Regenerative medicine in cardiovascular disease: from tissue engineering to tissue regeneration
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CHAPTER 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS
BACKGROUND

Diseases of the heart and circulatory system (cardiovascular disease or CVD) are the major cause of death in the Western world, causing over 4.35 million deaths a year in Europe alone [1]. Thereby, CVD causes nearly 50% of all deaths in Europe, and is estimated to cost the European Union economies €169 billion a year [1]. Furthermore, the prevalence of CVD is increasing because of improved treatment options with higher survival rates and because of the increasing elderly population [2]. Although cardiovascular medicine has achieved many breakthroughs, ischemic heart diseases (IHD), such as myocardial infarction (MI) and heart failure (HF) remain among the most important health challenges worldwide [3]. MI, due to coronary artery disease (CAD), is currently one of the major contributors to the development of HF [4]. In a multicenter heart failure treatment trial, comprising over 20,000 patients, CAD was the underlying etiology in 68% of cases [5]. Aggressive treatment strategies focusing on early reperfusion of the culprit artery and modifying the risk profile of patients improve mortality after MI [6]. However, if reperfusion is not accomplished in time, tissue ischemia is followed by irreversible loss of cardiomyocytes accompanied by scar formation, leading to progressive ventricular remodeling and HF [4]. The annual incidence of HF ranges from 1 to 5 per 1000 people and doubles each decade over the age of 45 [7]. Despite advances in medical and surgical treatment, prognosis remains poor with a median survival after establishing the diagnosis of HF of only 1.7 years in men and 3.2 years in women [8,9]. Although valvular heart disease (VHD) is less frequent than CAD it is an increasing problem often requiring intervention. Rheumatic disease used to be the most frequent etiology of valve disease in previous decades, but nowadays degenerative origin is by far the most frequent cause in the Western world [10,11]. The two most frequent valvular disorders are currently calcified aortic stenosis (AS) and mitral regurgitation (MR), whereas aortic regurgitation (AR) and mitral stenosis (MS) are less frequent [12]. AS is the most common valvular disease in the western world [13]. It is associated with significant morbidity and mortality, and valve replacement is often required [14]. As AS is a typical problem of the elderly patient, the number of patients increases due to the ageing population [14]. Surgical replacement of diseased human heart valves by mechanical and tissue valve substitutes is now commonplace, with approximately 275000 valve replacements carried out worldwide every year [15]. Importantly, it is estimated that the prevalence of heart valve disease, and as a consequence the need for replacement will increase threefold over the next 50 years [16]. However, although valve replacement improves the prognosis of patients with valvular disease, current valve prostheses have serious limitations and inherently lack the sophisticated design and function of the native valve. Both the epidemic proportions of CVD and the growing number of patients with valvular diseases warrant the development of alternative treatment regimens.
Regenerative Medicine

Regenerative Medicine (RM) is an emerging field focusing on unraveling the mechanism underlying complex regeneration processes in nature with the goal of providing elements required for in vivo repair, to create replacements interacting with the living body, and to stimulate the body’s own regenerative capacity [17,18]. RM aims at using strategies and methodologies used in curing conditions as opposed to treating them [19]. Current RM has its origin in observations made in nature that certain species have regenerative capabilities. For example, urodele amphibians, display the ability to regenerate a variety of body parts, including limbs, tail, jaw and retina [20]. Probably the best known example of this phenomenon is the tail regeneration in amphibians, where the missing part, including all differentiated tissue types, is re-grown from the site of transection [21] (Figure 1). This phenomenon has also been described in invertebrates, where e.g. transection of a planarian worm results in a regenerated tail structure [20]. Furthermore, examples of complex tissue regeneration are observed in higher species. Examples are the seasonal re-growth of deer antlers [22], and the distal fingertip regeneration in children [23]. Moreover, at the first two trimesters, human skin wound healing is characterized by complete regeneration and the absence of scar formation [24]. Interestingly, this ability to regenerate is lost in the third trimester.

Ultimately, RM should provide treatment options for diseases and/or injuries not treatable by conventional medicine and even new biologic drug therapies. The field of RM encompasses three areas of technology, 1) tissue engineering; 2) stem cell treatment; and 3) cloning [25]. Tissue engineering uses the principles of cell transplantation and scaffold design, in order to develop biological substitutes able to restore or maintain normal function.

Figure 1. Regeneration of the forelimb in an amphibian after amputation at distal (mid-radius and ulna; shown at left) or proximal (mid-humerus; shown at right) sites. The original limb is shown at the top and the regenerated limb at the bottom of the image. The photographs were taken at 7, 21, 25, 28, 32, 42 and 70 days after amputation. (adapted from Brockes et al., Science 1997;276:81-7)
REGENERATIVE MEDICINE IN HEART VALVE DISEASE: TISSUE ENGINEERING APPROACHES

Although heart valve repair is currently the preferred method of treating patients with severe valvular disease [26,27], a substantial number of valves are (especially in case of aortic valve disease) not suitable for repair and require replacement. Therefore, valve replacement is the most common surgical procedure in patients with advanced valvular disease [28]. Since their clinical implementation in 1965, extensive experience has been gained with mechanical and bioprosthetic valve prostheses [29]. However, although valve prostheses improve survival and quality of life [30], each specific prosthetic valve has limitations (figure 2).

Mechanical Valves

Mechanical prostheses, such as caged ball, tilting disk, or hinged semicircular rigid flap valves have excellent durability but include a high risk of thromboembolic complications (approximately 4% per patient a year [31]) necessitating lifelong anticoagulant therapy [32]. Therefore, the use of mechanical valves is relatively contraindicated among patients with an active lifestyle or a wish for pregnancy. Furthermore flow-profiles across the valves are (depending on the design of the valve) often far less than optimal.

