** Furthermore, the functionality and plasticity of the generated endothelia were assessed by fabricating living blood vessels based on umbilical cord-derived progenitor cells **(Chapter 4).** Biological activity of tissue engineered cardiovascular replacements based on umbilical cord blood-derived endothelial progenitor cells and Wharton's Jelly-derived cells was investigated by exposing tissue engineered heart valve leaflets to mechanical and/or biochemical stimuli as described in **Chapter 5**. Due to the limitation that prenatal tissue sampling of Wharton's Jelly has not been established as a routine procedure, yet, other extra-embryonic-situated fetal tissues were studied with respect to future clinical realization of the pediatric tissue engineering concept. Thus, chorionic villi that today are already harvested routinely for diagnostic purposes were investigated as a new cell source.

Combined with umbilical cord blood-derived endothelial progenitor cells they might enable the realization of the pediatric tissue engineering concept (**Chapter 6)**. In order to further reduce the risk of prenatal cell harvest and enable the fabrication of tissue engineered cardiovascular replacements from one biopsy amniotic fluid-derived fetal progenitor cells isolated from routinely obtained amniotic fluid samples were used as single cell source. The isolation and characterization of amniotic fluid-derived fetal progenitor cells as well as the prenatal fabrication of heart valve leaflets based on these cells is described in **Chapter 7**. In the last chapter, **Chapter 8**, advantages and limitations of these newly developed approaches are discussed, particularly, whether they might lead to a novel generation of cardiovascular replacements for the repair of congenital heart defects.

Chapter 2

Human umbilical cord blood-derived endothelial progenitor cells – A new cell source for pediatric cardiovascular tissue engineering

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"Umbilical Cord Blood Derived Endothelial Progenitor Cells for Tissue Engineering of Vascular Grafts."

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2.1 Abstract

A substantial limitation of present pediatric cardiac surgery is the lack of appropriate materials for the repair of congenital defects. To address this shortcoming, tissue engineering is a scientific field that aims at in vitro fabrication of living, autologous grafts with the capacity of growth, repair, and regeneration. Here we focused on tissue engineered vascular grafts using human umbilical cord blood-derived endothelial progenitor cells (EPCs), as a non-invasive cell source for pediatric applications.
EPCs were isolated from 20 ml fresh human umbilical cord blood by Ficoll gradient centrifugation and cultured in endothelial basal medium containing growth factors. After proliferation and differentiation cells were analysed by immunohistochemistry and seeded onto three-dimensional (3D) biodegradable vascular scaffolds (porosity > 95%, n=22). Twenty-four hours after seeding the vascular grafts were positioned into a pulse-duplicator-system and grown for 48h under biomimetic conditions. A second group was grown 6 days statically and an additional 6 days biomimetically. Controls were cultured statically. Analysis of the grafts included immunohistochemistry, histology and scanning electron microscopy.
Pre-seeding, differentiated EPCs indicated constant endothelial phenotypes including acetylated low-density lipoprotein, cluster of differentiation 31, von Willebrand factor, and endothelial nitric oxide synthetase. Seeded EPCs established favorable cell-to-polymer attachment and proliferation into the 3D tubular scaffolds. Both conditioned and static cellular constructs demonstrated positive staining for cluster of differentiation 31, von Willebrand factor, and expression of endothelial nitric oxide synthase.
Human umbilical cord-derived EPCs indicated exceptional growth characteristics used for tissue engineering of vascular grafts. These cells demonstrated a constant endothelial phenotype and related functional features. Based on these results EPCs seem to be a promising autologous cell source with regard to cardiovascular tissue engineering, particularly for the repair of congenital defects.

2.2 Introduction

A substantial limitation of present pediatric surgery is the lack of appropriate autologous cardiovascular graft materials for the repair of congenital defects. This shortcoming is addressed by tissue engineering is a scientific field aiming at in vitro fabrication of living, autologous grafts with the capacity for growth, repair, and regeneration.

An important functional component of native vessel walls is the endothelium. Various cell sources have been investigated in tissue engineering for their ability to form neoendothelium in vitro, e.g. vascular-derived cells (Niklason *et al.*, 1999). These cells require an invasive surgical procedure associated with the sacrifice of intact vascular donor tissue. Optimally, a cell source should be easily accessible and exhibit high growth and repair potential. A potentially promising cell source is endothelial progenitor cells (EPCs), a subpopulation of stem cells in human peripheral blood. EPCs are a unique circulating subtype of bone marrow cells differentiated from haemangioblasts, a common progenitor for both haematopoetic and endothelial cells. These cells manifest the potential to differentiate into mature endothelial cells. Currently, EPCs have been investigated for the repair of injured vessels, neovascularisation or regeneration of ischemic tissue (Kawamoto *et al.*, 2001; Kocher *et al*., 2001; Assmus *et al*., 2002; Pesce *et al.*, 2003), endothelialization of decellularized grafts in an animal model (Kaushal *et al*., 2001), coating of vascular grafts (Shirota *et al.*, 2003a) and seeding of hybrid grafts (Shirota *et al*., 2003b).

In previous studies, we have demonstrated the feasibility of using human bone marrow-derived cells for tissue engineering of functional cardiovascular replacements such as heart valves (Hoerstrup *et al*., 2002a). However, in most congenital applications the need for surgical correction is early after birth making these and frequently used vascular-derived cells less appropriate. Therefore, cells which could be obtained before birth would be ideal for tissue engineering purposes by providing sufficient time for the in vitro generation of autologous replacement material ready to use at or shortly after birth. Cells can be harvested by e.g. ultrasound-guided chordocentesis. Little is known regarding the feasibility and use of EPCs for in vitro engineered endothelium in humans. We present the results of human umbilical cord blood-derived EPCs as an alternative and less invasive autologous cell source with regard to tissue engineering of endothelialized vascular grafts tissue. For pediatric applications, EPCs indicate potential as a future therapeutic option.

2.3 Materials and methods

2.3.1 Isolation of EPCs

The human umbilical cord vein of newborns (n=6) was punctured and 20 ml of fresh blood was obtained directly after delivery. Immediately, EPCs were isolated from mononuclear cells by density gradient (Histopaque–1077, Sigma Diagnostics, Inc., St. Louis, MO) after differential centrifugation described by Asahara et al. (Asahara *et al*., 1997). Isolated cells were cultured in endothelial basal medium (EBM-2; Cambrex Bio Science Verviers S.P.R.L., Verviers, Belgium) containing growth factors and supplements according to the manufacture information (vascular endothelial growth factor (VEGF) 0.5 ml, human fibroblasts growth factor 2.0 ml, human recombinant long-insulin-like growth factor-1 0.5 ml, human epidermal growth factor 0.5 ml, gentamycin and amphotericin 0.5 ml, hydrocortisone 0.2 ml, heparin 0.5 ml, ascorbic acid 0.5 ml and 2% fetal bovine serum). After 4 days, attached cells were reseeded. After 7 days, the medium was changed to EBM-2 containing the same growth factors but 20% fetal bovine serum. The attached cells were characterized by the expression of vascular endothelial growth factor receptor-2 (VEGF receptor-2; kinase insert domain-containing receptor/fetal liver kinase-1 [KDR;Flk-1])/fragment crystallizable [Fc] chimera; [Sigma Aldrich Co., St. Louis, MO]) lectin from ulex europeaus-1 (UEA-1) (Sigma Aldrich Co., St. Louis, MO), and cluster of differentiation 34 (CD34) (immunoglobulin glycoprotein 1 [IgG1], clone QBEND/10; Serotec, Kidlington, Oxford,UK). After 3 weeks the endothelial phenotype was confirmed by immunohistochemistry. The confluent monolayers were stained with antibodies to von Willebrand factor (vWF, polyclonal; Dakocytomation Corp., Carpinteria, CA), cluster of differentiation 31 (CD31) (IgG1/kappa, Clone JC/70A; Dakocytomation Corp., Carpinteria, CA), CD34 (IgG1, Clone QBEND/10; Serotec, Kidlington, Oxford, UK) and examined for uptake of acetylated low-density lipoprotein (Dil-Ac-LDL, Biomedical Technologies, Soughton, MA) and expression of endothelial nitric oxide synthase (eNOS, polyclonal; Transduction Laboratories, Inc., Lexington, KY).

2.3.2 Seeding and three-dimensional (3D) in vitro culture

Characterized cells were expanded up to passage 4 and exposed to laminar shear stress using rotating culture flasks. Thereafter, EPCs were seeded onto biodegradable vascular scaffolds (n=22; 3 Mio cells/cm^2). For assessment of cell attachment and in-growth, two different scaffold materials were used for the fabrication of the vascular scaffolds (0.5 cm diameter and 2.5 cm length; Figure 2.1): a nonwoven mesh (polyglycolic-acid [PGA] thickness: 1.0 mm, specific gravity: 69 mg/cm^3, Albany International, Mansfield, MA) coated with poly(4-hydroxybutyric acid (10% wt/vol P4HB; TEPHA, Cambridge, MA) and a 3D porous biodegradable polyurethane foam (Degrapol®, porosity >95%; Department of Materials, Federal Institute of technology, Zurich, Switzerland). Before seeding, the scaffolds were cold gas sterilized and incubated for 24h in growth medium.

Two sets of experiments differing with regard to in in vitro culturing conditions were performed: group 1 (n=6 vessels, polyurethane scaffolds, 72 h in vitro time), and

group 2 (n=16 vessels, PGA, 12 d in vitro time). Group 1 was cultured statically in nutrient medium (EBM-2) for 24 h in the 3D biodegradable system under humidified incubator conditions (37°C, 5% CO_2). Afterwards, two vessels were directly analyzed for the cell-to-polymer attachment. Two vessels were transferred to a biomimetic flow system for further 48h as described by Hoerstrup et al. (Hoerstrup *et al*., 2000b). Corresponding controls were cultured statically (n=2). For group 2 the cells were seeded onto a non-woven PGA-mesh and were cultured statically in the incubator system for 6 days. Thereafter, six scaffolds were positioned in the biomimetic flow system, whereas the others were kept under static conditions as a control group for an additional 6 days.

Figure 2.1: *Example of a biodegradable vascular scaffold (biodegradable polyurethane foam, porosity > 95%, 0.5 cm diameter, 2.5 cm length).*

2.3.3 Analysis

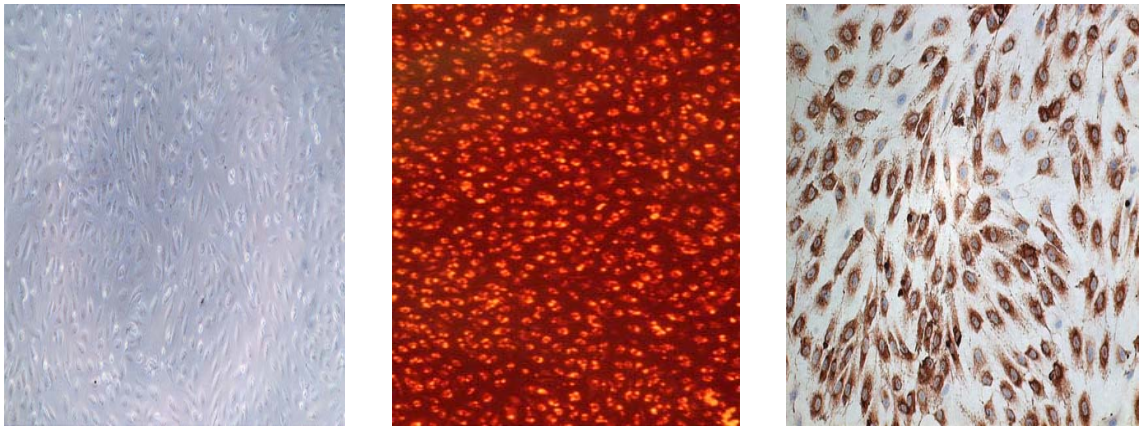
To document the presence of EPCs and cell-to-scaffold attachment, fixed (4% formalin) samples of each group were examined histologically by eosin-hematoxylin (H&E) and hematoxylin-sudan (H&S) staining. To confirm the phenotype of EPCs after in vitro culture, explanted scaffold samples of each group were characterized by immunohistochemistry (vWF, CD31 and eNOS staining) as in the pre-seeding analysis. Samples for electron microscopy (SEM) were collected from each group and fixed in 2% glutaraldehyde (Sigma St. Louis, MO) for 24 h and gold sputtered.

2.4 Results

2.4.1 Morphology of EPCs

EPCs could be isolated from all of the blood samples. After isolation, the plated cells were initially round. The total amount of isolated cells in 20 ml was $3x10^6$. After 4 days, cells were attached and formed clusters. Two different types of EPCs were found:

spindle-like shaped cells (70%) and polymorph cells (30%). Polymorph EPCs formed clusters and established satisfactory growth under in vitro conditions. After 3 weeks, they differentiated into mature endothelial cells forming cobblestone monolayers (Figure 2.2a), whereas the spindle-like shaped cells detached in the meantime.



a) native *b) LDL staining* *c) vWF staining*

Figure 2.2: *(a) Differentiated native endothelial progenitor cells after 3 weeks. (b) Immunofluorescence staining demonstrated the uptake of acetylated low-density lipoprotein (Ac-LDL) (magnification= x50). (c) von Willebrand factor (vWF) confirms the endothelial phenotype (magnification= x100).*

2.4.2 Phenotype of EPCs

Differentiated EPCs indicated an endothelial phenotype. They stained positive for lectin from ulex europeus, VEGF receptor-2, vWF, CD31, CD34, eNOS, and indicated Ac-LDL-uptake (Figure 2.2b-c). Differentiated EPCs kept this phenotype when cultivated on both types of 3D scaffolds both in the biomemetic flow system and under static conditions (Figure 2.3d-i).

2.4.3 Cell-to-polymer attachment

Histological examination of the seeded tubular grafts revealed good cell-to-polymer attachment in both types of scaffold. After 24h and 48h, differentiated EPCs demonstrated suitable growth in the 3D scaffolds (polyurethane foam) in both the dynamic and the static controls (Figure 2.3b-f). A similar picture was observed after 12 days of in vitro culture (PGA/P4HB mesh) under dynamic and static conditions (Figure 2.3g-i)

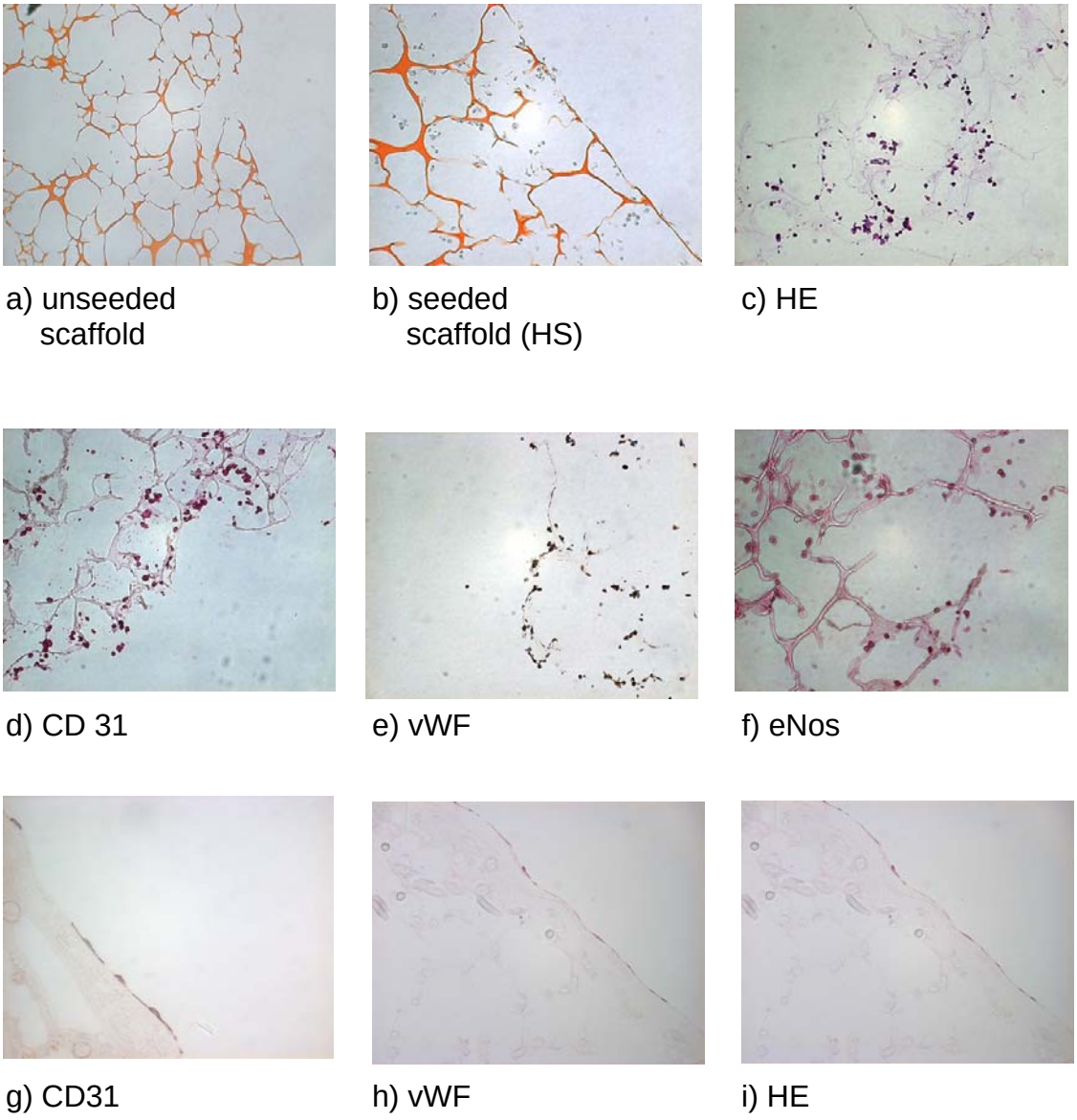


Figure 2.3: *Endothelial progenitor cell (EPCs) formation on the seeded scaffolds: histology and immunohistochemistry illustrate cell-to-polyurethane foam attachment under static conditions (24h after seeding): (a) unseeded scaffold, (b) seeded scaffold, (c) hematoxylin & eosin [H&E] staining and with stable endothelial phenotype of attached EPCs under dynamic conditions on polyurethane foam 48h after seeding: (d) cluster of differentiation 31 [CD31], (e) von Willebrand factor [vWF], (f) endothelial nitic oxide synthethase [eNOS] and on polyglycolic-acid (PGA) meshes 12 days after seeding: (g) CD31, (h) vWF, (i) HE, magnification = x20.*

2.4.4 Analysis

H&E staining revealed a monolayer of differentiated EPCs on the lumen of the vascular scaffolds after 12 days. Immunhistochemistry confirmed the endothelial phenotype of the neo-tissue (Figure 2.3g-i). Analysis by SEM demonstrated endothelial tissue formation with favorable cell-to-polymer attachment in all groups (Figure 2.4).

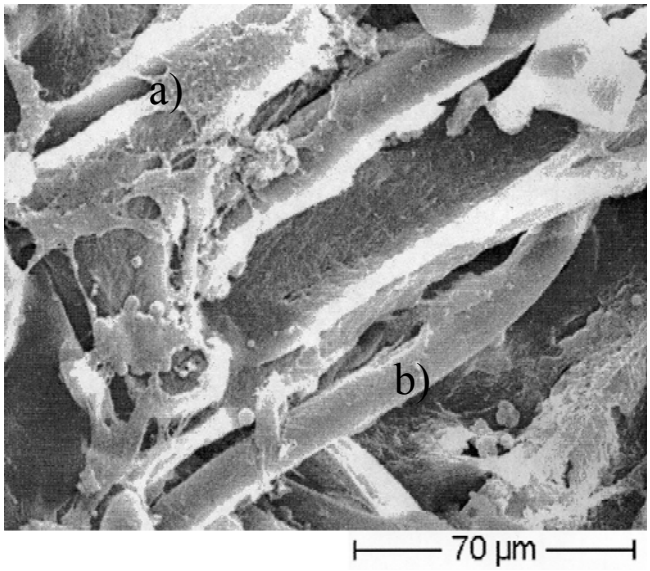


Figure 2.4: *Cell-to-polymer attachment (polyglycolic-acid [PGA] meshes). In the scanning electron microscope (measurement = 70µm): (a) differentiated endothelial progenitor cell, (b) fiber of PGA/P4HB scaffold.*

2.5 Discussion

One of the present limitations of pediatric cardiac surgery is the lack of appropriate graft materials for the repair of congenital defects. Tissue engineering is a scientific field that address this shortcoming using in vitro fabrication of living, autologous grafts with the capacity for growth, repair and remodeling. Previous approaches using cell sources for cardiovascular tissue engineering were burdened by the disadvantage of necessitating an invasive surgical procedure for cell harvesting. EPCs are a subpopulation of bone marrow cells circulating in the peripheral blood. Thus, they can be obtained easily by venous puncture and without the sacrifice of intact donor tissue. The results presented demonstrated and confirmed that EPCs can be isolated from fresh human umbilical cord blood. Isolated EPCs differentiated in culture into mature endothelial cells and were expandable in vitro. Two different types of EPCs were indicated: polymorph and spindle-like cells. The polymorph EPCs demonstrated remarkable growth characteristics and constant phenotype, as is also described by Hur and associates (Hur *et al*., 2004), whereas the spindle-like EPCs revealed limited

proliferation properties. EPCs exhibited properties similar to those of angioblasts which can be defined as migratory endothelial cells that manifest the capacity to circulate, proliferate and differentiate into mature endothelial cells (Coffin *et al*., 1991; Robert *et al*., 1996; Caprioli *et al*., 1998).

The present study demonstrated that differentiated human umbilical cord blood-derived EPCs seeded on vascular scaffolds formed neo-tissue in both a biomemetic and static in vitro environment. These tissues were characterised as endothelial monolayers with related functions e.g. the production of eNOS indicating features of functional endothelium. Because of their stable phenotype and their growth and repair potential human umbilical cord blood-derived EPCs seem to be a promising cell source for tissue engineering of vascular grafts, particularly for pediatric applications, where EPCs could adjust to the specific growth requirements of the cardiovascular system during childhood.

The focus of this study was to investigate the attachment of EPCs on an implantable biodegradable scaffold. Based on previous animal studies using PGA/P4HB scaffolds (Hoerstrup *et al*., 2000a) and the subsequent successful transfer of the methodology to human cell systems (Hoerstrup *et al.*, 2001; Hoerstrup *et al.*, 2002a), we investigated the EPC attachment by including these established scaffolds in the present study.

A critical limitation of the availability of EPCs may be the low number that exists in the peripheral blood, because they are a scant circulating population. However, previous studies revealed that umbilical cord blood contains increasing amounts of EPC that approach ten-fold compared with the blood of adults (Peichev *et al*., 2000; Murohara *et al.*, 2000) potentially providing sufficient cell numbers for tissue engineering purposes.

In many cases the congenital defect is detected by ultrasound at approximately 16-20 weeks of gestation. One option regarding the tissue engineering concept involves obtaining the EPCs before birth using cordocentesis. This would allow enough time for isolation, differentiation and fabrication of the graft material. In some instances a successful chirurgical correction of this type of defect depends on the weight of the infant and so cannot be performed directly after birth (e.g. tetralogy of Fallot). In these occurrences a second option exists in which the blood can be obtained from the umbilical cord directly after birth and the isolation and cultivation of EPCs can be performed at that time. After differentiation cells can be deep-frozen and stored until operating conditions are optimal.

Our results indicate that human umbilical cord blood-derived EPCs seem to be a promising autologous cell source for cardiovascular tissue engineering because of their physiological properties and easy accessibility. The possibility of prenatal cell harvesting may allow the preparation of autologous grafts for use at birth and it may overcome the limitations of present-day graft materials for treatment of congenital defects.

Acknowledgement

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Chapter 3

Living patches engineered from human umbilical cord-derived fibroblasts and endothelial progenitor cells – A feasibility study

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3.1 Abstract

A major shortcoming in contemporary congenital heart surgery is the lack of viable replacement materials with the capacity of growth and regeneration. Here, we focused on living autologous patches engineered from human umbilical cord-derived fibroblasts and endothelial progenitor cells (EPCs) as a ready-to-use cell source for paediatric cardiovascular tissue engineering.

EPCs were isolated from 20 ml fresh umbilical cord blood by density gradient centrifugation and myofibroblasts were harvested from umbilical vessels. Cells were differentiated and expanded in vitro using nutrient media containing growth factors. Before seeding, cell-phenotypes were assessed by immunohistochemistry. Biodegradable patches fabricated from synthetic polymers (PGA/P4HB) were seeded with myofibroblasts followed by endothelialization with EPCs. All patches were cultured in a perfusion bioreactor. A subgroup of patches was additionally stimulated by cyclic strain. Analysis of the neo-tissues comprised histology, immuno-histochemistry, extracellular matrix (ECM) analysis and biomechanical testing.

Endothelial phenotypes of EPCs before seeding were confirmed by Ac-Dil-LDL, CD 31, von Willebrand Factor (vWF) and eNOS staining. Histology of the seeded patches demonstrated layered viable tissue formation in all samples. The cells in the newly formed tissues expressed myofibroblast markers, such as desmin and alpha-SMA. The EPCs derived neo-endothelia showed constant endothelial phenotypes (CD31, vWF). Major constituents of ECM such as collagen and proteoglycans were biochemically detected. Stress-strain properties of the patches showed features of native-analogous tissues.

Living tissue engineered patches can be successfully generated from human umbilical cord derived myofibroblasts and EPCs. This new cell source may enable the tissue engineering of versatile, living, autologous replacement materials for congenital cardiac interventions.

3.2 Introduction

A major shortcoming in today's surgery of congenital cardiovascular defects is the lack of living replacement materials with the capacity of growth and regeneration. Currently, pediatric surgical treatment requires the application of synthetic or non-autologous bioprosthetic materials, such as patches and vascular conduits (Mayer, 1995; Schoen and Levy, 1999). These materials are burdened with substantial disadvantages including obstructive tissue ingrowth and calcification of the replacement (Endo *et al.*, 2001). These limitations and the lack of growth typically cause re-operations of pediatric patients with cardiovascular defects associated with increased morbidity and mortality. To address the above mentioned problems cardiovascular tissue engineering is a new scientific field aiming at in vitro fabrication of living, autologous grafts with the capacity of growth, repair, and regeneration.

Important functional features of ideal replacement materials for the repair of congenital defects comprise a living matrix with native-analogous biomechanical properties and a thrombo-resistant surface. In previous studies, we have demonstrated the feasibility of using human umbilical cord-derived myofibroblasts as a cell source for engineering of functional pediatric tissues (Hoerstrup *et al*., 2002b). The umbilical cord blood is a known source for endothelial progenitor cells (EPCs) differentiated from haemangioblasts, a common progenitor for both haematopoetic and endothelial cells. These cells have the potential to differentiate into mature endothelial cells and have been successfully utilized in non-tissue engineering applications such as for the repair of injured vessels, neo-vascularization or regeneration of ischemic tissue (Kawamoto *et al.*, 2001; Kocher *et al*., 2001; Assmus *et al*., 2002; Pesce *et al*., 2003) as well as coating of synthetic vascular grafts (Shirota *et al*., 2003a). Recently, animal-derived EPCs have been used for the endothelialization of decellularized grafts in animal models (Kaushal *et al.*, 2001) and for seeding of hybrid grafts (Shirota *et al.*, 2003b).

Little is known about the feasibility and utility of umbilical cord-derived EPCs for in vitro engineered endothelium in humans. Here, we investigate human umbilical cord blood-derived endothelial progenitor cells (EPCs) as a source for in vitro generation of functional endothelia covering living tissue engineered patches as a simplified model for pediatric cardiovascular tissue engineering.

3.3 Material and methods

3.3.1 Isolation of myofibroblasts from human umbilical cord tissue

Cells were obtained from umbilical cords of healthy individuals after informed consent was obtained from the participants. Myofibroblasts were isolated from umbilical cord tissue of newborns. Several pieces of umbilical cord tissue (size ≈ 8 mm^3)were washed with PBS, placed in petri-dishes and cultured in a humidified incubator (37°C, 5% CO_2) in advanced DMEM medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (PAN Biotech, Germany), 2 mM Glutamax (Gibco) and Gentamycin (50µg/ml, PAN). Myofibroblasts were expanded up to passage 11.

3.3.2 Isolation of EPCs from human umbilical cord blood and differentiation into endothelial phenotype

The human umbilical cord vein of newborns was punctured directly after delivery and 20 ml of fresh blood were obtained. Immediately, EPCs were isolated from mononuclear cells by density gradient centrifugation (Histopaque–1077, Sigma), followed by differential centrifugation as described by (Asahara *et al*., 1997). Isolated cells were cultured in endothelial basal medium (EBM^{TM}-2, Cambrex), containing growth factors and supplements provided by the supplier: Vascular Endothelial Growth Factor (VEGF), human Fibroblasts Growth Factor (hFGF), human recombinant long-Insulin-like Growth Factor-1 (R3-IGF), human Epidermal Growth Factor (hEGF), Gentamycin and Amphotericin (GA-1000), Hydrocortisone, Heparin, Ascorbic Acid and 2% Fetal Bovine Serum. After 4 days, attached cells were reseeded and after 7 days, the medium was changed to EBM-2 containing the same growth factors but 20% FBS. After 3 weeks of culturing, the endothelial phenotype was confirmed by immuno-histochemistry. The confluent monolayers were stained with antibodies for von Willebrand factor (vWF, polyclonal; DAKO A/S), CD31 (IgG1/kappa, Clone JC/70A; DAKO A/S), CD34 (IgG1, Clone QBEND/10; Serotec) and examined for uptake of Ac-LDL (Dil-Ac-LDL, Biomedical tech.) and expression of endothelial nitric oxide synthase (eNOS, polyclonal; Transduction Laboratories Inc.).

