Redefining endothelial progenitor cells using a proteomics approach

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REDEFINING ENDOTHELIAL PROGENITOR CELLS USING A PROTEOMICS APPROACH

A thesis submitted for the degree of Doctor of Philosophy to
King’s College London

By
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The James Black Centre
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ABSTRACT

The concept of endothelial progenitor cells (EPCs) has attracted considerable interest in cardiovascular research, but despite a decade of research there are still no specific markers for EPCs and results from clinical trials remain controversial. To identify membrane proteins, which could serve as potential markers for EPCs, an alternative proteomic method was adopted to obtain sufficient membrane material for proteomic analysis. Microparticles (MPs), the intact vesicles formed from the plasma membrane, were harvested from the conditioned medium of EPC cultures and their protein composition was analysed by liquid chromatography–tandem mass spectrometry. Surprisingly, the platelet-specific integrin alpha IIb emerged as the most abundant integrin in EPC cultures. Subsequent experiments confirmed that the conventional methods for isolating peripheral blood-derived mononuclear cells (PBMNCs) lead to a substantial contamination with platelets. Notably, platelets readily disintegrate into platelet MPs when activated during PBMNCs isolation conditions. These platelet MPs are taken up by the PBMNCs, which acquire “endothelial” characteristics (CD31, von Willebrand factor [VWF], lectin-binding), and angiogenic properties. In a large population-based study (n = 526), platelets emerged as a positive predictor for the number of colony-forming units and early outgrowth EPCs. This study provides the first evidence that the cell type consistent with current definitions of an EPC phenotype may arise from an uptake of platelet MPs by mononuclear cells resulting in a gross misinterpretation of their cellular progeny. In addition, the release of platelet MPs at sites of vascular injury may play a role in orchestrating tissue repair and contribute to the activation of an angiogenic programme in monocytes by inducing the expression of non-coding regulatory RNA, known as microRNAs, which act as translational repressors. The uptake of platelet MPs by mononuclear cells induced the release of the CXCL7 chemokine which in turn guided a de novo induction of miR-885-5p in mononuclear cells. The increased expression of miR-885-5p resulted in the targeted reduction of the actin-bundling protein LCP-1, facilitating the adhesive and migratory ability of mononuclear cells. These findings demonstrate the advantage of using an unbiased proteomic approach to assess cellular phenotypes and advise caution in attributing the benefits in
clinical trials using unselected bone marrow mononuclear cells (BMCs) to stem cell-mediated repair.
DEDICATION

In loving memory of my parents...
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ABBREVIATIONS

1D SDS-PAGE: 1 dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis
2DE: 2-dimensional gel electrophoresis
CFU: colony forming unit
CM: conditioned medium
Cy2/Cy3/Cy5: fluorescent dyes of the cyanine dye family
DAPI: 4',6-diamidino-2-phenylindole (double stranded DNA staining)
DIGE: difference in gel electrophoresis
EPC: endothelial progenitor cell
HUVEC: human umbilical vein endothelial cell
IPG strip: Immobiline DryStrip Gels
LC/MS/MS: liquid chromatography, tandem mass spectrometry
LTQ: Linear Trap Quadrupole
miRNA: micro RNA
MP: microparticle
mRNA: messenger RNA
NOD/SCID: non obese diabetic/severe combined immunodeficient mice
O/N: over night
PBMNC: peripheral blood mononuclear cell
PBS: phosphate buffered saline
RT-PCR: reverse transcription
SILAC: Stable Isotope Labelling With Amino Acids In Cell Culture
THP-1: human acute monocytic leukaemia cell line
TNF-α: Tumour necrosis factor alpha
VEGF: vascular endothelial growth factor
vWF: von Willebrand factor
CHAPTER I

INTRODUCTION
1.1 Stem cell therapy for cardiovascular disease?

Cardiovascular diseases (CVD) are the leading cause of death worldwide (Lopez et al., 2006) (Diwan and Dorn, 2007). Major progress has been made in the diagnosis and treatment of CVD. However, cardiac injury cannot be reversed. Hence, the concept of stem cell-based therapeutic strategies is attracting considerable attention. Stem cells have the capacity for self-renewal, infinite ex vivo proliferation and their ability to differentiate into specialized cells. Stem cells can be classified into two major categories: embryonic stem cells (ESC) and non-embryonic (somatic or adult) stem cells. ESCs have the ability to differentiate into cells from all three embryonic germ layers (Thomson et al., 1998), and they can give rise to all cardiac cell types (Yamada et al., 2008). In contrast, adult stem cells are restricted in their differentiation potential to cell lineages of the organ, in which they are located. For example, hematopoietic stem cells (HSC) can give rise to mature blood cells, mesenchymal stem cells (MSC) are multipotent and differentiate into a variety of cells including osteoblasts, chondrocytes, and adipocytes. Ethical concerns, the risk of teratoma formation as well as rejection from the immune system are obstacles that need to be overcome before any potential use of ESCs for stem cell therapy. Thus, the cardiovascular community focussed on the therapeutic application of adult stem cells (Stastna et al., 2009).

Potential mechanisms of stem cell action to treat heart disease may include a) re-muscularisation of the heart through transdifferentiation of stem cells into viable cardiomyocytes, b) reduction of the cardiac muscle remodelling, c) angiogenesis and arteriogenesis through endothelial progenitor cells (EPCs), d) beneficial effects derived from the paracrine mediators secreted by the transplanted stem cells, e) stem cell fusion to improve the function of cardiomyocytes f) mobilization of stem cells from the bone marrow, g) immunomodulation and h) promotion of the endogenous tissue repair via resident cardiac stem cells (CSCs) (Mollmann et al., 2009, Zimmet and Krum, 2008). The transplanted stem or progenitors cells could engraft into the injured myocardium, transdifferentiate into cardiac cells and generate new cardiomyocytes resulting in improvement in cardiac function (Orlic et al., 2001). Besides myocardial
regeneration, angiogenesis is an alternative therapeutic objective. Mature endothelial cells possess limited proliferative and reparative capacity (Urbich et al., 2005b) and thus transplantation of cells that could restore the integrity of the endothelial monolayer or contribute to the formation of new blood vessels, may complement cardiac cell based therapeutic approaches (Chavakis et al., 2010). Finally, soluble factors released by the transplanted cells may induce cytoprotection and enhance proliferation of resident cardiac progenitors to mediate cardiac repair or alter the immune-inflammatory response (Figure I.1).
Figure I.1. **Mechanisms and potential problems of cardiac stem cell therapy.** Adapted from (Segers and Lee, 2008).
1.2 Candidate cell types for cardiac repair

1.2.1 Skeletal Muscle Myoblasts

Human autologous skeletal myoblasts were among the earliest cell types used for injection into ischemic myocardium (Pagani et al., 2003). Myoblasts, often called satellite cells, are localized beneath the basal membrane of mature muscular fibres where they lie dormant until stimulated to differentiate into myotubes by injury or disease (Buckingham and Montarras, 2008). Skeletal myoblasts are resistant to ischemia and can be harvested from the host, expanded \textit{in vitro} and reimplanted (Murry, Wiseman et al. 1996). Studies in animal models have shown promising improvement in left ventricular function on both systolic and diastolic performance (Leobon, Garicin et al. 2003; Laflamme and Murry 2005; Menasche 2007). However, skeletal myoblasts do not differentiate fully into cardiomyocytes \textit{in vivo} and do not integrate electrically in synchrony with the surviving cardiomyocytes and thus may pose a serious risk of ventricular arrhythmias (Reinecke, Poppa et al. 2002; Fouts, Fernandes et al. 2006). Additionally, there is a significant delay of 3-4 weeks between the harvest of skeletal muscle from the host, the culture and the expansion of the cells \textit{in vitro} and the transplantation to the patient. Moreover, the efficiency of the engraftment of the injected cells is extremely low, about 90% of the cells will die after transplantation (Suzuki et al., 2004, Pagani et al., 2003).