Bioprosthetic Valves

Currently there are three types of commercially available bioprosthetic valves: (1) porcine xenograft valves, (2) bovine pericardial valves, and (3) allograft or homograft valves [33]. The porcine xenograft valve is fabricated from an intact pig aortic valve preserved in low concentration glutaraldehyde solution and mounted on a prosthetic frame. The bovine pericardial valve consists of 3 separate pieces of glutaraldehyde treated calf pericardium attached to a stent and sewing cuff, and is configured very similar to that of the porcine xenograft. Both types of valves are crosslinked in glutaraldehyde and treated with various other chemical agents to reduce their antigenicity and to minimize their tendency to calcify [34]. Human tissue valve prostheses have been introduced as homograft valves, defined as a valve from an individual or equal species. They are usually cryopreserved as complete aortic or pulmonary roots and trimmed before implantation [35,36].

One of the major limitations of the porcine and bovine xenografts is their progressive structural failure, which is more often seen in younger recipients [37,38,39]. Although, homograft valves include a greater durability and demonstrate better resistance to infective endocarditis [40], their availability is limited due to organ scarcity.
All clinically available valve prostheses basically represent non-viable structures and lack the ability to grow repair, or to remodel [41]. This imposes potentially severe problems, especially in the pediatric population. Moreover, with the currently available valves, approximately 60% of the recipients will suffer from a serious valve-related complication within 10 years post-operatively [42].

Figure 2. Different artificial valve prostheses. From left to right; S. Jude Medical® and Starr-Edwards aortic valve mechanical valves, Medtronic Freestyle stentless porcine bioprosthesis, human cryopreserved homograft, and Carpentier-Edwards PERIMOUNT bovine tissue heart valve.

**Tissue Engineering**

Tissue engineering of heart valves aims to overcome the above described limitations of currently available heart valve prostheses. Tissue engineering is one of the main components of regenerative medicine, and follows the principles of cell transplantation, materials science, and engineering towards the development of biological substitutes that can restore, maintain, or improve normal function [25,43]. A tissue engineered heart valve potentially has several advantages, like non-thrombogeneicity, resistance to infection and cellular viability. It promises to be a living implant with a potential to grow and last a lifetime, like most native valves do [33]. The initial and crucial step is to obtain an optimal valve matrix. Tissue engineering strategies for heart valves therefore can be divided into two categories: engineering of completely artificial valves from biodegradable synthetic polymers, and decellularization of biological heart valves from allogeneic or xenogeneic donors, seeded with autologous cells either in vitro or in vivo.

**DIFFERENT APPROACHES TO VALVULAR TISSUE ENGINEERING**

*Synthetic Scaffolds*

Biodegradable materials break down as a result of macromolecular degradation with dispersal in vivo, but are not eliminated from the body [44]. A biodegradable valve scaffold can thus provide a temporary matrix until repopulating cells produce their own matrix proteins. The use of biodegradable materials is especially appealing from a developmental standpoint, with the ability to create a variety of sizes and lengths in order to tailor the valve for individual patients.
Several biodegradable polymers have been demonstrated to be useful in cardiovascular tissue engineering. Polyglycolic acid (PGA) and polylactic acid (PLA) and their copolymers have been widely used in TE [45]. However, these materials have been abandoned for heart valve TE because of their stiffness and short degradation times. Furthermore, they cause a significant local inflammatory response [46,47]. Recent advances include more compliant materials like polyhydroxyalkanoates (PHAs) [48] or combinations of PGA and poly-4-hydroxybutyrate (P4HB) [49,50]. However, in most animal studies, these scaffolds have been implanted in the pulmonary artery only, because the degrading scaffold cannot sustain the higher LV pressures until the new valve has been regenerated [49,50,51]. Furthermore, none of these scaffolds have been used in a clinical setting.

**Decellularized Biological Scaffolds**

Another approach to heart valve TE involves the (bio)-chemical decellularization of homo- or xenografts. Xenogeneic and allogeneic cellular antigens are capable of inducing an inflammatory response or immune-mediated tissue rejection, whereas components of the extracellular matrix (ECM) are well conserved among species and are well tolerated even by xenogeneic recipients [52]. Decellularized heart valves are particularly attractive because of the natural anatomical geometry, which may serve as a template for cellular attachment and retaining many of the mechanical and structural properties of native valves such as tensile strength and ECM composition [53]. The goal of a decellularization protocol is to remove all cellular and nuclear material while minimizing any adverse effect on the composition, biological activity, and mechanical integrity of the remaining ECM [54]. Various decellularization methodologies have been explored for the purpose of heart valve tissue engineering.

**Enzymatic Decellularization Methods**

Enzymatic techniques for heart valve decellularization include the use of protease digestion and nucleases. The proteolytic enzyme Trypsin is one of the most commonly used enzymes in valvular decellularization protocols. It displays its effect by cleaving the peptide bonds on the carbon side of arginine and lysine if the next residue is not praline [54]. Nucleases, such as endonucleases and exonucleases, catalyze the hydrolysis of the interior and terminal bonds of ribonucleotide or deoxyribonucleotide chains, ultimately leading to the degradation of RNA and DNA [54]. Steinhoff et al., demonstrated that a 48-hour Trypsin/EDTA treatment resulted in an almost complete decellularization of ovine pulmonary valve conduits [55]. This protocol was later also successfully applied to human aortic and pulmonary allografts, resulting in complete decellularization [56]. However, others reported (applying the same protocol) only incomplete decellularization of porcine aortic and pulmonary roots [57,58].
Detergent Decellularization Methods

Detergents are amphipathic molecules that can solubilize membrane proteins by creating a mimic of the natural lipid bilayer environment normally inhabited by the protein [59], and can therefore disrupt cell membranes and bonds responsible for intercellular and extracellular connections. Detergents are classified according to their structure, and generally fall into three categories:

1) Non-ionic detergents have a relatively mild effect on the ECM structure, and have therefore been used extensively in decellularization protocols. Non-ionic detergents disrupt lipid-lipid and lipid-protein interactions, but not protein-protein interactions, leaving proteins in their functional conformation [59]. Non-ionic detergents include Triton X-100 and the Tween family of agents. The group of Bader, was the first to use Triton X-100 for the decellularization of porcine aortic valves [60]. Also Numata et al. showed sufficient cellular removal in porcine pulmonary allografts after Triton X-100 treatment [61]. However, Kim et al. could still detect cells after Triton X-100 treatment in porcine heart valve leaflets [62]. Furthermore, Booth et al. also could not achieve complete decellularization of porcine aortic valves after Triton X-100 or Tween 20 treatment [63].