3.3.3 Fabrication of scaffolds

Scaffolds were fabricated from a non-woven polyglycolic-acid mesh with randomized distributed fibers (PGA, thickness: 1.0 mm, specific gravity: 69 mg/cm^3, porosity > 95%. Albany Int.), cut into 2.3 cm^2 patches (Figure 3.1) and coated with poly-4-hydroxybutyric acid (10% w/v P4HB, TEPHA Inc., Cambridge, MA) by dipping. Three patches each were attached to a ring-shaped support of 20 mm diameter and 15 mm length. After drying in an exicator, the scaffolds were cold-gas sterilized.

3.3.4 Seeding of scaffolds

Cells were expanded as monolayers either in 175cm^2 tissue culture flasks (TRP, Milian, Geneva, Switzerland) or in roller bottles (Costar, 850 cm^2/bottle) using the Cellroll system (Integra biosciences) at a speed of 0.5 to 1 min^{-1}. At confluency, cells

were harvested by treatment with trypsin / EDTA (0.25%) and seeded onto the biodegradable PGA/P4HB patches by using fibrin as a cell carrier (Mol *et al*., 2005a). Briefly, 3.5 x 10^6 cells/100mm^3 of scaffold were resuspended in a sterile thrombin solution (Sigma, 10 U/ml medium) and kept on ice in a volume that equals half of the void volume of the scaffold. The same volume of sterile, ice-cold fibrinogen solution was added (10 mg/ml medium) and after carefully mixing, the cells were dripped onto the scaffold.

The patches were positioned into a bioreactor system and perfused with culture medium supplemented with 1 mM ascorbic acid-2-phosphate. After 7 days, the cultivation conditions were altered. Patches of group 1 (n=9) were continued to be perfused only, while patches of group 2 (n=9) were additionally exposed to cyclic straining performed about 12 mmHg resulting in a straining of 10-15% by using a pressure applicator connected to a pressure air chamber. After another 14 days, all patches were taken out of the bioreactor system and were endothelialized with EPCs (1.5 x 10^6 cells/cm^2) on both sides. Patches were kept at humidified incubator conditions (37°C, 5% CO_2) in EBM-2, containing growth factors and 20% FBS. Endothelialization was repeated after 24h. After additional 24h, the patches of both groups were placed back into the bioreactor system and exposed for 4 subsequent days to perfusion or perfusion and cyclic straining respectively. After culturing, the patches were cut into samples and portioned out for analysis.

3.3.5 Tissue formation analysis

Histology

Samples were fixed in 4% phosphate buffered formalin (pH 7.0) and paraffin-embedded. Sections of 3-5 µm of each group were examined histologically by Eosin-Hematoxylin (H&E), Hematoxylin-Sudan (H&S), and Trichrom-Masson staining. Selected samples were characterized by immunohistochemistry. Vimentin, α-SMA, and desmin were used for analysis of the myofibroblast phenotype and vWF, CD31 and eNOS staining to validate the phenotype of the EPC derived endothelial cells.

Quantification of extracellular matrix elements

Collagen

As an indicator for collagen formation, hydroxyproline content was determined of dried samples as described by (Huzar *et al*., 1980). Briefly, tissue samples were hydrolyzed in 50 to 100 µl 4 M NaOH (Fluka, Switzerland) in an autoclave at a temperature of 120°C for 10 minutes. The solution was neutralized by adding an equal volume of 1.4 M citric acid (Fluka). Chloramin-T (Riedel-de Haën (Fluka) 62 mM) was added and the samples were allowed to oxidize for 25 minutes. Aldehyde/perchloric acid solution (1 M) was subsequently added and the chromophore was allowed to develop at 65°C for 15 minutes. The absorbance of the obtained solutions was determined at 570 nm. The amount of hydroxyproline present in the hydrolysates was determined from a standard curve using known amounts of trans-4-hydroxy-L-proline (Sigma).

Glycosaminoglycans
To further specify the extra cellular matrix, glycosaminoglycans were detected colorimetrically using 1,9-di-methyl-methylene blue stain (Farndale *et al*., 1986) following complete papain digestion of the constructs in 100 mM sodium phosphate pH 6.5, 5 mM cysteine, 5 mM EDTA and 125µg/ml papain (Sigma).

Determination of cell number
The amount of cells growing on the constructs was indirectly determined by measuring the DNA content from the same papain digested samples using Hoechst dye (Bisbenzimide H 33258, Fluka) (Kim *et al.*, 1988) and a fluorometer (Fluostar, BMG, Offenburg, Germany, 355 nm excitation / 460 nm emmission). Calf thymus DNA (Sigma) was used as a standard.

Testing of mechanical properties
Measurements of the mechanical properties were performed with freshly harvested patches (15x5x1mm) using a uniaxial tensile tester (Instron) equipped with a 10 Newton load cell. Tensile stress and strain were recorded and the Young´s modulus was calculated.

3.4 Results

3.4.1 Macroscopic appearance of the patches

All patches showed homogenous tissue formation and dense surfaces (Figure 3.1).

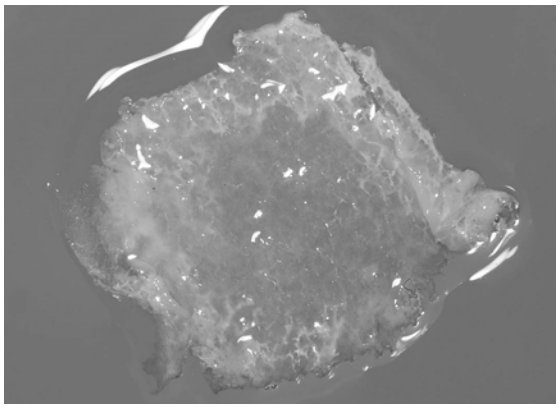

Figure 3.1: *Tissue engineered patch based on human umbilical cord cells.*

3.4.2 Phenotype of myofibroblasts

Myofibroblasts showed positive staining for vimentin and expression of α-SMA and desmin (Figure 3.2a-b).

3.4.3 Morphology and phenotype of EPCs

EPCs could be isolated from all blood samples. After isolation, the plated cells were initially round. After 4 days, cells were attached and formed clusters. Two different types of EPCs were found: spindle-like shaped cells (80%) and polymorph cells (20%). Polymorph EPCs formed colonies and showed good growth under in vitro conditions. After 3 weeks, they differentiated into mature endothelial cells forming cobblestone monolayers.

Before seeding onto the scaffolds, differentiated EPCs showed an endothelial phenotype. They stained positive for vWF, CD31, CD34, eNOS, and showed Ac-LDL-uptake. Differentiated EPCs kept this phenotype when cultivated under both cyclic strain and perfusion in the bioreactor flow system (Figure 3.2e-f).

3.4.4 Histology

Histological examination of the seeded patches revealed good cell-to-polymer attachment. Myofibroblasts showed good ingrowth into the PGA/P4HB scaffolds under cyclic strain. A similar picture was observed after culturing under perfusion. EPCs demonstrated a good attachment on the neo-tissue formed by myofibroblasts in both groups of culturing conditions.

H&E staining revealed organized tissue-formation with good extracellular matrix formed by myofibroblasts in the inner part of the patches (Figure 3.2c). Trichrom-Masson-staining highlighted elements of extracellular matrix (Figure 3.2d). An endothelial cell lining formed by differentiated EPCs was found on surfaces of all patches. Immunohistochemistry confirmed the endothelial phenotype of the neo-tissue (Figure 3.2e-f).

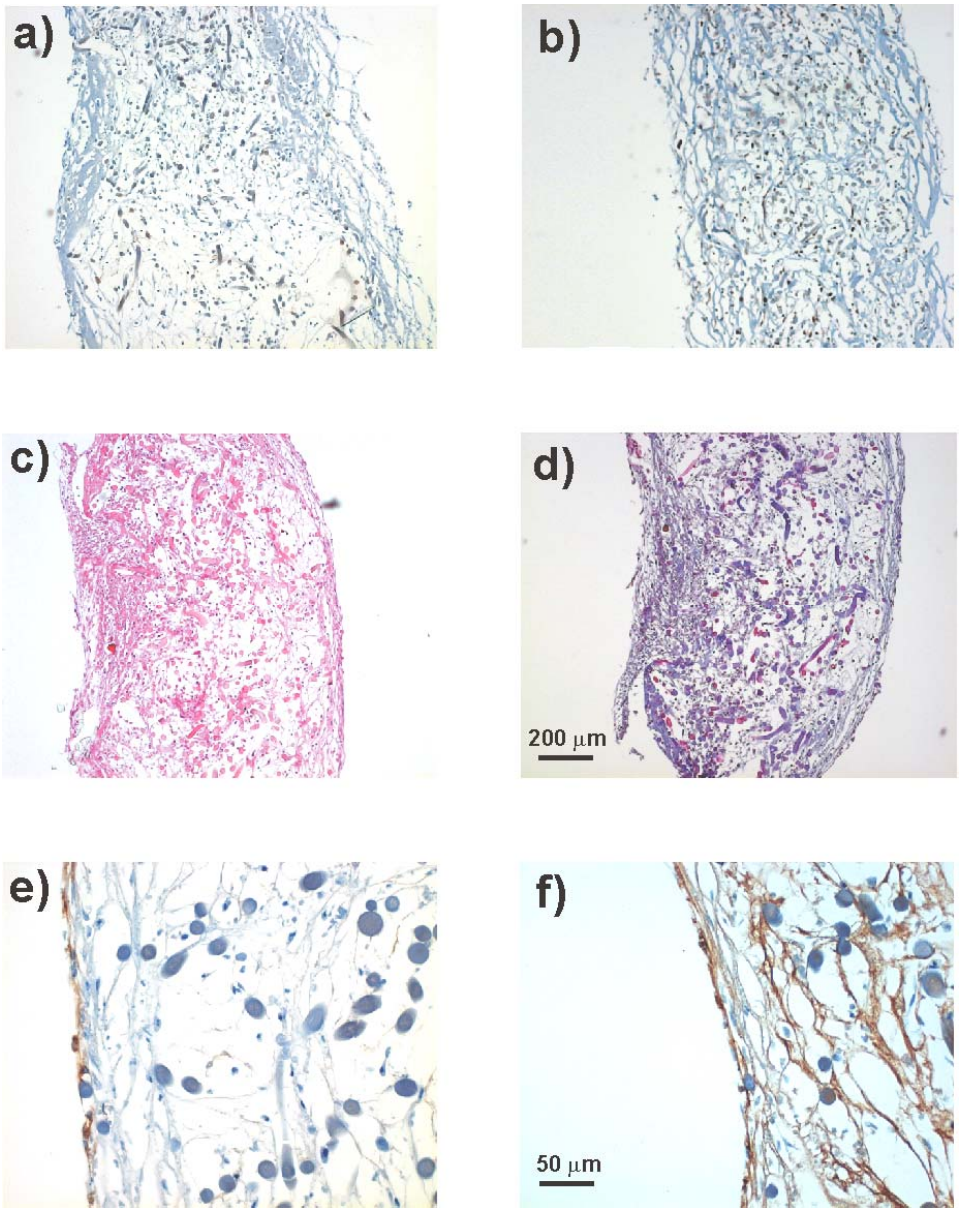

Figure 3.2: *Histological and immunohistological characterization. Myofibroblasts of the new tissue showed positive staining for a) desmin and b) α-SMA (magnification 50x). Hemalaun & Eosin staining c) demonstrated organized tissue formation with production of extracellular matrix components. In Trichrom-Masson staining d) collagen fibres are blue (magnifications 50x). Expression of CD31 on surfaces of patches and f) vWF, respectively confirm the endothelial phenotype of the neo-endothelium formed by EPCs (magnification 200x).*

3.4.5 Quantification of extracellular matrix elements

A summary of the extracellular matrix composition is given in Figure 3.3. The collagen content of both groups were comparable (strained 4.06 µg/mg ± 1.92, n=8, perfused 4.21 µg/mg ± 0.44, n=8). The s-glycosaminoglycan content was higher in the strained than in the perfused patches (6.44 µg/mg ± 1.45; n=4 versus 4.65 µg/mg ± 0.61; n=5) and the cell number was significantly higher in the strained patches (3.14 µg/mg ± 1.02; n=4 versus 1.24±0.35; n=5; Student's t-test $p<0.05$).

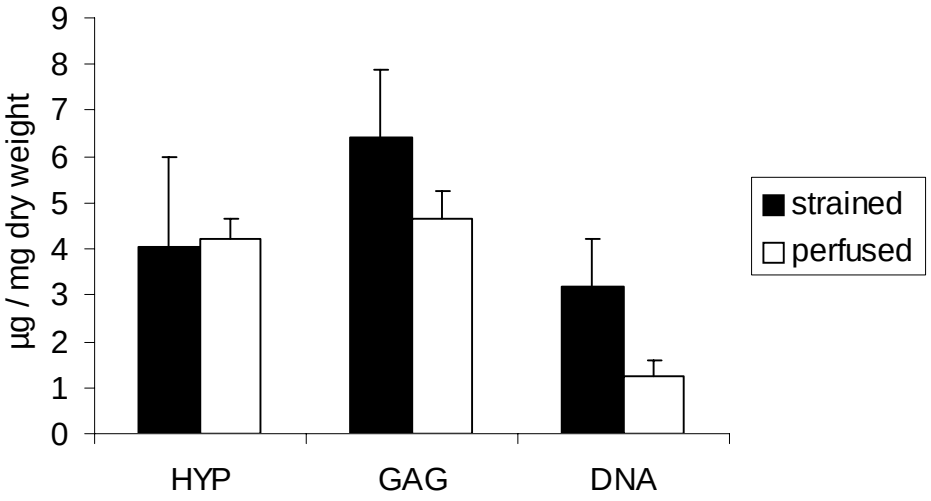

Figure 3.3: *Extracellular matrix analysis. HYP, hydroxyproline; GAG, sulfated glycosaminoglycans; DNA (cell number). Averages are given in µg/mg of dry weight. The error bars represent the standard deviation.*

3.4.6 Tensile testing

Tissue engineered patches showed normal stress-strain profiles in both groups of culturing conditions (Table 3.1). Only strain at break showed significantly differences between the groups.

Table 3.1: *Mechanical properties of perfused or perfused and strained tissue engineered patches.*

Samples	**Tensile strength (MPa)**	**Young`s modulus (MPa)**	**Strain at break**
Strained 1	.20	.92	.38
Strained 2	.17	.74	.33
Strained 3	.11	.49	.34
Strained 4	.22	1.07	.28
Strained Mean ± SD	.18 ± . 05	. 80 ± .25	.33 ± .04
Perfused 1	.30	.74	.56
Perfused 2	.17	.50	.60
Perfused 3	.17	.30	.64
Perfused 4	.08	.24	.50
Perfused Mean ± SD	.18 ± .09	.44 ± .23	. 57 ± .06

3.5 Discussion

For surgical treatment of congenital cardiac defects such as augmentation of the hypoplastic, stenotic right ventricular out flow tract or the pulmonary artery in tetralogy of Fallot, for the closure of a complete AVSD, enlargement of the aorta in the Norwood I procedure synthetic or bioprosthetic replacement materials are commonly used. Such non-autologous materials have been associated with substantial complications including calcifications, obstructive tissue ingrowth, infections, and thrombogenicity (Ben-Sachar *et al.*, 1981; Mayer, 1995; Endo *et al.*, 2001). The availability of living graft materials with the potential to grow, regenerate and to adapt to the changes in the developing cardiovascular system would have fundamental advantages over currently used replacement materials.

In the present study, we investigated the feasibility to utilize human umbilical cord blood-derived endothelial progenitor cells for in vitro fabrication of living autologous patches. We further investigated the influence of different in vitro culture conditions on tissue maturation and functionality of the newly formed endothelia.

As demonstrated previously (Hoerstrup *et al.*, 2002b), umbilical cord derived myofibroblasts showed exellent ingrowth into the biodegradable scaffolds and organized tissue formation with the production of extracellular matrix proteins such as collagen and proteoglycans. This myofibroblasts based matrix formation represents a

crucial prerequisite for the successful creation of mechanically competent, surgically implantable replacement materials. Isolation of the endothelial progenitor cells from umbilical cord blood samples was easily performed and the cells were expandable in sufficient amounts for tissue engineering purposes. After seeding, the endothelial progenitor cells formed functional endothelial layers on the surfaces of the myofibroblast-derived neo-matrices of all patches. Most importantly, the EPCs showed constant endothelial phenotype during the whole tissue engineering process and expressed functional features such as eNOS. In previous studies we and others have demonstrated that mechanical conditioning of growing tissue engineered cardiovascular structures, such as heart valves and blood vessels results in enhanced and more mature tissue formation (Niklason *et al*., 1999). Accordingly, the patches were cultured in a custom designed bioreactor in vitro system. We have chosen two bioreactor protocols using perfusion and cyclic strain as mechanical stimulation to increase the production of extracellular matrix and mechanical strength of the patches. By comparing the culture conditions, we found higher contents of extracellular matrix proteins such as proteoglycans as well as cell number when patches were exposed to cyclic strain. However, collagen content was not significantly different. These results indicate the important influence of strain stimulation on early matrix formation as recently described by Mol et al. (Mol *et al*., 2003).

The mechanical profiles of all patches showed features of native cardiovascular tissues demonstrating a non-linear mechanical behaviour. In contrast, the scaffold material itself exhibits linear behaviour indicating that the measured mechanical properties are relating from the neo-tissues. However, the mechanical strength did not reach physiological values during the investigated in vitro time, restricting the current patches to low pressure applications such as in reconstruction of the RVOT. Interestingly, we observed a higher Young`s Modulus representing a higher stiffness in the cyclically strained compared to perfused tissues. This difference in stiffness of the patches may result from more mature extracellular matrix formation reflected by increased proteoglycans content and a higher degree of cross-links among collagen fibres to be assessed in further experiments.

In summary, in this feasibility study we created living autologous human patches based on umbilical cord-derived cells, representing a versatile replacement material for congenital cardiac surgery. The endothelial progenitor cells isolated from the umbilical cord blood showed excellent expansion capacities, constant endothelial differentiation, and formation of endothelia with functional properties. Apart from being a potent cell source for differentiated endothelial cells, human endothelial progenitor cells may enable to use tissue engineered replacement materials directly after birth, since they can be harvested prenatal e.g. by ultrasound-guided chordocentesis. This approach would provide sufficient time for the in vitro generation of autologous replacement material ready to use at or shortly after birth and is currently investigated in animal models.

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Chapter 4

Functionality and plasticity of endothelia generated from human umbilical cord-derived progenitors

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"Engineered Living Blood Vessels: Functional Endothelia Generated from Human Umbilical Cord-Derived Progenitors."

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4.1 Abstract

Tissue engineered living blood vessels (TEBV) with growth capacity represent a promising new option for the repair of congenital malformations. Here, we investigate the functionality of TEBV with endothelia generated from human umbilical cord blood–derived endothelial progenitor cells (EPCs).

TEBV were generated from human umbilical cord-derived myofibroblasts seeded on biodegradable vascular scaffolds, followed by endothelialization with differentiated cord blood-derived EPCs. During in vitro maturation the TEBV were exposed to physiological conditioning in a flow bioreactor. For functional assessment, a subgroup of TEBV was stimulated with TNF-α. Controls endothelialized with standard vascular endothelial cells were treated in parallel. Analysis of the TEBV included histology, immunohistochemistry, biochemistry (extracellular-matrix-analysis, DNA) and biomechanical testing. Endothelia were analyzed by flowcytometry, immunohistochemistry (CD31, vWF, thrombomodulin, tissue factor, and eNOS).

Histologically, a three-layered tissue organization of the TEBV analogous to native vessels was observed and biochemistry revealed the major matrix constituents (collagen, proteoglycans) of blood vessels. Biomechanical properties (Young`s Modulus 2.03 ± 0.65 MPa) showed profiles resembling those of native tissue. EPCs expressed typical endothelial cell markers CD31, vWF and eNOS comparable to standard vascular endothelial cells. Stimulation with TNF-α resulted in physiological up-regulation of tissue factor and down-regulation of thrombomodulin expression.

These results indicate that TEBV with tissue architecture and functional endothelia similar to native blood vessels can be successfully generated from human umbilical cord progenitor cells. Thus, blood-derived progenitor cells obtained before or at birth may enable the clinical realization of tissue engineering constructs for pediatric applications.

4.2 Introduction

Currently available replacement materials for the repair of congenital malformations lack the potential of growth and remodeling and consequently require re-operations with increased morbidity and mortality over the pediatric patients` lifetime. Furthermore, thrombogenicity and graft occlusion still represent a major limitation of currently used cardiovascular grafts (Ben-Shachar *et al*., 1981). As these are either synthetic or bioprostethic (Mayer, 1995) patients have to deal with severe side-effects such as life-long anticoagulation therapy and increased risks for infections and thromboembolism (Endo *et al*., 2001). Autologous living replacement materials with functional bioactive endothelium closely mimicking its native counterpart could avoid these severe complications. A promising strategy to generate such viable autologous cardiovascular replacements with growth and regeneration potential in vitro is by tissue engineering. In vitro as well as in vivo studies on tissue-engineered cardiovascular constructs have shown promising results (Shin`oka *et al*., 1995; Niklason *et al.*, 1999; Hoerstrup *et al*., 2000a). Recently, bone marrow-derived progenitor cells have been successfully applied in cardiovascular tissue engineering (Hoerstrup *et al*., 2002a; Sutherland *et al*., 2005) and first clinical experiences have been reported (Shin`oka *et al.*, 2005). Furthermore, peripheral blood-derived ovine endothelial progenitor cells have been successful used for the coating of vascular ePTFE grafts (Kaushal *et al*., 2001) and first clinical trials have been carried out using antibody coated coronary stents for endothelial progenitor cell capture in vivo (Aoki *et al*., 2005).

For congenital applications, the autologous tissue replacement should ideally be available at or shortly after birth in order to prevent secondary damage to the immature heart. Thus, cells would have to be harvested during pregnancy. Here, bone marrow-derived cells may not be the optimal choice as prenatal harvest is difficult and bears high risks. The umbilical cord appears to be a more promising cell source as prenatal cell harvesting could be realized by cordocentesis without harming intact fetal structures. In previous studies, we have demonstrated the feasibility of using human umbilical cords as a cell source for the engineering of pediatric tissues (Hoerstrup *et al.*, 2002b; Schmidt *et al.*, 2005). Furthermore, we reported that human umbilical cord blood-derived endothelial progenitor cells formed endothelia-like structures on the surfaces of biodegradable vascular grafts (Schmidt *et al*., 2004).

Here, we investigate the structural and functional characteristics of complete living tissue engineered blood vessels (TEBV) fabricated from Wharton’s jelly with endothelia generated from human umbilical cord blood-derived endothelial progenitor cells (EPCs).

4.3 Material and methods

4.3.1 Cell harvest and isolation

Cells were harvested after delivery from fresh human umbilical cord tissue and blood of healthy individuals after informed consent was obtained from the participants (approved by the Ethics Committee STV1-2005).

Human umbilical cord-derived myofibroblasts

Pieces of umbilical cord tissue (size ≈ 8 mm^3)were obtained from Wharton's jelly by excision biopsy and placed in culture dishes as described before (Hoerstrup *et al*., 2002b). Outgrowing cells were expanded up to passage 5.

Human umbilical cord blood-derived endothelial progenitor cells (EPCs)

EPCs were isolated from the mononuclear fraction of 20 ml fresh blood that was obtained by puncturing the human umbilical cord vein directly after delivery using density gradient centrifugation (Histopaque–1077, Sigma Chemical Company, St. Louis, MO). EPCs were cultured and differentiated in endothelial basal medium (EBM^{TM}-2, Cambrex, Walkersville, MD), containing growth factors and supplements (Vascular Endothelial Growth Factor (VEGF), human Fibroblasts Growth Factor (hFGF), human recombinant long-Insulin-like Growth Factor-1 (R-3-IGF-1), human Epidermal Growth Factor (hEGF), Gentamycin and Amphotericin (GA-1000), Hydrocortisone, Heparin, Ascorbic Acid, and 2% Fetal Bovine Serum) (Schmidt *et al*., 2004).

Standard vascular endothelial cells

Human umbilical cord vein-derived endothelial cells (HUVEC) were isolated using a collagenase installation technique. The umbilical cord veins were incubated in 0.2% collagenase (Collagenase A, Roche Diagnostics GmbH, Mannheim, Germany) solved in serum-free medium. After 20 min, the cell suspension was centrifuged. Cells were cultured and expanded in EBM^{TM}-2 medium containing the above mentioned growth factors and supplements.

4.3.2 Fabrication of tissue engineered blood vessels (TEBV)

Biodegradable tubular scaffolds (n=8; inner diameter 0.5 and length 3 cm) were produced by heat application welding technique from a non-woven polyglycolic-acid mesh (PGA, thickness: 1.0 mm, specific gravity: 69 mg/cm^3, Albany Int., Albany, NY) and dip-coated with poly-4-hydroxybutyric acid (1% w/v P4HB, TEPHA Inc., Cambridge, MA). After sterilization with ethanol, human umbilical cord-derived myofibroblasts (3.5×10^6 cells/cm^2) were seeded on the inner surfaces of the vascular tubes using fibrin as a cell carrier (Mol *et al*., 2005a). After 14d culturing under static conditions in EBM-2 medium containing the above mentioned growth factors, vessels (n=4) were endothelialized with differentiated cord blood-derived EPCs (1.5×10^6 cells/cm^2). Controls (n=4) were endothelialized with HUVEC, respectively. After endothelialization, all TEBV were kept at humidified incubator conditions (37°C, 5%

CO_2) for additional 24h and implanted into a pulse duplicator system (Hoerstrup *et al.*, 2001). Briefly, the pulse duplicator system consisted of a bioreactor connected via a silicon tubing with a medium reservoir. The pulsatile flow of the nutrient medium (EBM-2 medium containing the above mentioned growth factors) was generated by periodic expansion of a highly elastic membrane, de- and inflated using an air pump. Tissue engineered vessels were connected in parallel to the medium reservoir-bioreactor circulation. The pulsed flow of nutrient medium was directed through their inner lumen exposing them to flow and shear stress (125 ml/min at 30mmHg) for 7 subsequent days.

4.3.3 Exposure to inflammatory stimuli

For functional plasticity assessment of endothelial cells in response to inflammatory stimuli representative samples of TEBV were exposed to TNF-α (Sigma). EPC-derived endothelia were exposed to TNF-α (10 ng/ml medium) for 20h. Controls endothelialized with standard vascular endothelial cells as well as monolayers of both cell types seeded into chamber slides (Falcon) were treated in parallel.

4.3.4 Analysis of the TEBV

Histology and immunohistochemistry

After fixation in 4% phosphate buffered formalin (pH 7.0) and paraffin-embedding, 5-7 µm sections of TEBV were examined histologically by Hematoxylin-Eosin (H&E) and Masson's Trichrome staining. Cell phenotypes of the neo-tissues were validated by immunohistochemistry using the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ) with Ventana reagents for the entire procedure. Primary antibodies against the following antigens were applied: vimentin (clone 3B4), desmin (clone D33), CD31 (clone JC/70A), von Willebrand factor (vWF; affinity purified rabbit antibodies; all from DakoCytomation, Glostrup, Denmark), α-smooth muscle actin (α-SMA, clone 1A4; Sigma) and endothelial nitric oxide synthase type III (eNOS; affinity purified rabbit antibodies; BD Transduction Laboratories, San Diego, CA). Primary antibodies were detected with the Ventana iVIEW DAB detection kit, yielding a brown reaction product. For eNOS, the signal was enhanced with the amplification kit.

Quantitative evaluation of extracellular matrix elements

As an indicator for collagen, the content of hydroxyproline was determined of dried samples (Huzar *et al.*, 1980). Sulphated glycosaminoglycans (GAG) were detected colorimetrically using papain digested samples and 1,9-di-methyl-methylene blue (Farndale *et al.*, 1986). Human saphena vein tissue served as native vascular control.

Determination of cell number

Cell numbers were determined from the papain digests after 50× dilution in TNE-buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH7.4) and labelling of the DNA using Hoechst dye (Bisbenzimide H33258, Fluka, Buchs, Switzerland) (Kim *et al*., 1988). After 10 min incubation in a dark environment, the fluorescence was measured by fluorometry (Fluostar, BMG, Offenburg, Germany, 355 nm excitation / 460 nm emission). The amount of DNA was calculated based on a standard curve using calf thymus DNA (Sigma) and compared to the DNA amount of native vascular tissue.

Scanning Electron Microscopy

Representative samples of TEBV of all groups were fixed in 2% glutaralaldehyde for 24 h. After preparation, samples were sputtered with gold and investigated with a Zeiss Supra 50 VP Microscope (Zeiss, Jena, Germany).

Assessment of physiological properties of endothelia

Monolayers of stimulated and unstimulated differentiated EPCs were trypsinized and analyzed for the expression of Tissue Factor (TF) and thrombomodulin (TM) by flowcytometry (FACS). All samples were stored at 4°C following trypsinisation. FACS-analysis was performed using antibodies against TF (Clone l, Acris, Hiddenhausen, Germany), TM (Clone 1009, DakoCytomation, Glostrup, Denmark), CD31 and vWF. Primary antibodies were detected with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies (Boehringer Mannheim, Indianapolis, IN). Analysis was performed on a Becton Dickinson FACScan (Sunnyvale, CA). Irrelevant isotype-matched antibodies (IgG1MOPC-21, Sigma Chemical Company, St. Louis, MO) served as negative controls and stimulated as well as unstimulated HUVEC as positive controls. Fixation and permeabilization for detection of vWF was performed using 70% ethanol. Geometric mean fluorescence intensity ratios (MFIR) were calculated as $MFI_{\text{antibody of interest}}/MFI_{\text{corresponding control antibody}}$.