In humans, skeletal muscle transplantation was first performed to a patient with severe ischemic heart failure and resulted in viability and contraction of the graft as well as symptomatic improvement (Menasche et al., 2001). However, larger clinical trials that followed confirmed the concerns regarding ventricular arrhythmias and the requirement of an implanted cardioverter (Dib et al., 2005). Therefore, skeletal myoblasts in clinical trials have not succeeded; one of the most notable large-scale clinical trials (placebo-controlled trial), Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC), was ended prematurely because of lack of efficacy. The results were disappointing as no significant benefit was shown between the treatment group versus the placebo (Menasche et al., 2008) suggesting that it is unlikely that skeletal myoblast will be able to truly regenerate the myocardium.
1.2.2 Embryonic Stem Cells (ESCs)

ESCs are the pluripotent cells derived from the inner mass of the blastocyst that have an unlimited capacity for self-renewal and indefinite expansion *in vitro* (Thomson, Itskovitz-Eldor et al. 1998; Itskovitz-Eldor, Schuldiner et al. 2000). More importantly, they can differentiate into all three germ layers and specific growth conditions can dictate their differentiation towards a desired cell type. Detailed protocols for enriched generation of cardiomyocytes, vascular smooth muscle cells and endothelial cells from ESCs have been developed and theoretically ESCs could be used to replace entire organs including the myocardium (Laflamme and Murry, 2005). Cardiomyocytes derived from ESCs showed the hallmarks of primary cardiomyocytes. They share molecular markers, similar structure, electrophysiological and contractile properties to that of native cardiac cells (Xu et al., 2008, Rosamond et al., 2008) (Reviewed by(Hansson et al., 2009). It is worth mentioning, however, that ESC-derived cardiomyocytes exhibit a fetal phenotype (Kehat, Kenyagin-Karsenti et al. 2001) and thus far, attempts to obtain a more mature phenotype have failed.

Even though many preclinical studies in rodent models have shown promising results (reviewed in(Passier et al., 2008), the use of ESCs for cell based therapeutic approaches in humans faces major hurdles. Besides the obvious ethical concerns, derivation of ESCs does require the destruction of a human embryo, they are allogeneic and thus face immune rejection after transplantation (Draper et al., 2002). Additionally, due to their pluripotent state they are highly teratogenic. None of the established differentiation protocols can exclude contamination of the derived cell types with undifferentiated ESCs that can induce tumour formation (Lensch et al., 2007).

1.2.3. Induced Pluripotent Stem (iPS) Cells

Induced pluripotent stem cells derived from reprogrammed adult somatic cells may represent a novel source of cells for therapeutic approaches. Like ESCs but with less ethical concerns (Wernig et al., 2007), iPS cells can be used to generate theoretically large quantities of functional cardiomyocytes (Narazaki et al., 2008). The reprogramming of somatic cells into a pluripotent-like state can
be achieved by overexpression of a group of reprogramming factors e.g. OCT3/4, SOX2, c-MYC and KLF4 as shown in the pioneering study by Yamanaka in mouse fibroblasts (Takahashi and Yamanaka, 2006). This epigenetic reprogramming was applied shortly afterwards to human somatic cells yielding human iPS cell lines with similar characteristics to human ESCs (Takahashi et al., 2007). Moreover, recent studies have succeeded in differentiating human iPS into myocytes with cardiac-specific molecular, structural and functional properties. Zwi et al used a combination of retroviral reprogramming of fibroblasts with OCT4, SOX2, c-MYC and KLF4 supplemented with hTERT and SV40 large T while Zhang et al used a lentiviral-mediated transduction with OCT4, SOX2, NANOG and LIN28 both shown similar cardiomyocyte differentiation potential (Park et al., 2008, Zwi et al., 2009, Zhang et al., 2009). The approaches to generate iPS cells are changing with rapid pace, techniques using small molecules to increase the efficiency of generating iPS cell lines as well as methods using non-integrating transgenes have succeeded in generating human iPS cells devoid of any genetic manipulation, raising hopes for the future of these cells for therapeutic applications (Yu et al., 2009, Kamp and Lyons, 2009).

1.2.4 Cardiac Stem Cells (CSCs)

The heart was traditionally thought to be post-mitotic (Joggerst and Hatzopoulos, 2009). Recently, resident populations of stem cells have been identified in the heart. CSCs or cardiac progenitor cells (CPCs) are multipotent cells previously found in the foetal heart and give rise to cardiomyocytes, smooth muscle cells and endothelial cells (Moretti, Caron et al. 2006; Zhou, Ma et al. 2008). CSCs can be isolated and expanded from the patient with a biopsy procedure (Smith, Barile et al. 2007). Thus, they do not face the same challenges as ESC, such as immune rejection or the risk of teratoma formation. Injection of CSCs into the infarcted myocardium in mice has been associated with short-term benefits (Messina et al., 2004); pre-activation of CSCs with insulin growth factor 1 (IGF-1) and transplantation after myocardial infarction boosted the formation of small coronary arteries, reduced infarct size and improved function (Tillmanns
et al., 2008). However, several different cell types have been proposed for therapy:

1) The so called side population (SP) cells stain negative for the Hoechst dye 33342 and Rhodamine 123. These cells were previously identified in the bone marrow and the skeletal muscle and later in the heart (Challen and Little, 2006, Martin et al., 2004). Isolated SP cells supposedly give rise to cardiomyocytes *in vitro* and *in vivo* and there is evidence that these cells are mobilised after cardiac injury (Oyama et al., 2007).

2) Another population of SP cells that express the stem cell factor receptor c-kit has also been shown to have regenerative potential after transplantation and to improve ventricular function (Beltrami et al., 2003, Dawn et al., 2005, Bearzi et al., 2007).

3) A third stem cell population in the heart expresses the stem cell antigen 1 (Sca-1) and have been shown to differentiate into cardiomyocytes around the injury area (Oh, Bradfute et al. 2003). Furthermore, a subpopulation of Sca-1 positive CD31 negative cells could differentiate into both endothelial cells and cardiomyocytes *in vitro* and home to injured myocardium was reported (Wang et al., 2006).

4) Finally a fourth population of CSCs expressing the transcription factor Islet 1 (Isl1) could differentiate into lineages of the right atrium of the adult heart (Laugwitz et al., 2005).

Currently, it is still unknown which of these CSCs, if any, possesses real cardiogenic potential and to what extent they might possibly contribute to repair.

### 1.2.5 Bone Marrow-Derived Stem Cells (BMSCs)

BMSCs comprise a heterogeneous population of cells that can be isolated either from the mononuclear fraction after density centrifugation (Sieveking and Ng, 2009)). BMSCs have the capacity to differentiate and proliferate into a variety of cell types including hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and endothelial progenitor cells (EPCs). A subset of bone marrow-derived hematopoietic stem cells were the first adult cells being reported to differentiate into adult cardiomyocytes when implanted in infarcted hearts in
mice (Reviewed in (Leri and Kajstura, 2005). However, further studies by several groups failed to detect permanent engraftment and transdifferentiation of transplanted BM-derived HSC. Instead cell fusion of BM-derived donor cells with recipient cardiomyocytes has been suggested as a compensatory mechanism (Noiseux et al., 2006, Nygren et al., 2004, Alvarez-Dolado et al., 2003).

1.2.5.1 Haematopoietic Stem Cells (HSCs)

HSCs are isolated from the bone marrow and can be mobilized and home to sites of injury. Currently HSCs are isolated through selective sorting for cell markers such as c-Kit, Sca-1, CD38 and CD34 positive and Lin negative. It is a major caveat that there is no specific marker to unambiguously identify the subpopulation of these cells. A number of clinical studies showed either no benefit or only a small improvement in cardiac function (although clinically significant) when HSCs were injected or mobilised from endogenous reservoirs (Abdel-Latif et al., 2007). Furthermore, Orlic et al. successfully attempted to mobilize HSCs from the bone marrow using a systemic administration of the cytokine granulocyte-colony stimulating factor (G-CSF) and stem cell factor (SCF), which eventually formed cardiomyocytes and capillaries (Orlic et al., 2001). Takahashi et al. demonstrated that G-CSF alone was sufficient to elevate levels of BMSCs and enhance neovascularisation at sites of hind limb ischemia (Takahashi et al., 1999).