2) Ionic detergents effectively solubilize both the cytoplasmatic and nuclear cellular membranes, but have a propensity to denature proteins by disruption of protein-protein interactions [59]. For heart valve decellularization, the two most commonly used ionic-detergents are sodium dodecyl sulfate (SDS) and sodium deoxycholate. Several authors have demonstrated complete decellularization of porcine pulmonary [63,62,64] and aortic valves [57,58] after SDS treatment. Ueda et al. used a combination of SDS and the non-ionic detergent Triton X-100 with successful decellularization [65]. Sodium deoxycholate treatment has been successfully applied in porcine aortic [63] and pulmonary valve leaflets [66].

3) Combination of an ionic and a non-ionic substance has been used to decellularize valve preparations. For example, sodium deoxycholate with Triton X-100 resulted in complete decelluarization of porcine aortic [57,58,67] and pulmonary valve conduits [57,58].

Commercially Available Patented Decellularization Methods

To date only two commercially available decellularized heart valve prostheses are available. CryoLife Inc from Kennesaw Ga, has developed a patented procedure called the SynerGraft technology that includes cell lysis in hypotonic solution, enzymatic digestion of nucleic acids, and washout in isotonic, neutral buffer, which is able to produce acellular porcine [68] and aortic [69] and pulmonary [70] human homografts. In addition, recently also the AutoTissue LTd® method [71] has become available, which involves the use of a solution of 1% deoxycholic acid and ethanol 70%, and is able to produce completely acellular cryopreserved human homografts [72].
EFFECTS OF DECELLULARIZATION ON THE EXTRACELLULAR MATRIX

Although the focus of the different cell extraction protocols is primarily its efficiency in removing all cellular elements, it should be noted that chemically induced cell extraction can be quite detrimental to the valvular matrix [73]. Decellularisation can result in degradation or denaturing of the matrix proteins or leave toxic residues which can detrimentally affect recellularization and mechanical function [74]. In addition, it was recently demonstrated that the remaining extracellular matrix composition could be involved in early calcification and incomplete repopulation of transplanted decellularized valves [75]. Different cell extraction protocols could also all have different effects on the remaining ECM [63,76].

CELL SEEDING OF VALVE SCAFFOLDS

There is variability in potential strategies and sources of cells. They include the repopulation of scaffolds with appropriate cell types in vitro, or the use of synthetic or decellularized scaffolds, without in vitro repopulation prior to implantation, which can attract endogenous cells. In the later approach the repopulation of the valve scaffold takes place in vivo after the implantation.

Pre-seeding of Valve Scaffolds

Different cell types have been successfully applied for reseeding of synthetic and biological scaffolds. Venous endothelial cells have been preferred due to the simple access to peripheral venous segments. Porcine and goat jugular [62,64] and human saphenous [60,56,58] endothelial cells have also been used for the in vitro repopulation of biological scaffolds. However, other authors have used porcine femoral artery endothelial cells [61], and myofibroblasts and endothelial cells derived from the wall of carotid arteries have been used in synthetic [77,49] and acellular [55] matrix scaffolds. More recently, also ovine and human mesenchymal stem cells have been investigated for the feasibility of tissue engineering [50,78].

Non-seeded Valve Scaffolds

Leyh et al demonstrated that decellularized porcine xenograft valves were repopulated in vivo within 24 weeks with interstitial myofibroblasts and endothelial cells after transplantation in the pulmonary circulation of a sheep model [75]. In Contrast, Goldstein et al. found progressive recellularisation of the matrix with fibroblasts and myofibroblasts without endothelialization in the same model [79]. Others showed partial degeneration of unseeded porcine valves in contrast to valves that were in vitro seeded with myofibroblasts and endothelial, were complete histological reconstitution was observed [55]. Interestingly Kim et al. found in a goat model no histological differences between decellularized porcine valve leaflets with or without seeding of autologous endothelial cells [64]. Therefore, role and necessity of in vitro pre-seeding remains controversial.
Clinical Experience with Tissue Engineered Valves

Despite promising results in animal experiments, to date there have been only a limited number of clinical studies. Dohmen et al described in 2002 the first successful implantation of a 1% deoxycholic acid decellularized cryopreserved pulmonary allograft in the right ventricular outflow tract (RVOT) of a 43 year old patient that underwent a Ross procedure, with good clinical outcome at one year of follow-up [80]. These valve grafts were seeded with autologous vascular endothelial cell, and conditioned in a bioreactor [80]. The results of the first 50 consecutive patients receiving these decellularized xenografts also showed positive results, with normal right ventricular-pulmonary artery pressure gradients [81]. This method (AutoTissue Ltd®) was modified, with the addition of ethanol 70% to the protocol [71]. In a series of 20 patients receiving decellularized (n = 11) or cryopreserved (n = 9) allografts during a Ross procedure, the decellularized valves showed normal function up to 18 months of follow-up and showed a substantial reduction in immune-response compared to cryopreserved valves [72]. To date more than 171 patients underwent a Ross procedure with reconstruction of the RVOT using decellularized allografts or xenografts [82], and its value has recently also been demonstrated in more complex congenital aortic valve disease [83].

