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VASCULAR PROGENITOR CELLS IN ARTERIAL REMODELING

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Picture on the front by *Gail Robinson, March 20, 2006*, presents a microscopic look at a lab dish whose contents are derived from human embryonic stem cells

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The important thing is not to stop questioning.
Curiosity has its own reason for existing.
One cannot help but be in awe when he contemplates
the mysteries of eternity, of life,
of the marvelous structure of reality.
It is enough if one tries merely to comprehend a little
of this mystery every day. Never lose a holy curiosity.

(Albert Einstein)

to all of you with never-ending life curiosity…

ABSTRACT

Cardiovascular disease is the leading cause of global mortality and physical disability mainly due to the complications such as myocardial infarction or stroke. Physiological healing reaction takes place in the diseased vessel wall aimed to repair the vessel after an injury. There are two factors essentially important for clinical improvement of vascular diseases. The first one is protection of the vascular damage, and the second one is repair of injured, ischemic and regenerating tissues to restore and maintain their function. The existing paradigm states that vascular progenitor cells are found in the vasculature and contribute to repair of injured blood vessel. However, the mechanism underlying the integration of these cells into the vasculature, their origin and specific functions has been unclear. This thesis presents a new understanding to this concept. Using human cardiac biopsies and animals models of arterial injury, we investigated whether progenitor cells can affect arterial repair and which mechanisms could be responsible for their action. Indeed, we have shown that adult vascular progenitor cells exist and possess a potential that extends beyond the cell types of their resident tissue. The vascular progenitor cells can be recruited either from bone marrow or blood vessel tissue in response to inflammation and migrate towards the sites of injury. Although, these cells are able to inhibit intimal hyperplasia, their contribution to formation of intimal lesion is not equal.

We provide evidence that inflammation and monocyte chemoattractant protein 1 (MCP-1) are pivotal in the recruitment of recipient-derived cells of smooth muscle cell phenotype into arterioles of transplanted human hearts. The number of these cells in the arterioles correlated strongly with the number of CD45-positive leukocytes and the grade of rejection, confirming that inflammation is strongly related to the recruitment of circulating progenitor cells into the graft vessels. This knowledge may be useful to design protocols for increase of progenitor cell numbers to limit tissue damage and facilitate healing at sites of tissue injury.

In our hands, bone marrow-derived cells, which are known source of stem cells and their progenitors, supported early stages of arterial injury and thereafter were eliminated from the artery wall. These cells localized in the arterial intima and the majority of them were of endothelial phenotype. Furthermore, bone marrow-derived cells were not able to fuse however could differentiate into vascular cells to adjust in the vessel wall and meet the demands and needs of their new microenvironment. Interestingly, local delivery of bone marrow-derived endothelial cells to the sites of arterial injury resulted in decrease of the intimal lesion area. Taken together, our results indicate the importance of these cells in the inhibition of early stages of intimal formation.

Further, we showed that enhanced inflammation in rat arterial allograft by acute infection with Cytomegalovirus (CMV) led to enhanced local MCP-1 production in the vasculature. Interestingly, CMV potentiated inflammation mainly in the adventitia, which resulted in migration of adventitial cells towards the intima and more rapid and severe intimal hyperplasia. Our findings increase understanding of the role of pathogens, such as CMV, in vascular remodeling and highlight that adventitial cells are able to migrate *in vivo* towards the sites of arterial injury, most likely in response to MCP-1.

We identified mesenchymal tissue-derived progenitor cells in vascular adventitia (Sca-1/CCR2, c-kit/CCR2) that contributed to vascular remodeling in a rat model of transplant vasculopathy. Early proliferation of cells in the adventitia coincided with an increase in the number of apoptotic cells in the media, and both proliferation and apoptosis were associated with inflammation. Inflammation and MCP-1 production were pivotal in the migration of mesenchymal progenitor cells derived from adventitia towards the intima.

In summary, this thesis presents novel evidence showing that inflammation and MCP-1 are important for recruitment of vascular progenitor cells to the sites of arterial injury and suggests tissue-derived mesenchymal progenitor cells, here from arterial adventitia, as a key source of cells for vascular repair. We believe that knowledge presented here not only increases understanding of vascular pathology but also provides unique value for understanding of unraveled aspects of tissue repair process.

CONTENTS

1 LIST OF PUBLICATIONS

This thesis is based on the following original articles and manuscripts, which will be referred to in the text by their roman numerals:

I. Religa P*****, **Grudzinska MK***, Bojakowski K, Soin J, Nozynski J, Zakliczynski M, Gaciong Z, Zembala M, Söderberg-Naucler C. Host-derived smooth muscle cells accumulate in cardiac allografts: role of inflammation and monocyte chemoattractant protein 1.
PLoS One, 2009 January; 4(1): e4187

II. **Grudzinska MK**, Bojakowski K, Soin J, Stassen F, Söderberg-Naucler C, Religa P. RCMV increases intimal hyperplasia by inducing inflammation, MCP-1 expression and recruitment of adventitial cells to intima.
Herpesviridae, 2010 December; 1:7; doi:10.1186/2042-4280-1-7

III. **Grudzinska MK**, Nowak G, Reinecke H, Murry CE, Söderberg-Naucler C, Religa P. Bone marrow derived endothelial cells differentiate to cells of arterial intima and inhibit early stages of intimal formation.
Manuscript submitted

IV. **Grudzinska MK**, Bojakowski K, Soin J, Wolmer N, Reinecke H, Murry CE, Söderberg-Naucler C, Religa P. Migration of adventitial progenitor cells mediated by Monocyte Chemoattractant Protein -1 is a major source of intimal hyperplasia in transplant arteriosclerosis.
Manuscript submitted

*****These authors shared first authorship

2 LIST OF ABBREVIATIONS

BM	Bone marrow
BMC	Bone marrow cell
CMV	Cytomegalovirus
EC	Endothelial cell
ECM	Extracellular matrix
EM	Electron microscopy
EPC	Endothelial progenitor cell
FISH	Fluorescent *in situ* hybridization
HCMV	Human cytomegalovirus
HSC	Hematopoietic stem cell
IGF-1	Insulin-like growth factor 1
IP-10	Interferon γ-induced protein 10
LCM	Laser capture microdissection
LSCM	Laser confocal scanning microscopy
MCP-1	Monocyte chemoattractant protein 1
MOI	Multiplicity of infection
SDF-1	Stromal-derived factor 1
MSC	Mesenchymal stem cell
PC	Progenitor cell
RANTES	Regulated upon activation, normally T-expressed, and presumably secreted
RCMV	Rat cytomegalovirus
RT PCR	Real time PCR
SC	Stem cell
SDF-1	Stromal-derived factor 1
SMC	Smooth muscle cell
TGF-β	Transforming growth factor β1
TNF-α	Tumor necrosis factor-α
VEGF	Vascular endothelial growth factor

3 AIMS

The general objective of this thesis was to increase our knowledge of how somatic and stem cells contribute to vascular remodeling in vessel injury. There are two factors essentially important for clinical improvement of vascular diseases. The first one is protection of the vascular damage, and the second one is repair of injured, ischemic and regenerating organs, to keep and restore organ function.

The specific objectives were to make initial observations in the clinical scenario in order to further investigate the mechanisms of biological phenomenon with help of animal models and cell culture systems:

- to understand the clinical importance and molecular mechanism of the recruitment of recipient-derived cells of smooth muscle cell phenotype to the arterioles of transplanted human hearts

- to investigate how specific pathogenic situation (CMV infection) modulates arterial remodeling and migration of vascular cells in a rat aortic transplant model, and to understand the mechanisms of interaction between the virus and host immune response initiating alterations in the vascular wall

- to identify the predominant cell types of bone marrow origin in the arterial remodeling in a murine model of arterial injury by catheterization, and to investigate the incorporation of these cells into vascular wall and their role in tissue repair

- to identify the predominant vascular cell types and their origin in the intimal hyperplasia induced by allogeneic-immune response in a rat aortic transplant model and to investigate the mechanisms responsible for their targeting to the vascular intima

4 GENERAL INTRODUCTION

Normal tissue function depends on adequate supply of oxygen and nutrients, and blood vessels constitute the elaborate transport system by which these necessities are delivered throughout our bodies. Around one hundred and fifty years ago, Virchow explained most vascular diseases in terms of cellular mechanisms. Many of his explanations remain valid today. Nonetheless, as recently as the 1970s, the vessel wall has been seen as a conduit, containing blood provided by the heart around a circuit optimized to distribute and exchange oxygen, hormones, nutrients, carbon dioxide and wastes.

4.1 BLOOD VESSELS

Most of our knowledge of the origin of blood vessels has been derived by investigators studying avian or mouse embryos. These studies show that the first elements of the vessel wall to form are aggregates of endothelial cells (ECs) surrounding hematopoietic cells. Once aggregates of endothelial precursors form, they organize themselves into hollow tubes. The primitive vascular tubes form in positions ready to be connected to create a primitive vascular circuit (1). The vascularization of most organs occurs by formation of new branches which extend from existing vascular network. Interestingly, the origin and branching pattern of the blood vessels is determined by ECs which originate from primitive precursors found during gastrulation. Thereafter, smooth muscle cells (SMCs) begin to coat the vascular tubes however the origin of SMCs remain mysterious.

All blood vessels apart from capillaries are composed of three layers: tunica intima, tunica media and tunica adventitia (Figure 1). The tunica intima consists of a single layer of ECs lining the vessel lumen and forming a barrier against blood. The ECs form a tight monolayer and rest on a basement membrane, a fine meshwork made up of extracellular matrix components, mainly collagen type IV, laminin, and heparin sulfate proteoglycans (2). The tunica media contains SMCs and represents the muscular component of blood vessels. SMCs are responsible for synthesis of elastin, collagen and other extracellular matrix components. The tunica adventitia is the outmost layer of blood vessels and contains fibroblasts and connective tissue. The adventitia consists of vasa vasorum, a lymphatic system, and some nerve fibers involved in vasomotor control. For a long time, the tunica adventitia has been considered as an inactive component of blood vessels involved in structural support of the tunica media. Recently it has been demonstrated that the adventitia plays a role in vascular remodeling and the development of vascular diseases, such as atherosclerosis, restenosis and transplant vasculopathy (3-7).

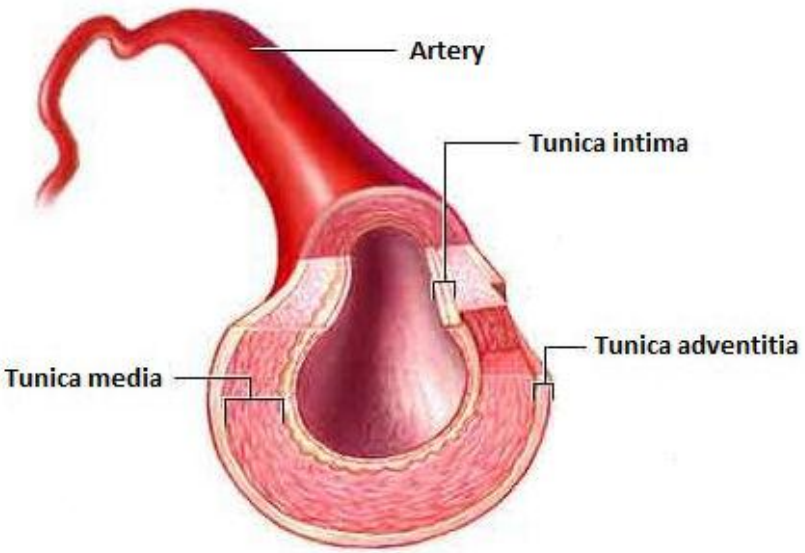

Figure 1. Structure of the arterial wall (adapted from Diseases & Conditions, 2003, A.D.A.M., Inc.).

4.2 TISSUE INJURY AND REGENERATION

The repair of tissue injury is one of the most complex biological processes that occur during human life. After an injury, multiple intracellular and extracellular pathways immediately become activated and are synchronized to restore tissue integrity. Cellular components of the immune system, the blood coagulation cascade and the inflammatory pathways are activated. Many types of cells, including immune cells, endothelial cells, fibroblasts undergo changes in gene expression and phenotype, leading to cell proliferation, differentiation and migration (8). If this response is successful, the repair processes must be shut down in a precise sequence as recovery progresses.

In general, the repair process occurs in almost all tissues after exposure to almost any destructive stimuli. Thus, the sequence of events that follows for example a myocardial infarction is quite similar to that following burn or mechanical injury. In organ systems, the normal response to injury occurs in three overlapping but distinct stages: inflammation, new tissue formation and remodeling. The first stage, inflammation, occurs immediately after tissue damage, and components of the coagulation cascade, inflammatory pathways and immune system are needed to prevent further tissue damage and to remove dead and dying tissues. Monocytes are recruited to the site of injury and differentiate into macrophages. Macrophages are thought to be crucial for coordinating later events in the response to injury (9) however their importance in tissue repair is not completely understood. The second stage of tissue repair, new tissue formation, is characterized by cellular proliferation and migration of different cell types. During the third stage of repair, remodeling, all of the processes activated after

injury should turn down and cease. In addition, the extracellular matrix is actively remodeled.

Humans have the ability to completely restore the original tissue during prenatal development however it is lost during adult life (10). How regeneration occurs and why we lose this ability remain a mystery (11). In humans, problem with tissue repair can manifest as either delayed or excessive healing. The ultimate solution to both problems is likely to be delivery of cells that retain the ability to elaborate the full complexity of biological signaling, together with environmental factors that can regulate their functions. Thereby, one of the emerging solutions is an implementation of stem cells (SCs) and their progenitor cells (PCs). After delivery to the injured tissue, these cells can themselves provide the proper sequence of factors to accelerate tissue repair and regeneration however the phenotype, homing and specific function of these cells in tissue regeneration has been not fully understood.

4.3 THE ROLE OF INFLAMMATION IN TISSUE INJURY AND REPAIR

Inflammation plays a critical role in the vascular response to injury. In particular, mechanical injury using techniques such as balloon angioplasty and stenting results in complex inflammatory reactions which influence proliferation of vessel wall constituents such as endothelial cells, smooth muscle cells, and extracellular matrix (ECM) proteins. Inflammatory cells are recruited to the injured vessel wall initially as a reparative mechanism. However, this infiltration of inflammatory cells has been described in many forms of arterial remodeling and has been proven to contribute to the progression of the disease (12, 13). Leukocytes serve as the primary inflammatory cells however also platelets produce a number of important inflammatory mediators. Thus, the mechanisms that regulate endothelial cell migration, smooth muscle cell activation, and ECM protein production are key components in the inflammatory response to vascular injury (Figure 2).

Atherosclerosis is responsible for approximately 50% of all deaths in the developed world. The concept that atherosclerosis develops in response to vascular injury and involves inflammation and vessel remodeling is now well accepted (14). Spontaneous atherosclerosis results from vascular injury induced by multiple insults including hypercholesterolemia, diabetes, smoking, and hypertension. Effective treatment strategies for stenotic atherosclerotic lesions include percutaneous interventions such as balloon angioplasty and stenting. However, these procedures are associated with a significant recurrence rate. Mechanical injury has been shown to provoke a distinct pathological response that is significantly different from spontaneous atherosclerosis (15). Mechanical injury may also result in vascular remodeling with vessel constriction and a reduced vascular lumen as a result of scarring of the outer layer of the vessel. These responses are collectively referred to as restenosis. Furthermore, transplant vasculopathy, an immunologic phenomenon, is driven by an inflammatory milieu consisting of multiple cell types that contribute to vascular cells proliferation with their subsequent obstruction. Examination of cellular infiltrates in the transplanted arteries

has shown a predominant T-cell population mainly localized in the intima and adventitia (16).