Evaluation of mechanical properties

Mechanical properties of TEBV tissues were analyzed using a uniaxial tensile tester (Instron 4411, Massachusetts, USA) equipped with a 10 Newton load cell. The crosshead speed was set to correspond to an initial strain rate of 1 min^{-1}. The recorded tensile force and displacement were transformed into stress-strain curves.

4.4 Results

4.4.1 Analysis of TEBV

Macrostructure

Figure 4.1A shows the vascular scaffold of about 3 cm length and 0.5 cm diameter before seeding. After in vitro culturing the surfaces were covered densely with cells and the lumen was open (Figure 4.1B). A homogenous wall thickness of 0.3 cm was observed. TEBV were macroscopically intact and pliable. Furthermore, they were amenable for suturing manipulation.

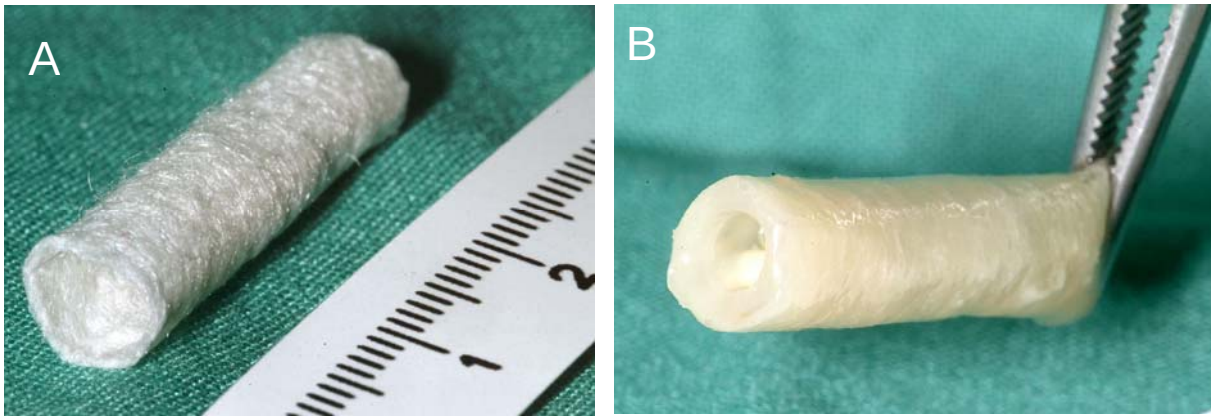

Figure 4.1: *Macroscopic appearance of TEBV: Vascular scaffolds of 0.5 cm diameter and 3 cm length fabricated from PGG/P4HB before seeding (A) and after in vitro culturing (B). TEBV were intact, pliable and densely covered with cells (B). The lumen was open and the wall thickness was homogenous at about 0.3 cm.*

Tissue microstructure

Histology revealed a cellular tissue in the H&E staining (Figure 4.2A) with beginning collagen synthesis as demonstrated by Masson's Trichrome staining (data not shown). Morphologically, a three-layered tissue architecture with an outer layer consisting of loosely arranged cells was observed. Cells in the middle layer were organized more compactly and those in the inner monolayer were longitudinally oriented (Figure 4.2A). Immunohistochemistry revealed expression of α-SMA in the middle layer (Figure 4.2B) and of CD31 in the inner layer (Figure 4.2C). Additionally, in the middle layer some cells expressing CD31 were found (Figure 4.2C). The distance of these cells from the inner endothelial layer is visualized by higher magnification (Figure 4.2D). Vimentin could be seen throughout the whole tissue, whereas desmin could not be detected (data not shown). No difference in tissue formation or phenotype was found between TEBV endothelialized with differentiated EPCs and HUVEC (data not shown).

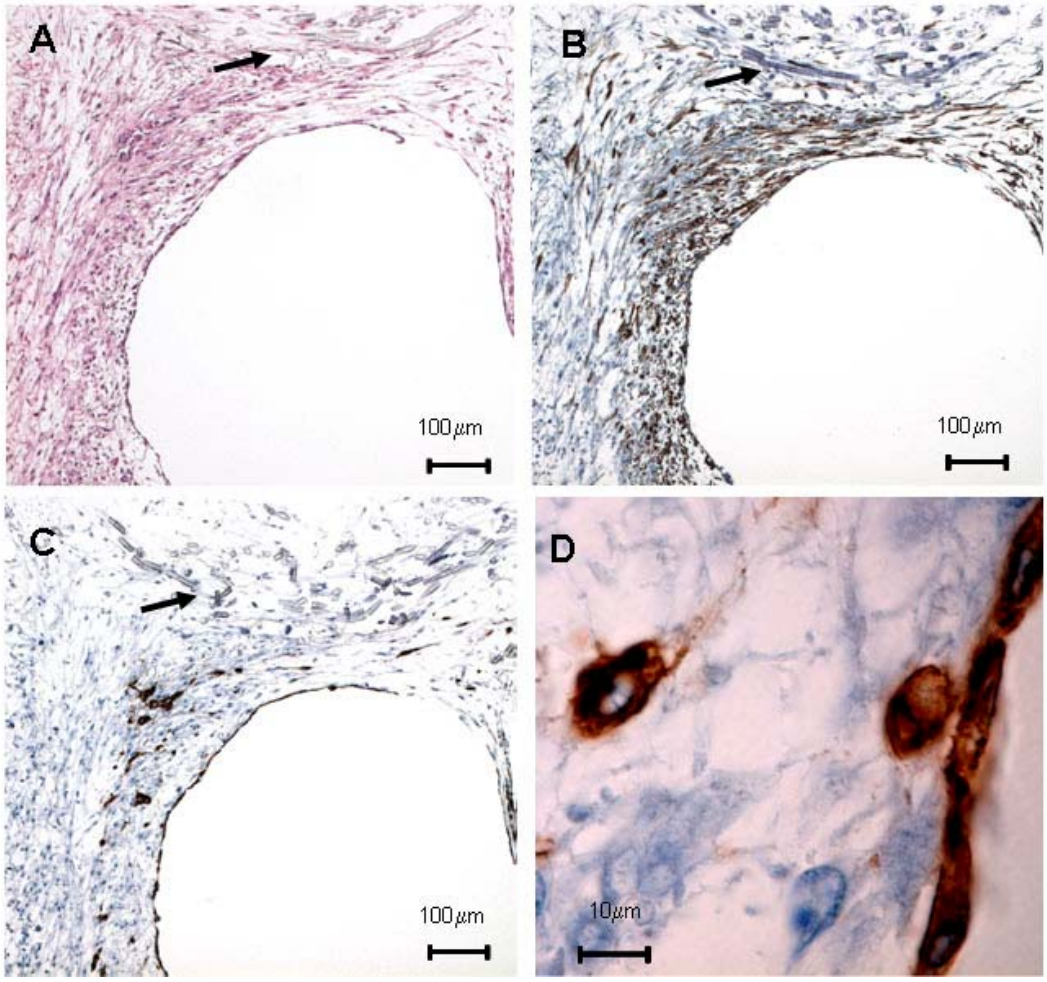

Figure 4.2: *Histology and immunohistochemistry of TEBV: (A) H&E staining revealed a cellular tissue with an outer layer consisting of loosely arranged cells and a middle layer organized more compactly. The innermost layer consisted of endothelial cells. (B) Cells in the middle layer expressed α-SMA. (C) A CD31-positive cell layer lined the lumen of the TEBV. Additionally, some cells expressing CD31 could be found in the middle layer. (D) Higher magnification of CD31 positive cells distant from the luminal cell lining. Arrows point at remnants of scaffold.*

Surface morphology

Scanning electron microscopy demonstrated smooth endothelialized inner surfaces for all TEBV. Both, EPC-derived (Figure 4.3A) and HUVEC-derived neo-endothelia (Figure 4.3B) were intact.

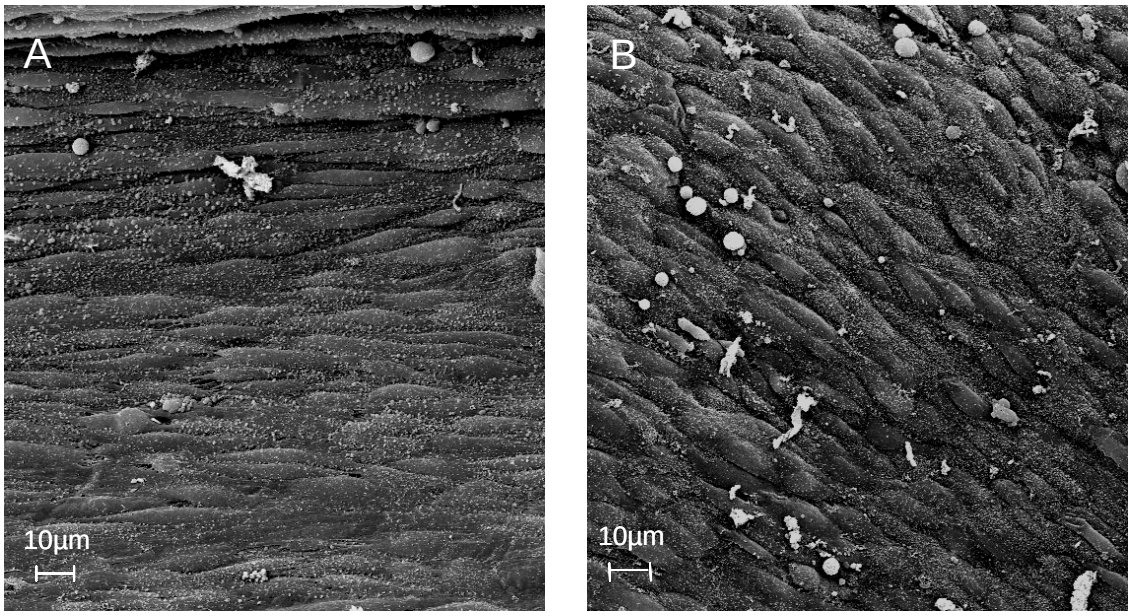

Figure 4.3: *Morphology of the inner surfaces: Scanning electron micrographs show smooth inner surfaces of the TEBV densely covered with endothelial cells derived from EPCs (A) and HUVEC (B).*

Extracellular matrix analysis

Biochemistry revealed the major extracellular matrix constituents (Figure 4.4). Up to 62% of native tissue glycoaminoglycans (7.4 µg/mg) was present in the TEBV (4.55 ± 1.67 µg/mg). The cell number expressed as total DNA amount in the TEBV (3.87 ± 2.30 µg/mg) was 90% of that of native tissue (4.30 µg/mg). However, the average amount of hydroxyproline was only 0.5 ± 0.32 µg/mg corresponding to 1% of the amount found in native tissue (50 µg/mg).

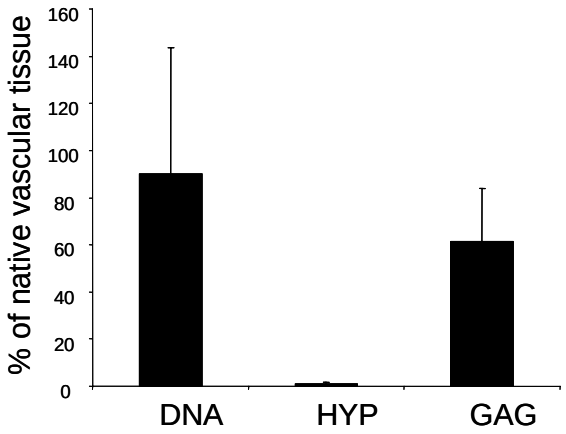

Figure 4.4: *Quantification of extracellular matrix elements and cell number: Total cell number (represented as DNA) in TEBV was 90% of native tissue values, glycoaminoglycans (GAG) and collagen (hydroxyproline,HYP) were 62% and 1%, respectively.*

4.4.2 Assessment of physiological properties of endothelia

Figure 4.5 shows the antigene expression pattern of differentiated EPCs compared to HUVEC determined by flowcytometry. Differentiated EPCs (purple curves) expressed the typical endothelial cell markers CD31, TM and vWF (data not shown for the latter). Furthermore, they lacked the expression of TF. HUVEC demonstrated a similar expression pattern. Stimulation of differentiated EPCs with TNF-α (black curves) resulted in a tendency of up-regulation of TF (MFIR-TF unstimulated = 0.50±0.36 vs. MFIR-TF stimulated = 1.56±0.32) and down-regulation of TM (MFIR-TM unstimulated = 1.57±1.10 vs. MFIR-TM stimulated = 0.96±0.09). The same tendency could be observed with HUVEC (MFIR-TF unstimulated = 0.73±0.20 vs. MFIR-TF stimulated = 1.39±0.77) and down-regulation of TM (MFIR-TM unstimulated = 1.20±0.56 vs. MFIR-TM stimulated = 1.02±0.75). These findings were consistent in three independent experiments. Furthermore, immunohistochemistry demonstrated constant expression of eNOS and vWF in the unstimulated and stimulated TEBV (data not shown).

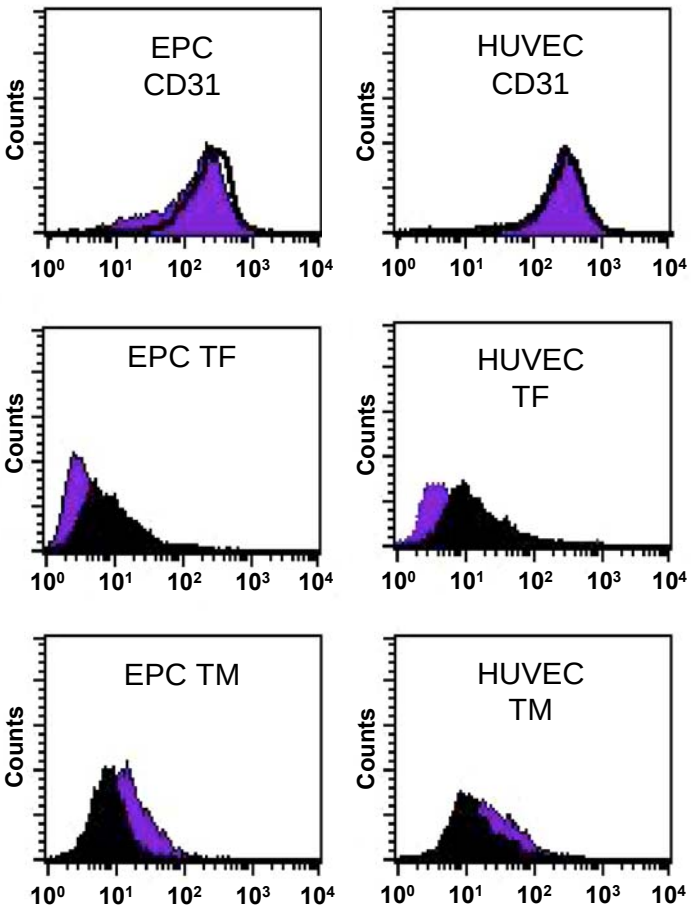

Figure 4.5: *Functional plasticity and bioactivity of endothelia cells: Both, EPC-derived endothelial cells and HUVEC expressed CD31 and TM but lacked the expression of TF (grey). After stimulation with TNF-α (black) the expression of TM was decreased whereas the expression of TF was up-regulated. CD31 expression was not affected.*

4.4.3 Biomechanical properties

Stress-strain curves of TEBV showed profiles that were comparable to those of native tissues. Table 4.1 summarizes the mechanical properties that were similar for TEBV endothelialized with EPC-derived EC and HUVEC. For both groups of TEBV, the Young`s Modulus was about 2.0 MPa, the tensile strength was 0.19 MPa, and the strain at maximal stress 0.18%.

Table 4.1: *Mechanical properties of TEBV.*

	Young`s Modulus (MPa)	Tensile strength (MPa)	Strain at max. stress %
TEBV endothelialized with EPC	1.99±0.43	0.22±0.06	0.18±0.03
TEBV endothelialized with HUVEC	2.08±0.93	0.16±0.06	0.17±0.10

4.5 Discussion

Successful tissue engineering of entire blood vessels based on umbilical cord-derived cells could be demonstrated. The generated vessels showed a three-layered tissue architecture similar to the structure of native blood vessels with an endothelium lined intima, a media containing smooth muscle cells and an adventitia with connective tissue. The outer layer of the TEBV showed characteristics of connective tissue with beginning collagen and glycosaminoglycans production. Cells in the middle layer expressed α-SMA, a marker of smooth muscle cells, indicating the development of a media. This stratified architecture with specific cell phenotypes may be related to the transdifferentiation of Wharton's Jelly-derived cells as the presence of mesenchymal progenitor cells with multilineage potential in the Wharton's jelly of human umbilical cords is reported (Wang *et al.*, 2004; Sarugaser *et al.*, 2005). The applied flow and shear stress and/or growth factors in the medium may enhance this effect. The lack of desmin indicates that the transdifferentiation into smooth muscle cells and the tissue maturation has not been completed yet.

The inner layer was formed by functional EPC-derived endothelial cells which expressed typical makers of endothelial cells such as vWF, CD31, eNOS and TM. Furthermore, the absence of TF and the presence of TM indicate that the neo-endothelia may have non-thrombogenic characteristics. Biologically active TF plays an

important role in the pathogenesis of thrombosis (Belting *et al.*, 2005). Thrombomodulin has been demonstrated to be an important modulator of endothelial thromboresistance (Esmon, 2004). The physiological response to TNF-α (Esmon, 2004) demonstrates the functional plasticity of the endothelia. This is in analogy to native endothelia, whose phenotypic characteristics "vary in space and in time" as well as in response to inflammatory stimuli (Aird, 2004).

The presence of endothelial cells in the middle layer (Figure 4.2C) may be explained by beginning VEGF-guided migration (Eichmann *et al*., 2005; Gerhardt *et al.*, 2003) of the endothelial cells from the inner lumen surface to the outer layers in order to form a network providing a basis for the vasa vasorum.

Beginning collagen synthesis was detected in TEBV, even though it was still low at 1% of the native tissue value. This may indicate beginning tissue maturation which will continue in vivo (Hoerstrup *et al.*, 2000a) but might restrict the TEBV to low pressure applications. Alternatively, collagen content could be increased prior to implantation by exposing the neo-tissues to higher mechanical stimulation in a biomimetic system (Mol *et al*., 2003) or by diminishing the static culture time for the benefit of the bioreactor phase as it has been reported that the collagen content increases with the time of applied pulsatile stress (Niklason *et al.*, 1999; Hoerstrup *et al.*, 2000a; Hoerstrup *et al.*, 2001). However, the mechanical characteristics of the TEBV suggest that the neo-tissues would function also in vivo. Corresponding in vivo tests in an animal model will be performed.

In summary, these results indicate that TEBV with three-layered tissue architecture and functional endothelia similar to native blood vessels can be successfully generated from human umbilical cord progenitor cells. Thus, umbilical cord-derived progenitor cells obtainable before or at birth combined with the possibility to manufacture scaffolds of any diameter will allow the fabrication of living autologous TEBV tailored to the anatomy of the patient. This approach may enable the realization of the tissue engineering concept for pedriatic applications and will be investigated in future in vivo studies.

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Chapter 5

Assessment of biological activity of tissue engineered cardiovascular pediatric replacements based on umbilical cord-derived progenitors

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"Engineering of Biologically Active Living Heart Valve Leaflets Using Human Umbilical Cord-Derived Progenitors."

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5.1 Abstract

This study demonstrates the engineering of biologically active heart valve leaflets using prenatally available human umbilical cord-derived progenitor cells as the only cell source. Wharton's Jelly–derived cells and umbilical cord blood-derived endothelial progenitor cells were subsequently seeded on biodegradable scaffolds and cultured in a biomimetic system under biochemical and/or mechanical stimulation. Depending on the stimulation, leaflets showed mature layered tissue formation with functional endothelia and extracellular matrix production comparable to native tissues. This demonstrates the feasibility of heart valve leaflet fabrication from prenatal umbilical cord-derived progenitor cells as a further step to overcome the lack of living autologous replacements with growth and regeneration potential for the repair of congenital malformation.

5.2 Introduction

Nowadays, approximately 275.000 patients undergo heart valve replacement each year (Schoen, 2001) and have to deal with severe side-effects such as life-long anticoagulation therapy, increased risks for infections and thromboembolism as the currently used replacement materials are either synthetic or bioprostethic (Mayer, 1995; Schoen and Levy, 1999). Glutaraldehyde-fixed or cryopreserved biological valves do not require an anticoagulation therapy, though, but represent non-viable prostheses suffering from structural dysfunction due to progressive tissue deterioration (Hammermeister *et al*., 1993; Schmidt and Baier, 2000). Thus, they necessitate re-replacement within 10–15 years. Because of a higher immunological competence their durability is even less in younger individuals. Also, their availability is limited. Being non-viable structures, all clinically available valve prostheses, except the pulmonary autograft, lack the ability to grow, to repair, or to remodel.

These disadvantages could be avoided by valve replacements that closely mimic their native counterparts and comprise adequate mechanical function, durability, good haemodynamic performance, as well as the absence of immunogenic, thrombogenic, and/or inflammatory reactions (Harken *et al*., 1962). Particularly, pediatric patients would benefit from living replacement materials with growth and regeneration potential as the currently used non-living prostheses necessitate re-operations due to their lack of growth (Mayer, 1995).

Tissue engineering aims to overcome the limitations of currently used prostheses by the in vitro fabrication of living autologous tissue replacements with the capacity of growth, remodeling and regeneration. In previous studies, the concept of engineering living, functional heart valves from ovine vascular-derived cells has been demonstrated in animal models (Hoerstrup *et al.*, 2000a). Furthermore, the in vitro methodology has been transferred successfully from animal to human by using human cell sources including bone marrow–derived mesenchymal cells (Hoerstrup *et al.*, 2002a). However, the ideal cell source for the in vitro fabrication of living autologous biologically active heart valve replacements for the repair of congenital malformation in newborns has not been identified, yet. Besides easy access, the ideal cell source must allow prenatal cell harvesting as soon as the cardiovascular defect is detected, in order to have the autologous, tissue engineered replacement ready to use at or shortly after birth and to prevent secondary damage to the newborn's immature heart. In addition, an ideal cell source should provide all types of cells required for the tissue engineering process. The obtained cells should have a high potential of growth as well as characteristics similar to those of their native counterpart.

A promising cell source is the umbilical cord as it provides a variety of cell types, including progenitor cells (Wang *et al.*, 2004; Sarugaser *et al.*, 2005) and allows easy sampling of both tissue and blood-derived progenitor cells e.g. by cordocentesis and biopsy using today's advanced operative fetoscopic technology (Kohl *et al*., 1997; Kohl *et al*., 2000; Quintero *et al*., 2001; Hirose *et al.*, 2003). Particularly, endothelial progenitor cells (EPCs), which are obtainable from the peripheral and the umbilical cord blood (Griese *et al*., 2003; Hur *et al*., 2004; Urbrich and Dimmeler, 2004) are a

promising cell source for the fabrication of autologous antithrombogenic surfaces on tissue engineered heart valve replacements. They have been used successfully for the repair of injured vessels, the neo-vascularization and regeneration of ischemic tissue (Kawamoto *et al.*, 2001; Assmus *et al.*, 2002; Pesce *et al.*, 2003; Schachinger *et al.*, 2004) as well as for the coating of synthetic vascular grafts (Shirota *et al.*, 2003a), endothelialization of decellularized grafts in animal models (Kaushal *et al.*, 2001) and for seeding of hybrid grafts (Shirota *et al.*, 2003b).

In previous studies, we have demonstrated the feasibility of using human umbilical cord–derived cells for the engineering of cardiovascular constructs such as vascular grafts and patches (Hoerstrup *et al.*, 2002b; Schmidt *et al.*, 2004; Schmidt *et al.*, 2005) resulting in layered immature, proliferative tissues with beginning matrix formation. Here, we focus on the in vitro engineering and maturation of living autologous biological active heart valve leaflets based on the umbilical cord as the only cell source as a further step towards the clinical realization of the pediatric tissue engineering concept.

5.3 Materials and methods

5.3.1 Isolation and phenotyping of cells

Tissue and blood were harvested from fresh umbilical cords of healthy individuals after informed consent was obtained from the participants.

Human Wharton's jelly-derived myofibroblasts (WMF)

Pieces of umbilical cord tissue (size ≈ 8 mm^3)were obtained from Wharton's jelly by excision biopsy and placed in culture dishes as described before (Hoerstrup *et al.*, 2002b). Outgrowing cells were expanded up to passage 11 and the cell phenotype was characterized by immunohistochemistry.

Human umbilical cord blood-derived EPCs

EPCs were isolated from the mononuclear fraction of 20 ml fresh blood that was obtained by puncturing the human umbilical cord vein directly after delivery using density gradient centrifugation (Histopaque–1077, Sigma Chemical Company, St. Louis, MO). EPCs were cultured and differentiated in endothelial basal medium (EBM^{TM}-2, Cambrex, Walkersville, MD), containing growth factors and supplements (Vascular Endothelial Growth Factor (VEGF), human Fibroblast Growth Factor (hFGF), human recombinant long-Insulin-like Growth Factor-1 (R-3-IGF-1), human Epidermal Growth Factor (hEGF), Gentamycin and Amphotericin (GA-1000), Hydrocortisone, Heparin, Ascorbic Acid, and 2% Fetal Bovine Serum). Cultured cells were investigated for the expression of VEGFR-2 (KDR, Flk-1/Fc Chimera, human recombinant, Sigma), lectin (from ulex europeus, UEA-1, Sigma), and the uptake of ac-LDL (Dil-Ac-LDL, Biomedical Tech., Stoughton, MA). After 3 weeks, the endothelial phenotype was analyzed by immunohistochemistry.

Immunohistochemistry
WMF and EPCs were cultured in chamber slides (Falcon). After confluency, cells were washed with buffer and fixed with ethanol. Immunohistochemistry was performed using the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ) with Ventana reagents for the entire procedure. Primary antibodies against the following antigens were applied: vimentin (clone 3B4), desmin (clone D33), CD31 (clone JC/70A), Ki-67 (clone MIB-1), von Willebrand factor (vWF; affinity purified rabbit antibodies; all from DakoCytomation, Glostrup, Denmark); α-smooth muscle actin (α-SMA, clone 1A4; Sigma) and endothelial nitric oxide synthase type III (eNOS; affinity purified rabbit antibodies; BD Transduction Laboratories, San Diego, CA). Primary antibodies were detected with the Ventana iVIEW DAB detection kit, yielding a brown reaction product. For eNOS, the signal was enhanced with the amplification kit. Slides were counterstained with hematoxylin and glass coverslipped.

5.3.2 Fabrication and seeding of scaffolds

Heart valve leaflet scaffolds were fabricated from a non-woven polyglycolic-acid mesh (PGA, thickness: 1.0 mm, specific gravity: 69 mg/cm^3, Albany Int., Albany, NY). PGA sheets were cut into 2.3 cm^2 leaflets (n=18), dip-coated with Poly-4-Hydroxybutyric acid (1% w/v P4HB, TEPHA Inc., Cambridge, MA) and three each attached to ring-shaped supports (diameter 20 mm). After drying, the scaffolds were sterilized with ethanol and the leaflets were manually checked for mobility by repetitive up and down movement. WMF (3.5×10^6 cells/cm^2) were seeded individually onto each leaflet using fibrin as a cell carrier (Mol *et al.*, 2005a).

5.3.3 Biochemical and mechanical conditioning protocol

After 15 min at humidified incubator conditions, one part of the heart valve leaflets (n=6) was cultured statically as a baseline, whereas the other part (n=12) was positioned into a Diastolic Pulse Duplicator, described in detail before (Mol *et al*., 2005b). Briefly, the Diastolic Pulse Duplicator consisted of a bioreactor, in which the valve leaflets were placed, and a connected medium reservoir. The leaflets were perfused continuously (4ml/min) with medium from the reservoir using a roller pump. Biological activity of leaflets was investigated by response to biochemical and/or mechanical stimulation. Two groups were formed with n=6 leaflets each: Group 1 with the addition of the above mentioned growth factors (VEGF, hFGF, R-3-IGF-1, hEGF as well as Hydrocortisone and Heparin) to the basal medium, group 2 without. After 7 days, both groups were divided into two subgroups. One group was continuously exposed to perfusion while the other was exposed to perfusion and cyclic straining (mechanical stimulation). For cyclic straining a dynamic pressure difference (12 mmHg; 1 Hz) over the leaflets was applied by releasing compressed air via a magnetic valve. After additional 14 days, all valves were removed from the bioreactor and endothelialized with EPC-derived cells (1.5×10^6 cells/cm^2) on both leaflet sides.

After seeding, leaflets were kept at humidified incubator conditions (37°C, 5% CO_2) for additional 48h and placed back into the bioreactor for 5 subsequent days under exposure of the same biochemical and/or mechanical stimulation as before. Static controls were treated in parallel.