The commercially available SynerGraft (CryoLife Inc) cryopreserved decellularized pulmonary homograft valve has been successfully implanted in the RVOT of 22 patients with evidence for reduction of an immunologic response, but no difference in transvalvular pressure gradients, compared to cryopreserved pulmonary homografts [70]. Interestingly, in one patient who died 5 weeks after SynerGraft pulmonary homograft implantation, histopathology showed integrity of the ECM with only an early nonspecific inflammatory phase of recellularization, with no signs of any specific immune response [84]. However, whether this observation precedes a possible repopulation of the valves remains to be determined.

More recently, also acellular aortic valve conduit homografts have been developed with the SynerGraft technology, which after transplantation in an initial 22 patients showed low transvalvular gradients similar to cryopreserved homografts, and almost absent allosensitization as determined by panel reactive antibody measurements [69]. Importantly, Miller et al demonstrated that in an aortic SynerGraft homograft, explanted after 2 years, due to a cardiac transplantation, endothelial and smooth muscle cell differentiation in a subset of cells, with a virtual absence of calcification and inflammation [85].

In contrast, Simon et al reported in the first series of 4 patients receiving SynerGraft decellularized porcine xenograft valves for RVOT reconstruction, early severe valvular complications, resulting in the death of 3 patients [68]. Histological examination revealed severe inflammation with degeneration of the leaflets and vascular wall, indicating that the xenogeneic collagen matrix is capable of eliciting a strong immune response in humans [68].
REGENERATIVE MEDICINE IN ISCHEMIC HEART DISEASE: CELL BASED THERAPY

Myocardial infarction (MI) results in acute ischemia, and is accompanied by an irreversible loss of cardiac muscle with formation of scar tissue, eventually leading to progressive ventricular remodeling and heart failure [4]. None of the current available therapies address the underlying cause of the remodeling process (damage of myocardium and vasculature in the infarcted area). Furthermore, no currently available treatment option is able to restore viability and function of necrotic myocardium. Although cardiac transplantation and the use of mechanical LV assist devices are an option for patients with end-stage heart-failure, they are associated by a high comorbidity or limited effectiveness [86,87]. Recent years, several animal studies and a few clinical trials have been published using stem- or progenitor cells as potential therapeutic options ischemic heart disease.

Stem and Progenitor Cells

The zygote, and the cells derived from the first two divisions are called totipotent because they can form both the embryo and parts of the placenta, and are considered the most primitive cells in an organism [88]. In the embryo, the blastocyst then gives rise to the embryonic stem cells that can form all the cell types derived from the three germ layers, and are called pluripotent. Stem cells are primitive cells that are by definition clonogenic, capable of self-renewal and differentiation into all lineages (pluripotent) [89]. Most stem cells found in adult organs are already committed to one or more specific lineages and are therefore referred to as multipotent progenitor cells. The initial thought was that the progeny of a stem cell undergoes an irreversible change during early embryogenesis, making them committed to a specific lineage of undifferentiated progenitor cells. However, recent evidence suggested that adult progenitor cells can differentiate into mature cell types outside their original lineage, in response to specific cues, a characteristic called plasticity [90].

Different Cell Types for Regenerative Therapy

Potentially, a variety of stem and progenitor cell populations can be used for cardiac regeneration, each with its own profile of advantages, limitations, ethical considerations and practical use in the clinical setting.
EMBRYONIC STEM CELLS

Embryonic stem (ES) cells are totipotent stem cells derived from the inner cellular mass of the blastocyst-stage embryo. In contrast to other progenitor cells, ES can give rise to all cell types of the body, including cardiomyocytes [91]. ES cells are able to restore cardiac function in animal models of MI with evidence for long term benefit [92]. However, multiple issues prevent the therapeutic application of ES cells in patients, including ethical issues centering on the use of human embryos, the observation of teratoma formation in immune compromised animals [93], and the need to use allogeneic cells. Possibly, the application of somatic cell nuclear transfer (SCNT), involving the transfer of the nucleus of a somatic cell into an enucleated donor oocyte (“therapeutic cloning”), may provide a means of producing immune-compatible ES cells for the treatment of IHD [94].

SKELETAL MYOBLASTS

Skeletal myoblasts (SM) or satellite cells are progenitor cells located under the basal membrane of muscle fibers, where they serve as a reservoir of regenerative cells for skeletal tissue [95,96]. SMs can be easily obtained from patients and cultured in vitro where they maintain native morphological and functional characteristics [97]. These cells can engraft as shown in clinical and experimental studies of MI with improvement of systolic and diastolic cardiac function [98,99,100,101,102]. However, SMs fail to electromechanically couple with resident cardiomyocytes when transplanted into ischemic myocardium [103,104]. This may result in the formation of arrhythmogenic foci and the occurrence of life threatening arrhythmias [105,106]. Therefore, new options to enhance electromechanical and functional coupling need to be explored for these cells to be used in cardiac cell-therapy.

CARDIAC STEM CELLS

The heart has been traditionally viewed as a static organ, without the capability of repairing damage. However, evidence has accumulated suggesting that the heart contains a pool of cardiac stem cells (CPCs) that display an endogenous regenerative potential [107]. Over the past few years these cells have been phenotyped using different antigenic approaches. Beltrami et al. isolated lineage negative c-kit positive (lin-/c-kit+) cells from the adult rat heart that could differentiate into cardiomyocytes, smooth muscle cells and endothelial cells in vitro, and are able to regenerate myocardium after MI [108]. Later, also murine stem cell antigen-1 (Sca-1) positive cells that were either CD31 positive [109] or negative [110] were found, with in vitro and in vivo myocardial differentiation potential. Messina et al reported on the identification of cardiospheres from murine hearts, expressing SCA-1, c-kit, KDR and CD31, and capable of transdifferentiation in vitro, as well as myocardial and vascular regeneration after MI [111]. Also several authors reported on a pool of cardiac cells that were identified by their ability to efflux the Hoechst 33342 dye;
these cells were found to express SCA-1 but not CD31, and had the capability to differentiate into cardiomyocytes in vitro [112,113]. Finally, Laugwitz et al isolated a population of cardiac stem cells from post natal mouse hearts using the isl-1 transcription factor [114]. When co-cultured with neonatal cardiomyocytes these cells showed electrical and contractile properties evocative of neonatal cardiomyocytes [114].