There are many factors involved in the vascular response to injury. However, inflammatory mediators appear to play a key role in the initiation and progression of this response. This vascular response to injury involves a complex network of interactions that begins as a beneficial reparative process but may ultimately result in detrimental vascular changes. Inflammatory mediators include cell adhesion molecules, cytokines, chemokines and growth factors that direct the recruitment of inflammatory cells including monocytes/macrophages, neutrophils, and T-lymphocytes. These mediators have multiple and diverse effect on the constituents of the vessel wall, including ECs, SMCs, and ECM proteins.

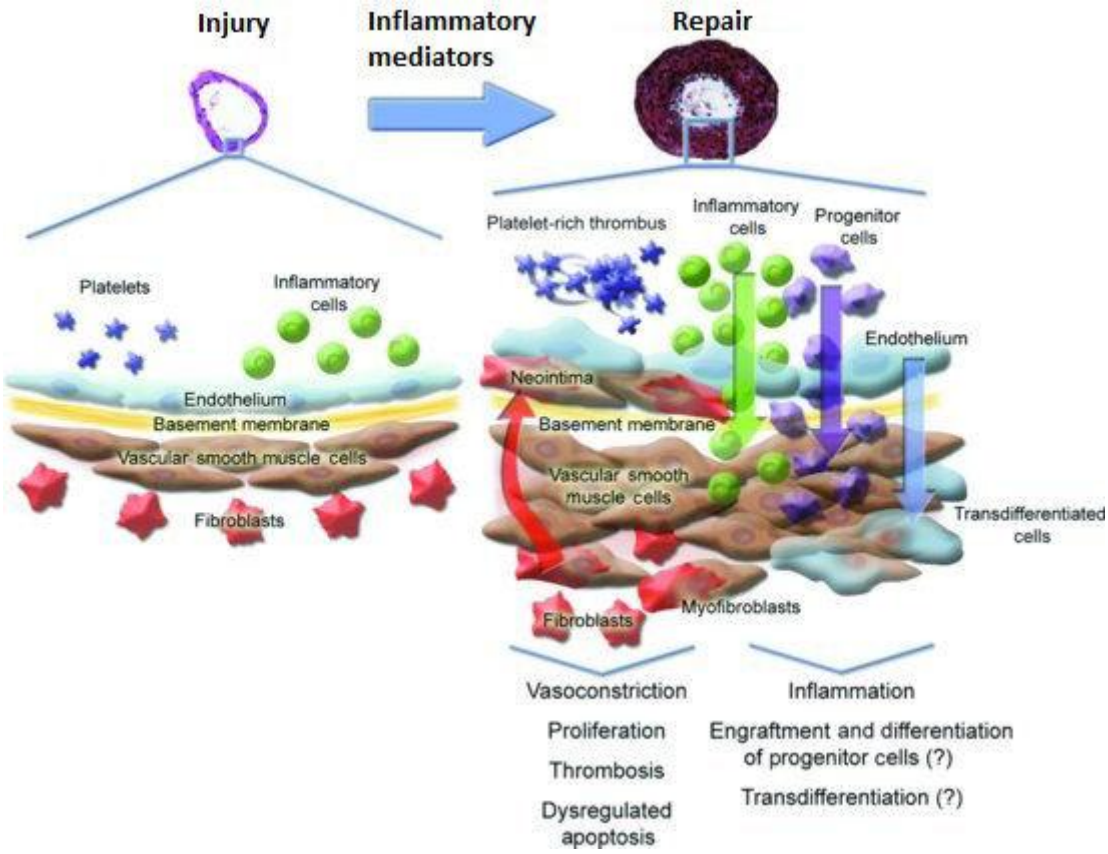

Figure 2. Vascular response to injury (modified after Loscalzo, J., Comprehensive Physiology, 2011).

Cytomegalovirus in the development of vascular disease (Paper II)

Cytomegalovirus (CMV) belongs to the family of herpesviruses. This family is divided to α, β, γ subfamily depending on the biological properties such as replication cycle, site of latency and growth ability in culture. Herpesviruses are large DNA viruses and share the ability to establish latency after a primary infection. CMV sustains a balance

with the host immune system and remains in a normal, immunocompetent host for the duration of host life without causing acute damage.
CMV is not highly infectious. To be infected it is necessary to come in direct contact with a large amount of viral particles. In the immunocompetent individual, a primary CMV infection results in subclinical symptoms similar to those of a common influenza infection, such as mild fewer, fatigue and moderate pain. In some individuals, a more severe pathology occurs, including adenopathy, hepatitis and splenomegaly. These differences in displayed symptoms are most likely due to viral load, viral phenotype, site of viral entry or host immune system composition.
CMV infects many cells, such as blood-, endothelial-, epithelial-, neuronal-, stromal cells, SMCs and fibroblasts. Apart from the acute effects of CMV, many reports have been published recently on the role of persistent infection in inflammatory diseases such as atherosclerosis, restenosis, transplant vasculopathy (17). CMV infection in macrophages and ECs affects cellular processes that may contribute directly to vascular disease. For example, CMV antigens activate the immune system and hence may drive an inflammatory process in the arterial wall. It is hypothesized that a local injury (for example, allospecific injury or balloon angioplasty) can reactivate latent CMV in cells in the vessel wall or in cells recruited to the site of injury. The virus then initiates an acute infection and inflammation, which may trigger the migration and proliferation of vascular cells. In vitro, human CMV (HCMV) infection mediates vascular SMC migration that is dependent on the expression of the virally encoded chemokine receptor homologue US28 (18). Deletion of the US28 rat homolgue R33 reduces the capacity of CMV to accelerate chronic rejection and transplant vasculopathy in a rat model (19).
Thus, CMV replication and the local inflammation in the vasculature stimulate each other, and thereby support viral persistence but simultaneously damage the tissue. Once the CMV replication has been initiated by the milieu at the site of inflammation, it begins to influence the pathologic process. Through further stimulation of immune responses the cells where virus resides, the local inflammation is further potentiated. By these mechanisms, it is the host immune response and not the viral infection *per se* that causes tissue damage (20-22).

Rat Cytomegalovirus (RCMV)

For CMV, there is a species specific variant for each mammal. This phenomenon creates a problem when trying to establish animal models for CMV, since the species variants differ and the most differences lies in the virus-host cell interaction. Common animal models used today include mice, rats, guinea pigs and monkeys. The rat cytomegalovirus (RCMV) has been used to study various aspects in the development of vessel wall pathology (23-25).

The Maastricht strain of RCMV was first isolated in 1982 from the salivary glands of wild brown rats and passed in rat embryo fibroblasts in vitro as well as in laboratory rats (26). Sequencing of the entire RCMV genome was completed nearly 20 years later (27) and was found to be collinear with that of HCMV or MCMV (27-29). Interestingly, although the vast majority of the genes encode viral proteins, the RCMV

genome was also found to encode proteins that show homology to host proteins which thereby appear to be hijacked from the host genome by an ancestral virus. These include R33 and R78, both encoding G-protein-coupled receptors (30), r144, encoding a major histocompatibility class I heavy chain homologue (31) and r131 and r129, both encoding homologues of CC chemokines (27, 32).

The RCMV model has proven to be suitable for studying the CMV-mediated development of vascular disease, particularly in atherosclerosis, arterial restenosis and transplant vasculopathy. The use of this model resulted in substantial data showing a stimulatory role of CMV in the pathogenesis of various types of vascular disease. RCMV was found to exert its detrimental effects both directly, through dysfunction of cells in the vascular wall, and indirectly, through modulation of the inflammatory and immune responses. Also, CMV seems to affect all stages of the vascular disease. In immunocompromised rats, RCMV causes a widespread infection of most tissues and various cell types (33), however the effects of CMV infection in transplant vasculopathy in rats and mice have so far only been studied after systemic infection (34-39). In this thesis, **(Paper II)**, we investigated the impact of a local CMV infection on cellular activation and arterial graft morphology in transplant vasculopathy. To do so, we used a rat model, in which the aorta or adventitia were locally infected with RCMV *ex vivo* after collection from the donor rat and before transplantation into the recipient rat. This model aims to mimic the effects of a severe systemic infection on the allograft and gives insight into which cells in the allograft become infected, and enable investigation of the cellular immune response against the virus, and its impact on vascular remodeling. We found that RCMV influenced arterial wall of the allograft by increased apoptosis of SMCs in the tunica media and decreased extracellular matrix deposits, which indicate that virus directly or by interacting with host immunity reduces the integrity of arterial wall and thereby increases tissue vulnerability to injury, inflammation and uncontrolled remodeling. Moreover, RCMV potentiated inflammation mainly in the adventitia, which resulted in migration of adventitial cells towards the intima that most likely contributed to increased intimal hyperplasia. Our findings suggest that the virus itself creates pro-inflammatory environment and is able to alter cellular processes involved in the development of vascular diseases. These data are discussed in details in the next chapters.

5 ARTERIAL REMODELING (PAPER II, III, IV)

The *arterial remodeling* is a part of a healing reaction in the vessel wall in majority of vascular diseases, such as transplant arteriosclerosis, restenosis, atherosclerosis, hypertension, diabetes. It is a broadly used term, which basically describes structural changes of the arterial wall as a response to various stimuli, such as wall shear stress, hypoxia, immunological or mechanical injuries, leading to changes in a vessel size and luminal width (13, 40).

The *arterial remodeling* is an active process of structural alterations of the vascular tissue and involves changes in at least four cellular processes: cell growth, cell death, cell migration, and production or degradation of extracellular matrix (41, 42). The arterial remodeling involves narrowing of the vessel lumen caused, as previously thought, by abnormal expansion or thickening of the intima (43). However, remodeling can result in increased (outward remodeling) or decreased arterial cross-sectional diameter (inward remodeling) with either thinning or thickening of the vessel wall (40, 44) depending on the causative mechanism. In many diseases, such as restenosis after percutaneous intervention, cardiac allograft vasculopathy, or pulmonary arterial hypertension, arterial remodeling plays a central role in causing a reduction in the luminal diameter (40, 45). In atherosclerosis, however, outward remodeling can occur and leads to an increase of the luminal diameter and can compensate for an increased plaque load (46). Thereby, every layer of the arterial wall can be affected by the remodeling process, including intimal hyperplasia, medial hyper- or hypotrophy, and adventitial fibrosis (Figure 3).

For clinical reasons, remodeling is generally defined as any change in arterial size (enlargement or contraction), independent or dependent on intimal formation (45). Intimal formation and inward remodeling cause major clinical problems after percutaneous interventions of obstructed and stenotic arteries, which often results in significant luminal narrowing and ischemia.

The first events of vascular remodeling are: activation, migration and proliferation of cells with SMCs phenotype triggered by injury. These cells can be derived from the vessel itself and circulation to the newly formed intima (neointima), perhaps via platelets and inflammatory cells chemokine-chemokine receptor interactions. In this thesis, we showed that both, bone marrow (BM)-derived cells of endothelial phenotype **(Paper III)** and adventitial progenitor cells **(Paper IV)** were able to migrate to the sites of vascular injury. However, BM-derived cells in our hands contributed only to the early stages of vascular remodeling, whereas the cells of adventitial origin were a major constituent of developed intimal lesion. Thus, the intima grows in size due to continuous cells migration and proliferation, and deposition of extracellular matrix (ECM). Moreover, cells in pathologic lesions may have distinct chemokine receptor expression that influences further cells recruitment and activation.

Under the influence of arterial pressures, any loss of medial SMCs or medial ECM degradation in excess of synthesis can cause positive remodeling. Conversely, collagenous scarring of the media results in a stiffer vessel with negative effect on remodeling. Furthermore, the balance between cellular proliferation and apoptosis, and the synthesis or degradation of ECM in the vascular wall, can be potentiated by increased inflammation, for example by CMV infection **(Paper II)**. This specific situation can contribute to an earlier and enhanced destruction of the media layer, as shown by us in a rat model of transplant vasculopathy **(Paper II)**. Interestingly, CMV decreases the collagen content in the allografts and upregulates expression of MMP-2 **(Paper II)**, which regulates collagen turnover in the vessel wall and is thought to be altered in vascular disease (47, 48).

In injured vessel, infiltrating macrophages can contribute to medial SMCs apoptosis. They can direct matrix remodeling through their production of MMPs (matrix metalloproteinases). Conversely, a relative overproduction of tissue inhibitors of metalloproteinases potentially inhibits ECM degradation and thereby contributes to negative remodeling. Indeed, inflammation and leukocyte infiltration in the injured vessel are key initiators of both vessel damage and healing. Leukocyte recruitment is dependent on local production of cytokines and chemokines, expression of adhesion molecules, and changes in the ECM, resulting in recruitment of cells of SMC phenotypes into the intima (49). Inflammation damages the endothelium, exposing SMCs to fibronectin and growth factors, such as PDGF-BB that phenotypically modulate the cells and activate metalloproteinases (50). Metalloproteinases also contribute to the degeneration of ECM and damage of the cells, which facilitate cellular infiltration and inflammation. This inflammatory process activates mechanisms of tissue repair, resulting in further recruitment of inflammatory cells and production of ECM (51), **(Paper II)**, and appears to be a key event in the initiation of intimal formation.

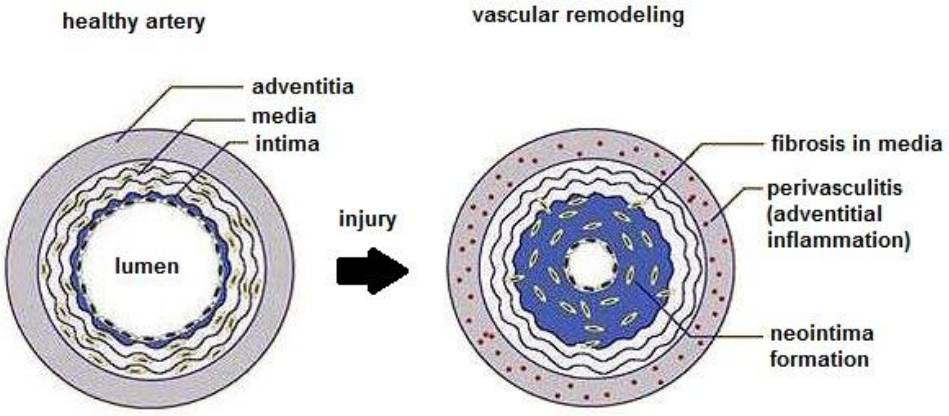

Figure 3. Schematic presentation of the alterations in a blood vessel's geometry during vascular remodeling (observe that intimal lesion is concentric with equal distribution in transplant vasculopathy, whereas restenotic intimal lesion is concentric however local, according to the mechanical damage of the arterial wall).

5.1 INTIMAL HYPERPLASIA

Intimal hyperplasia directly means an increase in the number of cells in the intima. It is, however, accompanied by an increase also in the amount of extracellular matrix. Intimal hyperplasia occurs physiologically in closure of the ductus arteriosus after birth (52) and during involution of the uterus. Diffuse intimal thickening also occurs naturally with age in the human aorta (53).
Intimal hyperplasia occurs pathologically in hypertensive pulmonary arteries (54). Intimal thickenings containing largely vascular SMCs can also be generated iatrogenically after injuring the luminal surface of arteries during percutaneous transluminal angioplasty (55). Intimal thickening also occurs in homograft transplanted organs (56) and in veins used as arteriovenous fistulas or arterial bypass conduits (57, 58). Last, but not least it is also observed in atherosclerosis.