5.3.4 Analysis of the engineered heart valve leaflets

After the in vitro culture time, representative samples of all leaflets were analyzed using the following techniques:

Histology and immunohistochemistry

After fixation in 4% phosphate buffered formalin (pH 7.0) and paraffin-embedding, sections of 5-7 µm of each group were examined histologically by Hematoxylin-Eosin (H&E), Trichrom-Masson, and Movat pentachrome staining. Cell phenotypes of the neo-tissues were validated by immunohistochemistry that was performed as described above. For paraffin sections, antigen retrieval was done for all antibodies by heating with cell conditioner 1 (CC1, Ventana), except for CD31, where predigestion with protease 1 (Ventana) for 4 min was required.

Quantitative evaluation of extracellular matrix (ECM) elements

As an indicator for collagen, hydroxyproline content was determined of dried samples (Huzar *et al.*, 1980). Sulphated glycosaminoglycans (GAG) were detected colorimetrically using papain digested samples and 1,9-di-methyl-methylene blue (Farndale *et al.*, 1986). Native human heart valve tissues served as controls.

Determination of cell number

Cell numbers were determined from the papain digests after 50× dilution in TNE-buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4) and labeling of the DNA using Hoechst dye (Bisbenzimide H33258, Fluka, Buchs, Switzerland) (Kim *et al.*, 1988). After 10 min incubation in a dark environment, the fluorescence was measured by fluorometry (Fluostar, BMG, Offenburg, Germany, 355 nm excitation / 460nm emission). The amount of DNA was calculated based on a standard curve using calf thymus DNA (Sigma).

Evaluation of mechanical properties

Mechanical properties of leaflet tissues (15×5×1 mm) were analyzed by using a uniaxial tensile tester (Instron 4411, Massachusetts, USA) equipped with a 10 Newton load cell. The crosshead speed was set to correspond to an initial strain rate of 1 min^{-1}. The recorded tensile force and displacement were transformed into stress-strain curves. Young's moduli were determined from the latter by linear fits to maximum slopes.

Scanning Electron Microscopy

Representative samples of leaflets of all groups were fixed in 2% glutaralaldehyde for 24 h. After preparation, samples were sputtered with gold and investigated with a Zeiss Supra 50 VP Microscope (Zeiss, Jena, Germany).

Statistical analysis
The data were analyzed using SYSTAT version 10 (SPSS, Chicago, IL). Analysis of variance was performed to estimate the means and the significance of the categorical factors (biochemical and mechanical stimulation). Post-hoc pairwise mean differences were estimated using the Bonferoni test and the nonparametric Kruskal-Wallis test was performed. A p-value <0.05 was considered as significant.

5.4 Results

5.4.1 Morphology and phenotypes of cells

WMF: Outgrowing WMF expressed vimentin and α-SMA but lacked desmin. This staining pattern reflects a fibroblast-myofibroblast-like phenotype (VA type).
EPCs: After isolation, plated cells were initially round shaped. After 4 days, cells were attached and formed clusters. Two different types of EPCs were detected: spindle-like cells (80%) and polymorph cells (20%). Spindle-like cells died within 14 days, whereas polymorph EPCs (Figure 5.1A) proliferated by forming colonies (Figure 5.1B). Outgrowing cells of these colonies stained positive for VEGFR-2 (data not shown) and demonstrated binding of UEA-1 (Figure 5.1C). Cells showed good proliferation under in vitro conditions and formed confluent cobblestone endothelial cell monolayers after 3 weeks (Figure 5.1D) with uptake of ac-LDL (Figure 5.1E), expression of CD31 (Figure 5.1F), vWF (Figure 5.1G) and eNOS (Figure 5.1H).

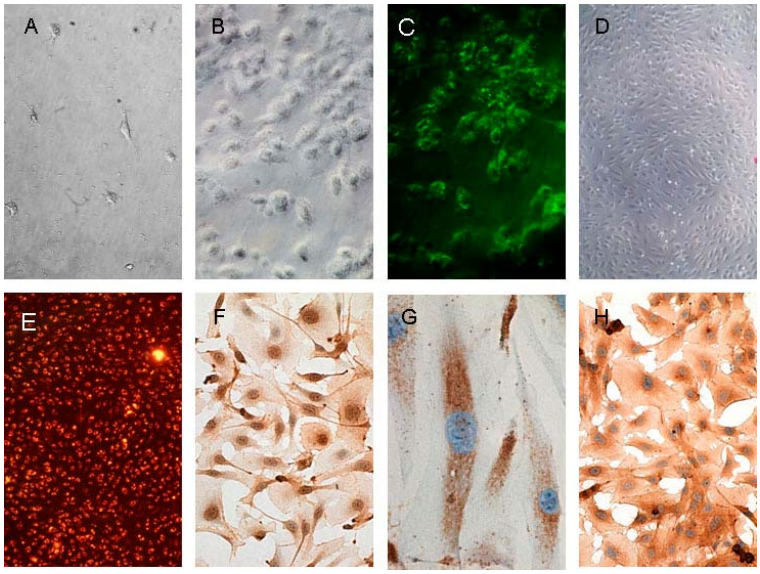

Figure 5.1: *Pre-seeding characterization of EPCs: 12d after isolation, polymorph EPCs began to proliferate (A, magnification: ×100). Outgrowing cells formed colonies (B, magnification: ×100) with binding of lectin (UEA-1) (C, magnification: ×100). After 3 weeks, EPCs formed a confluent monolayer (D, magnification: ×50) with uptake of ac-LDL (E, magnification: ×50), expression of CD31 (F, magnification: ×200), vWF (G, magnification: ×400) and eNOS (H, magnification: ×200).*

5.4.2 Analysis of the engineered heart valve leaflets

Macroscopic appearance

A macroscopic picture of the leaflets in the ring shaped support after 28 days in the bioreactor is presented in Figure 5.2. The leaflets were intact and pliable. Cells densely covered all leaflets.

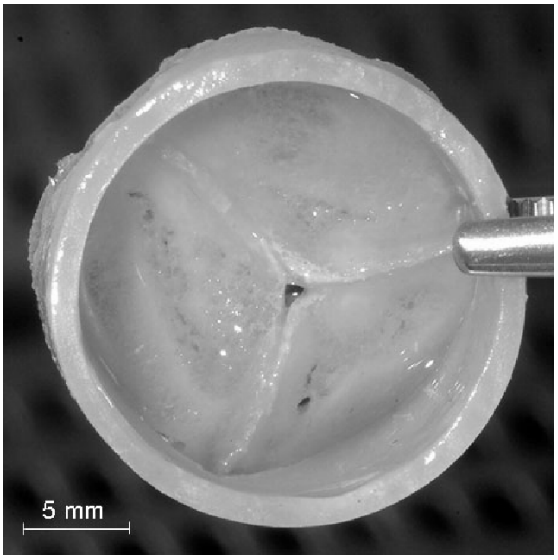

Figure 5.2: Tissue engineered heart valve leaflets with supporting ring after 21d in the bioreactor: Leaflets were intact and pliable and densely covered.

Histology and immunohistochemistry

Leaflets grown under static conditions demonstrated cellular ingrowth but little tissue formation in the H&E staining (Figure 5.3A) and no production of ECM elements in the Trichrom-Masson-staining (Figure 5.3B). In contrast, leaflets of all bioreactor groups showed cellular tissue formation with production of ECM. The tissue formation as well as cell phenotypes were independent of mechanical stimulation (perfusion/cyclic straining) but influenced by biochemical stimulation. In response to biochemical stimulation, H&E staining revealed tissue formation organized in a layered manner with dense outer layers and lesser cellularity in the inner part (Figure 5.3C). Trichrom-Masson-staining highlighted collagen at the outer layers (Figure 5.3E), whereas in the center loosely arranged ECM substance was detectable, characterized as GAG in the Movat staining (Figure 5.3G). In contrast, without biochemical stimulation leaflets (group 2) showed less tissue layering (Figure 5.3D), but more production of collagen in all parts of the tissue (Figure 5.3F) and less production of GAG (Figure 5.3H).

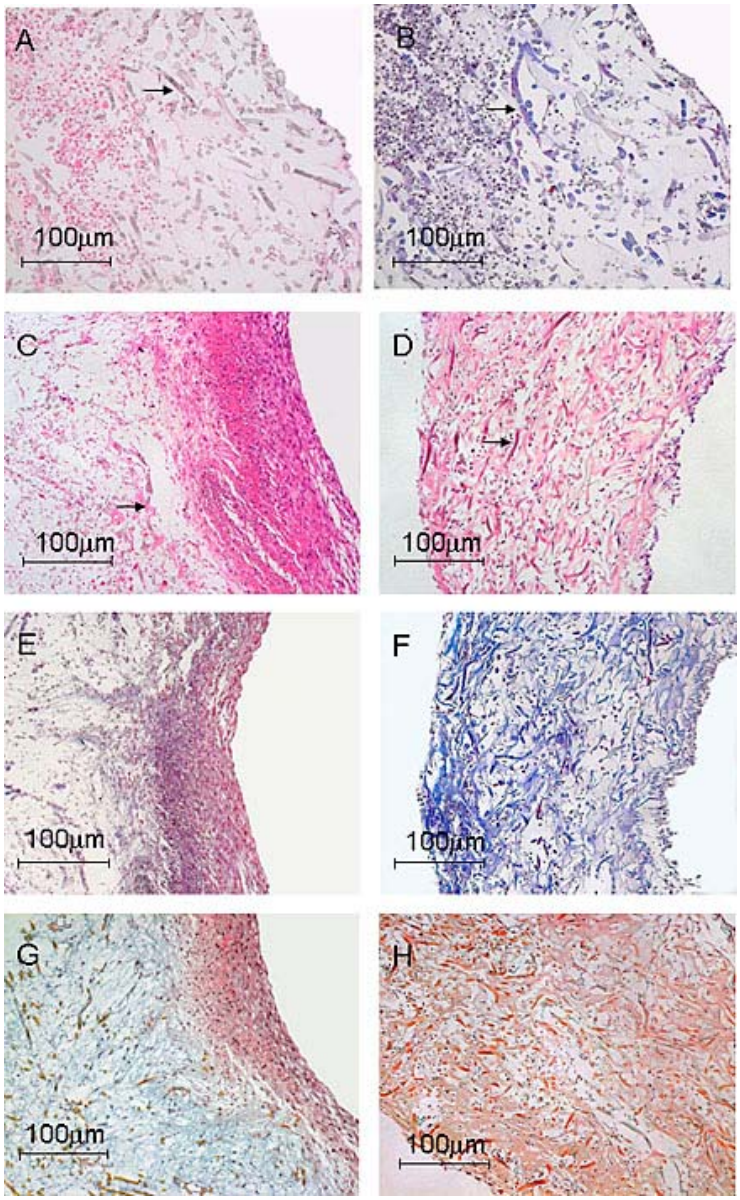

Figure 5.3: *Histology of tissues: H&E staining of statically cultured tissue engineered leaflets revealed cellular ingrowth with little tissue formation (A) and no collagen fibers could be detected in the Trichrom Masson staining (B). In contrast, leaflets cultured in the bioreactor (C-H) demonstrated cellular tissue formation and production of extracellular matrix elements. While histologies did not show an effect of mechanical stimulation on tissue formation, biochemical stimulation resulted in a more organized tissue formation as demonstrated by H&E staining (C). Collagen fibers in the outer layer and GAG in the middle part of the stimulated leaflets were highlighted bluish in the Trichrom Masson staining (E) and light blue in the Movat staining (G), respectively. In contrast, without biochemical stimulation leaflets showed less tissue organization in H&E staining (D), collagen fibers in all parts of the tissue in the Trichrom Masson staining (F) and less GAG in the Movat staining (H). Arrows point at remnants of scaffold.*

In detail examination of the outer tissue layers, biochemically stimulated leaflets of group 1 demonstrated denser cell layering in the H&E staining (Figure 5.4A) than those of the unstimulated group 2 (Figure 5.4B). An endothelial cell lining, highlighted brownish in the CD31 staining, was found on the surfaces of group 1 (Figure 5.4C) as well as of group 2 (Figure 5.4D) leaflets. Expression of vWF, and eNOS confirmed the presence of endothelial cells (data not shown). Beneath the endothelia, α-SMA positive cells were detectable in the more dense outer layer in group 1 leaflets (Figure 5.4E), whereas only a few α-SMA positive cells were present in the leaflets of group 2 (Figure 5.4F). Vimentin could be demonstrated throughout all leaflets whereas desmin was not expressed (data not shown). Ki-67 nuclear protein was detectable in the outer layers of the neo-tissue as well as in the inner parts of the leaflets of all groups (Figure 5.4G, H).

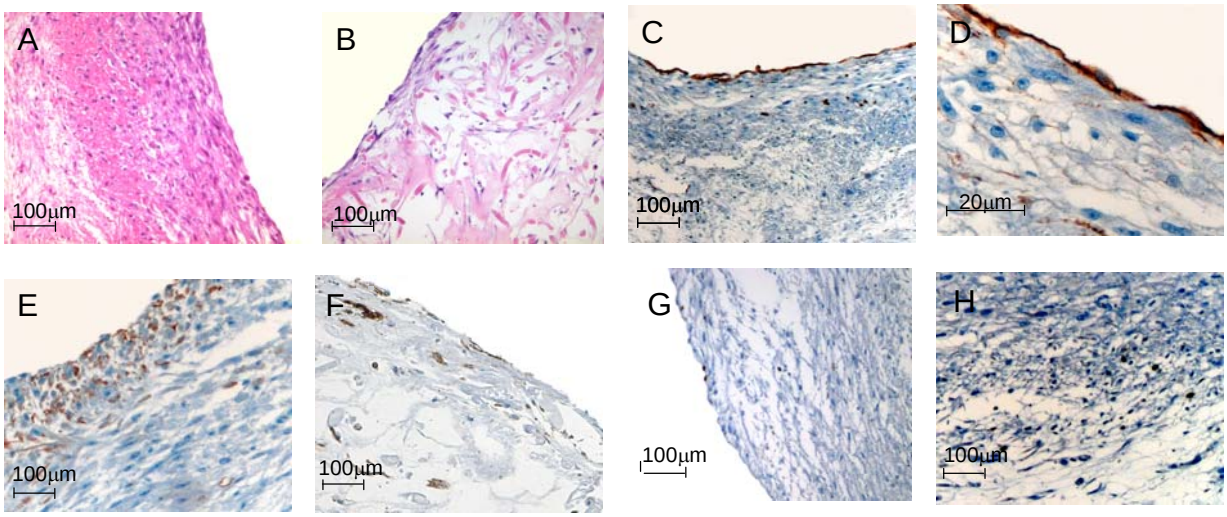

Figure 5.4: *Immunohistochemical characteristics of the tissues in response to biochemical stimulation: Detailed examination of the outer tissue layer by H&E staining showed denser cell layering for leaflets exposed to biochemical stimuli (A) compared to unstimulated leaflets (B). Expression of CD31, highlighted brownish, confirmed the endothelial phenotype of cells in the topmost layer of both stimulated (C) and unstimulated (D) leaflets. A subendothelial cell layer expressing α-SMA was formed under stimulation (E) but was not present without stimulation (F). Expression of Ki-67 nuclear protein demonstrated living, proliferating cells in all parts of the stimulated (G) and unstimulated tissues (H), respectively.*

Quantification of ECM elements

When leaflets were cultured statically few collagen could be detected (0.2% of native content). The amount of GAG was only 28% and the cell number was 54% of native tissue values. In contrast, in all leaflets of the bioreactor groups, the amount of GAG was comparable to that of native tissue but was higher in response to biochemical

stimulation. The amount of hydroxyproline reached about 16% of the native tissue content and was higher in cells cultured without growth factors. The cell number, expressed as DNA content, was similar for all culture conditions, reaching up to 65% of native tissue values. For both groups, mechanical conditioning showed no significant impact on the production of ECM elements. Figure 5.5A summarizes the content of extracellular matrix of leaflets of all bioreactor groups relative to native heart valve tissues. Figure 5.5B represents ECM elements per cell number and further stresses the response to biochemical conditioning on ECM production. Cells of group 1 produced significantly more GAG ($p<0.0001$) whereas those of group 2 produced significantly more collagen ($p<0.02$), expressed as hydroxyproline.

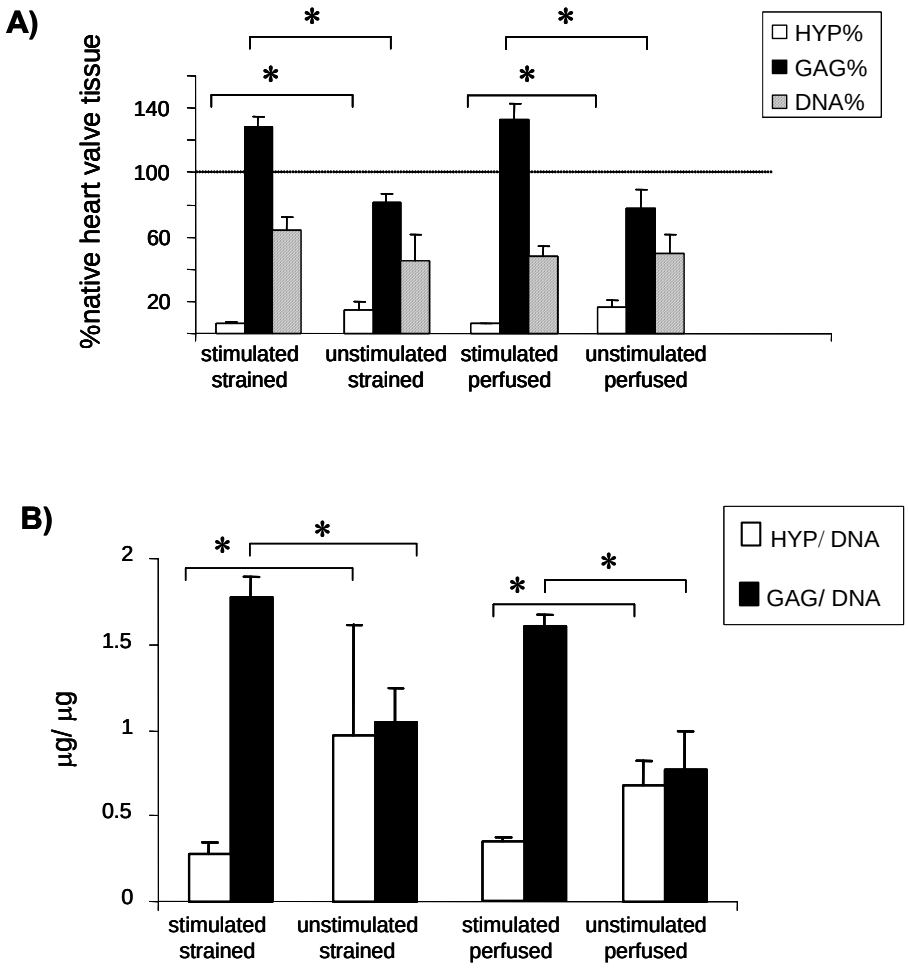

Figure 5.5: *Extracellular matrix analysis: (A) shows the amount of hydroxyproline (HYP, white), GAG (black) and cell number (DNA, patterned) of tissue engineered leaflets, which were either biochemically stimulated or not and strained or perfused, relative to native heart valve tissue and (B) contents of HYP and GAG per cell number. Means are given in µg matrix/µg DNA, error bars represent standard deviations, and asterisks significant differences.*

Mechanical properties

Table 5.1 summarizes the mechanical properties of the tissue engineered leaflets maturated in the bioreactor. Leaflets of group 2 were stiffer than those of group 1 as indicated by the higher Young`s Modulus. Mechanical stimulation influenced the mechanical properties of the tissue engineered valves independent of the medium as a trend of higher Young`s Moduli could be observed in response to cyclic strain.

Table 5.1: *Mechanical properties of tissue engineered heart valve leaflets.*

Group	Young`s Modulus (MPa)		Tensile strength (MPa)		Strain at max. Stress (-)	
1 strained	0.32 +/- 0.04	p=.017	0.08 +/- 0.01	p=.008	0.41 +/- 0.03	ns
1 perfused	0.05 +/- 0.03		0.01 +/- 0.01		0.62 +/- 0.02	
2 strained	1.44 +/-1.36	ns	0.16 +/- 0.11	ns	0.21 +/- 0.13	ns
2 perfused	0.36 +/- 0.42		0.04 +/- 0.02		0.35 +/- 0.12	

1: biochemically stimulated, 2: biochemically unstimulated

Scanning Electron Microscopy

All tissue engineered leaflets showed confluent endothelial surfaces. The configuration and orientation of EPCs was affected by mechanical but not by biochemical stimulation. Mechanically stimulated leaflets (Figure 5.6A) had a higher cell density compared to perfused ones (Figure 5.6B) but showed less parallel cell alignment.

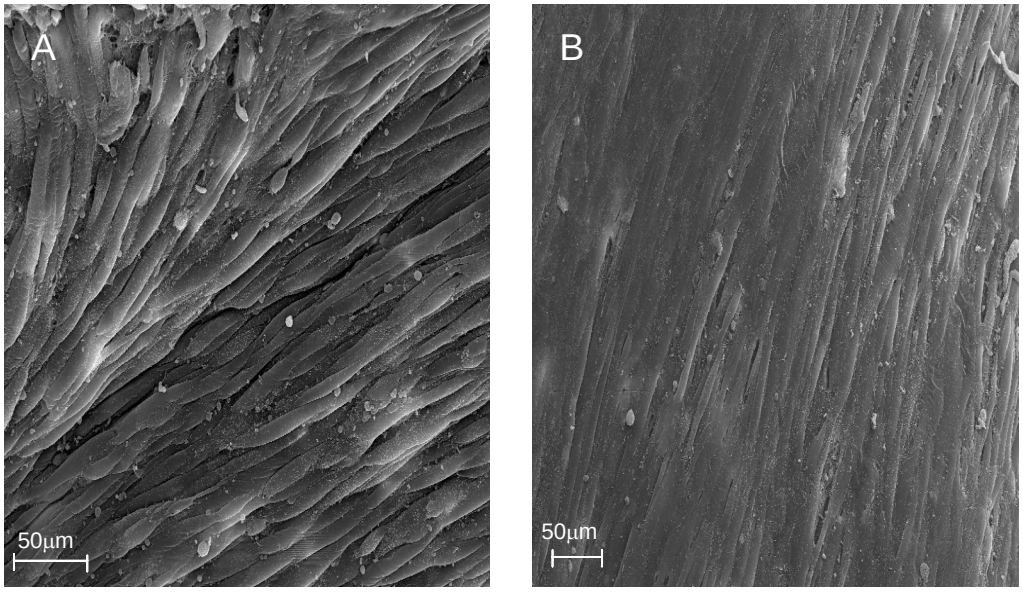

Figure 5.6: *Surface morphology: In scanning electron micrographs, mechanically stimulated heart valve leaflets (A) showed a higher cell density but less parallel alignment than perfused ones (B).*

5.5 Discussion

Pediatric cardiovascular tissue engineering aims at the fabrication of living, autologous growing replacements for the repair of congenital malformations in newborns. For this concept, cells are ideally obtained during pregnancy to have the tissue engineered replacement available at or shortly after birth, particularly to prevent secondary damage of the newborn's heart. A still unsolved problem for the fabrication of those replacements is the lack of appropriate prenatal cell sources (Hoerstrup *et al*., 2000a; Hoerstrup *et al*., 2002a; Sutherland *et al*., 2005).

In this study, umbilical cord-derived cells showed characteristics of a promising sole cell source for pediatric heart valve tissue engineering. Wharton's jelly-derived cells exhibited phenotypic profiles of a fibroblast-myofibroblast lineage indicating substantial similarity to human valvular interstitial cells (Messier *et al*., 1994). Moreover, WMF produced excellent ECM in amounts comparable to native tissue and other cells successfully used for in vitro and in vivo heart valve tissue engineering studies (Hoerstrup *et al*., 2000a; Hoerstrup *et al*., 2002a).

Isolated umbilical cord blood-derived EPCs differentiated into mature functional endothelial cells during cell expansion as described before (Asahara *et al*., 1997) and formed functional endothelial layers on the surfaces of tissue engineered leaflets even under cyclic strain. This observation provides indications that the human umbilical cord blood-derived differentiated EPCs may also function as a non-thrombogenic cell layer in an in vivo environment with changing mechanical stresses. Experiments with prosthetic vascular graft surfaces seeded with blood- or bone

marrow-derived EPCs have shown evidence supporting these assumptions (Kaushal *et al.*, 2001; Griese *et al.*, 2003). Future in vivo experiments will elucidate this aspect.

When tissue engineered constructs were allowed to respond to biochemical stimuli a mature layered tissue organization was found comparable to native heart valves and/or tissue engineered heart valves remodeled several weeks in vivo (Rabkin *et al.*, 2002). These are characterized by an outer layer (zona fibrosa) composed of collagen, a central layer (zona spongiosa) containing GAG and a layer characterized by the presence of elastin fibers (zona ventricularis) (Schoen, 1999). In accordance with other studies (Hoerstrup *et al.*, 2000a; Rabkin *et al.*, 2002) elastin could not be detected in the Movat staining used in this study, but might be present on mRNA level, which will be investigated in future studies. Furthermore, elastin is expected to be formed after implantation during the in vivo tissue remodeling process as shown in previous studies (Rabkin *et al.*, 2002). The cell phenotypes in the engineered leaflets resembled those of native tissue demonstrating α-SMA expression in the subendothelial layer of the zona fibrosa (Della Rocca *et al.*, 2000) and vimentin in all layers (Rabkin *et al.*, 2002). The changes of tissue formation, degree of maturation and cell phenotypes in response to biochemical stimulation in this study confirm the biological activity, vitality and remodeling capacity of cells, indicating a living adaptive tissue. Besides the biological functionality and activity, the results demonstrate the importance of biochemical stimulation for the maturation of human tissues. This positive response resulting in a more layered and consequently more mature tissue formation may be related to and enhanced by the presence of mesenchymal progenitor cells in the Wharton's jelly of human umbilical cords with multilineage potential (Wang *et al.*, 2004; Sarugaser *et al.*, 2005) as growth factors and cytokines such as VEGF, FGF, IGF-1, and EGF are known to influence tissue formation and differentiation. For instance, FGF orchestrates development by instructing different, uncommitted cell types to proliferate, differentiate and/or organize into specific tissue lineages (Dailey *et al.*, 2005). Furthermore, FGF as well as IGF-1 are known to stimulate collagen and GAG biosynthesis and play an important role in remodeling tissues derived from umbilical cord cells (Thomas *et al.*, 1987; Feld *et al.*, 1995; Bankowski *et al.*, 1996; Zhang *et al.*, 1996; Sobolewski *et al.*, 1997). Interestingly, in this study the collagen content in the biochemically stimulated leaflet were lower compared to unstimulated leaflets. In order to elucidate the molecular mechanism behind this observation further experiments are required. The formation of a subendothelial α-SMA positive layer in this study may be related to the presence of the growth factors or of heparin (both used as medium supplements) as the latter and its non-anticoagulant derivatives are known to influence the expression of α-SMA in fibroblastic cells (Desmouliere *et al.*, 1992).

When tissue engineered leaflets were exposed to mechanical stimulation no significant affection on ECM protein production or cell phenotype could be detected, here. Previous studies reported that human vascular derived-cells produced more collagen and GAG under cyclic strain (Mol *et al.*, 2003). This discrepancy may be explained by different cell properties and/or by differences in the straining protocol.

However, we observed an influence of mechanical stimulation on the mechanical properties of the tissue engineered leaflets. Strained leaflets revealed a

tendency to higher stiffness, in agreement with literature (Mol *et al.*, 2003). The differences in mechanical properties could be related to mechanical stimulation (Niklason *et al.*, 1999; Hoerstrup *et al.*, 2000a; Hoerstrup *et al.*, 2000b) generating a different tissue organization by increased crosslinks between the extracellular matrix elements.

All stress-strain profiles showed a non-linear mechanical behavior similar to native leaflet tissues. In contrast, the initial scaffold material itself exhibited linear behavior indicating that the measured mechanical properties can be mainly attributed to the newly formed tissues. However, it is difficult to estimate the exact contribution of the scaffold to the mechanical properties as the polymer was not fully degraded after 4 weeks in vitro culturing.

The mechanical strength did not reach physiological values (Shin`oka *et al.*, 1995) during the investigated *in vitro* cultivation time period suggesting further improvement of the mechanical loading protocol. This will be investigated in future studies and the functionality of the engineered tissues will have to be demonstrated. For future clinical realization of the pediatric tissue engineering concept, harvesting cells prenatally would be possible as already today umbilical cord blood is routinely obtained by cordocentesis for genetic analysis. Tissue sampling from Wharton's Jelly has not been established as a routine procedure, yet but should be possible as fetoscopic technology for tissue harvest is highly developed (Kohl *et al.*, 1997; Kohl *et al.*, 2000; Quintero *et al.*, 2001; Hirose *et al.*, 2003). It could be performed in an extended maneuver of the well established umbilical cord puncturing for blood harvest and has to be evaluated in animal studies.

In summary, the successful in vitro engineering and maturation of biologically active heart valve leaflets using umbilical cord as the only prenatal cell source has been demonstrated. The main findings of this work are: (1) the fabrication of autologous heart valve leaflets with Wharton's jelly-derived myofibroblasts and umbilical cord blood-derived EPCs is feasible, (2) the resulting tissues showed layered tissue organization similar to native heart valve leaflets, and (3) they demonstrated biological activity. The progenitor potential of the cells used in this study may increase the capacity for remodeling, repair and regeneration and may open up novel strategies for heart valve tissue engineering based on the umbilical cord as the only prenatal cell source.

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Chapter

6

The use of chorionic villi-derived progenitors for pediatric cardiovascular tissue engineering

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"Living Autologous Heart Valves Engineered from Human Prenatally Harvested Progenitors."