The possibility of isolation of CSC from adult tissue, together with their potent differentiation capacity may contribute to future human autologous cell transplantation studies. However, to date there have been no clinical trials using human cardiac stem cells.

HEMATOPOIETIC STEM CELLS

Hematopoietic stem cells are the best characterized stem cell populations in bone marrow [115]. Hematopoietic stem cells are negative for lineage markers (lin-), but express the hematopoietic markers CD34, CD45 and CD133, and the stem cell factor c-kit. In a landmark study in 2001, the group of Anversa first reported that murine lin-/c-kit+ bone marrow derived cells could differentiate into cardiomyocytes after MI [116]. However, other groups demonstrated that HSCs do not readily acquire a cardiac phenotype in the ischemic heart, but adopted hematopoietic fates, despite the observed attenuation in ventricular function [117,118]. More recently Kajstura and colleagues provided further evidence that c-kit+ bone marrow cells, when injected properly, can differentiate into cardiomyocytes and coronary vessels, with no differentiation into hematopoietic lineages [119]. However, cardiac differentiation of HSCs still remains controversial. Moreover, all of the above described studies have been performed with murine HSCs, and currently no studies with the human counterpart have been performed. Furthermore, the low percentage of HSC in BM and the lack of clinically usable expansion methods limit the therapeutic application of HSCs.

SIDE POPULATION CELLS

Side population (SP) cells are a subset of HSCs that can give rise to all hematopoietic cells and represent only 0.05 % of the bone marrow cell population [120]. SP cells are characterized by their ability to exclude the Hoechst dye via the ABCG2 transporter on the membrane [121]. Although they are isolated independently of the antigenic markers, they are enriched for CD34-/low, c-kit and Sca-1+ [122]. SP cells can migrate into cardiac myofibers and small vessels two to four weeks after myocardial infarction in a mouse model [123]. Future studies are needed to further elucidate this cell type in cardiac regeneration.

ENDOTHELIAL PROGENITOR CELLS

Endothelial progenitor cells (EPCs) share phenotypic and functional characteristics similar to the fetal hemangioblast [124,125]. These bone marrow derived cells express some cell surface
markers characteristic of mature endothelium (VEGFR2, Tie-2) and hematopoietic cell surface markers (CD34), and are negative for CD45 [126,127]. In addition, EPCs have also been shown to express CD133, a marker of immature HSC and EPCs [128]. EPCs are released from the BM and circulate in the peripheral blood, home to various tissues into foci of neovascularization [129], and can participate in the regeneration of tissue damaged by ischemia [130]. In rat myocardial infarction models, injection of human CD34+ EPC can induce blood vessel formation in the infarcted scar, and proliferation of the existing vasculature [131,124]. Currently, clinical trials using CD34+ BM derived EPC transplantation for angiogenesis after MI are in progress [132]. Of interest, recent studies showed that EPC function and differentiation capacity is inversely correlated with risk factors for IHD [133].

**Mesenchymal Stem Cells**

Mesenchymal stem cells (MSCs) are rare population of non-hematopoietic multipotent stem cells that can differentiate into a variety of mesodermal cell types, including muscle, fibroblasts, bone, tendon, ligament and adipose tissue [134]. MSCs are isolated by culture flask adherence and are, unlike their hematopoietic counterparts, negative for CD34 and CD45, but characteristically express CD29, CD44, CD71, CD90, CD105, CD106, CD120a, CD124, SH2, SH3 and SH4 [135]. MSCs can differentiate into cardiomyocyte cells after appropriate stimulation in vitro [136]. Moreover, when MSC were injected into the healthy mouse heart, differentiation into cardiomyocytes was demonstrated [137]. Furthermore direct injection of MSCs in the infarcted heart has been shown to improve left ventricular (LV) function in rats [138,139], dogs [140] and pigs [141,142]. Several possibilities have been proposed in the interpretation of the observed positive effects on the LV function after myocardial infarction. They include the differentiation of hMSCs into cardiomyocytes [141,143] and vascular cells [140,143] or a paracrine effect mediated by the MSCs [138,139]. Chen et al demonstrated in the only clinical MSC study to date that intra-coronary injection after acute MI resulted in a significant improvement in global and regional LV function compared to the medium treated patients [144]. Importantly, because MSC can be expanded rapidly in vitro, and are reported to avoid rejection by lacking MHC-II and B-7 costimulatory molecule expression [145], they might also be used in an allogeneic setting.

**Multipotent Adult Progenitor Cells**

A subpopulation of the BM-derived stem cell that co-purify with MSCs, originally described as mesodermal progenitor cells, was because of its with spectrum of differentiation renamed as multipotent adult progenitor cells (MAPCs) [146]. MAPCs are CD34-/133+/Flk1+ and have been postulated to comprise the principle endothelial progenitor in human BM [147]. Furthermore, in vitro MAPCs can be expanded indefinitely and differentiate into all three embryonic germ layers
When injected into the tibialis anterior muscle of NOD/Scid mice, human MAPCs have been demonstrated to differentiate into myocytes. However, to date MAPCs have not been tested in animal models of IHD.

**Bone Marrow-Derived Multipotent Stem Cells**

Recently multipotent BMCs, which can differentiate into all three germ layers, were clonally isolated from human BM. These cells are currently referred to as human bone marrow-derived multipotent stem cells (BMSCs), and do not express any of the characteristic marker panels (e.g. CD34, CD44, CD90, CD105 and CD117) defining HSC, MSCs and MAPCs. hBMSCs were found to acquire a cardiomyocyte phenotype in the ischemic mouse myocardium with attenuation of ventricular function. Difficulties in culturing techniques observed with hBMSCs have to be overcome before this cell type could be used in a clinical setting.