Intimal hyperplasia was first described by Carrel and Guthrie in 1906 when they reported that the anastomosis of veins implanted in the arterial circulation was covered with a tissue similar in appearance to endothelium (59). The animal models of intimal hyperplasia were introduced in the seventies (60). It is possible to mimic surgical interventions on the small animals and thereby trigger vascular pathology similar to this one in humans. The animal models are often criticized because they do not reflect all features of a complex disease. However they allow investigating mechanisms of disease pathology, which are not possible to test on human individuals. Injury procedures similar to endovascular surgery can be performed on rodents or pigs, as well as bypass creation using vein or synthetic materials. Organ transplantation can be performed by transplanting vein or arterial graft from another animal or species. Balloon angioplasty with stenting is widely performed on porcine coronary arteries which have similar anatomical and physiological characteristics with human coronary arteries (61), however can also be tested in rabbits and rats. In the various animal models either molecular and cellular mechanisms of intimal hyperplasia or the impact of new therapeutic strategies can be studied. One of the benefits of working with animals is the access to tissues, which can be harvested and analyzed at various time points critical for the disease progression. Therefore, they give profound insight into the different stages of the disease and enable to investigate molecules critical for the regulation of these distinct stages.

Interestingly, both migration and proliferation of cells responsible for intima formation are regulated by growth factors, which can be derived from adhering platelets, leukocytes, injured endothelial cells, SMCs (62) or surrounding tissue. The intima thereafter enlarges due to continuous cells proliferation and deposition of ECM (66). SMCs proliferation is believed to be stimulated by growth factors, such as PDGF-BB, angiotensin II, IGF-I released in an autocrine or paracrine manner by SMCs (2) and surrounding tissue. Finally, further intimal growth occurs due to ECM synthesis (67). The schematic cascade of events leading to the development of intimal lesion is presented on Figure 4. Interestingly, it has been suggested that cells of newly formed intima are of different origin. Indeed, circulating cells can invade the intima after vascular injury (68-72) and peripheral blood has been reported to contain endothelial- and fibroblast-like precursor cells of bone marrow origin (73). Furthermore, in a rat

model of transplant vasculopathy, recipient cells participated in the formation of intimal hyperplasia in the vessels of transplanted organ (74). Interestingly, in the studies of vein grafts (6) it has been proposed that adventitial fibroblasts and possibly progenitor cells residing in the adventitia of vein grafts can contribute to the formation of intimal lesion.

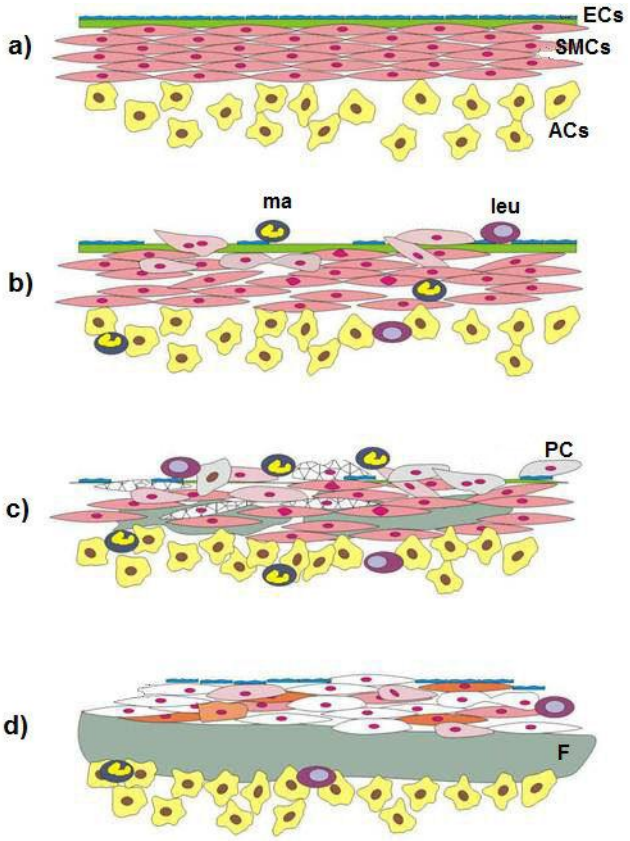

Figure 4. Development of intimal hyperplasia: a) normal artery, b) recruitment of inflammatory cells to an injured artery and mobilization of vascular progenitor cells from their niches, c) progressive apoptosis of the tunica media and formation of intimal hyperplasia by smooth muscle cells and their progenitors, inflammatory cells, and deposition of extracellular matrix, d) further accumulation of cells in the tunica intima and progressive fibrosis of tunica media. ECs, endothelial cells; SMCs, smooth muscle cells; ACs, adventitial cells; ma, macrophage; leu, leukocyte; PC, progenitor cell; F, fibrosis.

6 STEM CELLS

Stem cells (SCs) are undifferentiated cells that possess the ability to proliferate throughout postnatal life and thereby provide a source of progenitor cells that can differentiate into specialized cells in adult organism. SCs are demanding and require precise conditions to carry out their tasks, such as proliferation, differentiation, and functional integration into the cellular network. The fate of SCs can be directed by altering the extracellular milieu or by changing intracellular conditions. In the adult organism, SCs and progenitor cells (PCs) exist in tissues and organs to replace lost or injured cells. Adult SCs are found in many tissues, for example in the brain, heart, bone marrow, intestine, hair. They are maintained for long periods, since they have an unlimited self-renewing capacity compared to SCs in the embryo that can be defined as short term, as they only exist during the fetal life. SCs need mitogens to proliferate both *in vitro* and *in vivo.* Under culture conditions mitogens must be added to the medium to maintain cells with sustained self-renewing capacity. *In vivo*, SCs are protected or actively prevented from differentiation by their surrounding milieu, so-called niche. The niche consists of dedifferentiated cells that secrete factors and organize a rich environment for the SCs to maintain their unique status. The combination of the characteristics of these cells and their microenvironment shapes their properties and defines their potential (75). Proliferation of SCs may occasionally become uncontrolled and cells divide unrestrained forming tumors.

A basic tenet of modern biology is that adult SCs are restricted in their ability to generate only the differentiated cell phenotypes of the tissue in which they reside. Over the past several years, this assumption has been challenged by many published studies, indicating that some SC populations in the adult may possess a phenotypic potential that extends beyond the cell types of their resident tissue (76-81). This phenomenon is referred to as SCs plasticity or transdifferentiation. For example, circulating hematopoietic SCs appear to contribute to the liver, lung, gastrointestinal tract, blood vessels, and heart (82-86). Mesenchymal SCs, multipotential progenitor cells of the bone marrow stroma, have the capacity to supply blood, lung, liver, and intestine (87-89). SC populations found in the brain, skin and adipose tissue also display previously unsuspected multipotency (90-92).

6.1 VASCULAR PROGENITOR CELLS IN ADULTS

Tremendous interest has been focused on vascular regenerative biology in recent years. Differentiation of several distinct adult and embryonic precursor cell populations to vascular phenotype has been observed *in vitro* and *in vivo* (93). The first description of circulating adult endothelial progenitor cells showed that CD34+ peripheral blood mononuclear cells could differentiate into cells of endothelial phenotype *in vitro* (94). Further studies confirmed these findings demonstrating that a number of hematopoietic SC starting populations, for example CD133+ cells, can differentiate to endothelial cells (95-97). Last decade evidence has suggested that bone marrow (BM)-derived PCs

may contribute to vascular remodeling after injury (70, 98-101). Other studies have shown specifically that BM-derived hematopoietic or mesenchymal SCs can be recruited to the intima and participate in the pathogenesis of lesion formation (69, 102, 103). In the contrary, Hillebrands *et al* proposed the existence of a tissue-resident pool of vascular PCs outside of the bone marrow, a concept supported by the finding that endothelial cells in transplant arteriosclerosis may originate from sources other than bone marrow (104). Furthermore, abundant cells expressing SC markers such as c-kit, CD34, flk-1 (VEGFR-2) have been identified in the adventitia of murine aorta. Sca-1+ cells were found to differentiate into smooth muscle cells in PDGF-BB-supplemented culture conditions (6). Conclusive evidence that vascular PCs are found in the vasculature in health and disease is now available. However, the mechanism underlying the integration of these cells into the vasculature and their specific functions are still under debate.

6.1.1 Circulating progenitor cells (Paper I)

A constant population of peripheral circulating PCs has been described (94, 105). It is thought that this cell population can be isolated from peripheral mononuclear cells and have the ability to differentiate into other cell types, such as SMCs or ECs (73, 106). Briefly, these authors isolated mononuclear cells from the peripheral blood of healthy human subjects (73) and found that these cells expressed markers for PCs, such as CD34 and VEGFR, and in vitro differentiated into SMCs under PDGF-BB stimulation. Furthermore, Tanaka *et al* has shown in a murine model of arterial injury that a portion of cells in the injured arterial wall were derived from the pool of circulating peripheral cells and they expressed markers for SMCs and ECs (107). Interestingly, it has been suggested that vessels of transplanted hearts contain recipient-derived cells that migrate towards the graft through the circulation (108) and that recruitment of these cells is related to acute organ rejection (44).

In our study **(Paper I)** we have not indicated direct source of recipient-derived SMCs and their progenitor cells in the cardiac allografts however proposed that these cells may have reached graft vessels through the circulation. Moreover, we found that recipient-derived cells in the arterioles of each cardiac biopsy accounted for a mean of 3.4±2.3% of cells with SMC phenotype. It has been shown previously that recipient-derived SMCs appear in the graft early after transplantation and start to accumulate within 1 month (109). Since the number of these cells was not related to the time between transplantation and biopsy in our study, we hypothesized that recruitment of recipient-derived cells is facilitated by other factors, such as immune-mediated damage of the organ after transplantation (110). Indeed, the number of accumulated recipient-derived cells in the graft correlated strongly with the number of CD45-positive leukocytes and the grade of rejection, confirming that inflammation is strongly related to the recruitment of circulating PCs into the graft vessels.

6.1.2 Bone marrow cells (Paper III)

Bone marrow (BM) is primarly composed of cells of the blood cell lineages, for example myelocytes, lymphocytes, monocytes, megakaryocytes, erythrocytes, at various stages of differentiation. Together, stem cells (SCs) and progenitor cells (PCs) account for approximately 0.1% of total bone marrow cells (BMCs). Thus, it is only a small percentage of BMCs that are capable of repopulating the injured vessel wall. In humans, BM-derived SCs have been classified broadly into hematopoietic stem cells (HSCs) or non-hematopoietic SCs or mesenchymal SCs (MSCs) (111). HSCs are believed to be derived from a very early embryonic precursor, the hemangioblast. Although it remains controversial, many believe that the hemangioblast is a common precursor for both HPCs and endothelial progenitor cells (EPCs) (112, 113). EPCs are thought to be important in adult vasculogenesis and may also participate in vascular healing after injury, whereas MSCs has been described to differentiate into muscle cells, osteoblasts, chondrocytes, adipocytes (87).

Elucidation of pathophysiological mechanism and phenotypes of cells in intimal lesion is essential to optimize therapeutic strategies. Despite that BM-derived cells are one of the potential sources of cells in intimal hyperplasia (71), their role in this process has been unclear. Furthermore, the contribution of BMCs to intimal lesion has been reported to differ among investigators (99, 114) from range 5% to 60%, dependent on the type and severity of introduced injury (68, 69, 98, 99, 114). While intimal formation is a dynamic process, the presence of BMCs has been investigated in a completely developed intimal lesion. In this thesis **(Paper III),** we examined the contribution of BMCs and their distinct fractions at the intermediate stages of intimal formation in order to find whether these cells play a major role in this process and constitute a developed intimal lesion or rather are crucial only at earlier stages of arterial remodeling. To do so, we used a chimeric mice model (with bone marrow from GFP transgenic mice) to introduce injury of carotid artery by ligation. After one week, we found GFP-positive BMCs at the sites of arterial injury. Further, in a mouse model of balloon injury with a subsequent local delivery of BMCs and their distinct phenotypes to injured arterial bed **(Paper III)**, we found BMCs at early stages of intimal formation. Their number was constantly decreasing in time in the intima (10 fold fewer cells at day 14 compared to day 1 after injury), which indicates that BM-derived cells support arterial healing shortly after injury and afterwards are eliminated due to possibly low survival rate in the vascular wall or apoptosis. Interestingly, these cells expressed markers for SMC and EC both *in vivo* (in the arterial intima) and *in vitro* (in cell cultures of BMCs). Thus, our findings highlight the importance of bone marrow-derived cells only at the early stages of intimal formation and propose other sources of cells, outside of bone marrow, as a major cellular constituent of developed intimal lesion.

6.1.2.1 Hematopoietic stem cells

Hematopoiteic stem cells (HSCs) are defined by the ability to differentiate into cells of blood lineages. Some authors have proposed that these cells can differentiate into muscle cells (including SCs and cardiomyocytes), neural cells, hepatocytes, epithelial cells, pancreatic cells (83, 115, 116). Moreover, early studies by Sata *et al* have demonstrated that HSCs are important in intimal formation (98). However, in this study the conclusion was based on the contribution of entire BM-derived cells to vascular remodeling. Despite these findings, the ability of HSCs to differentiate into vascular SMCs is controversial (117). Indeed, when the same authors repeated their initial experiments using a single HSC, they found very few cells in the intima, indicating that the population of cells used in their original experiments may have been contaminated with other progenitor cells (PCs), such as mesenchymal stem cells (98, 117, 118).

6.1.2.2 Mesenchymal stem cells

In contrast to hematopoietic SCs, there is also a population of non-hematopoietic SCs or also called as mesenchymal stem cells (MSCs). They are believed to originate from bone marrow stromal cells and differentiate into myocytes, osteoblasts, chondroblasts and adipocytes (111). Friedenstein was the first person who identified multipotential stromal precursor cells (119). He described the isolation of spindle-shaped, clonogenic cells in monolayers cultures from bone marrow and defined them as colony-forming unit fibroblast (CFU-Fs). As a result of their suspected capacity for self-renewal and differentiation, BM-derived stromal cells were first considered as *stem cells* by Caplan and named MSCs (120). Currently, a precise definition of MSCs is elusive. Thus, MSCs are usually defined functionally, rather than by the presence of specific surface markers. They adhere to cell culture dishes and do not express the surface markers characteristic for HSCs (121). Although precise phenotypic characterization varies in different studies, it is generally accepted that MSCs are negative for CD11b, CD14, CD31, CD34, and CD45 but are positive for CD29, CD44, CD73, CD105, CD106, and CD166 (122-124). They can be expanded dramatically in culture, maintaining their growth and multilineage potential (122). Studies have shown that MSCs home to areas of injury after both local and systemic administration (111). These cells have been studied extensively in the context of cardiac tissue repair and are currently one of the cell types being studied in clinical trials of cardiac regeneration following myocardial infarction (125, 126). The contribution of MSCs to restenosis has only recently been explored (127).

Thus, MSCs are considered as one of the most promising cell types for therapeutic applications. Mechanisms responsible for this therapeutic role are not well understood, and may involve paracrine activity. The ability of MSCs to modulate the immune system opens a wide range of applications for inflammatory diseases. The use of MSCs for clinical purposes takes advantage of their low immunogenicity *in vitro* (128), in preclinical studies (129) and human studies (130), which supported the possible use of these cells obtained from allogeneic donors. Preclinical and clinical studies show promising results, but controversial results are still reported, indicating the need for further basic and preclinical investigation on their therapeutic potential.