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6.1 Abstract

Heart valve tissue engineering is a promising strategy to overcome the lack of autologous, growing replacements, particularly for the repair of congenital malformations. Here, we present a novel concept using human prenatal progenitor cells as new and exclusive cell source in order to generate autologous implants ready for use at birth.

Human fetal mesenchymal progenitors were isolated from routinely sampled prenatal chorionic villus specimens and expanded in vitro. A portion was cryopreserved. After phenotyping and genotyping, cells were seeded onto synthetic biodegradable leaflet-scaffolds (n=12) and conditioned in a bioreactor. After 21d, leaflets were endothelialized with umbilical cord blood-derived endothelial progenitor cells and conditioned for additional 7d. Resulting tissues were analyzed by histology, immunohistochemistry, biochemistry (amounts of extracellular matrix, DNA), mechanical testing and scanning electron microscopy (SEM) and were compared with native neonatal heart valve leaflets. Fresh and cryopreserved cells showed comparable myofibroblast-like phenotypes. Genotyping confirmed their fetal origin. Neo-tissues exhibited organization, cell phenotypes, extracellular matrix production, and DNA content comparable to their native counterparts. Leaflet surfaces were covered with functional endothelia. SEM showed cellular distribution throughout the polymer and smooth surfaces. Mechanical profiles approximated those of native heart valves.

Prenatal fetal progenitors obtained from routine chorionic villus sampling were successfully used as an exclusive, new cell source for the engineering of living heart valve leaflets. This concept may enable autologous replacements with growth potential ready for use at birth. Combined with the use of cell banking technology this approach may be applied also for postnatal applications.

6.2 Introduction

Many congenital cardiac heart valve defects require surgical repair. Although today's surgical strategies have substantially improved morbidity and mortality of children with cardiovascular malformations, there is a principle lack of autologous, living replacement materials. Currently used heart valve replacements comprise both synthetic or bioprostethic prostheses and lack the capacity of growth, frequently necessitating several re-operations over the patients` life time (Mayer, 1995). Furthermore, today's heart valve prostheses are associated with adverse side-effects such as life-long anticoagulation therapy and increased risks for infections and thromboembolism (Mayer, 1995; Schoen and Levy, 1999). These disadvantages may be potentially avoided by valve substitutes that more closely mimic their native counterparts as to adequate mechanical function, durability, growth potential as well as the absence of immunogenic, thrombogenic, and/or inflammatory reactions (Harken *et al.*, 1962).

Tissue engineering is a promising strategy to meet these requirements by in vitro fabrication of autologous, living heart valve replacements as has been demonstrated in previous animal studies (Shin`oka *et al.*, 1995; Hoerstrup *et al.*, 2000a; Sutherland *et al.*, 2005). Transfer of the methodology from animal to human systems revealed promising results using human cell sources such as bone marrow or umbilical cord tissues (Hoerstrup *et al.*, 2002a; Hoerstrup *et al.*, 2002b; Schmidt *et al.*, 2005). However, an ideal cell source enabling the fabrication of heart valve replacements ready for use in the early neonatal period has not been identified yet. Early surgical corrections of congenital heart malformations are critically important to prevent secondary damage to the infant's immature heart. Therefore, such a cell source would allow cell harvest in the prenatal period after the cardiac defect is detected (e.g. by ultrasound) in order to have the implant ready for use at or shortly after birth. Furthermore, the ideal cell source should be accessible easily and allow cell harvest without substantial risks for both the mother and the child and without sacrifice of intact infantile donor tissue.

A promising cell source is the human placenta enabling prenatal access to infant tissue. Particularly, its chorionic villi provide extra-embryonically situated fetal mesenchymal cells (Enders and King, 1988), including progenitor cells (Castelucci *et al.*, 1990) that are routinely obtained for prenatal genetic diagnostics by biopsy. The obtained tissue samples could also serve as a cell source for tissue engineering. Theoretically, one specimen could then be used for both diagnostics and the tissue engineering application.

In preliminary studies, we have demonstrated the feasibility of using umbilical cord blood-derived endothelial progenitor cells as a prenatally accessible cell source for the endothelialization of tissue engineered patches (Schmidt *et al.*, 2005) as prenatal ultrasound guided percutaneous umbilical cord blood sampling is already a routine and well established procedure. Here, we present a new concept using human prenatal progenitor cells derived from chorionic villi and umbilical cord blood as exclusive cell

source for the fabrication of living autologous heart valve leaflets as a further step towards the clinical realization of the pediatric tissue engineering approach.

6.3 Methods

6.3.1 Cell harvest and isolation

After informed consent was obtained from the participants, approximately 5 mg of tissue were obtained from accessory chorionic villus sampling of male infants that was harvested routinely for diagnostic purposes. Chorionic tissues were sampled using a transabdominal procedure between 11-13 weeks of gestation. Gestational age was confirmed according to the embryonic length assessment. After cleansing the skin with standard surgical preparation solution, a 18 gauge needle, 6 inches long was inserted carefully into the mid chorionic bed under continuous sonographic guidance. Through the 18 gauge needle placed in the chorionic bed, a second (20 gauge) needle was carefully inserted for aspiration of chorionic villi. 10-30 mg of chorionic tissue material were then aspirated and put into medium for further processing.

After routine genetic diagnostic assessment was performed, cells were isolated from the remaining chorionic villi of male infants by digestion. Briefly, chorionic villi were washed with serum-free medium and transferred to a centrifugation tube. Tissues were completely covered with collagenase (Collagenase A, Roche Diagnostics GmbH, Mannheim, Germany) solved in serum-free medium and incubated at 37 °C. After 60 min, cells were centrifuged and the supernatant discarded. Cells were suspended in trypsin/EDTA (PAN Biotech GmbH, Aidenbach, Germany) and incubated for 10 min at 37°C. Afterwards, cells were centrifuged again. After discarding the supernatant, cells were resuspended, cultured in Bio-AMF-1 (Biological Industries, Kiebutz Beit Hamek, Israel) and expanded. A portion of the cells were cryopreserved using cell medium containing 10% DMSO (Fluka, Buchs, Switzerland).

Endothelial progenitor cells (EPCs) were isolated from the mononuclear fraction of 20ml fresh umbilical cord blood after delivery using density gradient (Histopaque – 1077, Sigma Chemical Company, St. Louis, MO) and differentiated into endothelial cells using endothelial basal medium (EBM^{TM}-2, Cambrex, Walkersville, MD), containing growth factors and supplements [Vascular Endothelial Growth Factor (VEGF), human Fibroblasts Growth Factor (hFGF), human recombinant long-Insulin-like Growth Factor-1 (R-3-IGF-1), human Epidermal Growth Factor (hEGF), Gentamycin and Amphotericin (GA-1000), Hydrocortisone, Heparin, Ascorbic Acid, and 2% Fetal Bovine Serum] (Schmidt *et al*., 2005).

6.3.2 Phenotyping of cells

Immunohistochemistry

Freshly isolated as well as cryopreserved chorionic villi-derived cells were cultured in chamber slides. After confluency, cells were washed with PBS and fixed with 70% ethanol. Immunohistochemistry was performed using the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, Arizona). Primary antibodies against the following antigens were applied: vimentin (clone 3B4), desmin (clone D33), CD31 (clone JC/70A), Ki-67 antigen (clone MIB-1), von Willebrand factor (vWF; affinity purified rabbit antibodies; all from DakoCytomation, Glostrup, Denmark); α-smooth muscle actin (α-SMA, clone 1A4; Sigma Chemical Company, St. Louis, MO) and endothelial nitric oxyde synthase type III (eNOS; affinity purified rabbit antibodies; BD Transduction Laboratories, San Diego, CA). Primary antibodies were detected with the Ventana iVIEW DAB detection kit, yielding a brown reaction product. For eNOS, the signal was enhanced with the amplification kit. Slides were counterstained with hematoxylin and glass coverslipped.

Flowcytometry (FACS)

For quantification of antigen expression FACS-analysis was performed using antibodies against vimentin (clone 3B4), desmin (clone D33), α-smooth muscle actin (α-SMA, clone 1A4; all from DakoCytomation, Glostrup, Denmark). Cells were fixed and permeabilized using an inside stain kit (Miltenyi, Biotech, Bergisch Gladbach, Germany) following the manufacturer's instructions. Primary antibodies were detected with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies (Boehringer Mannheim, Indianapolis, IN). Analysis was performed on a Becton Dickinson FACScan (Sunnyvale, CA). Irrelevant isotype-matched antibodies (IgG1MOPC-21; IgG2a, cloneUPC-10, both Sigma Chemical Company, St. Louis, MO) were used as negative controls. Human vascular-derived fibroblasts served as positive controls.

6.3.3 Genotyping of cells

DNA was extracted from trypsinized cell cultures using the InstaGene™ Matrix (BioRad, Hercules, CA) according to the manufacturer's instructions. Fetal origin of the cells was determined by demonstration of male sex by quantitative fluorescent PCR and using the following microsatellite markers (STR markers): AMXY, P39, DXS981, DYS448, DXS6854, X22, XHPRT, DXS996 and DXS1283E (Mann *et al.*, 2001 and 2004).

6.3.4 Processing of tissue engineered heart valve leaflets

Scaffolds for heart valve leaflets (n=12) were produced from a rapidly biodegradable non-woven polyglycolic-acid mesh (PGA, thickness: 1.0 mm, specific gravity: 69 mg/cm^3, Albany Int., Albany, NY) dip-coated with Poly-4-Hydroxybutyric acid (1% w/v P4HB, TEPHA Inc., Cambridge, MA).

Three scaffold-leaflets each were assembled to heart valves using a ring-shaped device (diameter 20 mm). After sterilization in 70% ethanol, chorionic villi-derived cells were seeded onto the scaffolds (3.5×10^6 cells/cm^2) using fibrin as a cell carrier (Mol *et al.*, 2005). Constructs were positioned in a strain-perfusion bioreactor and perfused (4 ml/min) with endothelial basal medium containing the above mentioned growth factors and supplements. After 7d, two groups were formed with n=6 leaflets: one group was exposed to mechanical stimulation by cyclic straining (15 mm/Hg) in addition to perfusion and the other to perfusion only. After an additional 14d, leaflets were endothelialized with differentiated EPCs (1.5×10^6 cells/cm^2) on both leaflet sides and cultivated for an additional 7d under exposure of the same mechanical conditions. Thereafter, heart valves were explanted from the bioreactor and neo-tissues were analyzed.

6.3.5 Analysis of tissue engineered heart valve leaflets

Histology and immunohistochemistry

Representative samples of all leaflets were fixed in 4% phosphate-buffered formalin (pH 7.0) and paraffin-embedded. Sections of 5-7µm were examined by Hematoxylin-Eosin (H&E), Trichrom-Masson, and Movat pentachrome staining. Morphology and tissue organization of engineered heart valve leaflets were compared to native neonatal pulmonary heart valve leaflets. Cell phenotypes were validated by immunohistochemistry performed as described above. For paraffin sections, antigen retrieval was done for all antibodies by heating with cell conditioner 1 (CC1, Ventana), except for CD31, where predigestion with protease 1 (Ventana) for 4 min was required.

Quantitative evaluation of extracellular matrix (ECM) elements and cell number

As an indicator for collagen, hydroxyproline content was determined of dried tissue samples (Huzar *et al.*, 1980). Sulphated glycosaminoglycans (GAG) were detected colorimetrically using papain digested samples and 1,9-di-methyl-methylene blue (Farndale *et al.*, 1986). Cell numbers were determined from the same papain digests after 50× dilution in TNE-buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH 7.4) and labeling of the DNA using Hoechst dye (Bisbenzimide H33258, Fluka, Buchs, Switzerland) (Kim *et al.*, 1988). Native heart valve tissues served as controls.

Evaluation of mechanical properties

Mechanical properties of leaflet tissues (15×5×1mm) were analyzed by using a uniaxial tensile tester (Instron 4411), equipped with a 10 N load cell. The crosshead speed was set to correspond to an initial strain rate of 1 min^{-1}. The recorded tensile force and displacement were transformed into stress-strain curves.

Scanning Electron Microscopy
Representative samples of both tissue engineered and native leaflets were fixed in 2% glutaralaldehyde for 24h. After preparation, samples were sputtered with gold and investigated with a Zeiss Supra 50 VP Microscope.

Statistical analysis
The data were analyzed using SYSTAT version 10 (SPSS, Chicago, IL). Analysis of variance was performed to estimate the means and the significance of the categorical factors (straining and perfusion only).

6.4 Results

6.4.1 Morphology and phenotype of cells

Chorionic villi-derived cells were attached to the culture dishes 24h after isolation (Figure 6.1A) and started to proliferate (Figure 6.1B).
Cells showed fibroblast-myofibroblast-like morphology. Immunohistochemistry (Figure 6.2) and FACS-analysis (data not shown) revealed expression of vimentin (Figure 6.2A) and in subpopulations α-SMA (Figure 6.2B) but absence of desmin (Figure 6.2C). This staining pattern reflects a fibroblast-myofibroblast-like phenotype (VA type) comparable to interstitial cells detected in native heart valve leaflets (Messier *et al.*, 1994). No changes in phenotype were detected after cryopreservation (Figure 6.2D-F). After differentiation, EPCs showed typical cobblestone-morphology and expressed CD31, vWF and eNOS (data not shown).

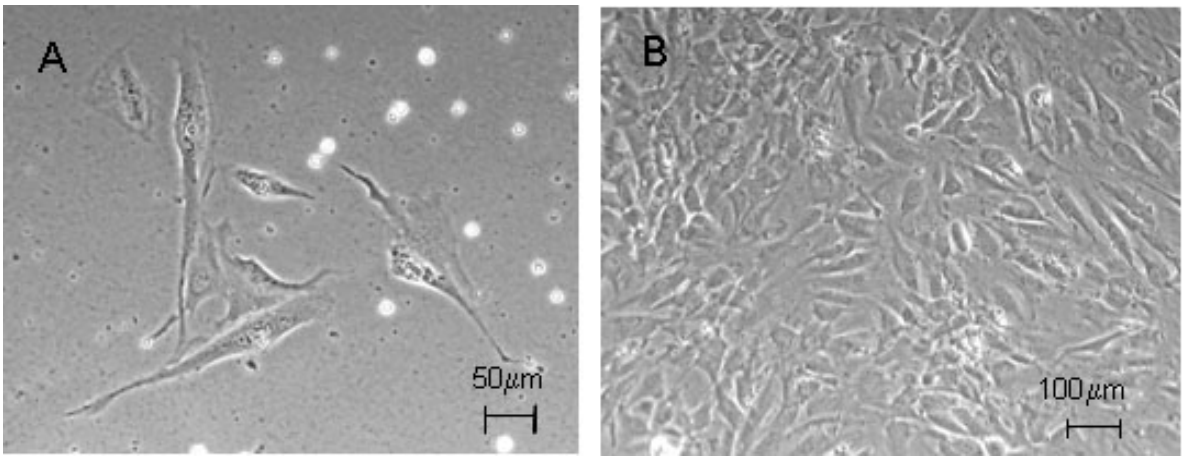

Figure 6.1: *Morphology of chorionic villi-derived cells: 1d after isolation, chorionic villi-derived cells were attached to the culture dishes (A) and began to proliferate. After 7d, cells formed a confluent monolayer (B).*

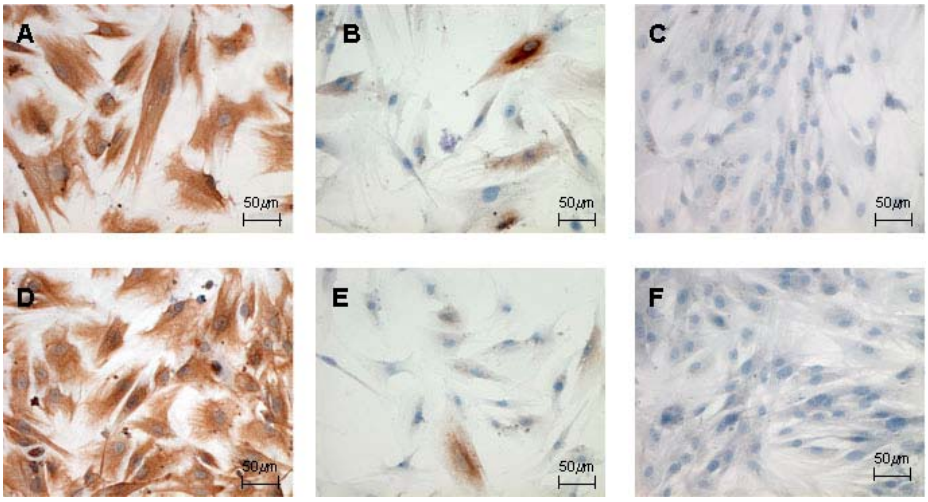

Figure 6.2: *Phenotype of chorionic villi-derived cells: After isolation, cells expressed vimentin (A) and α-SMA in subpopulations (B) but lacked desmin (C). No change of phenotype was detected after cryopreservation (D-F).*

6.4.2 Genotype of cells

Sex chromosome specific quantitative fluorescent PCR revealed only one allele for the x-chromosome specific STR-markers. Additionally, the y-chromosome specific markers were positive. This male genotyping result confirmed the fetal origin of the cells (Figure 6.3).

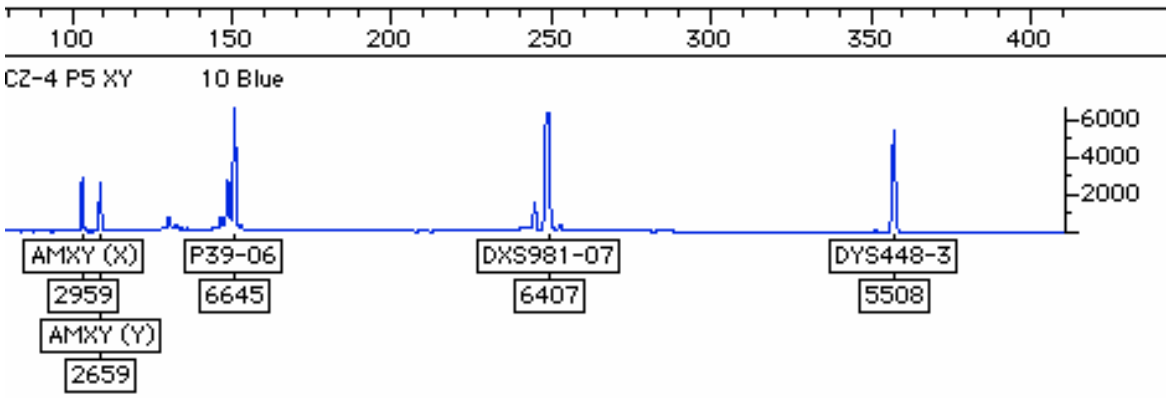

Figure 6.3: *Confirmation of male sex by quantitative fluorescent PCR: The polymorphism at the Amelogenin locus (AMXY) on both sex chromosomes exhibited a male pattern with two alleles (AMXY (Y) and AMXY (X)) with equal dosage. In addition, one allele of the y-chromosome specific STR marker DYS448 was present and all x-chromosome specific STR markers (only P39 and DXS981 shown in figure) exhibited single peaks indicating the presence of only one x- chromosome.*

6.4.3 Analysis of tissue engineered heart valve leaflets

Macroscopic appearance

A macroscopic picture of the leaflets assembled as a trileaflet heart valve after 28 days in culture is presented in Figure 6.4. The leaflets were intact and pliable. Cells densely covered all leaflets.

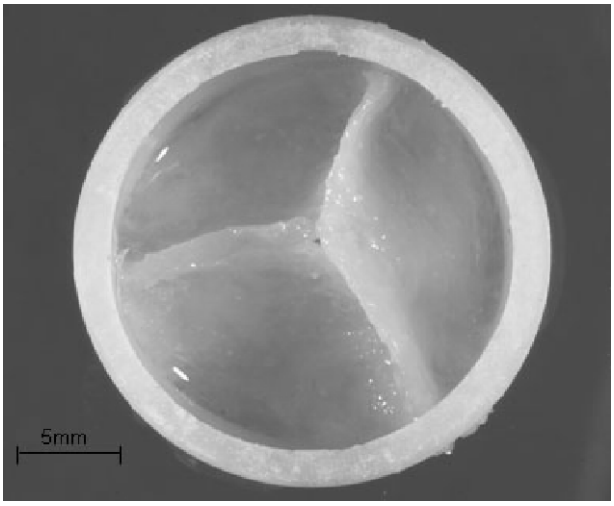

Figure 6.4: *Tissue engineered leaflets forming a trileaflet heart valve: Leaflets were intact, pliable and densely covered with cells.*

Histology and immunohistochemistry

Leaflets of all groups showed cellular tissue with production of ECM, independent of mechanical stimulation. H&E staining revealed tissue formation organized in a layered manner with dense outer layers and lesser cellularity in the inner part (Figure 6.5A) compared to native neonatal leaflets (Figure 6.5B). Trichrom-Masson staining highlighted collagen predominantly at the outer layers (Figure 6.5C), whereas in the center loosely arranged ECM substance was detected, characterized as predominantly GAG in the Movat staining (Figure 6.5E). In contrast, native leaflets demonstrated a more homogenous contribution of collagen fibers (Figure 6.5D) and GAG (Figure 6.5F). Cells expressed vimentin throughout all tissue engineered leaflets (Figure 6.5G) and native neonatal leaflets (Figure 6.5H) but lacked α-SMA expression in both tissue engineered leaflets (Figure 6.5I) and in native neonatal leaflets (Figure 6.5J). Also desmin was not detected (data not shown). Expression of CD31 demonstrated an endothelial cell lining, highlighted brownish on the surfaces of both tissue engineered (Figure 6.5K) and native neonatal leaflets (Figure 6.5L). Expression of vWF, and eNOS confirmed the presence of functional endothelial cells (data not shown).

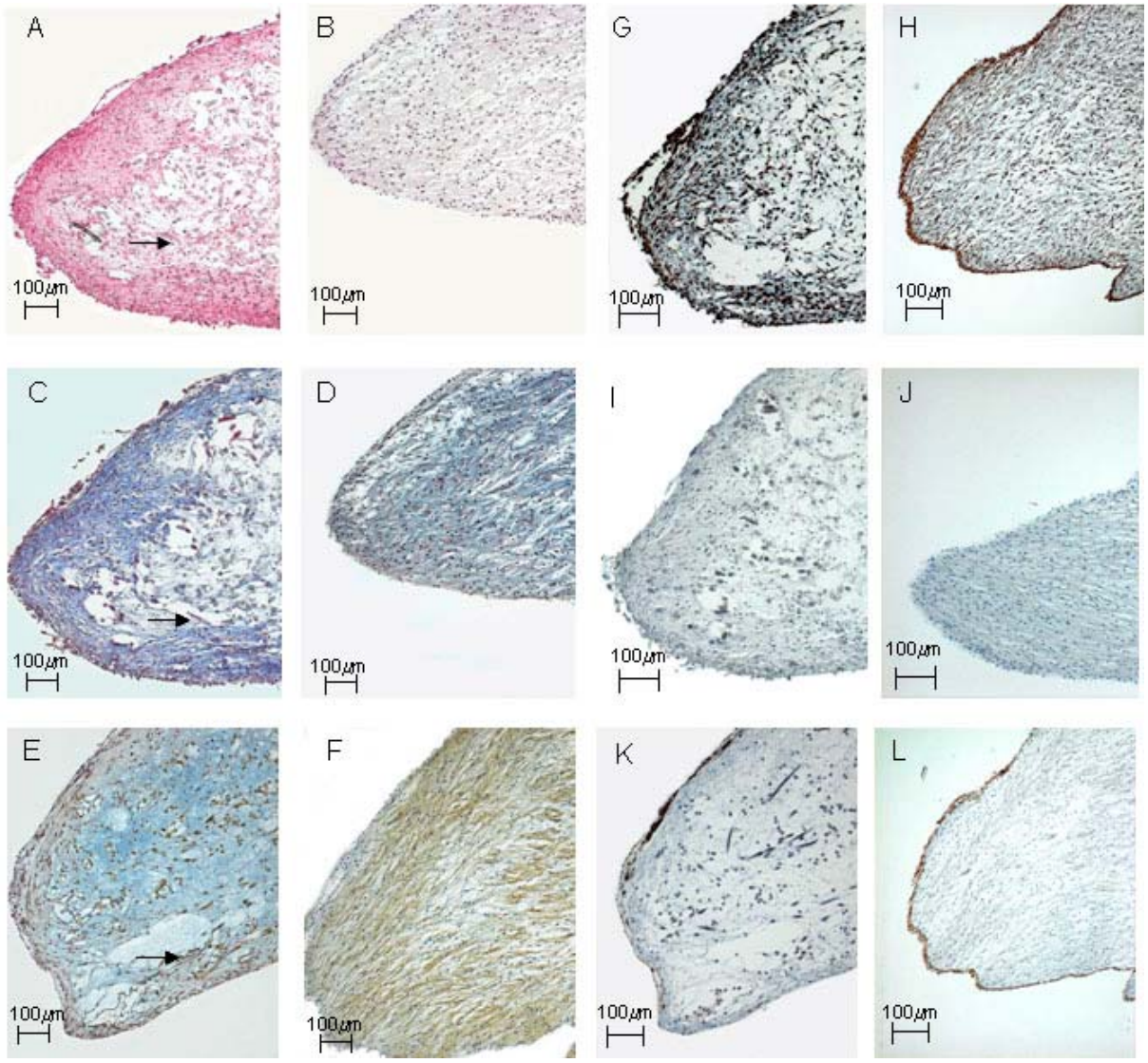

Figure 6.5: *Histological and immunohistochemical characteristics of tissue engineered leaflets: H&E staining demonstrated tissue organization of engineered leaflets (A) comparable to native neonatal pulmonary valve leaflets (B). Collagen fibers in the outer layer and GAG in the middle part of tissue engineered leaflets were highlighted bluish in the Trichrom-Masson staining (C) and light blue in the Movat staining (E), respectively. In contrast, native neonatal pulmonary leaflets showed more homogenous distribution of collagen fibers in the Trichrom-Masson staining (D) and GAG in the Movat staining (F). Both cells in tissue engineered leaflets (G) and in their native counterparts (H) expressed vimentin. In contrast, α-SMA could not be detected neither in tissue engineered (I) nor in native neonatal pulmonary heart valve leaflets (J). Expression of CD31, highlighted brownish, confirmed the presence of endothelia cells in the topmost layer of both tissue engineered (K) and native leaflets (L). Arrows point at remnants of scaffold.*

6.4.3.3 Quantification of ECM elements

Figure 6.6 summarizes the content of extracellular matrix of leaflets relative to those of native heart valve tissues. The amount of GAG was comparable to that of native tissue or in some samples even higher (strained leaflets 5.5±0.73 μm/mg; perfused 5.13±0.74 μm/mg; native 3.98±1.07 μm/mg). The amount of hydroxyproline reached up to 14% of native values (strained leaflets 4.62±2.11 μm/mg; perfused 3.10±1.54 μm/mg; native 54.04±6.25 μm/mg). The cell number, detected as DNA content, was up to 68% of native tissue values (strained leaflets 2.78±0.72 μm/mg; perfused 2.62±1.10 μm/mg; native 4.11±1.46 μm/mg). Mechanical conditioning showed no significant quantitative impact on the production of ECM elements.

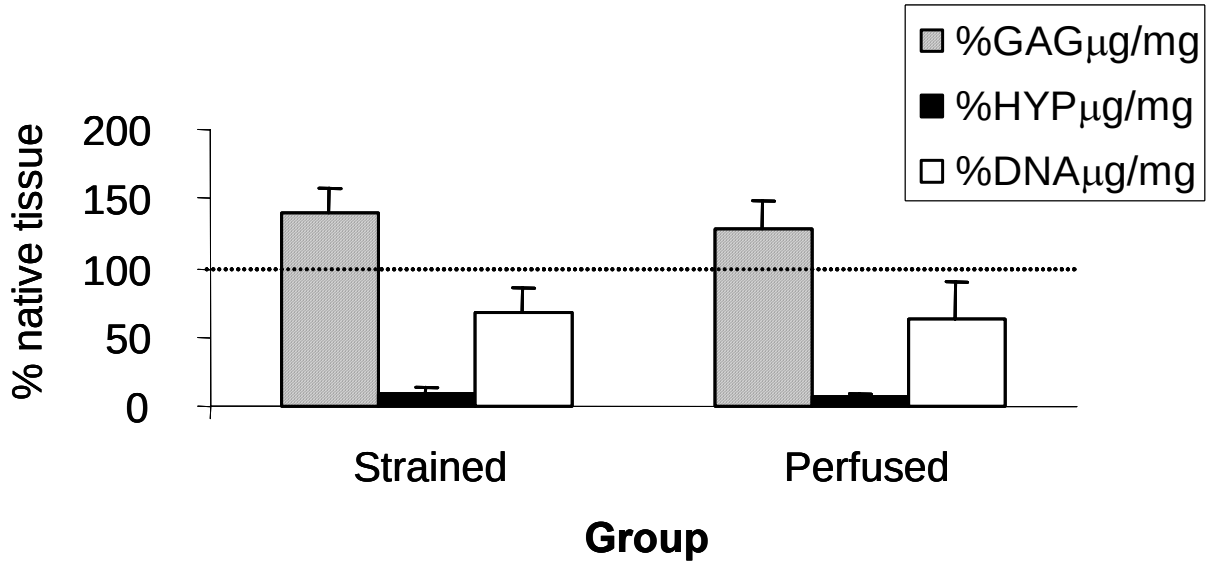

Figure 6.6: *Extracellular matrix analysis: Bars represent the mean amount of GAG (patterned), hydroxyproline (HYP, black), and cell number (DNA, white) relative to native heart valve tissue. Error bars represent standard deviations. Strain did not show significant impact on the degree of extracellular matrix production.*

Mechanical properties

Two representative stress-strain curves of strained (solid line) and perfused (dashed line) tissue engineered leaflets are presented in Figure 6.7. Mechanical stimulation resulted in stronger tissues as indicated by tensile strength (0.24±0.04 MPa versus 0.10±0.05 MPa; $p<0.001$) as well as strain at maximal stress (0.68±0.18 versus 0.46±0.14; $p<0.052$). Furthermore, mechanically stimulated leaflets were less pliable than those exposed to perfusion only as indicated by the higher Young`s modulus (0.59±0.17 MPa versus 0.33±0.13 MPa; $p<0.03$).