1.2.5.2 Mesenchymal Stem Cells (MSCs)

Mesenchymal stem cells are a subset of non-HSCs mainly found in the stroma of the bone marrow. They are isolated based on the lack of haematopoietic cell markers such as CD45, CD34 and CD14, the presence of specific molecules and antigens such as CD44, SH2/3/4 and STRO-1 and by their ability to adhere to plastic (Conget and Minguell, 1999, Alhadlaq and Mao, 2004). They have the potential to give rise to a variety of cells of mesenchymal origin such as osteoblasts, adipocytes, chondrocytes and even into tissues of non mesenchymal origin e.g. myoblasts when stimulated by appropriate cytokines.
and growth factors (Pittenger et al., 1999, Jiang et al., 2002, Minguell et al., 2001). Differentiation of MSCs into cardiomyocytes in vivo has also been observed but at very low rates (Amado et al., 2005).

The possible role of MSCs in cardiovascular regeneration is still under investigation. Many preclinical studies in animals have revealed a variety of benefits associated with MSCs derived from their ability to home to the heart, differentiate into cardiomyocytes and fibroblasts and even take part in the formation of coronary vessels (Silva et al., 2005, Vulliet et al., 2004). A potential advantage of MSCs is that they lack the major histocompatibility complex II and therefore appear to be less immunogenic than other stem cells when transplanted from one individual to another, potentially allowing allogenic cell therapy (Amado et al., 2005, Dai et al., 2005). Other advantages include improvement of the left ventricular function, decrease in the infarct size and increased vascular density of the myocardium (Toma et al., 2002, Chen et al., 2004b).

Interestingly, a clinical study on post-infarct patients demonstrated that administration of MSCs led to improved left ventricular function (Chen et al., 2004b). Currently there are on-going clinical trials devoted to MSCs therapy for myocardial infarction and chronic ischemic heart disease (Sievving and Ng, 2009). Recent studies have suggested that implanted MSCs could also be disadvantageous as insufficient engraftment and frequent calcification at the sites of injection was determined by two individual studies when they attempted to inject MSCs in infarcted hearts of transgenic mice (Breitbach et al., 2007, Yoon et al., 2004). Most importantly MSCs need to be seriously questioned after reports claiming that despite their cardiomyocyte differentiation potential, MSCs differentiated into bone-forming osteoblasts inside ventricular tissue of transplanted mice hearts (Breitbach et al., 2007, Yoon et al., 2004). MSCs have the ability to engraft in the heart and are activated in response to injury. However, there is an obvious cause for concern that needs to be addressed; differentiation into cell types other than cardiomyocytes is a crucial consideration for the use of MSCs as a therapeutic source for cardiac repair.
1.2.5.3 Endothelial Progenitor Cells (EPCs)

Numerous studies have demonstrated that EPCs exist among peripheral blood mononuclear cells and represent a subset of circulating haematopoietic cells found in the bone marrow. These cells are able to acquire certain features of mature endothelial cells and also have the capacity to differentiate into endothelial cells \textit{in vivo} (Gehling et al., 2000). A number of clinical trials have already tested the ability of bone marrow mononuclear cells (BMMNCs) to improve the repair of the denuded endothelium as well as the capacity of non-bone marrow derived EPCs to home in sites of vascular injury, decrease neointima formation and improve myocardial function (Abdel-Latif et al., 2007). The engraftment of these cells after injection into areas of infarcted myocardium is poor. There is evidence that their angiogenic potential might be attributed to the secretion of growth factors and not the ability of EPCs to differentiate into mature endothelium (Rehman et al., 2003, Yoshioka et al., 2005). No specific markers are currently available for the unambiguous characterization and isolation of EPCs. Therefore there is an urgent need to reassess their cellular progeny.
Figure 1.2. Sources of cells for cardiac cell therapy. Pluripotent stem cells can be isolated from different sources (blastula/iPS), expanded \textit{in vitro} and differentiated further into cardiac progenitors and mature cardiac cell types. Multipotent stem and progenitor cells can be isolated from either autologous or allogenic sources including the skeletal muscle of the heart, the bone marrow, the peripheral blood and the adipose tissue. Preparations such as bone marrow-derived cells have been used directly in clinical trials whereas other cell types needed to be differentiated \textit{in vitro} under specific conditions to give rise into new cardiomyocytes, endothelial cells and vascular smooth muscle cells. The improvement of cardiac function is mediated by various mechanisms including the differentiation of stem and progenitor cells into relevant cardiac cells. In addition the production of a variety of growth factors and cytokines by the transplanted cells mediates cardiac repair via a paracrine manner.
1.3 Challenges for the Clinical Application of Stem Cells

Stem cells have been introduced into clinical trials despite our lack of information regarding their true identity, phenotype, physiology and functionality. The major challenge facing cell therapy for cardiovascular diseases is to identify the most suitable type of stem or progenitor cell for transplantation (Wollert and Drexler, 2005). ESCs have probably the best regenerative and differentiation potential but feature the risk of immune reaction, teratoma formation and ethical barriers (Nussbaum et al., 2007). CSCs have the advantage that they could be isolated from the host, thus there is no need for immunosuppression. However, their isolation is difficult, and it is challenging to obtain them in sufficient numbers (Bearzi et al., 2007, Smith et al., 2007). Consequently, the majority of clinical trials were performed using bone-marrow derived cells due to easy accessibility and good safety profile despite the fact that their regeneration potential and identity remain highly controversial.

Besides the cell type, the efficacy of cell therapies also depends on the successful mobilization and homing of the cells to the desired sites of injury. Direct approaches include intramyocardial injection (either to endocardium via intracardiac catheters or to epicardium via surgical or thoracoscopic approach), percutaneous intracoronary infusion and peripheral intravenous infusion and indirect mobilization with peripheral delivery of cytokines (LaPar et al., 2009, Reventco and Morgan, 2009). However, direct stem cell delivery following these routes has revealed serious adverse effects such as embolic complications. Generally, cell loss and poor engraftment are the major problems that need to be addressed (Vulliet et al., 2004, Abdel-Latif et al., 2007, Menasche, 2007, Wollert and Drexler, 2005).

Thus far, clinical trials have been performed on patients with acute myocardial infarction (AMI) and with chronic heart failure. Both diseases require the use of different cell types and routes of delivery. As far as the therapeutic use of cells with patients after AMI is concerned, the most widely used cell type are currently bone marrow-derived cells. In the BOOST trial, autologous BMCs were delivered via intracoronary injection after myocardial infarction and showed an improvement in left ventricular ejection fraction (LVEF) after 6
months but not at 18 months after infusion (Wollert et al., 2004, Meyer et al., 2006). Data from the TOPCARE-AMI trial confirmed these results demonstrating improvement of LV function and absence of hypertrophy after 5 year follow-up (Leistner et al., 2011). One of the largest clinical trials (204 patients) using bone marrow-derived cells (REPAIR-AMI) delivered BMCs intracoronary into the infarct artery 3-4 days after reperfusion therapy. At 4 months follow-up there was an improved LVEF whereas after 1 year treated patients had a lower incidence of recurring MI and required less rehospitalisation (Schachinger et al., 2006b). In contrast, ASTAMI, another bone marrow cell trial, did not report any significant improvement after a 6 month follow-up (Lunde et al., 2005). Potential explanations that may have influenced the outcome might be the difference of the isolation procedures. Despite this, bone marrow-derived cells seem to be safe and overall most of the published trials have reported a 2.9-5.5% improvement in global LVEF, which suggest at best a very modest benefit on LV remodelling.