6.1.3 Endothelial line of progenitor cells (Paper III)

The phenotypic classification of endothelial progenitor cells (EPCs) still remains controversial. Indeed, a variety of cells types have been described as EPCs (131, 132) however they lead to considerable confusion in nomenclature. Additionally, EPCs may originate from multiple precursors including the hemangioblast, bone marrow progenitors and tissue resident stem cells (Figure 5). The characterization of these cells has also been hampered by the presence of circulating mature endothelial cells, hematopoietic and immune cells, as well as circulating EPCs of various developmental stages (132). Most importantly, there is an overlap in the expression of surface proteins used to identify putative EPCs and cells of hematopoietic lineage by flow cytometry (133). The majority of EPCs arose from a CD14+ subpopulation of peripheral blood cells but late outgrowth endothelial cells developed exclusively from the CD14- fraction (134). Urbich *et al*. reported that adherent cells deriving form CD14+ or CD14- mononuclear cells showed equal expression of endothelial marker proteins and capacity for clonal expansion as determined by measuring endothelial colony-forming units (135). Proteins commonly used to identify EPCs include CD34, CD133, flk-1 (VEGFR-2) and c-kit (CD11, the receptor for stem cell factor) (132). However, these markers are not specific and some CD34+/CD133+/VEGFR-2+ cells (an antigen combination commonly used to quantify EPCs) are positive for the common leukocyte marker CD45 and therefore likely to represent hematopoietic rather than endothelial precursors (136, 137). Amano et al. found that CD34-B220-CD3- VEGFR-2+ hematopoietic stem cell population in peripheral blood exclusively contained EPCs (138).

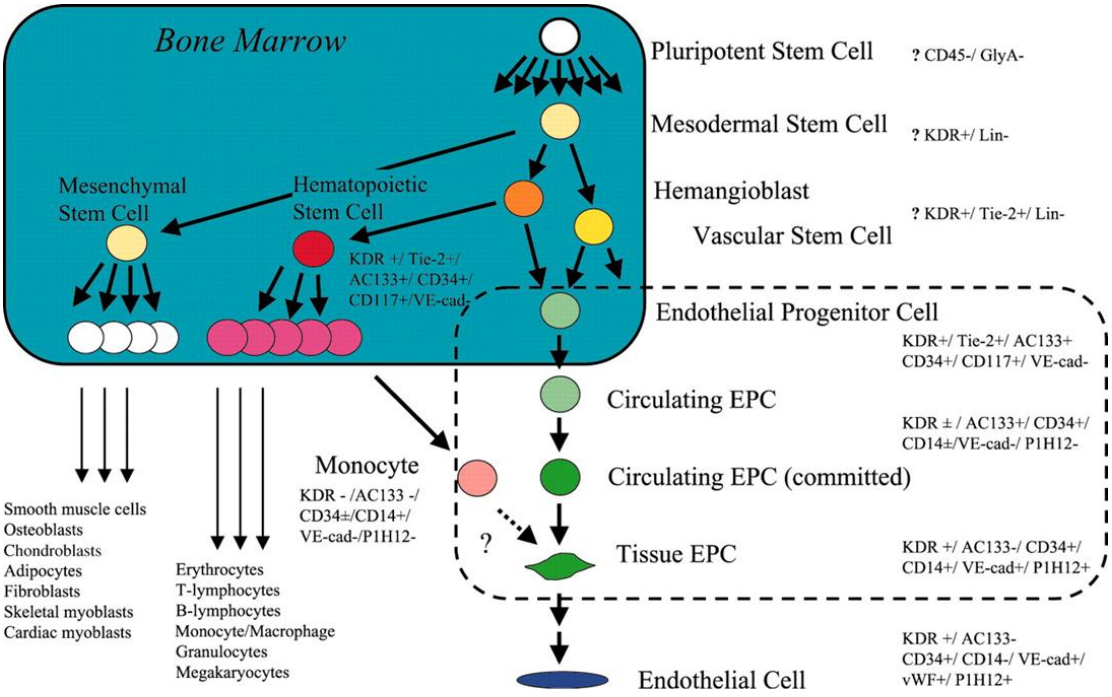

Figure 5. Putative cascade and expressional profiles of human bone marrow-derived endothelial progenitor cell differentiation (adapted from Asahara, T., American Journal of Physiology, 2003).

Interestingly, the first evidence that EPCs may contribute to neoangiogenesis came from studies by Asahara *et al*. In mice and rabbit models of ischemia, CD34 and Sca-1 population of mononuclear cells promoted new blood vessel formation in injured areas and lead to recovery of ischemic tissue (94, 139, 140). Reendothelialisation of injured vessels has also been shown using a rat balloon injury model. Indeed, accelerated repair of the denuded endothelium with decreased neointima formation following mobilisation of circulating progenitor cells has been observed (141). Interestingly, non-bone morrow derived EPCs also appeared to contribute to vascular repair. Spleen-derived mononuclear cells and cultured EPCs were recruited to the site of vascular injury resulting in an enhanced reendothelialisation and decreased intima formation after damage to an endothelium (142). Additionally, it has been shown that recipient-derived EPCs reconstituted endothelial layer in the model of vessel graft atherosclerosis (143). Thus, these data support the current hypothesis that EPCs reside in both the bone marrow (133, 144, 145) and tissue resident niches (146) and display ability to restore damaged endothelium. Indeed, it has been suggested that differentiation of progenitors is a form of cellular plasticity within the broad context of functional adaptation (147). Possibly, so called *endothelial progenitor cells* represent heterogeneous cell population containing certain cell-types that can express some endothelial cell markers and locate at the sites of new vessel formation and vascular remodeling. It still remained to be clarified how highly "EPCs" can differentiate into mature ECs phenotypically and functionally (148). Moreover, standardization of cell markers to identify putative EPCs is valuable to compare the number of circulating EPCs in patients from clinical trials at different institutes.

In this thesis, we studied the contribution of endothelial line of BMCs in vascular remodeling after injury **(Paper III)**. First, we identified which populations of cells derived from BM are in major importance for intimal formation. To do so, we isolated BMCs derived from GFP transgenic mice for distinct populations of lymphocytic origin (CD3), monocytic/macrophage origin (CD14), and endothelial origin (CD34, VEGFR-2) and delivered them locally to an injured artery. We found that cells expressing markers for endothelial cells (CD34, VEGFR-2) are the main phenotype of cells of BM origin contributing to early stages of intimal formation. Furthermore, we showed for the first time that BMCs did not fuse with vascular SMCs, however they incorporated to arterial wall in the mechanism of differentiation to SMCs or ECs. Interestingly, distribution of BM-derived cells in the intima was not equal and they trended to accumulate in the areas, where intima was thicker. In these areas BM-derived cells expressed mainly SMC markers. However, as mentioned previously, BM-derived cells have most likely reparative and protective function only at the early stages of intimal formation, and they are not a major cellular constituent of developed intimal lesion.

6.1.4 Smooth muscle progenitor cells

Two decades ago it was postulated that vascular SMCs originated from the undifferentiated mesoderm surrounding primitive endothelial tubes (149). Few years later, data from distinct experimental systems suggested that at least some endothelial cells are capable of giving rise to vascular SMCs (150, 151). Furthermore, work by

Williams and his colleagues (152, 153) strongly supported this hypothesis with the intriguing finding that adult endothelial cells may give rise to vascular SMCs. It initiated further interest in the origin of SMCs and their possible progenitor cells.
Thus, until now, it has been suggested that vascular smooth muscle progenitor cells (PCs) are both resident in the normal arterial wall and derived from a variety of sources outside of the vasculature. Smooth muscle PCs have been identified in the bone marrow (multipotent vascular smooth muscle PCs and MSCs), in the circulation (circulating vascular smooth muscle PCs), in the vessel wall (resident vascular smooth muscle PCs and mesangioblasts) and various extravascular sites (extravascular, non-bone marrow progenitor cells) (154). However, the molecular mechanism of mobilization, homing, and differentiation of putative smooth muscle progenitor cells has been still unclear. Furthermore, reported diversity of cells of smooth muscle phenotype observed in human lesions (155) suggests that there are most likely heterogeneous sources of SMCs and their progenitors (99). Identification of smooth muscle PCs has frequently required characterization and expression of specific SC- or PC antigens that have been also identified in a population of vascular SMC in the normal vessel wall or disease. These expression markers are listed in Table 1, together with the cell types currently identified to express them.

Table 1 Progenitor or stem-derived surface and cytoplasmic antigens in vascular SMCs and their progenitor cells (modified after Orlandi A *et al, Biochem Pharmacol, 2010*).

Antigen	Origin	Cell type
CD133	Transmembrane glycoprotein	hemangioblast, smooth muscle PC
CD34	Transmembrane protein	Smooth muscle PC, HSC
VEGFR-1	Transmembrane kinase receptor	intimal SMC, smooth muscle PC
VEGFR-2	Transmembrane kinase receptor	intimal SMC
c-kit (CD117)	Membrane receptor for Stem cell factor	Intimal SMC
CD45	Protein tyrosine phosphatase	Smooth muscle PC, HSC
CD14	Cytoplasmic endotoxin receptor	Smooth muscle PC
Sca-1	Transmembrane tyrosine kinase receptor	HSC, adventitial PC
Notch-1	Transmembrane regulatory receptor	Side population-derived SMC, intimal SMC
Stro-1	Trypsin-resistant cell surface antigen	MSC-derived SMC
SSEA1	Cell membrane antigen	MSC-derived SMC
CD105	Cell membrane antigen	MSC

Resident tissue mesenchymal stem cells in adult vessels (Paper II, IV)

Over the past number of years, the vascular adventitia has been increasingly implicated in the intimal formation after vascular injury. For example, in carotid artery vein grafts it has been shown that activated adventitial fibroblasts, which differentiated into myofibroblasts, were able to migrate towards the intima (5). Subsequent studies (156-158) showed that adventitial fibroblasts seeded in the carotid artery following balloon injury, were found in the intima. Similarly, Tomas J.J. *et al.* observed that adventitial myofibroblasts are tracked to the intima of venous grafts and contribute to the vascular healing (159). Interestingly, the presence of abundant cells within adventitia of the

aortic root of apoE-/- mice was observed (6). These cells expressed SCs markers, such as Sca-1 and c-kit, and were able to migrate towards the intima and differentiate to intimal SMCs.
Interestingly, satellite-like cells named "mesoangioblasts" can also be isolated from explants of murine dorsal aorta and are able to differentiate into skeletal muscle, smooth muscle and other mesenchymal cell types *in vitro* (160). Mesoangioblasts express both myogenic and endothelial cell markers (particularly VEGFR-2), and are phenotypically distinct from multipotent hemangioblasts (161), which express and can give rise to both hematopoietic and endothelial progenies (160). Mesoangioblasts, when incorporated into grafted host blood vessels, are successively dispersed by the circulation and appear integrated into a wide range of mesodermal-derived tissues, including cartilage, bone, smooth muscle and cardiac muscle (162).

The predominant source of cells in intimal lesions of injured vessels has for long been a matter of debate. Phenotypically modulated, synthetic SMCs in the media were previously considered the only source (2). However, SMCs of medial origin are often damaged by the inflammatory response and undergo apoptosis (109), and their migratory capacity is quite low *in vivo* (163), making them an unlikely source of intimal SMCs. Bone marrow PCs were also proposed as a major source of neointimal cells, however we **(Paper III)** and other investigators have been unable to confirm this possibility (6). Therefore, we hypothesized that progenitor cells in the injured vessel itself or adjacent vessel may play important role in intimal formation. We found in a rat aortic transplant model **(Paper IV)** that medial SMCs in the adjacent vessel changed their phenotype to synthetic but were not able to migrate to the allograft. Indeed, adventitial cells proved to be the main source of cells contributing to intimal hyperplasia. By two weeks after direct transplantation of labeled adventitia to previously transplanted allografts, over 50% of intimal cells derived from the transplanted adventitia. Our findings are in agreement with previous proposal that neointimal cells are from local sources in the vessels, rather than from circulating cells (164). Interestingly, the adventitia has been previously considered as a potential source of cells migrating towards the intima. However, such cells were difficult to trace, and thereby this hypothesis has been controversial (165). Furthermore, little is known about the phenotypes of adventitial cells that could contribute to intimal hyperplasia. Indeed, an adventitia contains Sca-1 and c-kit-positive mesenchymal PCs (166), and mesenchymal cells can differentiate into SMCs. In our study **(Paper IV)**, we found that approximately 20% of adventitial cells from rat aorta expressed Sca-1, and 4% expressed c-kit. Interestingly, both of these cells co-expressed CCR2, a receptor for MCP-1, which in our hands was the major chemokine involved in adventitial cells migration. When stimulated with platelet-derived growth factor BB (PDGF-BB), which is a potent mitogen for cells of mesenchymal origin, both CCR2/Sca-1 and CCR2/c-kit-positive cells could differentiate into SMCs *in vitro*. Thus, Sca-1 and c-kit-positive mesenchymal PCs in the adventitia could be a key source of neointimal cells.
Likewise, **(Paper II)**, we showed that contribution of adventitial cells to intimal lesion in a rat model of transplant vasculopathy was even potentiated while adventitia was infected with rat Cytomegalovirus (RCMV). This finding further strengthens the

hypothesis that cells derived from adventitia play pivotal role in vascular remodeling and that inflammation is important for their mobilization.
In contrast to rodents, there is very limited evidence for the presence of vessel wall-derived SCs in human vessels. A population of CD34+, $CD31^{low}$ cells has been identified in the space between media and adventitia of large and medium-sized human arteries and veins (167, 168). Cultured human vascular adventitial fibroblasts from pulmonary arteries were also found to contain a progenitor cell-like population (CD29+, CD44+, CD105+), negative for hematopoietic and endothelial cell markers (169). Interestingly, these cultured human vascular adventitial fibroblasts did not display the typical "hill-and-valley" growth pattern of normal medial vascular SMCs, but could undergo osteogenic, adipogenic and myogenic differentiation, the latter characterized by increased expression of SM-α-actin and calponin (169). Thus, even if the selective control of vascular SMC progenitors in the arterial wall may represent an attractive therapeutic possibility, more detailed studies to define their phenotypes and effects on vascular disease are required.

6.2 STEM CELL PLASTICITY

The adult stem cells (SCs) are restricted in their ability to generate only the differentiated cell phenotypes of the tissue in which they reside. Over the past several years, this assumption has been challenged by many published studies indicating that some SCs populations in the adult may possess a phenotypic potential that extends beyond the cell types of their resident tissue (88, 133, 170, 171), a phenomenon that is referred to as SCs plasticity or transdifferentiation. Transdifferentiation involves the conversion of a cell of a given tissue lineage into a cell of a distinct lineage, which is associated with the loss of expression of tissue-specific markers of the original cell type and the acquisition of markers and function of the transdifferentiated cell type. For example, circulating BM-derived SCs can transdifferentiate into unexpected phenotypes, such as skeletal muscle (172, 173), hepatocytes (174), epithelial cells (83), neurons (175), ECs (176), and cardiomyocytes (177), in response to tissue injury or placement in a new environment. Furthermore, it has been postulated that stem cells recognize tissue damage and migrate from a distance to the site of the injury (82, 178, 179).

Why should differentiated cells have the capacity to convert to other differentiated phenotypes? It has been believed that an answer to this question may come with an understanding of how cell phenotypes arose during evolution. During evolution, as more complex organisms developed, more distinct types of specialized cells were also generated. Two distinct pathways of phenotypic evolution could account for the generation of new specialized phenotypes. For example, new differentiated phenotypes arose directly from SC, or rather existing differentiated phenotypes underwent variation to yield new phenotypes. Among these two paths, the evolution of new phenotypes directly from undifferentiated SCs would require a more complicated selection mechanism. If however, the latter mechanism played major role during the earliest stages of multicellular evolution, then remnants of transdifferentiation may have persisted in higher species. Thus, there may be a division among differentiated cell

types according to their early or late appearance during evolution and thereby more primitive phenotypes would possess a greater capacity to transdifferentiate. For example, the most primitive monocyte phenotype is the SMC (180). It is intriguing that early embryonic cardiac myocytes transiently express several molecules that are hallmarks of differentiated SMCs, including smooth muscle α-actin, smoothelin and SM22*α* (181-183).