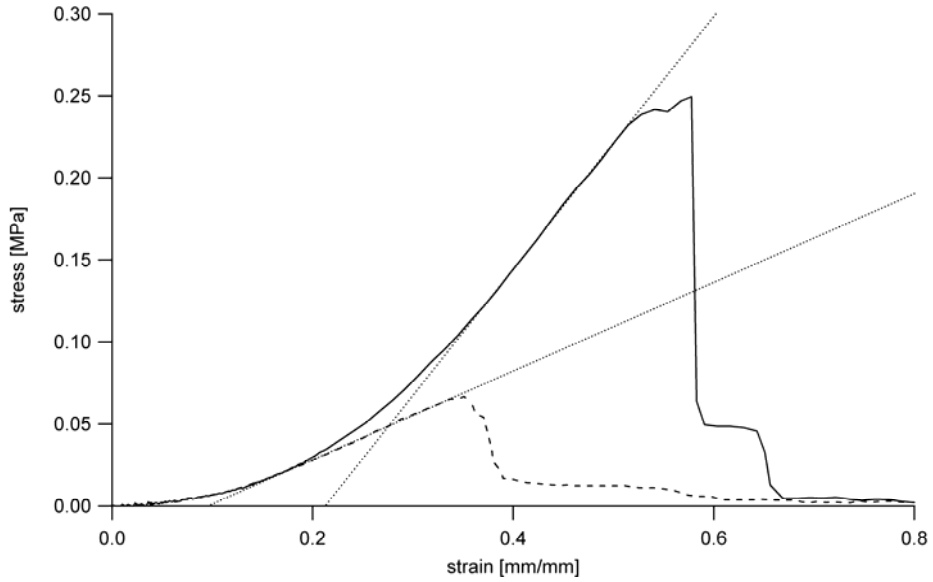

Figure 6.7: *Comparison of mechanical properties of strained and perfused leaflets: Mechanical properties of two representative samples of strained (solid line) and perfused (dashed line) tissue engineered leaflets are displayed as stress-strain curves. Strained leaflets were stronger but less pliable than perfused leaflets. The dotted lines represent the Young`s modulus.*

Scanning Electron Microscopy

Figure 6.8A shows a micrograph of the unseeded biodegradable leaflet scaffold. After 21d culturing in the bioreactor, seeded leaflets were completely covered with chorionic villi-derived prenatal cells (Figure 6.8B). Cells demonstrated complete ingrowth into the biodegradable scaffold. After endothelialization, tissue engineered heart valve leaflets demonstrated smooth surfaces (Figure 6.8C) comparable to native heart valve surfaces (Figure 6.8D). Magnification of the neo-endothelia and those of neonatal pulmonary heart valve leaflets are presented in Figure 6.8E and 6.8F, respectively.

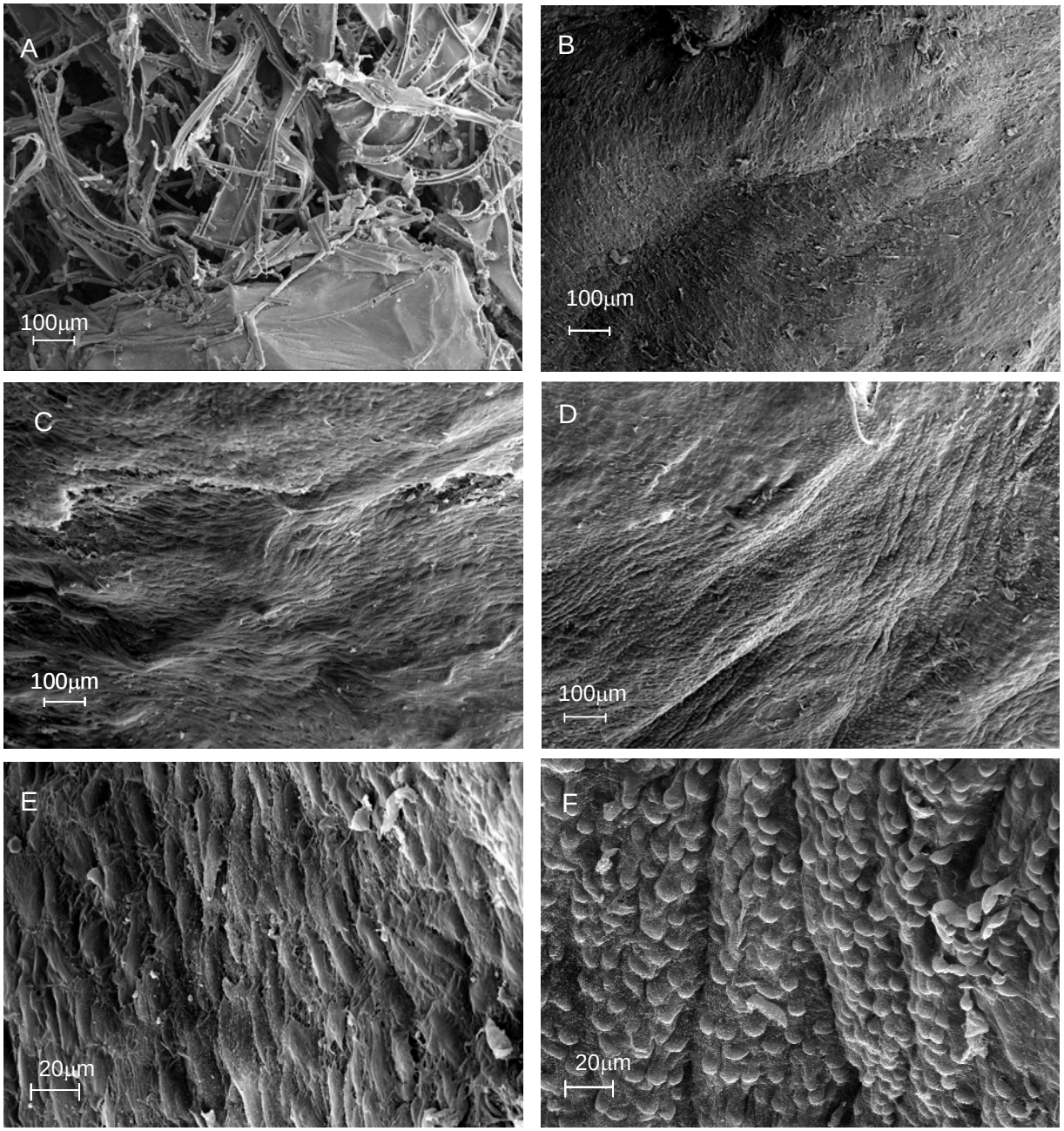

Figure 6.8: *Surface morphology: Scanning electron micrographs show the surfaces of unseeded scaffolds (A) and cellular distribution throughout the polymer 21d after seeding (B). After endothelialization tissue engineered leaflets demonstrated smooth surfaces (C) comparable to those of native heart valve leaflets (D). Magnifications revealed parallel cell alignment of neo-endothelial (E) and of native heart valve leaflet endothelia (F).*

6.5 Discussion

Pediatric cardiovascular tissue engineering represents a promising scientific concept to provide living heart valve replacements with the capacity of growth for the repair of congenital malformations. For this concept, cells are ideally harvested prenatally to enable the use of the engineered substitutes at or shortly after birth, in order to prevent secondary damage to the newborn's immature heart. So far, a major obstacle for the clinical realization of this concept is the lack of prenatal cell sources enabling cell harvest without substantial risks for the unborn child or mother and sacrifice of intact donor tissue.

Human chorionic villi represent a unique prenatal cell source providing minimally invasive access to fetal extra-embryonic mesenchyme sprouted out from the multi-lineage trophoblast (Enders and King, 1990). Villous development starts between days 12 and 18 post conception by cell sprouting and continues with local vasculogenesis out of the mesenchymal precursor cells (Castelucci *et al.*, 2000; Kaufmann and Kingdom, 2000). In the present study, cells were prenatally harvested from mesenchymal villi obtained for routinely diagnostic assessment. Today, this procedure is well established in the clinical routine with acceptable risks <1% for the mother and the unborn child (Rhoads *et al.*, 1989). Beside excellent expansion capacity, isolated cells exhibited phenotypic profiles of a fibroblast-myofibroblast lineage indicating similarity to human adult valvular interstitial cells (Messier *et al.*, 1994). After cryopreservation, cells showed stable phenotypes demonstrating the potential of cell banking for subsequent postnatal application.

When using chorionic villi-derived cells for the fabrication of heart valve leaflets, resulting tissues showed features of proliferating tissues with beginning collagen production and content of GAG approximating native values. Furthermore, in response to biomimetic conditioning cells expressed predominantly vimentin in the tissue engineered heart valve leaflets. Thus, cells exhibited phenotype profiles similar to those of native neonatal pulmonary heart valve leaflets (Rabkin *et al.*, 2002). The observed change in α-SMA-expression with regard to pre-seeding cells may be related to and enhanced by the progenitor potential of the chorionic villi-derived cells (Castelucci *et al.*, 2000).

Isolated umbilical cord blood-derived EPCs differentiated into mature functional endothelial cells during cell expansion as described before (Asahara *et al.*, 1997) and formed functional endothelial layers on the surfaces of tissue engineered leaflets also under mechanical stress. This observation indicates that the human umbilical cord blood-derived differentiated EPCs would also function as a non-thrombogenic cell layer in an in vivo environment within a range of mechanical stresses.

All stress-strain profiles showed non-linear mechanical behavior similar to native leaflet tissues. In contrast, the initial scaffold material itself exhibited linear behavior demonstrating that the measured mechanical properties were mainly attributed to the newly formed tissues. Exposure of the tissue engineered leaflets to mechanical stimulation (cyclic strain) resulted in stronger but less pliable tissues

compared to only perfused tissues, in agreement with other studies (Mol *et al.*, 2003). This observation indicates the positive effect of mechanical stimulation on the mechanical strength and may be related to a higher degree of cross-links among the collagen fibers. The mechanical strength of tissue engineered leaflets did not reach physiological values (Shin`oka *et al.*, 1995) during the investigated in vitro cultivation time period. This may be explained by the uncompleted collagen production suggesting further improvement of the mechanical loading protocol in vitro (Mol *et al.*, 2003; Niklason *et al.*, 1999). This will be investigated in future studies and the functionality of the engineered tissues in vivo will have to be demonstrated.

In summary, this study demonstrates the feasibility to use prenatally available human progenitors as an exclusive cell source for the fabrication of heart valve leaflets. The successful use of chorionic villi-derived mesenchymal progenitors and umbilical cord blood-derived endothelial progenitor cells may enable the clinical realization of autologous cardiovascular replacements with the capacity of growth ready for use at birth, as both can be accessed prenatally. Chorionic villi-derived mesenchymal progenitors were obtained from viable fetal specimens that are routinely sampled for genetic diagnostics while umbilical cord blood-derived endothelial progenitor cells can be harvested by prenatal percoutaneous ultrasound guided cord blood sampling.

Furthermore, prenatal not fully differentiated progenitor cells might have a higher potential to form heart valve tissues that are similar to the native counterparts in architecture and cell phenotypes. On the other hand, however, there is a potential risk that their immature stage might lead to tumor development by uncontrolled cell growth or differentiation via genetic alterations. This important aspect has to be considered and investigated in future in vivo studies. Using cell banking technology, the presented approach may be applied for postnatal applications as well.

Acknowledgements

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Chapter 7

Fabrication of pediatric cardiovascular replacements prenatally – Fetal amniotic fluid-derived progenitors as single cell source

The content of the chapter will be published in *Surgical Supplement to Circulation* (accepted):

"Prenatally Fabricated Autologous Living Heart Valves Based on Amniotic Fluid-derived Progenitors as Single Cell Source."

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7.1 Abstract

A novel concept providing prenatally tissue engineered human autologous heart valves based on routinely obtained fetal amniotic fluid progenitors as single cell source is introduced.

Fetal human amniotic progenitors were isolated from routinely sampled amniotic fluid and sorted using CD133 magnetic beads. After expansion and differentiation, cell phenotypes of CD133- and CD133+ cells were analyzed by immunohistochemistry and flowcytometry. After characterization, CD 133- derived cells were seeded onto heart valve leaflet scaffolds (n=18) fabricated from rapidly biodegradable polymers, conditioned in a pulse duplicator system and subsequently coated with CD 133+ derived cells. After *in-vitro* maturation, opening and closing behavior of leaflets was investigated. Neo-tissues were analyzed by histology, immunohistochemistry and scanning electron microscopy (SEM). Extracellular matrix (ECM) elements and cell numbers were quantified biochemically. Mechanical properties were assessed by tensile testing.

CD133- derived cells demonstrated characteristics of mesenchymal progenitors expressing CD44 and CD105. Differentiated CD133+ cells showed features of functional endothelial cells by eNOS and CD141 expression. Engineered heart valve leaflets demonstrated endothelialized tissue formation with production of ECM-elements (GAG 80%, HYP 5%, cell number 100% of native values). SEM showed intact endothelial surfaces. Opening and closing behavior was sufficient under half of systemic conditions.

The use of amniotic fluid as single cell source is a promising low-risk approach enabling the prenatal fabrication of heart valves ready to use at birth. These living replacements with the potential of growth, remodeling and regeneration may realize the early repair of congenital malformations.

7.2 Introduction

Many strategies have been applied in recent years to overcome the lack of living autologous replacement materials for the repair of congenital cardiovascular malformations as the current used prostheses are associated with adverse side-effects such as life-long anticoagulation therapy and increased risks for infections and thromboembolism (Mayer 1995; Schoen and Levy, 1999). Tissue engineering may have the potential to overcome these problems as is indicated by the successful implantation of tissue engineered cardiovascular constructs in animal models (Shin`oka *et al.*, 1995; Hoerstrup *et al.*, 2000a; Sutherland *et al.*, 2005; Hoerstrup *et al.*, 2006). Particularly, pediatric cardiovascular tissue engineering represents a promising approach focusing on the prenatal fabrication of living autologous materials with growth, repair and regeneration capabilities ready for use at or shortly after birth in order to prevent secondary damage to the immature heart.

With regard to heart valve replacements, two cell types are required for the tissue engineered constructs to match the characteristics of their native counterparts: a myofibroblast-fibroblast like cell type producing extracellular matrix that is mainly responsible for the mechanical properties of heart valves, and an endothelial cell type covering the valve surface representing an antithromobogenic and blood-compatible cell layer. These cells have been isolated from several human sources such as vascular-derived cells and bone marrow-derived cells (Schmidt *et al.*, 2006a). With respect to the pediatric cardiovascular tissue engineering concept, prenatally accessible cell sources have been investigated e.g. umbilical cord tissue (Schmidt *et al.*, 2006b) and chorionic villi (Schmidt *et al.*, 2006c) for myofibroblast-fibroblast-like cells and umbilical cord blood for endothelial-like cells (Schmidt *et al.*, 2006b and 2006c).

In order to further reduce the risk associated with prenatal cell sampling, both cell types should ideally be obtained in a single procedure from one source that is easily accessible. Such a cell source that could enable the fabrication of autologous heart valves prior to birth is the amniotic fluid as it contains fetal progenitor cells including hematopoetic progenitor cells (Toricelli *et al.*, 1993) and mesenchymal progenitor cells (In`t Anker *et al.*, 2003). Moreover, it is routinely obtained in low-risk procedure for genetic diagnostics. Here, a novel concept is introduced using prenatally obtained amniotic fluid as a single fetal cell source for the fabrication of living autologous heart valves prior to birth.

7.3 Methods

7.3.1 Amniotic fluid sampling

Amniotic fluid sampling was performed for diagnostic purposes (chromosomal abnormalities) between 16-18 weeks of gestation, gestational age being determined according to first trimester crown rump length. Patients were informed (written and orally) that amniotic cells would be used for research purposes as described in this manuscript (approved by the Ethics Committee STV20-2006) and written informed consent was obtained from the participants. After ultrasound assessment of fetal biometry and amniotic fluid index, ultrasound guided abdominal amniocentesis was performed under sterile conditions using a 22 G needle.

7.3.2 Cell isolation and differentiation

Cells were isolated from 4.5 ml of accessory amniotic fluid (n=9; mean geatation age 16 weeks, mean maternal age 37 years) that was not used for the diagnostics described above. Thereafter, amniotic fluid was centrifuged at 350g at room temperature for 10 min. After removing the supernatant, cells were sorted using a CD133 cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Briefly, cells were resuspended in phosphate buffered saline supplemented with 0.5% bovine serum (PAN Biotech, Aidenbach, Germany). Blocking reagent (100 µl / 10^8cells) was added and cells were labeled with CD133 MicroBeads (100 µl MicroBead dispersion / 10^8cells). After incubation at 4°C for 30 min, cells were washed by adding 10-20x the labeling volume of buffer and centrifugation at 350g for 10 min. The resuspended cell solution was injected into magnetic columns for cell separation. After CD133- cells were collected, the columns were washed with buffer and removed from the magnetic field. Subsequently, CD133+ cells were flushed out using a plunger supplied with the columns.

For proliferation and differentiation CD133– and CD133+ cells were cultured and non-attached cells were removed after 2d. In order to initiate endothelial differentiation, CD133+ cells were exposed to basal medium (EBM^{TM}-2, Cambrex, Walkersville, MD), containing growth factors and supplements, namely Vascular Endothelial Growth Factor (VEGF), human Fibroblasts Growth Factor (hFGF), human recombinant long-Insulin-like Growth Factor-1 (R-3-IGF-1), human Epidermal Growth Factor (hEGF), Gentamycin and Amphotericin (GA-1000), Hydrocortisone, Heparin, Ascorbic Acid, and 20% Fetal Bovine Serum. In contrast, the CD133- cell fraction was cultured in basal medium supplemented with hFGF, R-3-IGF-1, hEGF, GA-1000, Ascorbic Acid, and 20% Fetal Bovine Serum.

7.3.3 Phenotyping of cells

Immunohistochemistry

CD133- and CD133+ amniotic fluid-derived cells were cultured in chamber slides and immunohistochemistry was performed as described before (Schmidt *et al.*, 2006c) using the following primary antibodies: transcription factor Oct-3/4 (affinity purified goat antibodies; Santa Cruz Biotechnology, Santa Cruz, CA), CD44 (clone G44-26), endothelial nitric oxide synthase type III (eNOS) (affinity purified rabbit antibodies; both from BD Biosciences, Frankling Lakes, NJ), CD34 (clone QBEND 10; Serotec, Raleigh, NC), vimentin (clone 3B4), desmin (clone D33), CD31 (clone JC/70A), von Willebrand factor (affinity purified rabbit antibodies; all from DakoCytomation, Glostrup, DK), and α-smooth muscle actin (α-SMA, clone 1A4; Sigma, St. Louis, MO).

Flowcytometry (FACS)

For quantification of antigen expression, FACS-analysis was performed using antibodies against CD44 (fluorescein isothiocyanate (FITC)-conjugated, Clone MEM-85), CD105 (FITC-conjugated, Clone MEM-226; both from ImmunoTools, Friesoythe, Germany), CD34 (FITC-conjugated, Clone AC136; Miltenyi, Bergisch Gladbach, Germany), CD31 (Clone 158-2B3; NeoMarkers, Fremont CA) CD141 (thrombomodulin; Clone 1009; DakoCytomation, Glostrup, Denmark) and von Willebrand factor (vWF; Clone 3F2-A9; BD Bioscience, San Jose, CA). Irrelevant isotype-matched antibodies (FITC-conjugated IgG2a, clone 713; FITC-conjugated mouse IgG2b, clone PFR-02; both from ImmunoTools, Friesoythe, Germany; IgG1MOPC-21, Sigma Chemical Company, St. Louis, MO) served as controls. Unconjugated primary antibodies were detected with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies (Boehringer Mannheim, Indianapolis, IN). For detection of vWF, cells were fixed and permeabilized using an inside stain kit (Miltenyi, Biotech, Bergisch Gladbach, Germany) following the manufacturer's instructions. Analysis was performed on a Becton Dickinson FACScan (Sunnyvale, CA).

7.3.4 Genotyping of cells

DNA was extracted from amniotic fluid-derived cells directly after cell harvest as well as from cell cultures of CD133- and CD133+ cells using the InstaGene™ Matrix (BioRad, Hercules, CA) according to the manufacturer's instructions. In parallel, maternal DNA was extracted from peripheral blood samples using the EZ1 Blood Kit (Qiagen, Switzerland). Fetal origin of the cells and absence of maternal contamination was determined by comparing fetal and maternal microsatellite marker profiles obtained by quantitative fluorescent PCR (Mann *et al.*, 2001; Mann *et al.*, 2004). 13 autosomal and 8 sex chromosome specific STR markers as well as the deletion polymorphism at the Amelogenin locus were amplified by multiplex PCR. The PCR products were separated by capillary electrophoresis (ABI 310) and analyzed using the GeneScan and Genotyper software packages (Applied Biosystems, Foster City, CA).

7.3.5 Engineering of heart valve leaflet tissues

Heart valve leaflets (2.3 cm^2, n=18) were cut from rapidly biodegradable non-woven polyglycolic-acid meshes (PGA, thickness: 1.0 mm, specific gravity: 69 mg/cm^3, Albany Int., Albany, NY) and dip-coated with Poly-4-Hydroxybutyric acid (1% w/v P4HB, TEPHA Inc., Cambridge, MA).

Before evaporation of the solvent, three leaflet scaffolds each were attached to a ring-shaped device (20 mm diameter) fabricated from non biodegradable Fastacryl® (Vertex-dental, The Netherlands) using a trileaflet-shaped heart mould. Leaflets scaffolds were positioned onto the mold and the ring shaped device was placed on top. By dissolving the surface layer of the device the leaflet scaffolds were fixed to the edges of the ring. After evaporation of the solvent, the mould was removed and resulted valves were sterilized in 70% ethanol. Sterile scaffolds were seeded with CD133- derived cells (3.5×10^6 cells/cm^2) using fibrin as a cell carrier (Mol *et al.*, 2005a) and positioned in a Diastolic Pulse Duplicator, described in detail before (Mol *et al.*, 2005b). Continuous perfusion was applied (4 ml/min) using the basal medium containing the above mentioned growth factors and supplements for CD133- cell culture. After 7d, two groups were formed with n=9 leaflets each: to one group cyclic straining (15 mmHg) was applied in addition to perfusion while the other was exposed to perfusion only. CD 133+ derived cells were kept under humidified incubator conditions (37°C, 5% CO_2).

After an additional 14d, the leaflets were coated with CD133+ derived cells (1.5×10^6 cells/cm^2) on both sides. They were kept under static conditions for 24h and subsequently cultivated for an additional 7d exposed to the same mechanical conditions but in medium containing the above mentioned growth factors and supplements for CD133+ cell culture. Thereafter, heart valves were explanted from the bioreactor and neo-tissues were analyzed.

7.3.6 Analysis of generated heart valve leaflets

Assessment of functionality

The opening and closing behavior of heart valve leaflets was visualized in a custom-built in vitro set-up consisting of a computer-controlled pump, representing the left ventricle, and a systemic circulation (Geven *et al.*, 2004). Physiologic systemic flow profiles were generated and images of the opening and closure behavior were obtained using a high-speed video camera (Phantom v9.0; Vision Research Inc, New Jersey) up to half of systemic conditions.

Evaluation of tissue organization and phenotypes

Representative samples of all leaflets were fixed in 4% phosphate-buffered formalin (pH 7.0) and paraffin-embedded. Sections of 5-7µm were examined by histology using Hematoxylin-Eosin (H&E), Trichrom-Masson, and Movat pentachrome staining as to morphology and tissue organization. Cell phenotypes were displayed by immunohistochemistry using the primary antibodies and detection kits described above. Deparaffinization and antigen retrieval (CD31: predigestion with protease 1, all

other antibodies: heating with cell conditioner 1) was performed on the Ventana Benchmark staining system (Ventana Medical Systems, Tucson, AZ).

Quantitative assessment of extracellular matrix (ECM) elements and cell number
Hydroxyproline, an indicator for collagen content, was determined of dried tissue samples (Huzar *et al.*, 1980). Sulphated glycosaminoglycans (GAG) were detected colorimetrically using papain digested samples and 1,9-di-methyl-methylene blue (Farndale *et al.*, 1986). Cell numbers were determined from the same papain digests after 50× dilution in TNE-buffer (10 mM Tris, 100 mM NaCl, 1 mM EDTA, pH7.4) and labeling of the DNA using Hoechst dye (Bisbenzimide H33258, Fluka, Buchs, Switzerland) (Kim *et al.*, 1988). Native human heart valve tissues served as controls.

Evaluation of mechanical properties
Mechanical properties of leaflet tissues (15×5×1mm) were analyzed using a uniaxial tensile tester (Instron 4411, Massachusetts, USA), equipped with a 10N load cell, at strain rate of 1 min^{-1}. The recorded tensile force and displacement were transformed into stress-strain curves.

Scanning Electron Microscopy
Representative samples of tissue engineered leaflets were fixed using 2% glutaralaldehyde. After preparation, samples were sputtered with gold and investigated with a Zeiss Supra 50 VP Microscope (Zeiss, Jena, Germany). Native heart valve leaflets served as controls.

7.4 Results

7.4.1 Morphology and phenotype of cells

In total $2.6x10^5 \pm 1.2x10^5$ cells were isolated from 4.5ml amniotic fluid whereas 1% represented CD133+ cells. After 2d, about half of the amniotic fluid-derived CD133- amniotic fluid-derived cells were attached to the culture dishes (Figure 7.1A). After an initial phase of slow growth, cells started to proliferate and formed a confluent layer after 10d (Figure 7.1B). Immunohistochemistry revealed expression of CD44 (Figure 7.1C) and vimentin (Figure 7.1D) and a lack of Oct-3/4, CD34, α-SMA and desmin (data not shown). Furthermore, neither expression of eNOS (Figure 7.1E) nor CD31 or vWF (data not shown) could be detected after 28d. In contrast, CD133+ cells were initially round and demonstrated cluster-like formation after 2 days (Figure 7.1F). After 10d, cells formed a cobblestone-like morphology (Figure 7.1G) and expressed CD44 (Figure 7.1H) and vimentin (Figure 7.1I). Oct-3/4 and CD34 were not detected (data not shown). After 28d, cells started to express eNOS (Figure 7.1J) but lacked the expression of CD31 and vWF (data not shown).

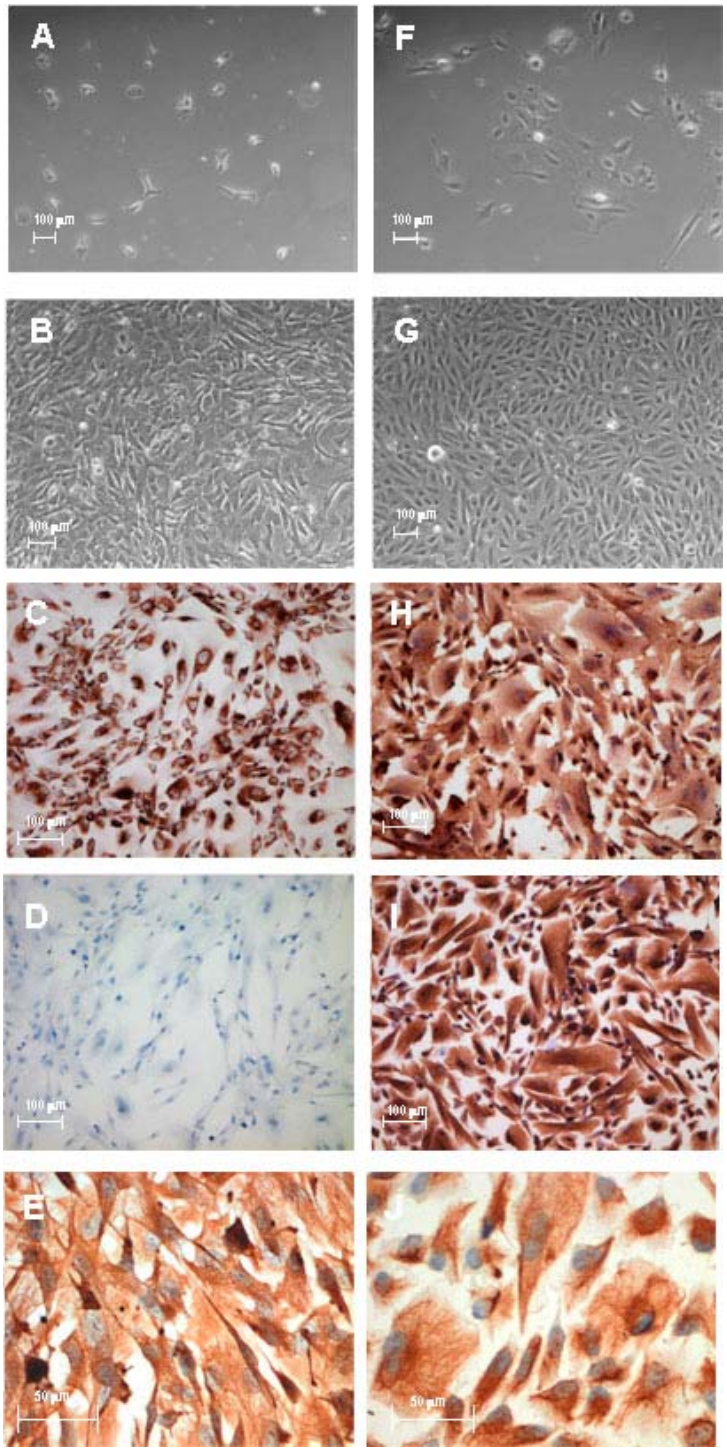

Figure 7.1: *Morphology and immunohistochemistry: After 2d, CD133- cells were attached to the culture plates (A) and formed a confluent layer after 10d (B). Immunohistochemistry revealed positive staining for CD44 (C) and vimentin (D) and negative staining for eNOS (E). CD133+ cells demonstrated cluster-like formation after 2d (F) and cobblestone-like morphology after 10d (G) with expression of CD44 (H) and vimentin. After 28d, CD133+ cells started to express eNOS (J).*

FACS analysis confirmed these staining patterns by displaying the expression of CD44 for most of the CD133- cells (Figure 7.2A) and partly for CD133+ cells (Figure 7.2B). Additionally, a fraction of CD133- cells demonstrated expression of CD105 (Figure 7.2C), whereas only a small amount of CD133+ cells was positive for CD105 (Figure 7.2D). CD34 was neither expressed by CD133- cells (Figure 7.2E) nor by CD133+ cells (Figure 7.2F). Furthermore, no CD141 expression could be observed on CD133- cells (Figure 7.2G) in contrast to the CD133+ cell fraction (Figure 7.2H). CD31 and vWF were not detectable in CD133- cells and CD133+ cells, respectively (Figure 7.2I-L).