On the other hand patients suffering for chronic heart disease may not benefit from the above mechanisms of repair e.g. enhanced neovascularisation and reduced cardiomyocytes apoptosis. Patients with ischemic disease suffer from long-established scars and thus they require regeneration of cardiac muscle. In an attempt to repair the myocardium, skeletal myoblasts were directly injected into the scarred region of LV during coronary artery bypass grafting and partially restored LV function (Menasche et al., 2001). Furthermore, in patients with post-infarction heart failure percutaneous injection of autologous skeletal myoblasts directly to the scar reduced the symptoms of heart failure but without obvious benefits regarding improvement of LV function (Smits et al., 2003). Nevertheless, the engraftment of myoblasts into post-infarction scars requires the placement of an internal defibrillator because patients may experience life-threatening arrhythmias due to the lack of the electrical coupling of skeletal myoblasts to the neighbouring cardiomyocytes (Leobon et al., 2003, Menasche et al., 2003). To investigate the feasibility of implanted autologous skeletal myoblasts, the Myoblast Autologous Grafting in Ischemic Cardiomyopathy (MAGIC) trial used cells derived from skeletal muscle biopsies including the placement of an implantable cardioverter-defibrillator. The 6 month report
revealed a failure to improve echocardiographic heart function and an increase in the incidents of early postoperative arrhythmic events (Menasche et al., 2008).
Table 1.1. Selected randomized clinical trials in patients with acute myocardial infarction or ischemic heart disease.

<table>
<thead>
<tr>
<th>Clinical Trial</th>
<th>No. of patients</th>
<th>Cell Type used</th>
<th>Patient Profile</th>
<th>LVEF</th>
<th>LVEDV</th>
<th>LVESV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Acute myocardial infarction</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strauer et al. (2002) (Strauer et al., 2002)</td>
<td>10</td>
<td>BMC</td>
<td>AMI</td>
<td>Increased</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>BOOST (2004) (Wollert et al., 2004)</td>
<td>60</td>
<td>BMC</td>
<td>AMI</td>
<td>Increased</td>
<td>NSD</td>
<td>NSD</td>
</tr>
<tr>
<td>TOPCARE-AMI (2004) (Assmus et al., 2002, Schachinger et al., 2004): 1 year results</td>
<td>59</td>
<td>BMC vs CPC</td>
<td>AMI</td>
<td>Increased</td>
<td>NSD</td>
<td>Decreased</td>
</tr>
<tr>
<td>Fernandez-Aviles et al. (2004) (Fernandez-Aviles et al., 2004)</td>
<td>20</td>
<td>BMC</td>
<td>AMI</td>
<td>Increased</td>
<td>NSD</td>
<td>Decreased</td>
</tr>
<tr>
<td>Chen et al. (2004) (Chen et al., 2004b)</td>
<td>69</td>
<td>MSC</td>
<td>AMI</td>
<td>Increased</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>REPAIR-AMI (2006) (Schachinger et al., 2006a)</td>
<td>204</td>
<td>BMC</td>
<td>AMI</td>
<td>Increased</td>
<td>NSD</td>
<td>NSD</td>
</tr>
<tr>
<td>ASTAMI (2006) (Lunde et al., 2005, Lunde et al., 2006)</td>
<td>100</td>
<td>BMC</td>
<td>AMI</td>
<td>NSD</td>
<td>NSD</td>
<td>N/A</td>
</tr>
<tr>
<td>Janssens et al. (2006) (Janssens et al., 2006)</td>
<td>67</td>
<td>BMC</td>
<td>AMI</td>
<td>NSD</td>
<td>NSD</td>
<td>NSD</td>
</tr>
<tr>
<td>MAGIC CELL-3DES (2006) (Kang et al., 2006)</td>
<td>82</td>
<td>PBMNC</td>
<td>AMI vs PMI</td>
<td>NSD</td>
<td>NSD</td>
<td>NSD</td>
</tr>
<tr>
<td>HEBE (2008) (van der Laan et al., 2008)</td>
<td>189</td>
<td>BMC vs PBMNC</td>
<td>AMI</td>
<td>N/A</td>
<td>N/A</td>
<td>NSD</td>
</tr>
<tr>
<td>FINCELL (2008) (Huikuri et al., 2008)</td>
<td>77</td>
<td>BMC</td>
<td>AMI</td>
<td>Increased</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>REGENT (2009) (Tendera et al., 2009)</td>
<td>117</td>
<td>BMC</td>
<td>AMI</td>
<td>Increased</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td><strong>Ischemic heart disease</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Meluzin et al. (2008) (Meluzin et al., 2008)</td>
<td>60</td>
<td>BMC</td>
<td>IHD</td>
<td>Increased</td>
<td>NSD</td>
<td>NSD</td>
</tr>
<tr>
<td>MAGIC (2008) (Menasche et al., 2008)</td>
<td>97</td>
<td>SMB</td>
<td>IHD</td>
<td>NSD</td>
<td>Decreased</td>
<td>Decreased</td>
</tr>
<tr>
<td>van Ramhorst et al. (2009) (van Ramshorst et al., 2009)</td>
<td>50</td>
<td>BMC</td>
<td>CIHD</td>
<td>Increased</td>
<td>NSD</td>
<td>NSD</td>
</tr>
<tr>
<td>TOPCARE-AMI: final 5-year results</td>
<td>55</td>
<td>BMC vs CPC</td>
<td>AMI</td>
<td>Increased</td>
<td>Increased</td>
<td>NSD</td>
</tr>
</tbody>
</table>

**Abbreviations:** LVEF: left ventricular ejection fraction, LVEDV: left ventricular end diastolic volume, LVESV: left ventricular end systolic volume, BMC: bone marrow-derived cell, CPC: circulating progenitor cell, MSC: mesenchymal stem cell, PBMNC: peripheral blood mononuclear cell, SMB: skeletal myoblast, AMI: acute myocardial infarction, PMI: post myocardial infarction, IHD: ischemic heart disease, CIHD: chronic ischemic heart disease, NSD: not significant different, N/A: not applicable.
1.4 Controversy about Endothelial progenitor cells

The endothelium forms the inner lining of the vasculature. Major cardiovascular pathologies such as hypertension, atherosclerosis and thrombosis result in endothelial dysfunction and/or injury. Therefore, regeneration of the endothelium is of major importance. Unfortunately, mature endothelial cells possess limited proliferative and repair capacity (Dimmeler and Zeiher, 2004). As a result, there is growing interest into EPCs because of their apparent ability to differentiate into ECs and to contribute to the recovery of the ischemic cardiac tissue by contributing to the repair of injured arteries and in postnatal neovascularisation (Rafii and Lyden, 2003).

EPCs were first described in 1997 by Asahara and colleagues (Asahara et al., 1997) who showed that purified CD34+ hematopoietic progenitor cells from adults can differentiate ex vivo to an endothelial phenotype. It has also been proposed that they play an essential role in both vascular formation (Pula et al., 2008a), vascular homeostasis as well as in wound healing and neovascularization (Aoki et al., 2004) providing a novel avenue for cell therapy (Isner and Asahara, 1999). Furthermore, a number of human clinical trials have tested the ability of bone marrow mononuclear cells (BMMNCs) to improve the repair of the denuded endothelium as well as the capacity of non-bone marrow derived EPCs to home to sites of vascular injury and decrease neointima formation.