6.2.1 Transdifferentiate or fuse? (Paper III, IV)

Cell–cell fusion (Figure 6) occurs in a wide range of developmental and pathological processes (184). The possibility that two different cell types can fuse, known as heterotypic fusion, and form a somatic cell hybrid was initially suggested back in 1965 (185). Homotypic somatic cell fusion occurs when two identical cells that are committed to the same fate fuse, and this has been shown to occur during the formation of myotubes, osteoclasts, placenta syncytiotrophoblasts and macrophage-derived giant cells (186). Thus, functional fused cells can contribute to several tissues in the body, and can continue to develop all life-generating cells with distinct biological properties.

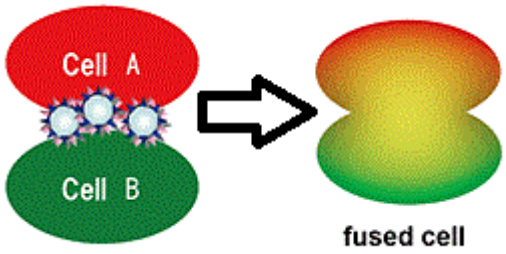

Figure 6. Cells fusion.

Interestingly, it has been shown that injury to a tissue can enhance spontaneous cell–cell fusion events. If one of the parent cells of a cell–cell fusion is highly plastic, such as a stem cell, and the other is a somatic cell, their fusion can be followed by reprogramming events that can generate new hybrid pluripotent cells. These, in turn, have the potential to differentiate and regenerate the damaged tissue.

Recently, it has been suggested that regeneration can occur not only through the transdifferentiation but also via cell–cell fusion (187-189). After such fusion, one of the two parental fusing cells takes over the developmental characteristics of the other. This implies that one of the parental cells has a dominant phenotype over the other, and that the resulting hybrids will have the features of only one of the parental cells. On the other hand, if one of the parental fusing cells is less differentiated and more plastic with respect to the other, new hybrid cells can arise that have a novel differentiation potential (190). These resulting hybrid cells might be reprogrammed, and therefore become pluripotent and can differentiate into cell types of different lineages.
Until now, the process of *in vivo* cell fusion and regeneration has been confirmed by contribution of BMCs to muscle fibers enhanced by the induction of muscle injury (116). BMCs have also been shown to have active roles in intestinal regeneration.

Transplanted BMCs can fuse with intestinal epithelium cells of the injured intestine in mice that had received γ-irradiation (191). Furthermore, cell fusion has also been shown to be a mechanism for liver regeneration.
Interestingly, the frequency of spontaneous cell fusion and reprogramming *in vitro* is quite low (one cell per $1 \times 104–1 \times 105$ cultured cells). Thus, if cell fusion serves to regenerate a tissue, it cannot be the unique regeneration mechanism. On the other hand, the *in vivo* role of spontaneous fusion is not fully understood, and it is possible that there are special conditions that can contribute to the enhancement of this phenomenon.

In the vascular system, studies of cardiac repair mechanisms revealed that hematopoietic cells can fuse with cardiomyocytes (192). However, there are no published data until now, that provide evidence for cell fusion between vascular PCs and intimal SMCs. It has been unclear whether cells that migrate to vascular intima and contribute to intimal lesion do so in the mechanism of cell transdifferentiation or rather fusion with intimal cells. In this thesis **(Paper III)**, we showed that BM-derived cells that contribute to early stages of vascular remodeling, were able to differentiate to ECs and SMCs, both *in vitro* and *in vivo*. Similarly, adventitial PCs, which in our hands were a major source of cells in neointima, could differentiate into SMCs **(Paper IV)**. In order to show it, we cultured adventitial cells co-expressing Sca-1/CCR2 or c-kit/CCR2 in the presence of PDGF-BB for 7 days and stained them for SM-α-actin. Interestingly, both of these cell phenotypes could differentiate into cells of SMC phenotype in the presence of stimulating factor, such as PDGF-BB.
To further examine whether intimal SMCs of adventitial origin can fuse with SMCs **(Paper IV)**, we performed a fusion assay between SMCs and adventitial cells using Cre/lox system (192). However, we found that neither cells positive for Sca-1 nor c-kit fused with SMCs, even under forced conditions with polyethyleneglycol (PEG) hybrid technique. Likewise, we showed for the first time, by using also Cre/lox system, that BMCs did not fuse with vascular SMCs. Our findings indicate that cells migrating to the sites of arterial injury, either derived from bone marrow or adventitia, differentiate to SMCs and ECs to adjust in the vessel wall and meet the demands and needs of their new microenvironment.

6.3 CELLS MIGRATION ASPECTS (PAPER I, II, III, IV)

Cell migration plays an essential role in a wide variety of biological phenomena. This process is involved in normal as well as pathological events. In the adult, cell migration is essential in homeostatic processes such as the immune response and repair of injured tissues (193). The pathological processes to which migration can contribute include vascular disease, chronic inflammatory diseases and tumor metastasis. Cell migration is a complicated process requiring precise regulation and integration of multiple signaling pathways (194).

6.3.1 How do stem cells find their way home?

Migration of stem cells through the blood to different organs or in the organ tissue requires active navigation, a process termed also "homing". Homing is a rapid, coordinated and multistep process and is required for seeding of the fetal bone marrow (BM) by hematopoietic stem cells (HSCs) during development. Homing has also important roles in adult tissue homeostasis, for example during stress-induced recruitment of leukocytes from the bone marrow reservoir and during stem cell mobilization, as a part of host defence and repair. The capacity of stem cells to migrate and invade is critical for functional integration even when cells are injected directly into the site of injury **(Paper II)**. Tissue damage leads to a dramatic increase in the levels of secreted chemokines, cytokines and proteolytic enzymes, such as MMP-9 (195), in many organs as part of the regeneration and repair process, which have profound impact on stem cells migration and repopulation. Thus, multiple molecules, including families of adhesion molecules and chemokines, provide signals for the dynamic trafficking of stem cells to the surface of injury (196), **(Paper I, II, IV)**. Chemokines are proinflammatory cytokines that function as potent chemoattractants for stem cells (197). They provide signals leading to selective recruitment of the cells at sites of inflammation. Interestingly, SDF-1/CXCR4 interactions and signaling have been implicated as a principal axis regulating retention, migration and mobilization of HSCs during homeostasis and injury (198). Using a mouse aortic transplantation model, Sakihama *et al* (199) show that SDF-1 is a critical molecular target for the progenitor homing. During the course of transplant arteriosclerosis, SDF-1 expression in the graft was upregulated, and the circulating stem cells expressing SDF-1 receptor, CXCR4, increased in the graft recipients. CXCR4+ stem cells, derived from transplant recipients, migrated into allografts through microvessels in the adventitia and then toward the luminal side. In support of a functional role for these molecules, *in vivo* neutralization of SDF-1 inhibited stem cell homing (200). Once stem cells are attracted by chemokine to the activated/damaged tissue, these cells may adhere to this activated tissue via highly expressed adhesion molecules.

In this thesis **(Paper I, II, III, IV)** we presented the ability of different cell phenotypes, including vascular progenitor cells, to migrate in response to vascular injury and inflammation. Interestingly, acute inflammation and leukocyte infiltration in the allograft are key initiators of both allograft damage and tissue healing. In the biopsies of transplanted human hearts, where donor and recipient were of different sex, we observed recipient-derived cells of SMC phenotype that localized in the arterioles of the allografts **(Paper I)**. The detection was done with help of real-time PCR, and confirmed with FISH for chromosome Y and immunostaining for SM-α-actin. Our findings in this study do not confirm the source of recipient-derived cells of SMC phenotype and their progenitors however propose, that these cells most likely reached the donor tissue through the circulation. Interestingly, it has been shown by other authors that reduction of circulating PCs in the blood seems to be related to the level of vascular damage (201-203). Furthermore, factors such as hypertension and diabetes appear to impair the migration of endothelial progenitor cells (203). Thus, the physiological status of the arteries in these diseases affects the number of circulating progenitor cells by influencing their maturation, release from the bone marrow, and

accumulation in the injured tissues (204-206). Furthermore, BMCs were found in the injured artery and they localized in the arterial intima (68, 69, 98, 99, 114), **(Paper III)**. Indeed, in the carotid injury model in chimeric mice with bone marrow from GFP transgenic mice, we showed with confocal microscopy that BMCs migrated to the sites of vascular injury and inhibited early stages of intimal hyperplasia by 1.4 fold **(Paper III)**.

Although SCs need sometimes long way to reach their destination, it has been shown by us **(Paper II, IV)** and others (3,6,7) that they can also migrate from the tissue adjacent to the injury. Interestingly, vascular adventitia has been implied as a source of cells that contribute to intimal hyperplasia in different vascular pathologies (5-7), and migrate in the vessel wall in order to localize in vascular lesions (207). To do so in our study, we transplanted rat male LEW adventitia labeled with fluorescent cell tracer into 4-week-old rat female F344 allografts that had been transplanted into LEW rats **(Paper II, IV)**. In this model, the labeled adventitia was not a direct target for allogenic inflammation. Two weeks after transplantation of labeled adventitia, we surprisingly found a massive migration of labeled adventitial cells towards the intima. This result was confirmed by RT PCR for SRY gene.

Our studies **(Paper I, II, IV)** showed that migration of cells important for vascular healing, especially progenitor cells, is potentiated by tissue inflammation and provide evidence that MCP-1 is a potent chemokine responsible for recruitment of these cells to sites of injury. These aspects are discussed in details in the following chapter.

7 CHEMOKINES IN VASCULAR PATHOLOGY

Chemokines are low molecular weight (8-14kDa), predominantly secreted proteins produced by a variety of cells. Chemokines act via chemokine receptors, which belong to the G-protein coupled receptor (GPCR) family (208). The classification of chemokines is based on their structural features - the number and spacing of the first two aminoterminal cysteines in a peptide sequence, which devides chemokines into 4 families: CXC, CC, C and CX3C (209). Chemokine receptors are also divided into 4 families depending on the spectrum of chemokines they bind (210).
The regulation of cell behaviour by chemokines is a complex process. The most important is undoubtedly their ability to induce chemotaxis, which is vital for the control of immune responses and constitutive cell trafficking (211, 212). Chemokines are involved in all stages of leukocyte migration into tissues through their functioning in concert with adhesion molecules (213). Thus, chemokines direct target subsets of effector cells to specific tissue sites of infection and inflammation and are therefore believed to play important roles in wound healing and clearance of infectious pathogens.

The pattern of chemokine receptors expression is different for different cells, while the expression of chemokines differs between tissues as well as between healthy and diseased states of the same tissue (214). This dictates differential recruitment of leukocyte subtypes in inflammatory and non-inflammatory (cell homing) conditions. At the same time different chemokines and their receptors seem to participate in different stages of leukocyte migration or at the same stage but different conditions/tissues (212, 213). Since chemokines will recruit cells of the immune system to sites of inflammation, these molecules have also been implicated in mediating tissue injuries in both acute and chronic inflammatory processes, and are believed to play a pathogenetic role in HCMV disease by attracting T cells and monocytes to an inflammatory site.
Besides regulating chemotaxis, chemokines regulate other processes. Some, for example, CCL2, CXCL8, CXCL10, CXCL16 possess mitogenic activity, while others, such as CCL3 inhibit cell proliferation. CXC CK can be either angiogenic or angiostatic depending on the presence or absence of the amino-terminal ELR (glutamid acid-leucine-arginine) motif (31). Several chemokines (CXCL4, CXCL9, CXCL10, CXCL11) have antimicrobial functions (215), while some chemokine receptors are utilized by human immunodefficiency virus to gain access to the cell (216). Given such a broad range of functions, it is not surprising that chemokines and their receptors are implicated in the pathogenesis of many diseases (217). Thus, different types of chemokines and their receptors have been demonstrated to be centrally involved in all phases of arterial remodeling and may therefore provide a valuable target for disease intervensions (218).

7.1 CCL2 (MCP-1) AND CCR2 (PAPER I, II, IV)

During inflammation, leukocytes release proinflammatory cytokines, for example, interleukines-1α and -6, tumor necrosis factor-α (TNF-α), MCP-1, stromal-derived factor 1 (SDF-1), and transforming growth factor β1 (TGF-β), that contribute both to initiation of the inflammation and to healing (219). The role of the MCP-1 (*Monocyte chemoattractant protein 1*) has been studied in experimental models of intimal formation. After acute mechanical injury MCP-1 mRNA expression is rapidly upregulated with a subsequent increase of the MCP-1 protein in the arterial wall and in the circulation (220-222). Interestingly, MCP-1 expression in SMCs can be increased by PDGF (220), which itself has also been shown to influence intimal hyperplasia (223). In the first report studying the functional role of MCP-1 in intimal hyperplasia, treatment with high doses of neutralizing MCP-1 antibody resulted in a significant reduction in intima thickening in a rat model of carotid injury (221). This effect of MCP-1 inhibition was associated with a reduced number of intimal SMCs, whereas the extent of leukocyte infiltration was unchanged (221). These results may imply a primary role of MCP-1 on SMCs accumulation but not inflammation in injury induced arterial remodeling. In a transgenic approach, intimal formation, intimal SMCs content and SMCs proliferation rates were clearly reduced after injury of femoral artery in $CCR2^{-/-}$ mice (224). Interestingly, in this model of arterial injury, leukocyte infiltration in the intima was absent. Similarly, in hypocholesterolemic apoE-/- mice, which were also $CCR2^{-/-}$, intimal lesions after wire-injury of the carotid artery were significantly diminished compared to $apoE^{-/-}/CCR2^{+/+}$ mice (222). Thus, in injury models where prominent inflammatory responses are induced, for example, endothelial denudation in hypercholesterolemic animals, MCP-1 mediated intimal formation is primarly due to an increased macrophage infiltration. In contrast, intravascular injury in non-hyperlipidemic animals accompanied by only little inflammatory infiltrates, a reduction in intima formation (12) is due to direct effects of MCP-1 on SMCs proliferation possibly in autocrine manner (225). Given the fact that MCP-1 upregulation occurs only transiently after vascular injury, the arrest function of MCP-1 in early monocyte recruitment, which has been shown to play a decisive role in intimal hyperplasia (226), is an important mechanism in MCP-1-induced vascular remodeling after injury.

Furthermore, in heterotopic cardiac transplantation models, MCP-1 expression was found to be upregulated in arterioles of cardiac allografts with transplant vasculopathy (227, 228) and appeared to be predominantly expressed by infiltrating monocytes (227). Moreover, intimal hyperplasia in cardiac allografts was reduced by MCP-1 neutralization (228). Interestingly, our study **(Paper I)** (229) provide evidence that inflammation and MCP-1 are pivotal in the recruitment of recipient-derived SMCs into transplanted hearts. In this study, we analyzed archived myocardial biopsies from heart transplant recipients in order to define the clinical factors that influence migration of recipient-derived cells to the arterioles of the allografts and potential factors involved in their migration. Since the number of leukocytes was associated with increased accumulation of recipient-derived SMCs in transplanted hearts, we further determined whether leukocytes can influence migration of SMCs. Indeed, in response to stimulation with leukocyte-conditioned medium, SMC migration increased by approximately 90%.