**Adipose Tissue Derived Stem Cells**

Adipose tissue contains different progenitor cells, including HSCs SPs and MSCs. Recently it was demonstrated that adipose tissue derived stromal cells, if cultured under specific conditions, could be differentiated into beating cells with a cardiomyocyte phenotype. Furthermore, adipose lineage cells can function as progenitors for endothelial cells that enhanced the neovascularisation in ischemic tissue. However, the exact molecular hallmarks of these cells remain to be established. The abundance and ease of excess to fat makes adipose tissue a potentially useful cell source in future cell-therapy approaches.

**Umbilical Cord-Derived Cells**

Several reports have indicated that the umbilical cord blood (UCB) contains both hematopoietic and mesenchymal progenitor cells. Furthermore, MSCs have been isolated from the Wharton’s jelly of the human umbilical cord. Recently a population of cells from human UCB, called unrestricted somatic stem cells, has been described that was negative for c-kit, CD34 and CD45, and was capable of differentiating in a variety of tissues, including cardiomyocytes. Interestingly, after injection in the ischemic heart of immuno-suppressed pigs these human unrestricted somatic stem cells improved regional and global function, improved perfusion, and reduced infarct size. Furthermore, UCB derived CD34+ cells were demonstrated to improve cardiac function in a rat model of acute MI after injection in the peri-infarct area, as compared to medium treated animals. However, to date, no clinical of UCB derived cells have been reported.
EPICARDIAL-DERIVED CELLS

The epicardium is a structure that comprises the outermost layer of the heart. It consists of mesothelial cells that during embryogenesis originate from the extracardiac pro epicardial organ (PEO) [163]. A subset of these epicardial cells can undergo epithelial-mesenchymal transformation (EMT), and migrate into the subepicardial, myocardial and subendocardial layers of the primitive heart, as well as the atrioventricular cushions [164,165,166,167]. These cells have been named EPDCs, and differentiate into perivascular and cardiac interstitial fibroblasts, as well as into coronary smooth muscle cells [164,166,168,169]. Furthermore, EPDCs do not only physically contribute to the developing heart; they also have a modulatory role in cardiogenesis [170,171]. EPDCs are involved in myocardial compaction [172,173], Purkinje fiber development [174] and coronary vessel formation [175,176]. Although EPDCs are crucial for proper cardiogenesis in the developing heart, limited information is available on the role of EPDCs in fetal and postnatal hearts.

Recently, an adult epicardial rat cell line was described that could be induced to undergo EMT in vitro, and from which a fraction had the capacity to form SMCs [177]. Furthermore, van Tuyn et al. demonstrated that human adult epicardial cells spontaneously undergo EMT early during ex vivo culture, express the cardiac markers GATA4 and cardiac troponin T, and obtain characteristics of SMCs after proper stimulation [178]. These results indicate that adult EPDCs retain part of their differentiation capacity present during embryonic development, making them progenitor cells. Recently, Winter et al. therefore hypothesized that adult human EPDC might also recapitulate their embryonic capacities in the ischemic myocardium, thereby positively modifying the surrounding myocardium [171]. However, to date no in vivo transplantation studies have been performed.

Cardiac Cell Therapy in Humans

The initial observed beneficial effects observed after different cell based therapies in small and large animal models generated excitement, and prompted investigators to initiate the first clinical studies. So far, cell-based therapies have been performed in patients with acute myocardial infarction, chronic ischemia and heart failure. In most studies, investigators have chosen the pragmatic approach of using bone-marrow mononuclear cell (BM-MNC) preparations containing different stem and progenitor cell populations.

CELL THERAPY IN ACUTE MYOCARDIAL INFARCTION

The use of BM-MNCs has been most extensively investigated in the setting of acute MI. Several small clinical studies, which all included patients with acute MI who had undergone primary PTCA and stent implantation, with infusion of the cells via the intra-coronary route showed
beneficial effects. However, most of these studies lacked an adequate control group and were not randomized in a blinded fashion.

Several larger scales randomized, controlled trials evaluating the therapeutic effect of BM-MNC transplantation for acute myocardial infarction have been performed recently, all with mixed results. Schachinger and colleagues reported in the largest BM-MNC therapy study to date (204 patients were randomized) the results of the Reinfusion of Enriched Progenitor Cells and Infarct Remodeling in Acute Myocardial Infarction (REPAIR-AMI) trial, a double-blind and fully controlled multicenter study that assessed the effect of intra-coronary infusion of BM-MNCs after successful PTCA for acute MI [179]. They observed a modest, but significant improvement in LVEF at 4 months in the BM-MNC treated patients as compared to those given placebo [179]. Of interest in the also randomized, but not blinded, Bone Marrow Transfer to Enhance ST-Elevation Infarct Regeneration (BOOST) trial, the relative improvement in LVEF observed 6 months after BM-MNC transplantation was no longer significant at 18 months, due to continuous improvement in the control group [180]. In contrast, in the double-blind randomized controlled trial by Janssens et al [181], no difference between control and treated groups was observed 4 months after BM-MNC transplantation. Furthermore, in the also recently published Autologous Stem Cell Transplantation in Acute Myocardial Infarction (ASTAMI) trial no significant difference in LVEF was found at 6 months after BM-MNC transplantation, as compared with the control group [182]. The observed discrepancies between different studies are at present not clear, but may relate to the methods used for cell preparation [183].