Besides, a number of studies have also demonstrated an inverse correlation between the numbers of circulating EPCs and the presence of atherosclerosis, cardiovascular dysfunction and mortality (Vasa et al., 2001b, Schmidt-Lucke et al., 2005, Werner et al., 2005). Also, the functional properties of EPCs such as migration, cell adherence, invasion and vessel formation appear to be attenuated in patients with increased cardiovascular risk. Hence, EPCs were proposed as a biomarker to predict cardiovascular outcomes (Tepper et al., 2002) (Reviewed in (Timmermans et al., 2009)).
As mentioned above the inconclusive results from the recent clinical trials have demonstrated that there is a need to better understand the biological properties of EPCs. By now, a PubMed search on EPCs returns thousands of publications (Deb and Patterson, 2010). Despite the immense explosion of interest in this area, there is neither a standard definition nor an accepted methodology for their enumeration (Povsic et al., 2009).

1.4.1 Characterization of EPCs

It is surprising that after a decade of research, there is no single marker or at least a combination of markers that unambiguously identify these cells. Instead, different criteria were used making comparisons between studies impossible. EPCs have been characterized by surface markers such as CD34, CD133, vascular endothelial growth factor receptor-2 (VEGFR2, KDR), CD31, von Willebrand Factor (vWF), VE-cadherin, CD146, c-Kit, CXCR4 (Asahara et al., 1997, Rafii, 2000, Peichev et al., 2000, Yin et al., 1997, Rafii et al., 2002). However, most of these markers are also expressed in other hematopoietic cells. CD31, KDR, CD34 and c-Kit are expressed in both hematopoietic stem and progenitor cells (Figure I.3A). CD34 is expressed by microvascular endothelial, hematopoietic progenitors (HPCs) and hematopoietic stem cells (HSCs). Moreover, the endothelial phenotype of these adherent cells has been defined by the uptake of acetylated LDL (acLDL) and the binding to lectins such as *Ulex europeas*, yet both are also characteristics of myeloid cells (Schmeisser et al., 2003).

The markers most commonly used for EPCs are CD34+, VEGFR-2+, CD133+. However, cells positive for these markers were never tested for their ability to generate new endothelial cells *in vitro or in vivo* (Peichev et al., 2000). Recently, Case et al, used hematopoietic and EC clonogenic assays and showed that these cells are not true EPCs, but HPCs that express the hematopoietic cell surface marker CD45. They also identified a rare subpopulation of cells, which are CD45-; they are devoid of hematopoietic activity and could be a source of EPCs (Case et al., 2007).
1.4.2 EPC Culture Methods

EPCs are isolated from the bone marrow or directly from the peripheral blood of the host by density centrifugation (Peichev et al., 2000); a popular method of choice because it is a fast and “stress”-free equilibrium method that fractionates on the basis of particle density when compared to other strategies such as high-speed fluorescence-activated cell sorting (FACS) sorters. Peripheral blood mononuclear cells (PBMCs) will settle on the interface when samples are spun over 1.077 g/ml density barriers such as Histopaque, Lymphoprep, Ficoll-Paque etc, creating a buffy coat. This cell layer is carefully washed but the expected yield of EPCs in such preparations is low (~0.5-1%). Hence, culture methods were developed to expand this rare cell population.

Currently, three main methods are used for the culture of EPCs (Figure I.3B). The first method widely known as the CFU-Hill colony is based on a modification of the cluster-forming assay. PBMCs are pre-plated on fibronectin-coated dishes for two days and the non-adherent cells are replated in the presence of specific serum supplements that gives rise to colonies within 7 days of culture (Hill et al., 2003). The second method, also known as early outgrowth EPCs, was described by Vasa et al 2001. Briefly, PBMCs were plated on fibronectin-coated dishes in the presence of endothelial growth factors and serum. On day 4 the non-adherent cells were removed from the culture and the adherent cells were tested for expression of endothelial progenitor markers. These cells are spindle-shaped and have the ability to secret an array of angiogenic cytokines (Vasa et al., 2001a, Vasa et al., 2001b). The third method is referred to as late outgrowth colonies; a cell population emerging late in culture that shows clear endothelial characteristics such as the cobblestone phenotype and mature endothelial cell markers.
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Figure I.3. A) Phenotypic characterization of EPCs. Markers used for isolation and identification of EPCs are also expressed on hematopoietic cells. Adapted from (Rafii and Lyden, 2003). B) Main methods for culture of EPCs. Culture of colony forming unit-Hill cells (CFU-Hill)/scale bar = 100um (A), Circulating angiogenic cells (CAC) or early outgrowth EPCs/scale bar = 200um (B), endothelial colony forming cells (ECFC) or late outgrowth EPC/scale bar = 400um (C) adapted from (Hirschi et al., 2008).
Concerns have been raised that monocytes can express a variety of endothelial-like proteins when cultured under specific conditions in vitro (Fernandez Pujol et al., 2000, Schmeisser and Strasser, 2002, Kalka et al., 2000). Addition of VEGF, FGF-2 and EGF in the growth medium gives rise to endothelial-like cells after 7-10 days in culture. These cells represent a unique form of macrophage differentiation, also known as angiogenic monocytes or circulating angiogenic cells (CACs) and have the ability to contribute to the formation of new vessels (Kalka et al., 2000, Rehman et al., 2003). Furthermore, Rehman and colleagues have demonstrated that EPCs derived from PBMNC fractions did not proliferate but release pro-angiogenic mediators such as VEGF, hepatocyte growth factor (HGF), granulocyte colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Rehman et al., 2003).

In addition, when CD14+ cells taken from peripheral blood were cultured in endothelial growth media, they expressed endothelial markers such as vWF and KDR (Urbich et al., 2003). A clonal analysis performed by Yoder et al (Yoder et al., 2007) revealed that CFUs are derived from the hematopoietic system, possess myeloid progenitor cell activity, and differentiate into phagocytic macrophages (Yoder et al., 2007, Hirschi et al., 2008, Gunsilius et al., 2000). Rohde et al demonstrated that CFUs formed as a result of a functional cross between T cells and monocytes (Rohde et al., 2007, Desai et al., 2009).

Collectively, findings from many different groups showed that early outgrowth EPCs fail to incorporate in the vasculature and to differentiate into endothelial cells in vivo (reviewed in (Richardson and Yoder, 2010), so it remains unclear how these cells promote angiogenesis. On the other hand, it has been shown that blood monocytes could mimic early outgrowth EPCs and mediate an angiogenic effect- similar to EPCs- but in a paracrine manner by releasing chemokines and other soluble factors (Rohde et al., 2006, Rehman et al., 2003). A hypothesis regarding a possibly answer to this matter may be that the majority of the early outgrowth EPCs (spindle-shaped cells) could be derived from either bone marrow or peripheral blood MNCs and are more likely to be related to CD14+ angiogenic macrophages than endothelial progenitors and promote angiogenesis by releasing angiogenic proteins. On the other hand, late EPCs (cobblestone shaped) may develop exclusively from angioblasts within the
CD14- fraction (Urbich et al., 2003) and is definite that mature into mature endothelial cells.
Table 1. Characteristics used to identify putative endothelial progenitor cells (taken from Padfield et al. 2010); CSF: colony-stimulating factor; N/A: not available; TNF: tumour necrosis factor