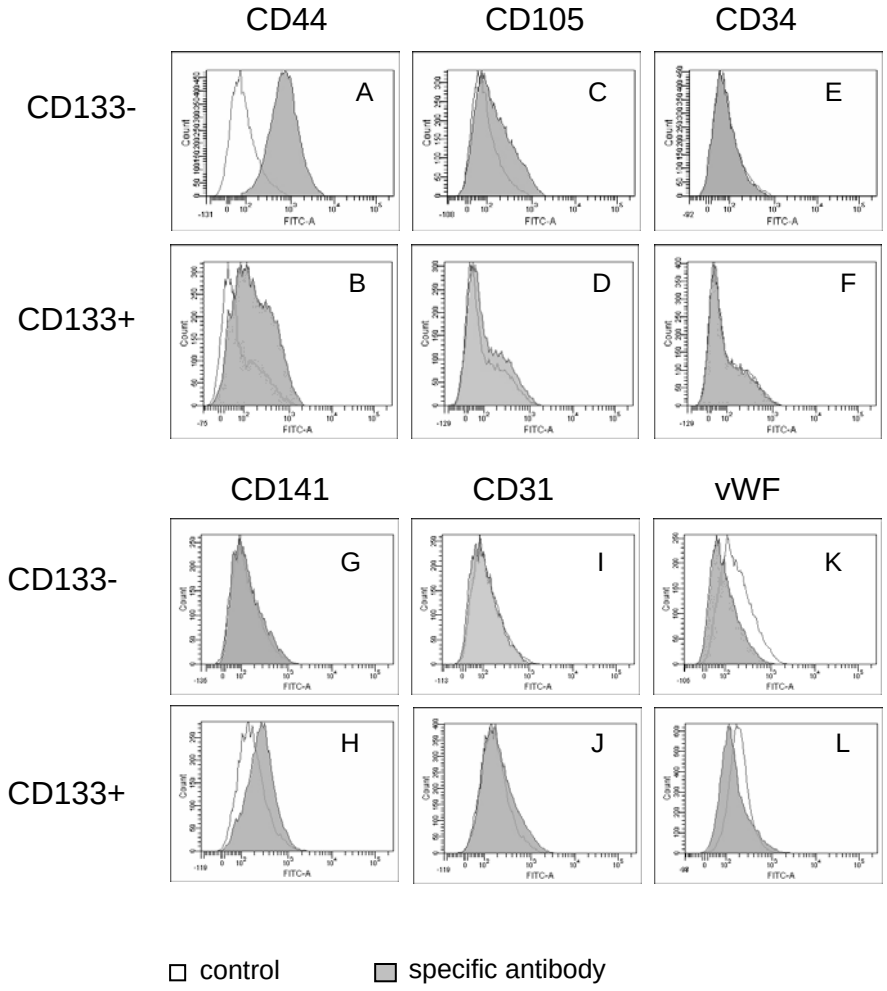

Figure 7.2: *Determination of amniotic fluid-derived cells phenotype by FACS: CD133- cells demonstrated expression of CD44 (A), CD105 (C) and a lack of CD34 (E). Moreover, they were negative for CD141 (G), CD31 (I) and vWF (K). In contrast, CD133+ cells expressed CD44 (B) and CD141 (H) and lacked the expression of CD34 (F), CD31 (J) and vWF (L). Few cells were also positive for CD105 (D).*

7.4.2 Genotype of cells

Quantitative fluorescent PCR revealed different autosomal STR profiles in all amniotic fluid cell-derived DNAs when compared to the corresponding profiles of maternal blood-derived DNAs (Figure 7.3A). Furthermore, in male fetal DNA only one allele for the X-chromosome specific STR-markers could be detected. Additionally, the Y-chromosome specific markers were positive (Figure 7.3B). No maternal admixture as evidence of contamination was found in the subsequent cell cultures thus confirming the pure fetal origin of CD133- and CD133+ cells.

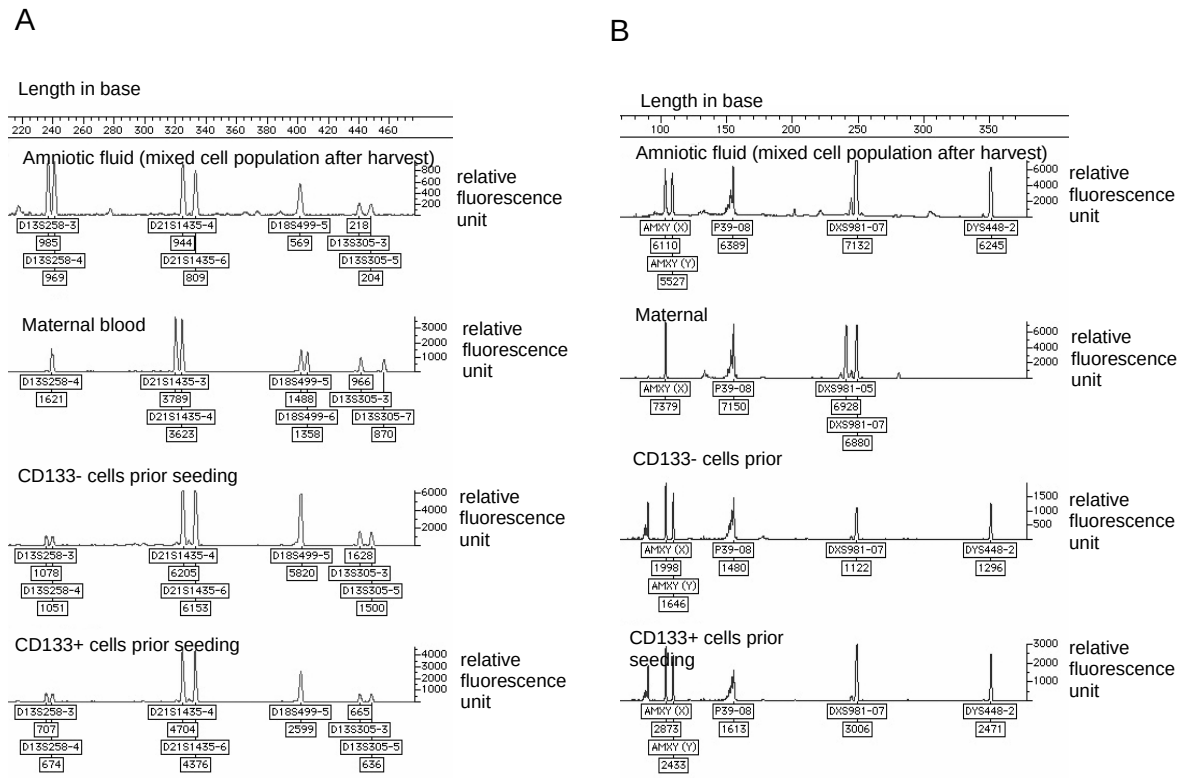

Figure 7.3: *Confirmation of fetal genotype and exclusion of maternal contamination by quantitative fluorescent PCR: The autosomal STR marker profiles (only D13S258, D21S143, D18S499 and D13S305 shown in Figure) obtained from the starting material (amniotic fluid cells after harvest) were identical to those obtained from CD133- and CD133+ cells, but were clearly different from the maternal blood profile. Furthermore, no contamination (indicated by additional maternal peaks) was detected (A). In male fetal DNA the polymorphism at the Amelogenin locus (AMXY) on both sex chromosomes exhibited two alleles ((AMXY (Y) and AMXY (X)) with equal dosage (similar peak height/area). In addition, the presence of only one allele for all X-chromosome specific STR markers (only P39 and DXS981 shown in Figure) as well as one allele of the Y-chromosome specific STR marker DYS448 is in agreement with a male profile without maternal contamination (B).*

7.4.3 Analysis of tissue engineered heart valve leaflets

Macroscopic appearance

Figure 7.4 demonstrates a photograph of a tissue engineered heart valve after 28d of culturing in the Diastolic Pulse Duplicator.

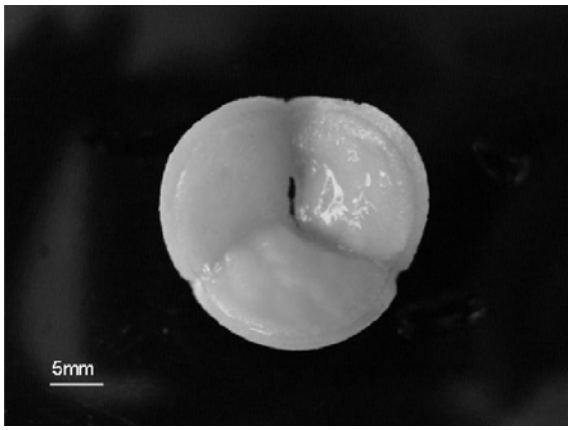

Figure 7.4: *Macroscopy: After 28d, tissue engineered leaflets were intact and densely covered with amniotic fluid-derived cells.*

The neo-tissue appeared to have homogeneous thickness with smooth surfaces. The leaflets were intact and pliable demonstrating a sufficient opening and closing behavior (Figure 7.5) under pulmonary pressure conditions (systolic pressure of ventricle = 25mmHg; enddiastolic pressure of ventricle = 2mmHg; systolic pressure of artery = 25mmHg; enddiastolic pressure artery = 4mmHg).

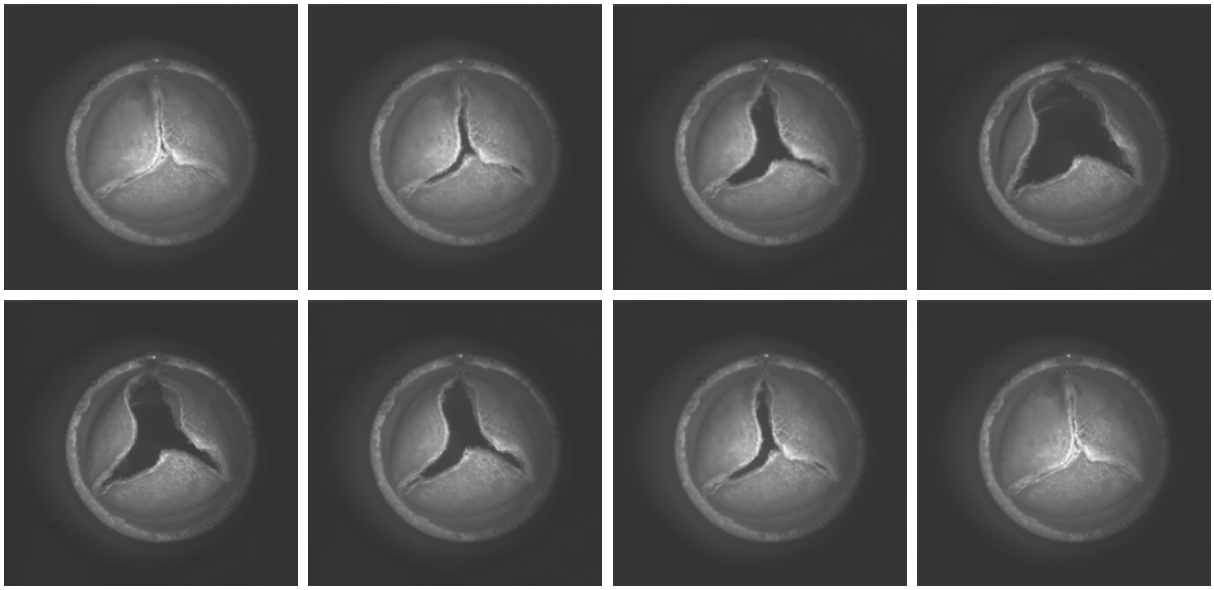

Figure 7.5: Opening and closing behavior: Tissue engineered heart valve leaflets demonstrated sufficient opening and closing behavior when exposed to half systemic conditions.

Histology and immunohistochemistry

Leaflets of all groups showed cellular tissue with production of ECM, independent of mechanical stimulation. H&E staining revealed tissue formation organized in a layered manner with dense outer layers and lesser cellularity in the inner part (Figure 7.6A). Expression of eNOS demonstrated an endothelial cell lining on the surfaces of the tissue engineered leaflets (Figure 7.6B). Trichrom-Masson staining highlighted collagen predominantly at the outer part (Figure 7.6C), whereas in the center loosely arranged ECM substance was detected, characterized as predominantly GAG in the Movat staining (Figure 7.6D). Additionally, vimentin was detected throughout the tissue, whereas α-SMA was not expressed (data not shown).

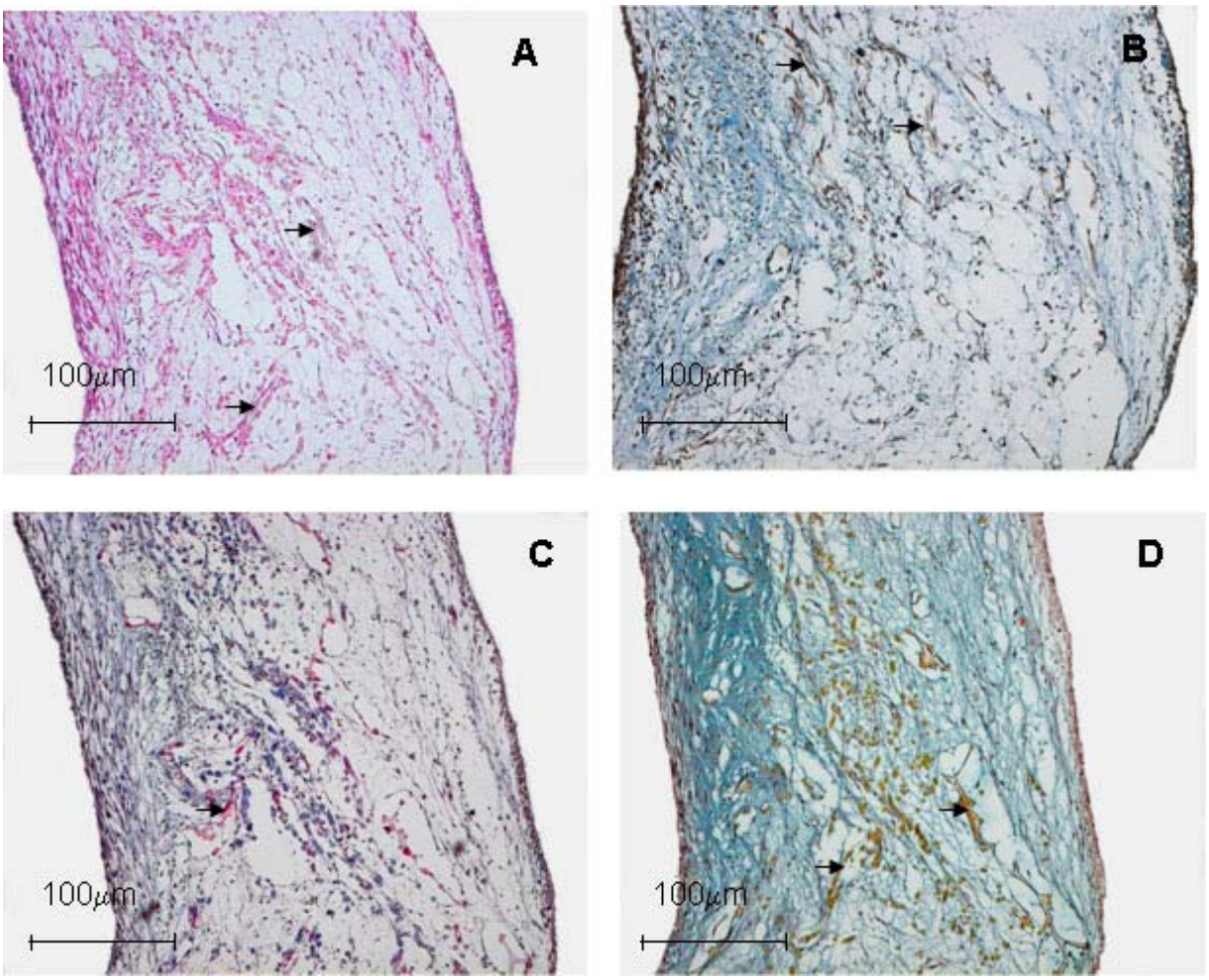

Figure 7.6: *Microstructure of tissue engineered leaflets: H&E staining of engineered leaflets revealed cellular tissue formation (A). Expression of eNOS, highlighted brownish, indicated the endothelial phenotype of cells in the topmost layer (B). Trichrom Masson staining demonstrated the presence of collagen fibers, highlighted bluish, in the outer part (C) and Movat staining showed GAG in the middle part the leaflets (D). Arrows point at remnants of scaffold.*

ECM elements and mechanical properties

Mechanical conditioning showed no significant quantitative and qualitative impact on the production of ECM elements or mechanical properties. Table 7.1 summarizes the content of ECM elements in the tissue engineered heart valve leaflets. The amount of GAG in tissue engineered heart valve leaflets reached values up to 80% of native values (strained leaflets 12.5±0.81 μg/mg; perfused 10.8±1.31 μg/mg; native 16.18 μg/mg). The amount of hydroxyproline was up to 5% of native values (strained leaflets 2.51±0.79 μg/mg; perfused 1.27±0.36 μg/mg; native 47.21 μg/mg). The cell number in all tissue engineered tissues, detected as DNA content, was comparable to that of native tissue (strained leaflets 4.12 ±0.77 μg/mg; perfused 3.47±1.70 μg/mg; native 4.11μg/mg).

Table 7.1: *Content of extracellular matrix elements and cell number in strained and perfused tissue engineered leaflets and native heart valve leaflets expressed as mean μg/mg ± standard deviation.*

leafletS	**GAG (μg/mg)**	**Hydroxyproline (μg/mg)**	**DNA (μg/mg)**
strained	12.5±0.81	2.51±0.79	4.12 ±0.77
perfused	10.8±1.31	1.27±0.36	3.47±1.70
native	16.18	47.21	4.11

The production of ECM elements was reflected in the mechanical properties, in specific, tensile strength: strained leaflets 0.05±0.02 MPa; perfused leaflets 0.07±0.03 MPa, strain at maximal stress: strained leaflets 0.61±0.11; perfused leaflets 0.65±0.20, Young's modulus strained leaflets 0.16±0.07 MPa; perfused leaflets 0.19±0.09 MPa.

Scanning Electron Microscopy

The tissue engineered heart valve leaflets demonstrated smooth surfaces when seeded with CD133- cells only (Figure 7.7A). When coated with CD133+ cells an endothelium-like formation was observed on the leaflet surfaces (Figure 7.7B).

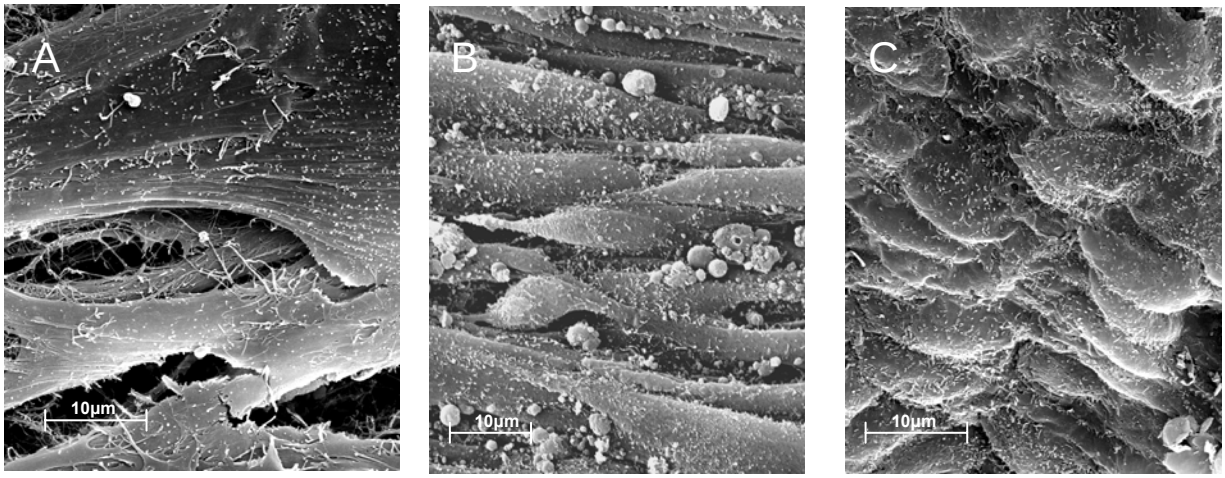

Figure 7.7: *Surface morphology: In SEM, tissue engineered heart valve leaflets based only on amniotic fluid-derived CD133- cells demonstrated smooth surfaces covered with extracellualr matrix elements producing cells (A) but an endothelium-like formation appeared after coating with CD133+ cells (B). (C) presents a magnification of a native heart valve leaflet surface.*

7.5 Discussion

In many congenital heart malformations corrective intervention is mandatory directly or shortly after birth to prevent secondary damage to the immature heart. Since such heart malformations are often detectable already during pregnancy (e.g. by routine ultrasound examination), the ideal pediatric tissue engineering paradigm would comprise prenatal cell harvest in order to provide time for the in vitro fabrication of an autologous living implant that is ready to use at birth. Amniotic fluid represents an attractive fetal cell source for pediatric cardiovascular tissue engineering as it enables easy prenatal access to fetal progenitor cells from all three germ layers in a low-risk procedure. Furthermore, previous studies have shown promising results based on these cells with regard to non-cardiovascular tissue engineering (Kunisaki *et al.*, 2006).
Here, we demonstrate that living autologous heart valve leaflets were successfully engineered using amniotic fluid-derived cells, namely CD133+, a non-committed progenitor cell population with the capacity to differentiate into endothelial cell lines, and CD133-.

Both CD133+ and CD133- cells demonstrated excellent growth capacity. When analyzed, they showed characteristics of fetal multipotent, but not pluripotent progenitor cells as indicated by the expression of CD44 and the lack of Oct-3/4, respectively. Furthermore, their fetal origin was confirmed by gene analysis. The staining pattern of CD133- cells, namely the expression of CD44 and CD105 and a lack of CD34, reflects a mesenchymal progenitor cell type. When used for the fabrication of autologous heart valve leaflets, CD133- exhibited characteristics of fibroblast-like cells by producing extracellular matrix elements.

Most of the isolated CD133+ cells expressed CD44 but lacked the expression of CD34. During cell expansion and differentiation using vascular endothelial growth factors, CD133+ cells showed characteristics of functional endothelial cells as indicated by the expression of CD141 and eNOS. This staining pattern suggests that CD133+ cells might be multipotential adult progenitor cells with the capacity to differentiate into endothelial cells under well defined conditions (Reyes *et al.,* 2001; Reyes *et al.,* 2002). Furthermore, the expression profiles may indicate that the CD133+ population is not homogenous. When seeded onto the surfaces of engineered heart valve leaflets, CD133+ cells formed functional endothelial layers. CD31 and vWF were not expressed during the culture time period indicating that the differentiation process might not have been completed suggesting modification of the culture conditions such as growth factor concentrations. Moreover, the functionality of the endothelial-like layers has to been proven in vivo.

The stress-strain profiles of tissue engineered heart valve leaflets showed non-linear mechanical behavior indicating that the the measured mechanical properties were mainly attributed to the newly formed tissues as the initial scaffold material itself exhibited linear behavior. The mechanical properties did not reach physiological values (Shin`oka *et al.*, 1995) during the investigated in vitro cultivation time period. This might be explained by the uncompleted collagen production suggesting further improvement of the mechanical loading protocol in vitro (Niklason *et al.*, 1999; Mol *et al.*, 2003). However, under half of systemic conditions tissue engineered heart valve leaflets demonstrated sufficient opening and closing behavior indicating that the engineered constructs might function in a low-pressure environment as pulmonary valve replacements.

In summary, here we demonstrated the feasibility to generate living autologous heart valve leaflets in vitro using amniotic fluid as a single cell source. The cell populations required for heart valve tissue, that is myofibroblasts-fibroblast like cells and endothelial cells could be successfully differentiated and expanded. The premature state of amniotic fluid-derived fetal multipotential progenitor cells may in addition represent an advantage regarding their regenerative capacity. This might be associated with an increased potential to form heart valve tissues that approximate their native counterparts in architecture and cell phenotypes under well defined conditions. However, the not fully differentiated stage might be a potential risk for tumor development by uncontrolled cell growth or differentiation. In this feasibility study focusing on the evaluation of a new fetal cell source for the prenatal fabrication of heart valve tissues a non biodegradable material was used for fixation of heart valve leaflets for technical simplicity. However, for in vivo studies the ring structure can be easily changed to biodegradable polymer materials. Furthermore, the applicability of the engineered heart valve tissues as to high pressure application has to be investigated using e.g. numeric flow-structure simulation. This important aspect has to be considered in future in vivo studies as well as their functionality with respect to long-term durability and adequate growth behaviour of this new class of replacements.

Acknowledgements

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Chapter

8

Discussion and conclusions

8.1 Introduction

Today, the repair of congenital malformations is limited by a lack of living autologous replacement materials. Thus, contemporary treatment comprises the use of non-living synthetic or bioprosthetic replacements. A native–analogous substitute would have fundamental advantages over the currently used non-living prostheses as it could grow with the patient and might avoid re-operations associated with increasing mortality and morbidity each time. Pediatric cardiovascular tissue engineering represents a promising strategy to overcome these limitations by focusing on the fabrication of replacements with growth, repair and regeneration capabilities.

One important prerequisite for generating such living replacements is an autologous cell source that provides the living part for the tissue engineered substitute. After biodegradation of the scaffold material, the temporary cell carrier, the extracellular matrix produced by the cells is responsible for the mechanical properties and functionality of the cardiovascular construct. The surface of the implant that is in contact with the surrounding blood flow has to function as an antithrombogenic and blood-compatible endothelial-like layer enabling a smooth blood flow through the engineered prosthesis without causing pathological phenomena such as thrombus formation or immunological reactions. Therefore, an ideal cell source for the fabrication of a long-lasting autologous cardiovascular replacement would provide cells with high quality for both, the extracellular matrix production and the endothelium maintaining their functionality over the patients` lifetime.

In this thesis, a series of studies was performed to investigate various human extra-embryonic situated progenitor cell sources with respect to their applicability as to the prenatal fabrication of pediatric cardiovascular tissue replacements. Thereby, the main goal was to define a prenatal progenitor cell source that might enable the realization of the pediatric cardiovascular tissue engineering concept. Starting from the clinically most critical criteria it was focused on fetal progenitor cell sources that can be reached in a minimally invasive procedure without high risks for both the mother and the unborn child. Ideally, cell harvest should be performed without harming or causing any dysfunctions of intact fetal structures. Therefore, a novel strategy using human prenatal progenitor cells obtained during pregnancy from routinely harvested tissues for prenatal diagnosis as new and exclusive cell source was developed in order to generate autologous living implants ready for use at birth.

8.2 Progenitor cells – When is the appropriate time point for harvest?

Progenitor cells, also named adult stem cells, have high potential to optimise the quality of living, autologous engineered tissues as they physiologically contribute to tissue development, repair and regeneration (Weisman, 2000; Grant *et al.*, 2002). Furthermore, as differentiation processes are not completed, progenitor cells have a high plasticity allowing guidance into various tissues under well defined culture conditions (Ferrari *et al.*, 1998). Thus, progenitor cells represent not-fully

differentiated cells with high proliferation capacity (Grant *et al.*, 2002). Furthermore, as differentiation processes are not completed, the use of progenitor cells might offer new strategies for cellular engineering leading to fetal-like tissues with a high potential to remodel, regenerate and grow. The development of those tissues might be closer to the natural, physiological development since the undifferentiated cells are passing through a developmental process which might closely match that of natural embryonic tissue. Ideally, for cardiovascular tissue engineering cells would be isolated in an early stage of differentiation in order to enable optimal guidance of these cells in vitro into cardiovascular tissues with architecture and functional properties resembling that of of their native counterparts.