Several studies also assessed the use of selected bone marrow cells in the setting of acute MI. In the TOPCARE-AMI trial, BM-MNCs were also compared with circulating blood-derived progenitor cells (mainly EPCs), with an improvement in LVEF in both the groups [184]. In another clinical trial, CD133+ BM cells that were isolated with a mouse antibody were injected into the infarct related artery [185]. Although an improvement of global contractility was observed, several of the patients developed a significant in-stent restenosis [185,186]. The therapeutic effects of intracoronary MSC transplantation after acute MI have been investigated by Chen et al, who demonstrated improvement of LV function and an attenuation of LV end-diastolic volume, compared with a randomized control group that received saline [187].

**CELL THERAPY IN PATIENTS WITH CHRONIC MYOCARDIAL ISCHEMIA**

Due to the diffuse nature of their disease, a subset of patients with coronary artery disease and chronic myocardial ischemia are not suitable for revascularization, and therefore experience refractory angina pectoris. Several small studies in patients with CAD have used trans-epicardial [188] and trans-endocardial [189,190,191] injections of BM-MNC in this group of patients, with improvements of anginal symptoms, exercise capacity, regional tissue perfusion and LV systolic function [188,189,190,191]. Beeres et al reported on the sustained effect on anginal symptoms,
perfusion and LV function for up to 12 months of follow up [192]. These studies therefore demonstrate the feasibility of BN-MNC injection in patients with chronic myocardial ischemia. However, none of these studies included a randomized control group. To determine the true efficacy of this approach, it should be tested in prospective randomized trials.

**CELL THERAPY IN PATIENTS WITH CHRONIC HEART FAILURE**

Several small clinical studies investigated the safety and feasibility of skeletal myoblast transplantation in LV scar tissue during CABG, all with significant improvements in LV function [105,106,193]. Furthermore, Smits and colleagues used a trans-endocardial approach to inject myoblasts as a stand-alone procedure in five patients with chronic heart failure [101], and observed an improvement in LVEF. However, in all of these initial studies episodes of ventricular tachycardies, that necessitated ICD placement, were observed [193,105,106,101]. It is therefore likely that skeletal myoblast transplantation increase the risk of ventricular tachycardies in patients with chronic heart failure. Another group used CD133+ BMC that were injected transepicardially into the infarcted borderzone of patients undergoing CABG 3-12 weeks after MI[194]. Perfusion and LVEF were improved after 6-8 months, however, in contrast to the myoblast studies, no ventricular arrhythmias were observed [194]. More recently, Assmus et al evaluated in The Transplantation of Progenitor Cells and Recovery of LV Function in Patients with Chronic Ischemic Heart Disease (TOPCARE-CHD) trial the effects of BMC or progenitor cells derived from the circulating blood (CPC) in patients who had a MI on average more than 6 years ago, and observed a moderate but significant improvement in the LVEF after 3 months [195].

**Mechanisms Mediating the Therapeutic Effects of Stem or Progenitor Cells**

Although almost all cell-based therapies have provided evidence for functional improvement or amelioration of LV remodeling, there has been much controversy surrounding the underlying mechanism. In the original concept, stem or progenitor cells were proposed to repair the damaged heart via transdifferentiation into cardiomyocytes [116]. Although initial studies using different stem and progenitor cells demonstrated that these cells could differentiate into cardiomyocytes, especially in the case of BM-derived cells, the ability of crossing lineage boundaries is under debate.

As mentioned earlier, plasticity indicates the ability of stem cells to differentiate into cell types other than their cell origin[89]. Although the group of Anversa demonstrated transdifferentiation of BM derived stem cells [119,116], this could not be reproduced by others [118,117]. Other groups demonstrated that the observed plasticity and repair of BM cells after transplantation in the ischemic heart is due to fusion with existing myocytes [196]. However, even if stem and progenitor cells could differentiate into functional cardiomyocytes, this alone would not be
enough to explain their observed beneficial effects. Murry and colleagues recently calculated that the cardiomyocyte deficit in MI induced heart failure is approximately one billion cardiomyocytes, and true regeneration would therefore require this amount of stem cells that are all synchronously contracting via electromechanical junctions with the host cardiomyocytes [197]. Although with current techniques this remains impossible to achieve, beneficial effect after cell-therapy are observed. Interestingly, even fibroblasts have been shown to improve the diastolic function of the ischemic rabbit heart [198]. Therefore, several alternative mechanisms of action have been proposed (Figure 3).

Skeletal myoblasts have been demonstrated to improve the passive mechanical properties of the infarcted region, resulting in the amelioration of ventricular remodeling [199]. Other investigators demonstrated in a rat model by atomic force microscopy that human MSC engraftment attenuates post-infarction remodeling by softening of the border zone area, thereby improving the elastic moduli resulting in a more compliant infarct scar [200]. Furthermore, BM derived EPC have been proposed to structurally enhance tissue perfusion by differentiating into endothelial cells at sites of neovascularization [201,124].

Another possibility is the so-called paracrine effect, referring to the production of local signal-
ing molecules by stem cells that act on the host myocardium [202]. It is becoming increasingly clear that cell therapy can also improve cardiac function by promoting angiogenesis [203,204], cardiomyogenesis [205] and reducing host myocardial apoptosis [124,206,207]. Interestingly, Gneecchi and colleagues demonstrated that most of the benefit observed after MSC transplantation could also be obtained after injecting the cell-free supernatant of MSC cell cultures [208]. Finally, other investigators suggest that cell therapy may stimulate endogenous resident stem cell migration and proliferation, facilitating the ability of the heart to regenerate itself [209]. At present, due to differences in animal models, delivery modes and cell types used, the relative contribution of each component is not clear, moreover, the beneficial effects observed after cell therapy could be due to multiple mechanisms.
CARDIAC PHENOTYPING IN REGENERATIVE MEDICINE: DIFFERENT METHODS FOR THE MURINE HEART

Recent developments in transgeneic mouse models of cardiac dysfunction have led to an increase in the use of these models to study cardiac function and disease. In addition, surgical techniques used to mimic cardiac pathology are becoming more common in laboratories around the world.