<table>
<thead>
<tr>
<th>Identifier</th>
<th>Distribution</th>
<th>Function</th>
<th>Hematopoietic</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>Leukocytes</td>
<td>A signaling molecule regulating leukocyte differentiation and proliferation</td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>Monocytes, macrophages, some neutrophils</td>
<td>Endotoxin receptor regulating inflammatory cytokine production such as TNF by monocytes</td>
<td></td>
</tr>
<tr>
<td>CD115</td>
<td>Monocytes, macrophages</td>
<td>Receptor for macrophage-CSF regulating myeloid proliferation and differentiation</td>
<td></td>
</tr>
<tr>
<td>CD117 (c-Kit)</td>
<td>Hematopoietic stem and progenitor cells</td>
<td>Receptor for stem cell factor; stimulates cellular proliferation</td>
<td></td>
</tr>
<tr>
<td>CD133</td>
<td>Hematopoietic stem and progenitor cells</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>CD34</td>
<td>Hematopoietic stem and progenitor cells, capillary endothelium</td>
<td>Intercellular adhesion molecule; binds E- and L-selectins and is thought to regulate leukocyte/endothelial interactions</td>
<td></td>
</tr>
<tr>
<td>CD31</td>
<td>Leukocytes, platelets, endothelium</td>
<td>Adhesion molecule thought to be important for transendothelial cellular migration to sites on acute inflammation</td>
<td></td>
</tr>
<tr>
<td>Acetylated low-density lipoprotein uptake</td>
<td>Macrophages, monocytes, endothelium</td>
<td>N/A; phagocytic process occurring in myeloid and endothelial cells</td>
<td></td>
</tr>
<tr>
<td>Ulex binding</td>
<td>Macrophages, monocytes, endothelium</td>
<td>N/A; histochemical stain</td>
<td></td>
</tr>
<tr>
<td>CD105</td>
<td>Endothelium, activated macrophages, smooth muscle</td>
<td>Constituent of transforming growth factor-beta receptor 1; important regulator of angiogenesis</td>
<td></td>
</tr>
<tr>
<td>CD141</td>
<td>Endothelium, smooth muscle, monocytes, and neutrophils</td>
<td>Binds thrombin and activates protein C and initiates anticoagulant pathways</td>
<td></td>
</tr>
<tr>
<td>von Willebrand factor</td>
<td>Endothelium, platelets</td>
<td>Hemostasis</td>
<td></td>
</tr>
<tr>
<td>Tie-2 (CD202)</td>
<td>Endothelium, monocytes, stem cells</td>
<td>Angiopoietin-1 receptor; regulates vessel remodelling and maintains vascular integrity</td>
<td></td>
</tr>
<tr>
<td>CD146</td>
<td>Endothelium, melanoma cells, dendritic cells</td>
<td>Intercellular adhesion molecule</td>
<td></td>
</tr>
<tr>
<td>E-selectin (CD62E)</td>
<td>Endothelium</td>
<td>Adhesion molecule regulating leukocyte/endothelial interactions and cell trafficking to sites of inflammation</td>
<td></td>
</tr>
<tr>
<td>CD144</td>
<td>Endothelium</td>
<td>Intercellular adhesion molecule regulating endothelial permeability and proliferation</td>
<td></td>
</tr>
<tr>
<td>VEGFR-2 (CD309, KDR, Flk1)</td>
<td>Endothelium</td>
<td>Regulation of endothelial adhesion and signaling; essential for embryonic vascular development</td>
<td></td>
</tr>
<tr>
<td>Endothelial nitric oxide synthase</td>
<td>Endothelium</td>
<td>Enzymatic generation of nitric oxide</td>
<td></td>
</tr>
</tbody>
</table>
1.5 Proteomic analysis of EPCs

The characterization of EPCs should be mandatory before embarking on clinical cell therapy trials and a proteomics-led approach may help to provide new insights. Proteomics offers a platform for the comprehensive analysis of proteins rather than investigating only individual cell surface markers.

1.5.1 Proteomics – general considerations

In the last decade of the 20th century, genomics and the functional genomic sciences revolutionised medical research. The key discovery was that the genetic specification of a human being – once assumed to be of almost limitless complexity – consisted of just 20,000 protein-coding genes, surprisingly similar to much simpler organisms such as C. elegans (2004). Therefore, it is not the number of genes, but the processing of the gene products that accounts for the biological complexity. Despite the sequencing of the genome, there remain a lot of unknowns at the protein level. For 35% of the protein encoding genes in the human genome, there is currently no evidence for the existence of these proteins at the protein level. Also, there are about 75,000 annotated post-translational modifications, but only half of them have been experimentally obtained. This shift in perspective led to the advance of “proteomic” sciences, and they are now beginning to influence cardiovascular research (Mayr et al., 2006, Arrell et al., 2001).

With conventional molecular biological approaches, studies on proteins can only be conducted on a limited number of proteins. Proteomics aspires to define the totality of protein concentrations, but currently it is impossible to resolve the entire complexity of the mammalian proteome. Proteomic technologies have advanced rapidly over the recent years (McGregor and Dunn, 2006) and enable us to monitor at least a proportion of the thousands of proteins in mammalian cells (Bendall et al., 2009). For low abundant proteins, such as plasma membrane proteins, transcription factors, etc, an enrichment step is essential. In this respect, whole genome arrays are a more mature technological platform. Transcript levels, however, are merely an indication of the protein
amount. Although protein and messenger RNA (mRNA) expression could be at some degree correlated, there is no correlation between protein and mRNA half-life. Therefore, the transcriptome is not linearly proportional to proteome and quantifying mRNA levels cannot suffice (Gygi et al., 1999).

Another important consideration is that current research relies heavily on the use of antibodies. Antibody-based techniques only probe for known proteins with the potential caveats of non-specific binding, epitope masking and cross-reactivity with proteins from different species. In contrast, mass spectrometry is considered the gold standard for protein identification. It does not rely on a priori assumptions, but provides an unbiased overview of protein expression. A mass spectrometry analysis, however, does require sufficient material and as yet cannot be applied to the single cell level (Newman et al., 2006). Neither does mass spectrometry reveal the spatial localization of the identified proteins unless different sub-proteomes are compared (Didangelos et al., 2010, Yin et al., 2010, Mayr et al., 2009b) or laser microdissection is used for protein collection (De Souza et al., 2004).

1.5.2 What techniques for what output?

In proteomics, the main current methods are either gel-based (two-dimensional electrophoresis (2-DE), gel-LC-MS/MS) or gel-free (shotgun proteomics) (Figure I.4). In gel-based proteomics, proteins are first separated by 2-DE or by SDS-PAGE prior to tryptic digestion and mass spectrometry analysis. In shotgun proteomics, the protein mixture is directly digested with trypsin. Relative quantitation is either performed at the protein (2-DE) or at the peptide level (gel-LC-MS/MS and shotgun proteomics). 2-DE visualizes proteins as discrete gel spots and spots with differential expression are subject to mass spectrometry analysis for protein identification. For many years, 2-DE has been the workhorse of proteomics (Dunn, 2000). By now, it is gradually superseded by mass spectrometry-based quantitation techniques. Yet, it can still deliver important insights and is more readily implemented in most laboratories. In gel-LC-MS/MS, gel bands covering the entire lane are excised and analysed by mass spectrometry. Without an isotope label, this approach does not allow multiplexing and requires more mass spectrometry time, but the gel-LC-MS/MS
approach can provide an in-depth characterization of the sample and an estimate of protein abundance when combined with label-free quantitation in simple mixtures. The most popular methods for isotopic labelling in shotgun proteomics are isobaric tagging for relative and absolute quantification (iTRAQ) and stable isotope labelling of amino acids in culture (SILAC) (Mann, 2006). Labelling with iTRAQ is performed at the peptide level. In contrast, SILAC involves supplementing the culture medium with either the light or the heavy isoform of a particular amino acid. Samples are combined immediately after harvesting thereby minimizing experimental variation. Every method has its advantages and drawbacks. The right choice depends on the complexity of the sample and on the type of proteins to be analysed.

1.5.2.1 Sensitivity

2-DE is biased to more abundant, soluble proteins. Very large, very small, and hydrophobic proteins are difficult to resolve. Therefore certain proteins, including membrane proteins, cytokines, transcription factors etc. are under-represented on 2-DE gels. Shot-gun proteomics provides a more comprehensive proteome coverage than 2-DE gel-based approaches but shows its strength at the required sensitivity only in simplified cell extracts. In tissues and whole cell lysates, shotgun proteomics is confronted with similar problems as in plasma. Since the peptides are selected for fragmentation based on their ion intensity, the more abundant peptides are more likely to be detected. If the proteome is not sub-fractionated, the complexity of the resulting peptide mixture can overwhelm the analytical capabilities of the mass spectrometer and interfere with quantitation. Previous quantitative comparisons applying shotgun proteomics to cardiac tissue had to exclude fractions containing myofilament proteins (Gramolini et al., 2008) to alleviate the severe dynamic range limitations stemming from the highly abundant contractile components.