Since MCP-1 is the major factor for monocyte recruitment to inflamed tissues, we hypothesized that MCP-1 might also be involved in the migration of recipient-derived SMCs. Indeed, in response to recombinant MCP-1, SMC migration increased to levels similar to those induced by leukocyte-conditioned medium. Moreover, this migration was inhibited by anti-MCP-1 and CCR2 antibodies added to leukocyte-conditioned medium. Interestingly, our *in vivo* experiments (229) confirmed these findings. We observed in murine heart transplant model, in which recipients received anti-MCP-1 antibodies, that anti-MCP-1 treatment significantly decreased either the number of CD45- and CD68-positive cells, or recipient-derived SMCs in the arterioles (229). Interestingly, MCP-1 was expressed to a much greater extent around small arterioles and in areas with increased cellular inflammation in the allografts.

Since adventitial PCs are a prominent source of recipient-derived cells in the graft and relevant constituent of intimal formation **(Paper IV)**, we aimed to investigate if MCP-1 has also an important role in their migration towards the intima. To do so, we used microDNA array assay to screen samples of rat allografts and found increased expression of the chemokines MCP-1, RANTES, SDF-1, and IP-10, and their receptors, CCR2, CCR5, CXCR4, and CXCR3. Moreover, we found that all these receptors were expressed at high levels in the adventitial cells. In our hands, Sca-1- or c-kit-positive mesenchymal SCs residing in vascular adventitia co-expressed a MCP-1 receptor, CCR2. Interestingly, staining of rat aortic allografts with anti-MCP-1 antibody revealed MCP-1 expression in the intima and adventitia at early phases of intimal formation, and prominent staining in the developed intima. CCR-2 was expressed in the adventitia at early stages of vascular remodeling, and in the intima and adventitia at advanced stages. We also observed **(Paper II)**, that MCP-1 is highly expressed in rat aortic allografts infected with CMV (7 fold increase compared to uninfected allografts), especially in the areas of the graft with high numbers of macrophages. Importantly, CMV also carries chemokines and chemokine receptor homologues that recruit and stimulate cellular infiltration, and thereby further potentiate inflammatory response. This scenario may contribute to the pro-inflammatory state in the vascular wall, resulting in release of inflammatory cytokines and chemokines that accelerate the inflammatory process and sustain viral replication (18) and uncontrolled vascular remodeling.

Taken together, these data suggest that inflammation and MCP-1 production are important for SCs migration towards the vascular intima and contribution to intimal hyperplasia. Thus, modulation of these pathways may lead to an improved understanding of not only vascular diseases but also the process of tissue repair.

7.2 CCL5 (RANTES), CCR1 AND CCR5

The CC-chemokine RANTES (*Regulated upon activation, normally T-expressed, and presumably secreted*) is stored in platelets and can be released upon platelets activation (230). Under flow conditions, platelet-derived RANTES can be immobilized on activated ECs in the presence of platelet P-selectin, and thereby supports monocyte adhesion and atherogenesis (231-233). RANTES has also been detected on endothelial

cells covering intimal lesions (232) and treatment of atherosclerotic apoE$^{-/-}$ mice with an antagonist for the RANTES receptors, CCR1 and CCR5, significantly reduced macrophage infiltration towards intima (233). In addition to a reduction in the macrophages content in the intima, CCR5 deficiency entailed an upregulation of the anti-inflammatory cytokine interleukin 10 (IL-10) in SMCs and was associated with a decrease in CD3+ T-lypmhocytes (234). Conversely, proinflammatory interferon-γ was increased in the intima of CCR1$^{-/-}$ mice, and its blockade unmasked a reduction in macrophage recruitment (234). In cardiac allograft vasculopathy, enhanced RANTES expression has been described in mononuclear cells infiltrating the coronary arteries, in endothelial cells of microvessels, and in intimal SMCs (235, 236). Furthermore, in a rat model of cardiac and aortic transplantation RANTES and its receptor, CCR5, along with interferon-γ-inducible protein (IP) 10-CXCR3 and MCP-1/CCR2, appear to be selectively upregulated in the arterial wall during intima formation (237).

7.3 CXCL12 (SDF-1) AND CXCR4

It has been shown that circulating progenitor cells (PCs) derived from bone marrow (BM) can be recruited to the injured arterial wall and contribute to vascular remodeling (98) and their mobilization from the bone marrow is regulated by the SDF-1 (*Stromal-derived factor 1*) (238). Transiently increased plasma levels of SDF-1 and an early and sustained upregulation of SDF-1 in SMCs following mechanical injury has been described in different models (99, 239-242) and has been shown to mediate the mobilization of circulating PCs (239). The effect of SDF-1 on PCs recruitment is dependent on the expression of the SDF-1 receptor CXCR4 in BMCs, since both intimal hyperplasia and SMCs content is diminished in hypercholesterolemic apoE$^{-/-}$ mice after BM reconstitution with fetal hematopoietic stem cells from CXCR4$^{-/-}$ mice (240). Interestingly, the infiltration of macrophages into the intima was not diminished by blocking the SDF-1/CXCR4 axis (239, 240). The injury-induced SDF-1 expression in arterial tissue and the degree of PCs recruitment to the intima appears to critically depend on the type of injury. For example, compared with carotid ligation, wire-induced injury produced the strongest SDF-1 expression (239) and was shown to correlate with the degree of apoptosis in SMCs.

BM-derived vascular PCs also contribute to intimal formation in transplant vasculopathy (71, 102, 108). In a murine aortic transplantation model, SDF-1 was upregulated in the adventitia and subsequently in the media and intima of aortic allogratfs (199). Interestingly, treatment with a neutralizing SDF-1 antibody effectively inhibited intimal formation in this model, and reduced the number of CXCR4-positive cells in the circulation and in the intima (199). These results suggest that SDF-1 promotes intima formation in transplant vasculopathy and after mechanical injury similarly by mobilization and recruitment of PCs.

7.4 CXCL10 (IP-10) AND CXCR3

IP-10 (*Interferon-γ- induced protein 10*) is a small cytokine belonging to the CXC chemokine family. IP-10 is secreted by several cell types in response to IFN-γ. These cell types include monocytes, endothelial cells and fibroblasts. IP-10 has been attributed to several roles, such as chemoattraction for monocytes/macrophages, T cells, NK cells, and dendritic cells, promotion of T cell adhesion to endothelial cells, antitumor activity, and inhibition of bone marrow colony formation and angiogenesis.
It has been previously demonstrated that IP-10 synthesis is markedly induced in reperfused canine (243) and murine (244) myocardial infarcts and suggested that its upregulation in the infarcted heart may be essential regulatory mechanism in infarct healing. Furthermore, it has been observed that blockade or depletion of CXCR3 severely attenuates recruitment of T helper lymphocytes type 1 (Th1) cells to the sites of inflammation (245, 246). In addition to enhanced infiltration of lymphocytes, the IP10 and its receptor are directly involved in the recruitment of hematopoietic PCs to an injured vessel. Interestingly, bone marrow–derived circulating PCs give rise to substantial numbers of intimal ECs and SMCs after endothelial denudation with controversial effects on intimal hyperplasia (98, 247, 248). CXCR3 is known to be expressed on CD34+ PCs derived from bone marrow and regulates adhesion and aggregation of these cells (249). As shown by double-immunofluorescence staining, c-kit+ circulating PCs expressed the chemokine receptor CXCR3 and were recruited to the site of the injured vessel. Likewise, blocking the CXCR3 axis significantly reduced the portion of c-kit+ cells in the intimal tissue after vascular injury. Beyond the recruitment of inflammatory T cells and circulating PCs, IP10 plays an essential role in the sustained induction of apoptosis after arterial injury.
T cell–induced apoptotic cell death of SMCs has previously been observed in a model of allograft atherosclerosis (250). In the model of wire-mediated arterial injury, the amount of apoptotic cells correlated positively with the number of recruited CD3+ T cells to the site of injury, emphasizing the impact of T cell–induced apoptosis in vascular remodeling (251).

7.5 IGF-1 AND IGF-1R

IGF-1 (*Insulin-like growth factor 1*) is produced primarily by the liver as an endocrine hormone as well as in target tissues in a paracrine/autocrine fashion. The primary action of IGF-1 is mediated by binding to its specific receptor, the Insulin-like growth factor 1 receptor (IGF-1R), present on many cell types. Binding to the IGF-1R, a receptor tyrosine kinase, initiates intracellular signaling. IGF-1 is one of the most potent natural activators of the serine/threonine protein kinase signaling pathway, a stimulator of cell growth and proliferation, and a potent inhibitor of programmed cell death. Interestingly, a variety of hormones and growth factors regulates IGF-1, IGF-1R expression in most tissues. Moreover, there is a crosstalk between IGF-1 signaling pathways and those of other growth factors and hormones. Thus, the physiological consequences of IGF-1 action are potentially altered by several mechanisms.
It has been shown that IGFs and their regulatory proteins, secreted by cells of the cardiovascular system, are growth promoters for arterial cells and mediators of

cardiovascular diseases (252-254) and changes in actions of these factors contribute to the progression of the disease. Interestingly, IGFs are potent stimuli of vascular SMCs migration (255, 256) and the effect appears to be mediated through IGF1R (256). IGFs also serve beneficial effects at the vessel wall after injury. A number of studies have shown that the production of elastin by vascular SMCs is increased by IGF-1 both *in vitro* (257) and *in vivo* (258). As a tunica media is stretched and damaged by balloon inflation, it is reasonable to assume that local IGF-1 production may play a role in regeneration of the elastin in the arterial wall.

In the early stages of restenosis, vascular SMC IGF-1 from human restenotic specimens is higher than in normal coronary vascular SMCs (259). However, restenotic tissue obtained several months after the intervention showed no IGF-1 mRNA expression (260). The predominant IGF-1 production and action in arterial media suggests that IGF-1 has a growth-promoting effect on SMCs after balloon injury. In fact, transgenic mice with IGF-1 overexpression show vascular smooth muscle hyperplasia (261).

8 METHOLOGICAL CONSIDERATIONS

8.1 ANIMAL MODELS

8.1.1 Transplantation procedure of murine heart

The vascularized cardiac hearts were harvested from Balb mice and transplanted into abdominal cavity of GFP transgenic mice recipient using a technique described previously (262, 263). Briefly, the donor aorta was attached to the recipient abdominal aorta by end-to-side anastomosis, and the donor pulmonary artery was attached to the recipient vena cava by end-to-side anastomosis. Heart recipients were monitored daily, and graft survival was examined by daily palpation through the recipient's abdomen wall. Cessation of beating was interpreted as rejection.
For further investigations, between days 5-10 after transplantation, mice received anti-MCP-1 or isotypic antibodies, and finally were collected for further investigations.

8.1.2 Transplantation of rat aorta

Rat aorta transplant model was used in this thesis to induce allogeneic-immune injury to the artery and further vascular remodeling in order to study cellular mechanisms responsible for intimal formation. Briefly, the infrarenal abdominal aorta was transplanted orthotopically from Fisher rats (F344) to Lewis rats (LEW), as allografts or isografts, using a surgical microscope. This technique was first described by Häyry P. *et al* as a model of transplant arteriosclerosis (264, 265). This transplant model is characterized by a low level of antigenicity, making immunosuppression unnecessary (266, 267). In this thesis, this model was further modified for three different reasons. First, in order to examine SMCs migration from an adjacent vessel, abdominal aorta from male LEW rats was transplanted into female LEW rats (isografts). Two weeks later, the F344 aorta was transplanted into the isograft to monitor side migration of the cells (Figure 7).
Second, in order to examine migration of adventitial cells towards the intima, adventitia was transplanted to the aortic abdominal allograft (see below). And third, to investigate the impact of local CMV infection on cellular activation and arterial graft morphology, a rat transplant model was used, in which aorta or adventitia were locally infected with rat CMV *ex vivo* and transplanted into the recipient rat.

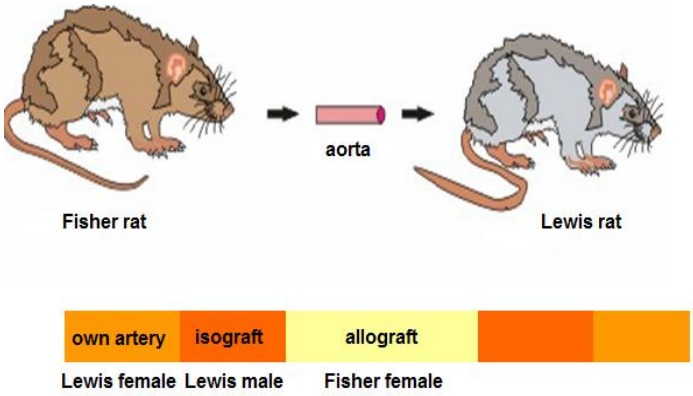

Figure 7. Modified rat aortic transplant model used to study side cell migration to the allograft.

8.1.3 Transplantation of adventitia

Transplantation of adventitia, as mentioned above, was used in order to study migration of adventitial cells *in vivo* towards the intima layer and their contribution in intimal formation. Briefly, four weeks after rat aortic allograft transplantation (F344 to LEW), isogenic adventitial cells (LEW to LEW) labeled with cell tracker, which does not leak to adjacent cells (Molecular Probes, Carlsbad, CA), were transplanted to the allograft by cuffing labeled adventitia around the previously transplanted allograft. Thereafter, two weeks after adventitia transplantation the allograft samples were collected and used for further investigations.

8.1.4 Bone marrow transplantation procedures

There are two methods for bone marrow (BM) transplantation for experimental purposes, *i.v.* transplantation of BMCs in suspension, or transplantation of vascularized BM by hind limb transplantation, both after sublethal animal irradiation. However, vascularized BM transplantation gives a better repopulation of hemo- and lymphopoiesis than transplantation of BMCs by infusion.
In this thesis, BM was transplanted from GFP transgenic mice to C57BL/6 mice to obtain chimeras by hind limb transplantation of syngeneic BM. The recipient mice were first irradiated with 8Gy to further receive vascularized BM by hind limb transplantation. The hind limb of the donor was amputated at the groin with the femoral and iliac arteries and veins dissected above this level to obtain long vascular stumps for anastomosis. The recipient hind limb was amputated at the mid-thigh level and the donor limb was attached by an intramedullary metallic stent in the femur. The blood vessels were connected by end-to-end anastomoses with muscles and skin sutured. The chimeric mice were further used to examine if BM-derived cells could participate in vascular remodeling after mechanical arterial injury by carotid ligation model, as described below.

8.1.5 Arterial injury in mice

8.1.5.1 Carotid artery ligation model

Mouse carotid artery ligation model was used as described elsewhere (268, 269) as a model of arterial injury in chimeric mice (C57BL/6 mice with BM from GFP transgenic mice) in order to investigate if BM-derived cells were able to migrate to the site of arterial injury (Figure 8). The choice of this type of chimeric mice allowed investigating the presence of green GFP-positive BM-derived cells in the injured arterial wall with help of confocal microscopy. Briefly, the left common carotid artery was exposed through a small midline incision in the neck. The artery was ligated proximal to the carotid bifurcation to disrupt blood flow. After 1 week post injury a 5 mm segment of the left carotid proximal to the suture was excised for further investigations.

8.1.5.2 Balloon catheterization of femoral artery

In this thesis, balloon injury of the mice femoral artery, followed by local infusion of BM-derived cells from GFP transgenic mice, was used in order to investigate the phenotype and contribution of BM-derived cells in the arterial remodeling (Figure 8). Briefly, the right femoral artery was exposed to the level of bifurcation through a transabdominal incision. Microclamps were placed on the lower aorta, left iliac artery, and the distal part of the right femoral artery. A 2-F Fogarty balloon catheter (Baxter, Deerfield, IL) was introduced into the right femoral artery, inflated, and withdrawn three times with rotation. Inflation was performed through the cannula and 1 ml of Ringer's solution with free outflow through the microincision. BMCs from GFP mice in RPMI medium were infused and incubated in the freshly injured arterial bed of C57BL/6 or C57BL/6 Nude mice for 15 to 20 minutes and blood flow was restored by removing the microclamps. Arteries were harvested at 1, 2, 7 and 14 days after surgery for further investigations.