Models of coronary artery ligation, resulting in acute myocardial ischemia, with subsequent heart failure, as well transverse aortic constriction to induce a pressure overloaded LV, have been developed. However, the extremely small size of the mouse heart (100 mg and 7 mm long axis) and fast heart rates (~500 beats per minute) under physiological conditions, require miniaturized and technically advanced methods to accurately assess the cardiac phenotype of these animals. Moreover, in the light of the increasing interest in regenerative medicine, especially the effects of cell therapy and its preclinical applications in small animals makes potential methods for murine cardiac phenotyping more important than ever. Several methods have been adapted for murine studies, including invasive pressure-volume loop measurements, magnetic resonance imaging (MRI) and echocardiology.

Assessment of Pressure-Volume Loops

The pressure-conductance catheter is a miniature (1.4F) catheter that is introduced into the LV of mice via the right coronary artery (Figure 4) [210]. This method is capable of simultaneously monitoring LV volumes and pressures, so that LV pressure-volume (PV) loops can be documented continuously.

In addition, also the time derivates of end-diastolic (dP/dt_{min}) and end-systolic pressures (dP/dt_{max}) can be obtained with this method. Moreover, pressure-volume relations are relatively independent of loading conditions and are therefore considered superior to quantify intrinsic ventricular function [211]. A disadvantage of this method is the invasive nature, resulting in the dead of the animal and therefore precluding longitudinal studies.

Figure 4. A 1.4F miniature pressure-conductance catheter containing four platinum electrodes and a solid-state pressure transducer used for measuring pressure-volume loops in mice.
Magnetic Resonance Imaging

MRI is a non-invasive technique that uses intrinsic contrast and is capable of obtaining true 3-D anatomical and function information. Combined with the high temporal resolution, which enables accurate assessment of cardiac function, MRI is the imaging modality of choice to study the effects of stem cell therapy [212]. In addition, the established clinical MR technique of infarct size determination by delayed contrast enhancement imaging after Gd-DPTA was recently adapted and validated in the mouse MI model [213]. Furthermore, being a non-invasive technique allows for serial investigations. Drawbacks of MR imaging in small animals are, that the experiments are costly and time consuming, making it difficult to use for large series of studies. Furthermore, no information on end-diastolic and end-systolic pressures, as well as several other indices of LV function can not be obtained with MRI. Despite these drawbacks, there is an increasing use of this promising technique in laboratories all over the world (Figure 5.)

Figure 5. Example of a currently available vertical 9.4-T (400 MHz), 89-mm bore nuclear magnetic resonance (MR) spectrometer (Bruker BioSpin, Rheinstetten, Germany) suitable for MR imaging in small laboratory animals, and used in the present thesis. (Picture was taken at the Gorlaeus laboratory, Leiden, The Netherlands).

Echocardiography

Ultrasound echocardiography is currently the most commonly used method for cardiovascular phenotyping in mouse models. Two-dimensional echocardiography (2D-echo) has been used in noninvasive cardiac phenotyping in normal and pathological mouse hearts [214,215]. However, this method requires geometric assumptions for calculation of LV volumes and mass, making it less reliable when the LV has an altered asymmetric shape (e.g. after MI). Recently, 3D-echo has been shown to be superior to standard 2D-echo, and even approaches the accuracy of MRI for determining LV parameters and infarct size [216].
AIM AND OUTLINE OF THE THESIS

The main aim of the present study was to explore two main components of regenerative medicine, namely tissue-engineering and stem cell therapy, for its applications in treating CVD. The focus was placed on ischemic heart disease and aortic valve disease. Furthermore, the effectiveness of different methods that could be used to determine changes in cardiac function after regenerative medicine applications is briefly described.

Part I of the thesis centers on different methods to produce acellular porcine and rat aortic valve scaffolds for the purpose of tissue-engineering. In Chapter 2 the effects of two different cell extraction techniques on the remaining extracellular matrix of porcine aortic valve leaflets was evaluated. In addition, the potential of reseeded endothelial cell to produce ECM molecules was assessed. Next Chapter 3 describes a novel method to produce acellular rat aortic valve conduits, and its effects on the remaining ECM. Furthermore, the in vivo behavior of these acellular valve conduits was studied in a heterotopic rat aortic valve implantation model. Finally Chapter 4 used this model to characterize the cellular immune response to cellular and acellular allogeneic aortic valves, and tried to correlate it to the histological and biochemical changes in the ECM collagen constitution.

Part II of the thesis focuses on the feasibility, in vivo behavior and functional evaluation of different cell types used for the treatment of acute myocardial infarction. Although several studies demonstrated the therapeutic regenerative potential of human MSCs from healthy volunteers, little information is available on the effects of hMSCs from hMSC derived from patients with ischemic heart disease. Therefore Chapter 5 investigated whether hMSC from IHD patients were able to engraft into the acutely infarcted myocardium of the immune-compromised NOD/scid mouse, differentiate into several cardiac cell types, and if these cells could preserve LV function. A 9.4T small animal MRI was used to assess LV function and remodeling. Chapter 6 uses the same model to describe the effects of forced expression of the cardiac transcription factor myocardin in hMSCs from IHD patients. Besides immuno-histochemical assessment of cellular engraftment and possible differentiation into cardiomyocytes, detailed hemodynamic measurements were performed with the 9.4T MRI and the invasive conductance catheter. Next in Chapter 7 the effects hEPDC transplantation on the acutely infarcted mouse myocardium were evaluated by MRI at two and 6 weeks after MI. Particular emphasis was placed on the possibility that these cells could recapitulate their embryonic capacities when injected in the ischemic myocardium.

Part III of the thesis addresses two methods used in cardiac phenotyping of small laboratory animals. Chapter 8 describes a head-to-head comparison between MRI and the conductance catheter for the assessment of LV volumes and ejection fraction in normal and failing mouse hearts.
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CHAPTER 1: GENERAL INTRODUCTION AND OUTLINE OF THE THESIS


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