1.5.2.2 Quantitation

The most quantitative 2-DE technique is difference in-gel electrophoresis (DIGE). DIGE involves fluorescent labelling of protein mixtures and can reliably quantify differences as low as 10% in protein expression. Importantly, there is no
real limit in the number of replicates that can be compared. Alternative multiplex quantitative mass spectrometry-based approaches enable the simultaneous analysis of up to eight samples (iTRAQ) or introduce isotope labels by metabolic labelling (SILAC). While a single peptide can unambiguously identify a protein, multiple peptides of the same protein are required for quantitation. Thus, not all of the identified proteins can be reliably quantified in a shotgun experiment. SILAC is considered the best technique to determine relative differences in peptide abundance, but a minimum of 5 population doublings is needed to achieve complete labelling, which limits its use for primary cells. Besides, metabolic labelling strategies in animals are prohibitively expensive. iTRAQ can be applied to any specimen, but the labelling step occurs rather late in the proteomics work-flow and may introduce additional experimental variation. In contrast, label-free quantitation is inexpensive and provides an estimate of protein expression based on spectral counts or ion intensities of the identified peptides. With increasing sample complexity, label-free quantitation is subject to quantification errors due to ion suppression (known as matrix suppression), which arises when particular peptides preferentially ionize in a complex mixture. Thus, quantitative changes may be misrepresented as a result of matrix effects, causing either suppression (underestimation) or enhancement (overestimation) of other peptides.

1.5.2.3 Post-translational modifications and protein degradation.

The gel-based separation can depict post-translational modifications and protein degradation as a shift in isoelectric point (2-DE) or molecular weight (2-DE and SDS-PAGE). Shotgun proteomics does not provide a map of intact proteins. The available material is usually insufficient to obtain spectra of all modified peptides of a particular protein by mass spectrometry and information on post-translational modifications can be lost (McGregor and Dunn, 2006). This problem has been partially overcome by new enrichment methodologies, i.e. for phosphopeptides, and technical advancements in mass spectrometry. An alternative peptide dissociation method, electron transfer dissociation (ETD), induces a softer peptide fragmentation process and preserves post-translational
modifications (Mikesh et al., 2006), that are labile in the customary fragmentation process (collision-induced dissociation, CID). A major concern for the comparison of diseased tissues is protein degradation by enzymes other than trypsin, which can interfere with a quantitation based on tryptic peptides in shotgun proteomics. Thus, there can be a trade-off between sensitivity and quantitative accuracy.
Figure I.4. Overview of proteomic methods. The main proteomic methods are gel-based (two-dimensional electrophoresis (2-DE), gel-LC-MS/MS) or gel-free (shotgun proteomics). In gel-based proteomics, proteins are first separated by two-dimensional electrophoresis (2-DE) or by SDS-PAGE prior to enzymatic digestion (usually with trypsin) and mass spectrometry analysis. In shotgun proteomics, the protein mixture is directly digested without gel separation. Relative quantitation is either performed at the protein (2-DE) or at the peptide level (gel-LC-MS/MS and shotgun proteomics). At the centre of any proteomic approach is the mass spectrometer. The current gold standard for mass spectrometry is nanoflow liquid chromatography tandem mass spectrometry (nLC-MS/MS).
1.5.3 Applications of proteomics to stem cell research

One can envisage several possibilities how the advent of novel proteomics technology may help the translation of stem cell therapy:

1) **Assessment of cell homology.** Proteomics can be used to compare cell similarity / diversity leading to the recognition whether the protein content of stem cell-derived cells recapitulates the protein profiles of their mature counterparts. It is this concern with the whole proteome that distinguishes a proteomics approach from traditional phenotyping using a selected panel of marker proteins. For example, comparative analysis of early outgrowth EPCs, late outgrowth EPCs, monocytes and human umbilical vein endothelial cells by 2-DE, showed that early EPCs are haematopoietic cells with a protein profile similar to monocytes, whereas the molecular fingerprint of late outgrowth EPCs corresponds to an endothelial phenotype (Medina et al., 2010, Didangelos et al., 2009). Similarly, a 2-DE comparison revealed that after platelet-derived growth factor-BB stimulation, the resident Sca-1\(^+\) stem cell population from the vasculature shared the proteomic characteristics of mature aortic SMC phenotype (Mayr et al., 2008). In contrast, the proteome of smooth muscle-like cells derived from Sca-1\(^+\) progenitors of embryonic stem cells (ESC) was clearly distinct from aortic SMCs, though the cells expressed a similar panel of smooth muscle markers (Yin et al., 2006).

Generally, there is a need for new markers that are not only able to assess the stage of the stem cell differentiation process, but to distinguish between mature and stem cell-derived cells. A DIGE comparison of endothelial colony-forming units (CFUs) and early-outgrowth EPCs identified thymidine phosphorylase to be among the main pro-angiogenic factors (Pula et al., 2009). Thymidine phosphorylase is an intracellular enzyme and highly expressed in certain tumour cells. The proposed mechanism of action is not clear, but it has been suggested that the protective effect of thymidine phosphorylase is mediated by the product of its catalytic reaction (2-deoxy-D-ribose-phosphate). This is in agreement with previous observations that supplementation of this metabolite or
thymidine phosphorylase-expressing tumour cells induce endothelial chemotaxis (Hotchkiss et al., 2003b, Hotchkiss et al., 2003a).

2) Identification of cell surface proteins. Many of the accepted markers for the identification and differentiation of stem cells are transcription factors and intracellular proteins. Surface markers are a prerequisite for selecting undifferentiated and differentiated stem cells. By identifying candidate proteins that allow purification of specific cell lineages from live heterogeneous populations of differentiated cells, proteomics would address a clinically relevant need for the translation of stem cells to therapies. Alas, the physiochemical characteristics of membrane proteins render analysis by proteomics challenging and high abundant intracellular proteins hamper the identification and quantification of membrane proteins present in low copy numbers. Additional enrichment steps, i.e. by using biotin/avidin labelling of surface proteins (Sidibe et al., 2007) or exploiting the fact that a majority of the cell surface proteins are glycosylated (Wollscheid et al., 2009), reduce sample complexity and facilitate the identification of membrane proteins by mass spectrometry.

Previous proteomic analysis conducted on plasma membrane proteins of mouse embryonic stem cells, established a reference catalogue of cell surface proteins expressed on undifferentiated mouse ESCs (Nunomura et al., 2005) and differentially expressed during early differentiation (Intoh et al., 2009). In a recent study, van Hoof et al applied a quantitative SILAC proteomics approach to compare the cell surface proteins of human ESC-derived cardiomyocytes and identified elastin microfibril interfacier 2 as a marker to sort stem cell-derived cardiomyocytes (Van Hoof et al., 2010). Similarly, Gundry et al have revealed new targets for the characterization of cell intermediates during skeletal myoblast differentiation into myotubes by using a proteomic approach capturing N-linked glycoproteins. In another study by Dormeyer et al (Dormeyer et al., 2008), the membrane proteome of human ESCs was analysed using only 500,000 cells. Their method involved an optimized digestion protocol that included a step of carbonate extraction and enzymatic deglycosylation. Although this method is not as specific as other membrane purification procedures, 237 plasma membrane proteins could be identified in the human ESC line HUES-7.
3) Identification of paracrine factors. In *in-vivo* as well as *in-vitro* systems, free and protein-bound secreted factors are distributed throughout the extracellular and intracellular environments. Modulation of the homeostasis among growth factors, hormones, proteases and extracellular matrix molecules, cell-cell interactions and intracellular compartments is critical in directing the differentiation of stem cells and the formation of tissue-like structures. However, little is currently known about the extracellular milieu produced by different stem cell populations. Proteomics is the method of choice for a large-scale analysis of protein secretion (Stastna et al., 2010), but many cell types require serum supplements for their survival. Protein concentrations in serum span 9 order of magnitude in linear dynamic range (Anderson and Anderson, 2002). Current proteomic technologies resolve 3-4 orders of magnitude. Consequently, they can array secreted factors at the required sensitivity only in serum-free medium. Otherwise, classical serum proteins mask the less abundant proteins. To minimize cross-contamination with bovine proteins, the cells have to be washed extensively with plain medium before the secreted factors are sampled. Despite these efforts to ensure that the collected conditioned medium contains no other extraneous proteins, except for the secreted or shed proteins, the cross-contamination with bovine proteins can vary depending on the cell type.