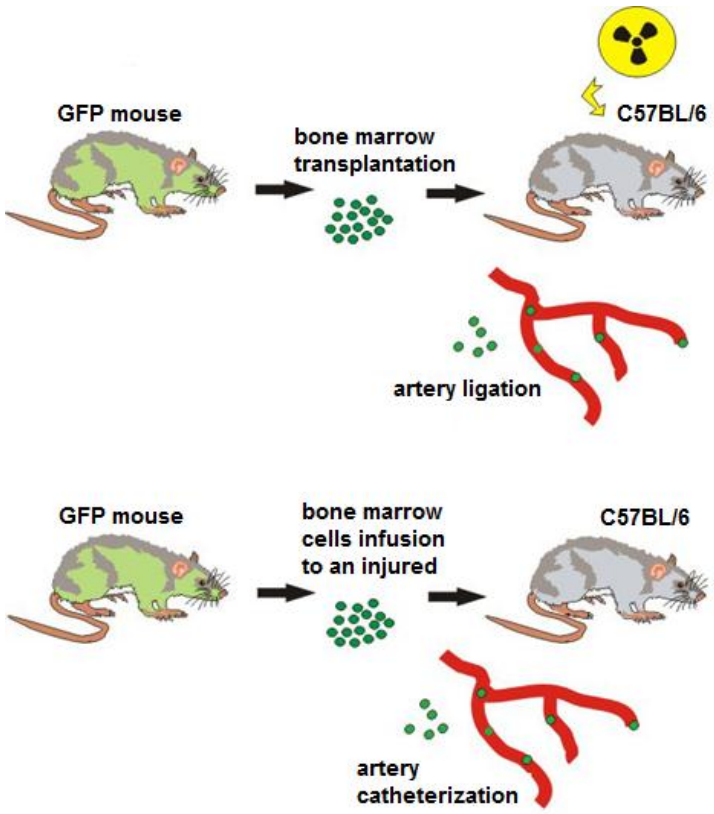

Figure 8. Murine models used to study a contribution of bone marrow-derived cells in arterial repair after whole bone marrow transplantation with subsequent carotid artery injury (upper picture); and after local delivery of bone marrow-derived cells to a previously injured femoral artery (lower picture).

8.2 CELL CULTURE TECHNIQUES

8.2.1 BMCs, SMCs and adventitial cells isolation and culture

Cell culture is the complex process by which cells are grown under controlled conditions. It is a fundamental component of tissue culture and tissue engineering, as it establishes the basics of growing and maintaining cells *ex vivo*, which can be used for further experimental studies or directly for the clinical needs. The concept of maintaining live cell lines separated from their original tissue source was discovered in the 19th century when Wilhelm Roux removed a portion of the medullary plate of an embryonic chicken and maintained it in a warm saline solution for several days, establishing the principle of tissue culture. Later, Ross Granville Harrison, working at Johns Hopkins Medical School and then at Yale University, published results of his experiments from 1907–1910, establishing the methodology of tissue culture. Indeed, cells can be isolated from tissues in several ways. They can be released from soft tissues by enzymatic digestion with enzymes, such as collagenase or trypsin, which break down the extracellular matrix. Alternatively, pieces of tissue can be placed directly in growth media, and the cells that grow out are available for culture. This method is known as explant culture. Further, cells are grown and maintained at an

appropriate temperature and gas mixture (typically, 37°C, 5% CO2 for mammalian cells) in a cell incubator. Culture conditions vary widely for each cell type, and variation of conditions for a particular cell type can result in different phenotypes being expressed. Aside from temperature and gas mixture, the most commonly varied factor in culture systems is the growth medium. Recipes for growth media can vary in pH, glucose concentration, growth factors, and the presence of other nutrients. The growth factors used to supplement media are often derived from animal blood, such as fetal calf serum (FCS). As cells generally continue to divide in culture, they generally grow to fill the available area or volume. Thus, they need passaging (also known as subculture or splitting cells), which involves transferring a small number of cells into a new culturing vessel. All manipulations carried out on culture cells rely on sterile technique. Sterile technique aims to avoid contamination with bacteria, yeast, or other cell lines. Manipulations are typically carried out in a biosafety hood or laminar flow cabinet to exclude contaminating micro-organisms.

In this thesis, cells derived from bone marrow, SMCs and adventitial cells originating from murine and rat arteries were isolated and manipulated in culture. BMCs were isolated as previously published (270, 271) by flushing the tibias and femurs with a 22 gauge needle using a syringe filled with RPMI-1640 medium. Flushing was done forcefully enough to break up clumps, but not so forcefully to damage the cells.

Thereafter, cells were incubated in serum-free EGM-2 medium (Cambrex) with PDGF-BB (50 ng/ml) or VEGF (50 ng/ml) in order to achieve a SMC- or EC phenotypes, respectively, already at 1 week of culture. For the studies based on local delivery of BMCs to the injured arteries, we used freshly isolated BMCs that were subsequently isolated for distinct phenotypes (CD34+, VEGF+, CD14+, CD3+) using fluorescence-activated cell sorting technique (see below), and immediately delivered to injured arteries. Rat medial SMCs and adventitial cells were isolated from a rat aorta by collagenase digestion and cultured for one passage in F12/10% FCS medium as previously described (109). The cultures were grown to confluency and used for migration experiments *in vitro* using Boyden chamber assay.

8.2.2 Cell fusion assay

Cell fusion is found in many sexually reproducing organisms that require fertilization or mating to propagate. Diverse organisms form multinucleate cells in organs such as muscles, placenta, bones, lens of the eye and stem cells, although most cells do not fuse during development. In recent years, it has become apparent that cells of different origin may fuse with one another, thus adopting properties of the fusion partner.

Here, cell fusion was detected by using Cre/lox system. In this system, β-galactosidase is activated when cells fuse, and blue staining is observed in cells after the addition of NTB substrate.

Fusion assays were performed to examine whether SMCs can fuse with adventitial fibroblasts, Sca-1-, or c-kit-positive cells. Briefly, SMCs from CMV-Cre mice and adventitial cells from flox-LacZ mice were isolated by collagenase digestion. The adventitial cells were stained with antibodies against Sca-1 or c-kit with or without antibodies against CCR2 and transferred to FACS tubes in order to obtain cell populations of desired phenotypes. These sorted adventitial cells were co-cultured with

CMV-Cre SMCs for 72h, and thereafter the substrate was added. After culturing, the cells were fixed and analyzed for β-galactosidase by X-Gal staining. CMV-Cre-negative SMCs were used as negative controls. As a positive control, the flox-LacZ SMCs and CMV-Cre SMCs were infected at a total of 300 viral particles per cell with adenoviruses encoding nuclear target LacZ and CRE, respectively. The integrity of the reporter was confirmed by X-Gal staining.
Similarly, cell fusion assays were also performed to examine whether SMCs can fuse with BMCs isolated for endothelial- and early myeloid cell markers (CD31, CD34, CD11b, VEGFR-2, CD34/CD31, VEGFR-2/CD11b).

8.2.3 Boyden chamber assay

The Boyden chamber is a useful tool to study cell chemotactic ability. It consists of a cylindrical cell culture insert nested inside the well of a cell culture plate. The insert contains a polycarbonate membrane at the bottom with a defined pore size. Briefly, cells are seeded in the top of the insert in serum-free media, while serum or similar chemoattractants are placed in the well below. Migratory cells move through the pores toward the chemoattractant below and can be stained or quantified in a plate reader.
In this thesis, the migration of SMCs and adventitial cells were measured in response to MCP-1, RANTES, SDF-1, IP10, and leukocyte conditioned medium. The migration inhibition experiments were also done using this method and antibodies against MCP-1 or its receptor, CCR-2. The cells (60,000/well) resuspended in culture medium were seeded into the upper chamber and chemoattractants of interest or their inhibitors (in inhibition experiments) were added in the lower chamber. After 6h of incubation, the medium was removed, and cells attached to the bottom of the filter were fixed in 99% methanol, stained with a Giemsa solution, and quantified in light microscope.

8.2.4 Fluorescence- activated cell sorting

Flow cytometry is a technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them by electronic detection equipment. It allows simultaneous multiparametric analysis of the physical and chemical characteristics of up to thousands of particles per second.
Briefly, a beam of light (usually laser light) of a single wavelength is directed onto a hydrodynamically-focused stream of fluid. A number of detectors are aimed at the point where the stream passes through the light beam: one in line with the light beam (Forward Scatter, FSC) and several perpendicular to it (Side Scatter, SSC) and one or more fluorescent detectors. Each suspended particle from 0.2 to 150 micrometers passing through the beam scatters the ray, and fluorescent chemicals found in the particle or attached to the particle may be excited into emitting light at a longer wavelength than the light source. This combination of scattered and fluorescent light is picked up by the detectors, and by analysing fluctuations in brightness at each detector (one for each fluorescent emission peak), it is then possible to derive various types of information about the physical and chemical structure of each individual particle. FSC correlates with the cell volume and SSC depends on the inner complexity of the

particle, such as shape of the nucleus, the amount and type of cytoplasmic granules or the membrane roughness.
In this thesis, a special type of flow cytometry, fluorescence-activated cell sorting, was used in order to isolate distinct populations of interest derived from BMCs and arterial adventitia. This technique allows sorting a heterogeneous mixture of biological cells into two or more containers, one cell at a time, based upon the specific light scattering and fluorescent characteristics of each cell. It is a useful scientific instrument, as it provides fast, objective and quantitative recording of fluorescent signals from individual cells as well as physical separation of cells of particular interest. Briefly, the cell suspension is entrained in the center of a narrow, rapidly flowing stream of liquid. The flow is arranged so that there is a large separation between cells relative to their diameter. A vibrating mechanism causes the stream of cells to break into individual droplets. The system is adjusted so that there is a low probability of more than one cell per droplet. Just before the stream breaks into droplets, the flow passes through a fluorescence measuring station where the fluorescent character of interest of each cell is measured. An electrical charging ring is placed just at the point where the stream breaks into droplets. A charge is placed on the ring based on the immediately-prior fluorescence intensity measurement, and the opposite charge is trapped on the droplet as it breaks from the stream. The charged droplets then fall through an electrostatic deflection system that diverts droplets into containers based upon their charge. In some systems, the charge is applied directly to the stream, and the droplet breaking off retains charge of the same sign as the stream. The stream is then returned to neutral after the droplet breaks off.

8.2.5 RCMV propagation

RCMV (RC 127, Maastricht strain, 2.1 x 10^6 pfu/ml; MOI, 3.5 x 10^{-2}) was isolated from wild rats (272). RCMV was propagated by infecting fibroblasts prepared from 17-day-old rat embryos. The supernatant was used for *in vivo* infection. Fibroblasts were cultured in flasks containing modified Eagle's minimum essential medium supplemented with L-glutamine, penicillin/streptomycin and 10% FCS. At confluency, the cells were infected with RCMV according to the standard viral culture techniques and maintained in culture medium (as above) supplemented with 2% FCS. After around 1 week, when a cytopathic effect was observed in at least 95% of the cells, the cells were detached from the bottom of the flask and the supernatant containing free RCMV was collected.

8.3 MOLECULAR BIOLOGY

8.3.1 Immunohisto- and immunocytochemistry

Immunochemical assays are based on a localization of antigens in tissue sections (immunohistochemistry) or cell cultures (immunocytochemistry) by the use of labeled antibody as specific reagents through antigen-antibody interactions that are visualized by a marker such as fluorescent dye, enzyme, radioactive element or colloidal gold. This technique has become a crucial technique and widely used in many medical

research laboratories as well as clinical diagnostics. While using the right antibodies to target the correct antigens and amplify the signal is important for visualization, complete preparation of the sample is critical to maintain cell morphology, tissue architecture and the antigenicity of target epitopes. The latter requires proper tissue collection, fixation and sectioning. Because of the method of fixation and tissue preservation, the sample may require additional steps to make the epitopes available for antibody binding, including deparaffinization and antigen retrieval. Additionally, depending on the tissue type and the method of antigen detection, endogenous biotin or enzymes may need to be blocked prior to antibody staining. Although antibodies show preferential avidity for specific epitopes, they may partially bind to sites on nonspecific proteins that are similar to the cognate binding sites on the target antigen. In the context of antibody-mediated antigen detection, nonspecific binding causes high background staining that can mask the detection of the target antigen. To reduce background staining, the samples are incubated with a buffer that blocks the reactive sites to which the primary or secondary antibodies may otherwise bind. Common blocking buffers include normal serum, non-fat dry milk, BSA or gelatin, and commercial blocking buffers with proprietary formulations are available for greater efficiency. For immunohistochemistry detection strategies, antibodies are classified as primary or secondary reagents. Primary antibodies are raised against an antigen of interest and are typically unconjugated (unlabelled), while secondary antibodies are raised against immunoglobulins of the primary antibody species. The secondary antibody is usually conjugated to a linker molecule, such as biotin, that then recruits reporter molecules, or the secondary antibody is directly bound to the reporter molecule itself. Reporter molecules vary based on the nature of the detection method, and the most popular methods of detection are with enzyme- and fluorophore-mediated chromogenic and fluorescent detection, respectively. With chromogenic reporters, an enzyme label is reacted with a substrate to yield an intensely colored product that can be analyzed with an ordinary light microscope. While the list of enzyme substrates is extensive, alkaline phosphatase (AP) and horseradish peroxidase (HRP) are the two enzymes used most extensively as labels for protein detection. An array of chromogenic, fluorogenic and chemiluminescent substrates is available for use with either enzyme, including DAB or BCIP/NBT, which produce a brown or purple staining, respectively, wherever the enzymes are bound. Fluorescent reporters are small, organic molecules used for immunohistochemical detection, such as FITC, TRITC, Alexa Fluors or Dylight Fluors. The direct method is a one-step staining method and involves a labeled antibody (e.g. FITC-conjugated antiserum) reacting directly with the antigen in tissue sections. The indirect method involves an unlabeled primary antibody that binds to the target antigen in the tissue and a labeled secondary antibody that reacts with the primary antibody. This method is more sensitive than direct detection strategies because of signal amplification due to the binding of several secondary antibodies to each primary antibody if the secondary antibody is conjugated to the fluorescent or enzyme reporter. Further amplification can be achieved if the secondary antibody is conjugated to several biotin molecules, which can recruit complexes of avidin-, streptavidin-enzyme. After immunohistochemical staining of the target antigen, a second stain is applied to help the primary stain stand out. Both chromogenic and fluorescent dyes are available to

provide a vast array of reagents to fit every experimental design, and include: hematoxylin, DAPI, propidium iodide.
In this thesis, most primary antibodies were non-conjugated and detected with appropriate pre-conjugated secondary antibodies. The stainings were performed on paraffin-embedded, frozen and whole tissue, as well as in the cell culture systems. HRP and AP systems were used for antibodies visualization, and developed with DAB as a substrate for HRP or BCIP/NBT as a substrate for AP. When double staining was performed, the first staining was developed with BCIP/NBT followed by incubation with HRP-conjugated anti-SM-α-actin antibodies, which were detected with DAB system. The sections were counterstained with QS-hematoxyline or Vector Red. Detection of antigens of most frozen tissue sections and cell cultures was performed with help of fluorescent reporters, such as FITC and Alexa Fluors. In the whole mount staining method, the detection was performed with cyanine dyes (*Cy*5)-conjugated antibody in the whole arterial segments.