With respect to cellular evolution, the least differentiated cell is the zygote which is capable of creating an entire being (totipotency). As development proceeds, the differentiation potential becomes more limited. The blastocyst provides cells in its inner cell mass that are self-renewing and can give rise to all three germ layers (pluripotency) of the developing embryo. These cells can be removed from the inner cell mass and are known as embryonic stem cells (Thompson *et al.*, 1998). In vitro, embryonic stem cells have the ability to proliferate indefinitely and seem to maintain their pluripotent properties by differentiating into any somatic cell type (Schuldiner *et al*., 2000; Odorico *et al*., 2001; Zhang *et al.*, 2001; Moretti *et al.*, 2006). On the other side, their indefinitely self-renewing capability and pluripotency are associated with an increased potential for malignancy by genetic alterations and mutations when cultured in vitro (Baker *et al*., 2007). Teratoma formation in vivo has been shown (Iskovitz-Eldor *et al*., 2000). In addition, control of the immunological development of these cells is also a significant problem (Tajima *et al.,* 2003; Kofidis *et al.*, 2005). In conclusions, embryonic stem cells might not be the appropriate cell source for pediatric cardiovascular tissue engineering. Furthermore, from an ethical point of view cell harvest is associated with harming embryonic structures as cells are obtained from the inner mass of blastocytes.

Multipotent progenitor cells are an ethical more acceptable cell source of not fully differentiated cells with the capacity to differentiate into various tissues in a well-defined in vitro microenvironment (Pittenger *et al.*, 1999; Rayes and Verfaillie, 2001; Jiang *et al.*, 2002). In particular, fetal progenitor cells derived from accessory extra-embryonic tissues such as umbilical cord, placenta (choronic villi) and amniotic fluid are less controversial sources for cells in early stage of differentiation (De Coppi *et al*., 2002; Mitchell *et al.*, 2003; Tsai *et al.*, 2004; Saraguser *et al.*, 2005; De Coppi *et al*., 2007) as they can be harvested without harming or destroying embryonic structures. Furthermore, harvesting of these cells for diagnostic purposes is widely accepted. Therefore, the use of fetal progenitor cells as investigated in this thesis might be a promising concept for the fabrication of pediatric autologous cardiovascular replacements with improved cell quality.

8.2.1 Endothelial progenitor cells (EPCs)

The first series of studies presented in this thesis focused on a prenatally available endothelial progenitor cell source for the generation of functional endothelia for pediatric cardiovascular replacements. The origin of EPCs is still not fully understood as it has been shown that both bone marrow-derived hematopoetic progenitor cells (Asahara *et al.*, 1997;) and non-hematopetic progenitor cells within the bone marrow (Reyes *et al*., 2002; Oswald *et al.*, 2004) as well as non-bone-marrow-derived cells such as adipose tissue-derived cells (Zuk *et al*., 2001; Planat-Bernard *et al.*, 2004), cardiac progenitors (Beltrami *et al*., 2003) and neural progenitors (Wurmser *et al*., 2004) can lead to endothelial cells. Also umbilical cord blood, that is already obtained prenatally for genetic analysis in the context of prenatal diagnostics, represent an attractive cell source for EPCs since circulating endothelial progenitor cells can be isolated from blood and differentiated in vitro into mature endothelial cells (Asahara *et al.*, 1997). Umbilical cord blood contains up to 10-fold higher number of EPCs compared to peripheral blood (Murohara *et al.*, 2000; Peichev *et al*., 2000). Moreover, umbilical cord blood-derived EPCs showed higher proliferation potential and expressed telomerase, a functional characteristic of early stem cells that is very low or absent in EPCs derived from adult peripheral blood (Ingram *et al*., 2004).

In this thesis, EPCs could be isolated from fresh umbilical cord blood and successfully differentiated in vitro. Isolated cells demonstrated colony-like formation and expressed markers that are characteristic for endothelial progenitor cells such as VEGF receptor-2. Furthermore, polymorph EPCs showed remarkable proliferation capacity. Experiments proving the capability of differentiated EPCs to form living endothelia on tissue engineered constructs demonstrated stable phenotype of these cells under biomimetic conditions by the expression of endothelial nitric oxide synthase (eNOS), a potent agent playing a critical role in vascular physiology and prevention of atherosclerosis (Naseem, 2005). Furthermore, the assessment of biological plasticity, which has been described in Chapter 4, displayed endothelial-like cell behavior with respect to antithrombogenic and thrombogenic markers (CD141, tissue factor). These results indicate the potential of umbilical cord blood-derived progenitor cells for application in the pediatric cardiovascular tissue engineering concept. Umbilical cord blood for these studies was harvested postnatally directly after birth in order to avoid any risks that might be associated with cordocentesis (Orlandi *et al.*, 1990) both for mother and the unborn child. Nevertheless, as cordocentesis is justified for diagnostic purposes today, the clinical realization of the concept using umbilical cord blood-derived EPCs would be possible.

In order to further reduce the potential risk associated with harvesting EPCs by cordocentesis endothelial progenitor cells were isolated from prenatally obtained amniotic fluid identified by the expression of CD133 as marker for EPCs (Asahara *et al*., 1997). Today, amniotic fluid sampling is a routine procedure for fetal tissue harvest for genetic prenatal diagnostic. Particularly, mid-trimester (15+0 - 16+6 gestation weeks) amniocentesis, the most common form of invasive prenatal fluid sampling, is a well established low risk technique (0.5%-1%; Wilson, 2000). Isolated amniotic fluid-derived fetal CD133+ cells initially demonstrated cluster-like formation and excellent

growth capacities during cell culture time. Phenotyping of these cells revealed a lack of Oct-3/4 and CD34, the expression of CD44 and endothelial markers such as eNOS, CD141. These results suggest that CD133+ cells might be multipotent progenitors (Rayes *et al*., 2001) but not pluripotent progenitors.

In contrast to the differentiated umbilical cord blood-derived endothelial progenitor cells, late endothelial markers like vWF were not expressed in amniotic fluid-derived CD133+ cells indicating that the cells were not fully differentiated during the investigated culture period. Further refinement of the culture conditions such as modification of growth factor and serum concentrations might lead to fully differentiated cells in future studies.

After coating of tissue engineered heart valve leaflets with CD133+ cells, surfaces demonstrated endothelial-like formation with the expression of eNOS indicating the presence of a functional endothelium. Thus, the feasibility of engineering endothelial from multipotent fetal progenitor cells prenatally could be demonstrated.

8.2.2 Mesenchymal progenitor cells

As two types of cells are required for the engineering of pediatric cardiovascular tissue replacements, the second part of this thesis focused on a prenatal cell source that provides mesenchymal-like progenitor cells. These cells can differentiate into cells that match the phenotype of cardiovascular interstitial cells, form tissue and produce extracellular matrix that is mainly glycosaminoglycans, elastin and collagen enabling tissue strength and adequate mechanical properties. In this thesis three different cell sources were investigated: Wharton`s jelly, chorionic villi and amniotic fluid. With respect to the progenitor potential chorionic villi-derived cells and amniotic fluid-derived cells might be more potent as they were harvested prenatally during the first trimester and mid-trimester of pregnancy, repectively. In contrast, Wharton`s jelly-derived cells were obtained postnatally as prenatal umbilical cord tissue sampling is not clinical routine, yet. Furthermore, although the presence of mesenchymal progenitor cells in the Wharton`s Jelly (Mitchell *et al.*, 2003; Wang *et al*., 2004;) and the perivascular tissue (Saraguser *et al*., 2005) has been reported the isolated cells might not be a homogenous mesenchymal progenitor population but a mixture of cells in different stages of differentiation.

However, after isolation and differentiation, cells derived from all investigated cell sources demonstrated myofibroblast-fibroblast–like phenotype comparable to native cells that are found in cardiovascular structures. When seeded onto cardiovascular scaffolds (PGA/P4HB) and exposed to cyclic strain, all cells demonstrated production of extracellular matrix elements, predominantly glycosaminoglycans. While amounts of glyocosaminoglycans reached native values, those of collagen were significantly lower. Regarding collagen production Wharton's jelly-derived cells produced up to 16% and chorionic villi-derived cells up to 14% of native values, whereas in amniotic fluid cell-derived tissues only 5% of native collagen amount was detected. In contrast, the content of DNA, an indicator for cell number, were higher in amniotic fluid-derived tissues (up to 100% of native values) compared

to chorionic villi cell-derived tissues (up to 68% of native values) and Wharton's jelly-derived tissues (up to 65% of native values). These results indicate that the amniotic fluid-derived tissues are in a stage of proliferation whereas in Wharton's jelly-derived and chorionic villi-derived tissues proliferation were completed and production of collagen has started as it has been demonstrated that myofibroblasts-fibroblasts commence collagen synthesis after completion of proliferation (Grotendorst *et al*., 2004). This phenomenon might be caused by a less mature cell differentiation stage of amniotic fluid-derived cells. This is supported by the absence of α-SMA expression indicating that amniotic fluid-derived cells did not exhibit an activated myofibroblast-fibroblast-like cell character but a miotogenic-active cell. The mechanical properties of resulting neo-tissues differed accordingly: chorionic villi-derived neo-tissues demonstrated a Young`s Modulus of 0.59 ± 0.17MPa, Wharton's jelly-derived neo-tissues of 0.32 ± 0.04 MPa, and amniotic fluid-derived tissues of 0.16 ± 0.07 MPa under the same culture conditions.

However, despite this difference in cell maturity and resulting degree of extracellular matrix formation it could be demonstrated that all investigated cell sources can successfully be used for the engineering of living cardiovascular tissue replacements.

As to the clinical application amniotic fluid represents the most promising cell source as both endothelial progenitor cells as well as mesenchymal progenitor cells were successfully isolated. Thus, the use of amniotic fluid might enable the prenatal fabrication of cardiovascular replacements based on a single cell source ready to use at birth.

8.3 Comparison with development of native fetal cardiovascular tissues

Cardiovascular tissues based on prenatal progenitor cells as fabricated and described in this thesis showed characteristics of mitogenic active tissues. They consisted predominantly of glycosaminoglycans and low collagen. In order to estimate the stage of development of the in vitro generated heart valve tissues, native counterparts from neonates were used for comparison as presented in Chapter 6. These native tissues demonstrated homogenous distribution of extracellular matrix elements without distinguishable layers. Furthermore, interstitial valve cells did not show characteristics of activated myofibroblast as they lacked the expression of α-SMA. When comparing the in vitro fabricated heart valve tissues with the native neonatal tissues, similar tissue formation could be observed but with differences in amount and distribution of collagen. As heart valve tissue development is a dynamic process during life (Aikawa *et al.*, 2006) this observation suggests that the in vitro fabricated leaflet tissues are less mature than the neonatal ones. Compared to postnatal heart valves, fetal tissues during intra-uterine development have much higher cellular densities. Furthermore, they differ in the extracellular matrix composition. After 14 weeks of gestational age, the extracellular matrix is predominantly composed of glycosaminoglycans with low collagen and elastin content. By week 20, a bilaminar structure with sparse, loose, and

unorganized collagen develops (Aikawa *et al.,* 2006). The well defined three-layered structure of heart valve leaflets containing elastin in the ventricularis and increased collagen in the fibrosa becomes apparent by week 36 but still remains incomplete compared with adult tissues (Aikawa *et al*., 2006).

Thus, the tissue engineered prenatal progenitor cells-derived heart valve tissues presented in this thesis resemble fetal heart valve tissues in an early stage of development according to week 20. With respect to the phenotype, in weeks 14 to 19 interstitial cells express α-SMA attributed to myofibroblasts mainly in a subendocardial location below the outflow surface (Aikawa *et al*., 2006). In this thesis, also Wharton's jelly-derived tissues demonstrated an accumulation of α-SMA positive cells beneath the endothelial surface. This observation indicates that Wharton's jelly-derived tissues are more mature than chorionic villi cell-derived tissues or amniotic fluid cell-derived tissues that lack the expression of α-SMA.

However, the use of fetal progenitor cells in an early stage of differentiation resulted in cardiovascular fetal-like tissues similar to their native counterparts. Thus, the use of fetal progenitors is a promising strategy for the engineering of nature-like cardiovascular structures by mimicking the embryonic development in vitro.

8.4 The impact of cell source, mechanical load and culture conditions on tissue development

Tissue formation during embryogenesis and regenerative processes demands highly coordinated cell migration and proliferation, followed by cellular differentiation, matrix synthesis and formation. The regulatory mechanisms that control these events appear to be linked in a cascade fashion. Early factors function as initiators of these complex biological processes (St. Johnston and Nusslein-Volhard, 1992; Horgan, 1999; Ng *et al*., 1999). Dysfunctional cascades lead to tissue malformation in the developing tissues. During tissue engineering, where cells are guided into functional tissues in vitro, similar processes might be passed through. Thus, stimuli that are applied to the developing and maturating tissue have to be controlled in a highly orchestrated manner in order to enable native-analogous tissue development and formation.

Various studies have demonstrated that the mechanical properties of tissue engineered constructs can be guided by mechanical load aiming at the production of properly aligned collagen fibers during in vitro maturation (Mol *et al.*, 2003; Mol *et al.*, 2006; Driessen, 2006; Stekelenburg, 2006; Ku *et al*., 2006). Also in the experiments carried out here, it could be shown that collagen production and alignment and consequently mechanical properties were influenced by mechanical load. However, although beginning collagen production was detected in all engineered cardiovascular tissues, the collagen production was significantly lower compared to values of in vitro fabricated tissues reported in the literature (Mol *et al.*, 2006) using a similar strain-based approach. This was reflected in the mechanical properties. Whereas Mol et al. (Mol *et al*., 2006) described a Young's Modulus up to 7.4 ± 1.6 MPa for tissue engineered heart valve leaflets based on mature vascular-derived myofibroblasts

obtained from adult patients, here the highest Young's Modulus for tissues maturated under cyclic strain and the same culture conditions was 1.44 ± 1.36 MPa for Wharton's Jelly derived cells. This indicates that besides the mechanical load also the cell source itself may play an important role. Furthermore, when the culture conditions were modified by using the same cell source and mechanical conditioning protocol but additional growth factors, namely IGF-1, EGF, VEGF and FGF, as described in chapter 5, collagen production and consequently mechanical properties changed (Young`s modulus 0.32 ± 0.04 MPa).

Moreover, as already mentioned above, mechanical properties of chorionic villi-derived tissues and amniotic fluid-derived tissues differed under these culture conditions which is an indication that the cell source itself is a crucial factor by responding in an individual and specific manner to mechanical and/or biochemical stimuli. This leads to the hypothesis that well defined prerequisites are required before stimulation results in enhanced collagen synthesis and formation. Grotendorst and others (Grotendorst *et al.*, 2004; Aikawa *et al.*, 2006) have demonstrated that a myofibroblast phenotype, reflected by expression of α-SMA and representing an activated fibroblast, has to be induced before up-regulation of collagen synthesis starts. Mature vascular-derived cells as were used by Mol et al. (Mol *et al.*, 2006) had an activated phenotype prior seeding. Thus, mechanical stimulation by the means of cyclic strain resulted in collagen synthesis whereas not fully differentiated cells as presented in this thesis responded mainly with proliferation and glyocosaminoglycans synthesis according to a native heart valve leaflet after during the first 20 weeks of development (Aikawa *et al.*, 2006). This indicates that the microenvironment with respect to mechanical and/or biochemical stimuli has to be defined individually for every cell source in order to enable optimal tissue development and formation.

8.5 Focus of future studies

Although the feasibility of engineering autologous fetal-like cardiovascular tissue replacements prenatally using fetal progenitor cells could be demonstrated, all the criteria of an ideal native-analogous prosthesis as described by Harken (Harken *et al.* 1962) are not matched, yet. Several issues have to be addressed in future studies in order to further improve the quality of the tissue engineered cardiovascular replacements based on prenatal progenitor cells and enable the fabrication of a prosthesis with long-term functionality for the repair of congenital malformation.

8.5.1 Improvement of mechanical properties

Long-term performance of tissue engineered replacements depends on their mechanical properties. Here, the mechanical properties did not yet reach physiological values (Shin`oka *et al.*, 1995) during the investigated in vitro cultivation period. Thus, future studies should focus on the improvement of mechanical properties by increasing collagen production and alignment. Therefore, investigations with respect to the response to mechanical forces and the biochemical environment of the particular

prenatal cells will be an important step towards a better understanding of the developing tissues and their composition.

One aspect of particular interest might be the modulation of cells towards activated myofibroblasts that synthesize collagen with tissue-specific allignment. Modifications in the mechanical loading protocol are one important issue and mathematic models might be a valuable tool predicting the tissue development in response to mechanical forces (Driessen, 2006). On the other hand, the results presented in this thesis suggest further refinement of the biochemical environment. TGF-beta, that is known to stimulate collagen synthesis by initiating the complex-building of connective tissue growth factor (CTGF) and IGF (Grotendorst *et al.*, 2004), or other biochemical factors such as insulin and plasmin (Neidert *et al*., 2002), might be promising candidates for improvement of cell matrigenic activity.

A further unsolved problem in cardiovascular tissue engineering in general is the production of elastin in vitro. The lack of elastin might be a risk for pathological creeping and for aneurysm formation if the in vivo remodelling process and the elastin production are delayed. Thus, the capability of fetal progenitor cells to synthesize elastin should be investigated.

8.5.2 Monitoring of cell differentiation and tissue development

Detailed differentiation processes of the progenitor cells guided by mechanical and/or biochemical stimuli or cell-to-cell and cell-to-scaffold interactions as presented in this thesis are unexplored. In general, major obscurities in tissue engineering are the tissue development in vitro and the in vivo remodelling processes after implantation. Furthermore, the fate that cells undergo in vitro after seeding and during tissue development is still unclear. A major obstacle for elucidating these aspects is the current use of invasive or destructive analysis methods. Once the tissue is fixed or destroyed only one state in tissue development can be displayed. Monitoring tissue growth variability such as cellularity, phenotypical characteristics, cell differentiation, localized ECM composition and microstructure over time is not possible.

The development of imaging methods e.g. molecular imaging may enable a non-invasive monitoring of tissue growth and development. Various studies have demonstrated the potential of this approach. Particularly, the application of traceable nano-scaled particles incorporated into the cells or attached to their surfaces (Aikawa *et al*., 2007) might open up new strategies and might bring important and valuable information as well as a better understanding of the tissue engineering process and remodelling phenomena. Using this method, early heart valve calcification has recently been shown in vivo (Aikawa *et al.*, 2007). Furthermore, molecular imaging could be used to track the presence, migration and proliferation as well as function of progenitor cells in vitro (Jaffer and Weissleder, 2004; Terrovitis *et al.*, 2006). Magnetically labelled mesenchymal stem cells have been followed by magnetic resonance imaging after injection into porcine myocardium in vivo (Hill *et al.*, 2003), and hematopoietic derivation of cardiac valve interstitial cells could be demonstrated (Visconti *et al.*, 2006). The use of magnetic resonance imaging for the monitoring of progenitor cells might be an attractive tool, particularly for the assessment of the cell fate in future

studies as in this thesis amniotic fluid–derived cells were magnetically labelled during the separation process. Thus, after seeding of the labelled cells their migration behavior and proliferation capabilities as well as their plasticity with respect to differentiation could be followed by magnetic resonance imaging and display important information on tissue development and the quality of resulting engineered cardiovascular constructs.

8.5.3 Assessment of tumor potential

The use of not fully differentiated cells for in vitro tissue engineering bears an increased risk for malignancy by genetic alterations and mutations as it has already been demonstrated for embryonic stem cells (Iskovitz-Eldor *et al*., 2000). However, when amniotic fluid-derived progenitor cells were injected into immunodeficient (SCID)/Beige mice, non of the implanted cell-lines formed tumors (De Coppi *et al*., 2007). Nevertheless, in order to avoid uncontrolled growth and differentiation, the differentiation processes of cells during in vitro culture have to be investigated in more detail with particular respect to chromosomal rearrangements, genetic alterations and cell cycle checkpoints.

8.5.4 Cryopreservation for postnatal use

Prenatal multipotent progenitor cells are a unique pool of cells in an early stage of development with high potential for repair and regeneration. Furthermore, the amount of undifferentiated multipotent cells in fetal tissues is increased up to 10-fold when compared to non-fetal tissues (Murohara *et al*., 2002; Peichev *et al*., 2002). The fact that fetal progenitor cells are only obtainable in a limited phase of life, namely during the first weeks of pregnancy, might restrict their usability for pediatric tissue engineering. However, cryopreservation of prenatal obtained fetal stem cells might offer a life-long availability. In this thesis, it could be shown that phenotypes of prenatal progenitor cells do not undergo changes after cryopreservation. This suggests that there is a potential for cell banking that might enable the use of these unique progenitor cells for later repair also. In future studies the potential of cryopreservation of this unique cell source should be investigated in more detail, in order to expand the versatility of fetal progenitor cells also for adult application.

8.5.5 In vivo studies

In this thesis the successful engineering of cardiovascular fetal-like tissues based on prenatal fetal cells has been demonstrated. Tissues demonstrated promising potential to serve as an autologous cardiovascular tissue replacement ready to use at birth. Particularly, the successful generation of bioactive living endothelia covering the surfaces of the engineered tissues is a crucial prerequisite for future implantation.

However, the final proof of functionality of the newly developed type of tissue replacement remains to be demonstrated in vivo. Therefore, future studies have to focus also on the transfer of methodology to animal models. The sheep would be the model of choice as it has already been used successfully in other studies evaluating

cardiovascular tissue engineered constructs (Hoerstrup *et al*., 2000; Sutherland *et al*., 2005; Hoerstrup *et al*., 2006)

8.5.6 Future clinical application

Today, only little clinical experience with tissue engineered cardiovascular tissue replacements exists. Reports on long-term experiences are limited. Some preliminary clinical studies have been initiated and recently, first long-term results have been reported by Shin`oka and co-workers (Shin`oka *et al.*, 2005). After successful reconstruction of a peripheral pulmonary artery using an autologous tissue engineered construct in a 4-years-old girl (Shin`oka *et al*., 2001), 24 vascular grafts have been implanted as an extra-cavopulmonary connection in children or young adults with an age ranging from 1 to 24 years (Shin`oka *et al.*, 2005). Those vascular grafts were fabricated from copolymers composed of l-lactide and ε-caprolactone (50:50) reinforced using PLLA and PGA. Before implantation, autologous bone marrow-derived cells that were obtained from the anterior superior spine were seeded onto the scaffolds and the outer layer was sprayed with fibrin glue. A follow-up was performed for 1.3 months and up to 31.6 months. During this time no graft-specific lethal complications were observed. Furthermore, no indication for graft rupture, thromboembolism, aneurysm formation or calcification was noted. The diameters of the implanted grafts increased about 110% ± 7% over time. These data demonstrate the successful transfer and application of one cardiovascular tissue engineering process in a clinical scenario and confirm the potential of autologous tissue engineered cardiovascular replacement as an alternative for currently used prosthesis. However, this approach is based on a slowly degradable scaffold material and a long time-follow-up is necessary to confirm the durability of fabricated constructs as well as careful evaluation of the engineered tissues after implantation.

For the approach presented in this thesis clinical management would start already prior to birth. As soon as the heart valve defect would have been detected by ultrasound cell harvest would be initiated. Depending on the gestation age the most adequate sampling procedure associated with the lowest risks for both mother and unborn child would be performed. Either chorionic villi and umbilical cord blood or, more preferable, amniotic fluid would be harvested Then, fetal progenitor cells would be isolated and differentiated and the engineering process of the cardiovascular tissue replacement would be initiated in order to have the living autologous tissue implant ready to use at or shortly after birth.

In conclusion, the approach presented in this thesis may enable the clinical realization of the pediatric tissue engineering concept by the prenatal fabrication of living autologous cardiovascular tissue replacements based on fetal multipotent progenitor cells and might lead to a new generation of cardiovascular replacements.

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Samenvatting

Menselijke prenatale voorlopercellen als bron voor het kweken van hart en vaat weefsel voor jonge kinderen

Weefselkweek is een veelbelovende techniek voor het vervaardigen van lichaamseigen en levende weefselvervangers voor de behandeling van aangeboren afwijkingen. Om een vervangend weefsel paraat te hebben direct na de geboorte is het van belang dat de cellen om het weefsel te vervaardigen op een eenvoudige manier tijdens de zwangerschap verkregen kunnen worden. Risico's voor de moeder en het ongeboren kind en aantasting van intacte weefsels moeten hierbij worden vermeden. Dit proefschrift beschrijft het gebruik van voorlopercellen uit verschillende extra-embryonale bronnen in de baarmoeder voor het kweken van cardiovasculaire weefselvervangingen.
Uit zowel het navelstrengweefsel als het –bloed, de vruchtwatervlokken (chorion villi) en het vruchtwater zijn voorlopercellen geïsoleerd. De verkregen cellen zijn vermeerderd en gedifferentieerd naar celtypen die geschikt zijn voor gebruik in cardiovasculaire weefselkweek. Differentiatie naar een myofibroblast-fibroblast fenotype is belangrijk voor de vorming van extracellulaire matrix (ECM) in de weefsels. Differentiatie in een endotheelcel fenotype is van belang voor de ontwikkeling van een weefsel oppervlak waar bloed niet aan kan hechten. Genotypering van de cellen is uitgevoerd om vast te stellen dat de cellen een foetale oorsprong hadden en niet van de moeder waren. De celfenotypen zijn gekarakteriseerd met flow-cytometrie en immunohistochemie. Voor het vervaardigen van cardiovasculaire weefselvervangingen zijn biodegradeerbare scaffolds (PGA/P4HB) gezaaid met tot myofibroblast-fibroblast gedifferentieerde voorlopercellen uit zowel de navelstreng, de chorion villi en het vruchtwater. Deze gezaaide scaffolds zijn vervolgens in bioreactoren blootgesteld aan zowel biochemische als mechanische conditionering om weefselvorming te stimuleren. In een later stadium zijn de oppervlakten van de gekweekte weefsels gezaaid met de tot endotheelcel gedifferentieerde voorlopercellen uit het navelstrengbloed of het vruchtwater. De weefselvorming is geanalyseerd met behulp van histologie, immunohistochemie (vimentine, desmine, α-SMA en Ki-67), biochemische assays, mechanische testen en scanning electron microscopie (SEM). De endothelia zijn geanalyseerd met immunohistochemie (CD31, vWf, thrombomoduline, tissue factor en eNOS).

Gedifferentieerde voorlopercellen uit alle bronnen vertoonden kenmerken van myofibroblast-fibroblast cellen, gekarakteriseerd door de expressie van vimentine, desmine en deels α-SMA. De voorlopercellen uit het navelstrengbloed en het vruchtwater konden gedifferentieerd worden in cellen met kenmerken van endotheelcellen, gekarakteriseerd door expressie van CD31, vWf, thrombomoduline, tissue factor en eNOS. De genotypering toonde aan dat de cellen allen afkomstig waren van het foetale weefsel en niet van de moeder. De expressie van eNOS toonde aan dat de gekweekte weefsels functionele endotheellagen bezaten. Expressie van Ki-67 bevestigde dat proliferatie van cellen ook in de weefsels nog doorging. De samenstelling van de extracellulaire matrix was vergelijkbaar met die van natuurlijke cardiovasculaire weefsels. De mechanische eigenschappen van de gekweekte weefsels bleven nog wel achter bij die van natuurlijke weefsels, maar het mechanische gedrag was desondanks veelbelovend. SEM liet goede ingroei van cellen zien en gladde oppervlaktelagen bedekt met endotheelcellen.
Voorlopercellen van het ongeboren kind uit verschillende bronnen in de baarmoeder zijn geschikt voor het kweken van levende cardiovasculaire weefselvervangingen. De meest veelbelovende celbron om een weefselvervanging klaar te hebben ten tijde van de geboorte is het vruchtwater, omdat deze cellen gedifferentieerd kunnen worden in zowel myofibroblasten als endotheelcellen.

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Curriculum Vitae

Dörthe Schmidt was born on April 13th, 1973 in Hilden, Germany. In 1992, she finished her secondary education ("Abitur") at the high school of Haan, Germany. Afterwards, she studied Medicine at the universities of Halle, Bonn, and Duisburg-Essen, Germany, and obtained practical training ("Praktisches Jahr") in Essen, Germany, and Zurich, Switzerland. Dörthe Schmidt graduated in Human Medicine from the University of Duisburg-Essen in 2001 and completed a dissertation (MD, with magna cum laude) in the field of Human Genetics on "Investigation of the clinical variability of Niemann-Pick Disease Type C with suggestion for a diagnostic score" with Prof. E. Passarge at the same university in 2002. Following her dissertation in medicine, Dörthe Schmidt obtained a clinical position in the Clinic for Cardiovascular Surgery at the University Hospital Zurich, Switzerland. She also joined the Laboratory for Tissue Engineering and Cell Transplantation (Regenerative Medicine) of Prof. S.P. Hoerstrup at the University Hospital Zurich in 2002 where she initially worked part-time in parallel to her clinical activity. In 2005 she became a full-time member of this research group and focused on fetal stem cells for pediatric cardiovascular tissue engineering in close collaboration with Prof. F.P.T. Baaijens at the Department of Biomedical Engineering at Eindhoven University of Technology, The Netherlands.