8.3.2 Detection of collagen

In this thesis, the Masson's trichrome staining was used in order to identify collagen deposits in the media layer of the arterial sections. In this method, collagen fibers are stained blue, nuclei are stained black and the background is stained red. Briefly, as the name implies, three dyes are employed selectively staining muscle, collagen fibers, fibrin and erythrocytes. The general rule in trichrome staining is that the less porous tissues are colored by the smallest dye molecule. Whenever a dye of large molecular size is able to penetrate, it will always do so at the expense of the smaller molecule. Masson's method is a sensitive technique for displaying fine collagen fibres, basement membranes, fibrin and hyaline.

8.3.3 In situ detection of chromosome Y

Detection of the SRY gene in chromosome Y was used for *in situ* visualization of recipient-derived cells in the arterioles of human cardiac allografts. The technique is based on fluorescent *in situ* hybridization (FISH) and primed *in situ* labeling (PRINS). FISH was described for the localization of nucleic acids in tissue by Pardue and Gall (273). This method involves three steps: fixation of the specimen, hybridization of the labeled probe to homologous fragments of genomic DNA, and fluorescent detection of the labeled probe. Here, FISH was performed using DNA Detector (KPL, Gaithersburg, MD) with the probe prepared using PCR DNA biotinylation kit (KPL, Gaithersburg, MD). *Cy*2-streptavidin and propidium iodide were used for staining of probe and nuclei. The sections were examined in a laser scanning confocal microscope. The *in situ* techniques of chromosome Y detection cannot be used for quantitative analysis of cellular origin because of difficulties to estimate sensitivity.

8.3.4 Laser capture microdissection

In order to distinguish the origin of SMCs in the arterioles of human cardiac allografts, where recipient and donor were mismatched in sex, these SMCs were microdissected in histological specimens using a laser capture microdissection (LCM) equipment (LCM, PixCEll II System, Arcturus Engineering, Mountain View, CA). The samples were prepared for immunohistochemistry as described above, dehydrated with ethanol followed by xylene and then anhydrous $CuSO_4$ in a dessicator. Approximately 200-400 cells were identified and microdissected. The percentage of host-derived cells among the captured cells was thereafter estimated by RT-PCR for the SRY gene (see below).

8.3.5 Real time PCR

In this thesis, real-time PCR (RT PCR) was used for the quantification of male cells in the studied material. RT PCR reflects the initial amount of the template and is the most specific, sensitive and reproducible methodology available for detection and quantification of genetic material. The RT PCR system is based on the detection and quantification of a fluorescent reporter present on a molecular probe. The signal increases in a direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emitted in each cycle it is possible to monitor the PCR reaction during an exponential phase, where the first significant increase in the amount of PCR product correlates to the initial amount of target template. Therefore, it is a true measurement of the amount of the substrate used. Primers and probes for multiplex RT PCR were designed using Perkin Elmer software (Foster City, CA). DNA was extracted using a commercial DNA extraction kit from Perkin Elmer (Foster City, CA). The SRY-specific probe was labeled with FAM and the probe for a 'house-keeping gene' (angiotensin II) was VIC-labeled. The reaction was performed in multiplex using the 7700 Sequence Detection System (Perkin Elmer). The quantification was performed by experimental determination of the cycle threshold values defined as the cycle at which the fluorescence exceeded 10 times the standard deviation of the mean baseline emission for the earlier cycles. Using the baselines determined for both of the genes, the fraction of the SRY gene with respect to the 'house-keeping gene' was found.

8.3.6 DNA microarray assay

A DNA microarray is a multiplex technology. It consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides, each containing picomoles of a specific DNA sequence (called probes). These can be a short section of a gene or other DNA fragment that is used to hybridize a cDNA or cRNA sample (called target). Probe-target hybridization is usually detected and quantified by detection of fluorophore-, silver-, or chemiluminescence-labeled targets to determine relative abundance of nucleic acid sequences in the target. Since an array can contain tens of thousands of probes, a microarray experiment can accomplish many genetic tests in parallel. Therefore arrays have dramatically accelerated many types of investigation.

In standard microarrays, the probes are attached via surface engineering to a solid surface by a covalent bond to a chemical matrix. The solid surface can be glass or a silicon chip (Affymetrix chip). DNA microarrays can be used to measure changes in expression levels, to detect single nucleotide polymorphisms (SNPs), or to genotype or resequence mutant genomes

In this thesis, RNA processing and hybridization to rat gene expression Affymetrix arrays were performed. In brief, the Affymetrix standard protocol was used to generate three labaled cDNA samples from each tissue pool using 5 μg of total RNA as starting material. Affymetrix Micro Array Suite Software (MAS) version 5.0 was used to analyze the scanned images, convert intensities to a numerical format, and obtain a signal value for each probe on the array. Target intensity values from each array were scaled to a value of 100 to allow different arrays to be compared. The statistical algorithms have been described in detail (274).

8.4 MORPHOMETRIC ANALYSES

This is a method whereby the amounts of particular tissue or subcellular components are quantified. These components may be, for example, particular cell types (at the tissue level), or the nucleus, mitochondria or secretory vesicles, for example, at the cellular level. To do this, the area of the particular component of interest and the total (tissue or cellular) area need to be determined. This used to be achieved by what was termed 'point counting', which involved the overlaying of an acetate sheet - with points regularly dotted upon it - over a photographic image. Nowadays, in the age of digital imaging, the areas of interest can be determined more quickly and accurately. Once these areas have been determined the actual volume fraction can be stated within the tissue or cell that a particular component occupies because in a very thin section area/area is equivalent to volume/volume. Briefly, in practice an initial digital image is taken and mapped for suitable contrast with image analysis software. Since a digital image is composed of pixels with a numeric value, the selected area of interest is easy quantified by the analysis system.

Here, the LeicaQWin software was used to measure cross-sectional areas of intima and media layer of rat aortic allografts in the images of tissue stained with hematoxylin. Furthermore, computer-assisted histomorphometric image analysis in Adobe Photoshope was used to determine collagen deposits, MCP-1 expression, MMPs content in the cross-sectional areas of the immunostained rat aortic allografts. The latter system was also helpful to estimate a number of GFP-positive BM-derived cells in the fluorescently stained sections of injured by balloon catheterization murine arteries.

8.5 MICROSCOPY

8.5.1 Confocal microscopy

Laser scanning confocal microscopy (LSCM) is based on point scanning principles. A laser beam is expanded to make optimal use of the optics in the objective and focused to a small spot by the objective lens onto a fluorescent specimen. The reflected light is deviated by a diachronic mirror while the emitted fluorescent light from samples is

passed through to the photodetector. A confocal aperture is placed in front of the photodetector to obstruct fluorescent light from point on the specimen that is not within the focal plane. These points are largely obstructed by the pinhole, whereas the focused object enters the center of the pinhole. In this thesis, LSCM was used to obtain high resolution fluorescence images of specimens for chromosome Y detection. This technique allowed qualitative visualization of male cells in the allografts and permitted localization of the host-derived cells in the grafts. Furthermore, the confocal microscopy technique was also used to detect fluorescently labeled adventitial cells that migrated towards intima, as well as the localization of GFP-positive bone marrow-derived cells in the vascular layers. All fluorescence images of cell cultures in order to detect SMC, EC and their progenitor phenotypes were obtained with LSCM.

8.5.2 Electron microscopy

Electron microscopy (EM) was used to analyze the ultrastructure of the transplanted vessels and the adjacent vessels with respect to cellular composition, topography and morphology. These studies allowed estimation of the phenotypic modulation of SMCs in the media layer of the graft and adjacent vessel that occurred early after transplantation. Tissue preparation for EM involves fixation, dehydration and embedding in low-viscosity epoxy resins that polymerize to give a material suitable for the preparation of ultrathin sections. The main goal of this process is to stabilize and preserve the fine structural details of the fixed cells as close to that of cells in the living tissues. Glutaraldehyde and osmium tetroxide have been applied as the most effective fixatives for EM. Glutaraldehyde stabilizes tissues by cross-linking proteins. Osmium tetroxide reacts with lipids and certain proteins but also provides electron density to the tissue. Block staining with uranyl acetate was used to further enhance the electron density of the material. Thin sections were cut with diamond knives on an ultramicrotome. SMCs were analyzed and classified as contractile or synthetic in phenotype according to standards described by Thyberg *et al* (275). During the process of phenotypic modulation, contractile SMCs lose contractile myofilaments whereas the amount of endoplasmatic reticulum and Golgi apparatus (synthetic organelles) increase. The density of different organelles and extracellular structures were determined by point counting morphometry.

9 CONCLUDING REMARKS

The existing paradigm states that vascular progenitor cells are found in the vasculature and contribute to repair of injured blood vessel tissue. However, the mechanism underlying the integration of these cells into the vasculature, the origin of these cells and specific functions are still under debate. This thesis presents a new understanding to this concept. Indeed, we were able to confirm that adult vascular progenitor cells exist and possess a potential that extends beyond the cell types of their resident tissue. The vascular progenitor cells migrate to the sites of vascular injury and are recruited either from bone marrow or blood vessel tissue in response to inflammation.
Although, the cells derived from these sources are able to inhibit intimal hyperplasia, their contribution to the formation of intimal lesion is not equal.

In **Paper I**, we provided evidence that inflammation and MCP-1 protein are pivotal in the recruitment of recipient-derived cells of smooth muscle cell phenotype into arterioles of transplanted human hearts. We have not indicated the source of these cells however proposed that they could have reached the graft through the circulation. Recipient-derived cells in the arterioles of each cardiac biopsy accounted for a mean of 3.4±2.3% of cells with smooth muscle cell phenotype. Since the number of these cells was not related to the time between transplantation and biopsy in our study, we hypothesized that recruitment of recipient-derived cells is facilitated by other factors, such as immune-mediated damage of the organ after transplantation. Indeed, the number of accumulated recipient-derived cells in the graft correlated strongly with the number of CD45-positive leukocytes and the grade of rejection, confirming that inflammation is strongly related to the recruitment of circulating progenitor cells into the graft vessels. This knowledge may be useful in designing protocols aiming to increase the number of progenitor cells in order to limit tissue damage and facilitate healing at sites of tissue injury.

In **Paper II**, we showed that enhanced inflammation in rat arterial allograft by acute infection with Cytomegalovirus (CMV) led to enhanced local MCP-1 production in the vasculature. Interestingly, CMV potentiated inflammation mainly in the adventitia, which resulted in migration of adventitial cells towards the intima and more rapid and severe intimal hyperplasia. Furthermore, we showed that RCMV increased smooth muscle cell apoptosis in the media layer of the allograft, which contributed to an earlier and enhanced destruction of this layer. These alterations led to further fibrosis and stimulation of intimal hyperplasia. Furthermore, decreased collagen content in the infected allografts and increased expression of MMP-2, which regulates collagen turnover in the vessel wall, indicate that virus directly or by interacting with host immunity reduced the integrity of the artery wall and thereby increased tissue vulnerability to injury, inflammation and uncontrolled remodeling. Our findings increase understanding of the role of pathogens, such as CMV in vascular remodeling and highlight that adventitial cells are able to migrate *in vivo* towards the sites of arterial injury, most likely in response to MCP-1.

In **Paper III**, we proposed that bone marrow-derived cells provided reparative and protective function at early stages of arterial injury and afterwards were eliminated possibly by apoptosis or had low survival rate in the artery wall. Bone marrow-derived cells localized in the arterial intima and the majority of them expressed endothelial cell markers (CD31, CD34, VEGFR-2) indicating their endothelial phenotype. Interestingly, the distribution of BMCs in the intima was not equal. In the few areas, where the intima was thicker, BMCs were of SMC phenotype and expressed mainly SM-actin and myosin. Furthermore, bone marrow-derived cells did not fuse with vascular cells however changed phenotype to SMCs or ECs to adjust in the vessel wall and meet the demands and needs of their new microenvironment. Interestingly, local delivery of bone marrow-derived endothelial cells to the sites of arterial injury resulted in 1.4 fold decrease in the area of intimal lesion, which indicate the importance of these cells in the inhibition of intimal formation, and thereby their role in vessel healing. Our results highlight the importance of bone marrow-derived endothelial cells in the early stages of intimal formation and propose that vascular progenitor cells derived from other sources than bone marrow might be a major constituent of developed intimal lesion. These findings newly define the role of bone marrow-derived cells in the development of intimal lesions after vascular injury and add to the current understanding of the pathogenesis of intimal formation.

In **Paper IV**, we identified mesenchymal tissue-derived progenitor cells in vascular adventitia (Sca-1/CCR2, c-kit/CCR2) that contributed to vascular remodeling in a rat model of transplant vasculopathy. Interestingly, early proliferation of cells in the adventitia coincided with an increase in the number of apoptotic cells in the media, and both proliferation and apoptosis were associated with inflammation. The predominant source of intimal cells in the vessels of transplanted organs has been for long a matter of debate. Previously, phenotypically modulated SMCs in the media were considered to be the only source. However, medial SMCs are often damaged by the allogeneic response and undergo apoptosis, and their migratory capacity is quite low *in vivo*, making them an unlikely source of intimal cells in transplanted organs. Bone marrow-derived cells have been also proposed to be a major source of cells in intimal lesion however we and others have been unable to confirm this possibility (**Paper III**). Therefore, we focused our studies on progenitor cells in the allograft or adjacent vessel. Medial SMCs in the adjacent vessel changed their phenotype to synthetic but did not migrate to the allograft. Instead, adventitial cells proved to be the main source of cells (over 50%) contributing to intimal hyperplasia. We found that approximately 20% of adventitial cells from rat aorta expressed Sca-1, and 4% expressed c-kit and were able to differentiate to SMCs. Thus, Sca-1- and c-kit-positive mesenchymal progenitor cells in the adventitia could be a key source of neointimal cells. We confirmed and strengthen our findings from **Paper I** and **Paper II** by showing that inflammation and MCP-1 production were pivotal also in the migration of mesenchymal progenitor cells derived from adventitia towards the intima. Thus, modulation of these pathways may lead to an improved understanding not only of the pathology of transplant vasculopathy and other vascular diseases, such as atherosclerosis or restenosis, but also tissue repair process.

In summary, this thesis presents novel evidence showing that inflammation and MCP-1 are important for recruitment of vascular progenitor cells to the sites of arterial injury and suggests tissue-derived mesenchymal progenitor cells, here derived from arterial adventitia, as a key source of cells for vascular repair. We believe that knowledge presented here not only increases understanding of vascular pathology but also provides unique value for understanding of unraveled aspects of tissue repair process. There is increasing enthusiasm for the use of adult stem cells for cell transplantation therapy and tissue engineering. Nevertheless, additional studies are needed in both cell culture systems and translational animal models to better define the phenotype of vascular progenitor cells, and characterize the specific function of these cells in the injured arteries. Furthermore, in considering preclinical animal studies for cellular therapies to evaluate the pharmacokinetic and pharmacological properties of the cells, the following parameters must be considered and understood to facilitate the design of human clinical studies:

1. What are the appropriate cell phenotypes, doses and the effect of increasing doses?
2. How will the cells be delivered into the artery, in what formulation?
3. How many treatments are likely to be required?
4. Do the implanted cells migrate into tissues other than the target artery tissue?
5. What is the tumorigenicity of the administered cells?

By moving from small molecule drug development to cellular therapy, the standard development model must be modified and adapted for the specific therapy under consideration. Design of a development program for a cell therapy would begin with these general areas but would be modified as the product is better defined.

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