Endothelial cells, for example, show a substantial carry-over of serum albumin. In a shotgun proteomics analysis of the secretome from human umbilical vein endothelial cells, 12-15% of all identified spectra corresponded to albumin peptides (Tunica et al., 2009). In contrast, SMCs tolerate serum starvation well and can be kept in serum-free medium for longer. In this setting, a shotgun proteomics strategy was able to mine deeper into the secretome and detected all cytokines present at concentrations >10 ng/ml (out of 27 tested in a multiplex assay). Only VEGF at 8 ng/ml was not identified. Similarly, a secretome analysis confirmed high levels of matrix metalloproteinase 9, interleukin-8, and cathepsins in endothelial colony-forming units (CFUs)(Hill et al., 2003), previously described as characteristics of early EPCs (Yoon et al., 2005, Pula et al., 2009). To increase the depth of proteomic profiling, Bendall et al. designed another approach to strategically identify low-abundance stem cell regulatory proteins of the human ESC secretome. By applying a MS-based proteomic method called iterative exclusion, the group successfully identified
previously undetectable growth factors, present at concentrations ranging from $10^{-9}$ to $10^{-11}$ g/ml (Bendall et al., 2009).

Another variable is the type of matrix the cells produce. In a recent study (Simper et al., 2010), our group compared the secretome of smooth muscle progenitors (SPC) with human aortic SMCs revealing a substantial overlap among the matrix proteins identified. SPCs, however, selectively retained certain proteins from bovine serum, including pigment epithelium-derived factor, a potent inhibitor of angiogenesis that binds to newly formed collagen and counters the effects of VEGF (Dawson et al., 1999). Pigment epithelium-derived factor was identified as bovine protein by mass spectrometry in the absence of corresponding mRNA expression in SPCs. Consistent with this finding, SPCs showed reduced invasive capacity and unlike EPCs, their conditioned medium had no angiogenic activity.

4) **Mechanisms of stem cell differentiation.** For stem cell-based therapies, it is essential that we gain knowledge on the molecular mechanisms controlling differentiation towards the cardiovascular lineage. The processes of stem cell renewal and differentiation are controlled by intrinsic factors regulated by extrinsic signals, whereby receptors act as transducers of these signals. Proteomics can be employed to dissect the mechanisms regulating the proteome of stem cells during self-renewal and commitment to the cardiovascular lineage. Of the different cellular sub-proteomes, those embedded in the plasma membrane have been of substantial interest as they regulate key biological functions such as cell-to-cell and cell–matrix interactions, transport, and signal reception/transduction. Signalling pathways governing differentiation are controlled by environmental cues, i.e. the binding of secreted ligands to membrane receptors. A proteomic approach targeting plasma membrane receptors as well as protein secretion may unravel key mechanisms regulating cardiovascular differentiation. A prerequisite is that stem cells can be expanded to obtain sufficient material for proteomic analysis. Unfortunately, many adult stem cells are scarce. Considering this, our group, like many others (Gundry et al., 2010, Van Hoof et al., 2006), opted to work with ESCs to understand molecular mechanisms determining their commitment to the cardiovascular lineage. For instance, Arrell et al elucidated the role of TNFα as a cardiogenic...
inducer in the endodermal secretome by using a combination of 2-DE and shotgun proteomics (Bendall et al., 2009). Subsequently, the same group used proteomic screens to decipher cardiogenic instructive signals in mouse ESCs that induced the expression and nuclear translocation of cardiac transcription factors (Behfar et al., 2007). Similarly, Williamson revealed the post-transcriptional regulation of mesoderm differentiation to endothelial and hematopoietic precursors, the haemangioblasts, in mouse ESCs (Williamson et al., 2008). The largest mouse ESC proteome reported to date, was published by Graumann et al (Graumann et al., 2008). In total, over 5,000 proteins were identified and two different proteomic methods were compared: gel-LC-MS/MS and shotgun proteomics after isoelectric focusing of tryptic peptides for pre-fractionation. The coverage in both methods was comparable and contained key stem cell markers.

Importantly, murine ES cells could be fully SILAC-labelled when grown feeder-free during the last phase of cell culture. Of course, large quantities of cells are required to achieve such coverage, i.e. up to 10 million. Notably, a recently developed proteomics sample processing and analysis platform, termed rare cell proteomic reactor (RCPR), can help to substantially reduce cell numbers: with this method, as little as 50,000 human ESCs were sufficient to identify over 2,000 unique proteins and quantify significant changes during early mesoderm development (Tian et al., 2010). Finally, a recent phosphoproteomic analysis in human ESCs has revealed >10,000 unique phosphorylation sites (Swaney et al., 2009), among which 5 were localized to Oct4 and Sox2. These two transcription factors are known to be important for stem cell pluripotency and to play a critical role in reprogramming adult cell lines to an ESC state (induced pluripotent cells, iPS cells) (Okita et al., 2007).

A major challenge of proteomics is the identification of scarce proteins. Generally, proteomic analysis of cell or tissue lysates results in the confident identification of the most abundant cytoplasmic proteins. Instead, many interesting proteins remain unidentified due to their relative scarcity. Regarding the identification of novel markers for cell characterization, membrane proteins, such as cluster of differentiation (CD) receptors and ligands, represent an important sub-proteome, which is used to differentiate cellular phenotypes. However, membrane protein enrichment in vitro is challenging and usually membrane protein preparations are contaminated with cytoplasmic proteins.
1.6 Microparticles

For this project, an alternative proteomic method was adopted to obtain membrane proteins: Microparticles (MPs) were harvested from supernatants of EPC cultures. MPs usually refer to intact vesicles formed from the plasma membrane and can be repeatedly harvested from the same cell culture. This is particularly advantageous for scarce cell populations. MP have heterogeneous density and size (0.1-1.0μm), are easily separated by differential centrifugation and originate from many cell types, including endothelial cells, platelets, monocytes, and SMCs (VanWijk et al., 2003, Mayr et al., 2009a).

40 years ago, microparticles (MPs) or microvesicles (MVs) were first described as ‘platelet dust’ by Wolf et al, when he unravelled the ability of eukaryotic cells to shed components off their plasma membrane into the extracellular space (Wolf, 1967). Since their initial discovery, MPs have generated increasing interest; currently, they are investigated in the context of many different diseases as these small secretory vesicles are released upon stimulation of the cell by various chemical or physical stimuli, such as cytokines, thrombin, mechanical forces or hypoxia (Boulanger et al., 2006, Morel et al., 2006b). These cellular fragments can originate from the shedding and blebbing of most cell types, including endothelial cells, platelets, monocytes, erythrocytes and smooth muscle cells (Jimenez et al., 2005, Caby et al., 2005). Interestingly, elevated numbers of different MPs can be found in individuals suffering from type 1 diabetes, acute coronary syndrome and myocardial infarction (Sabatier et al., 2002, Mallat et al., 2000). Notably, the presence of MPs in the bloodstream correlated to poor outcome (Morel et al., 2006a). Regarding vascular pathology, MPs express markers of vascular injury such as von Willebrand factor (vWF), E-selectin, and vascular cell adhesion molecule (V-CAM) (Boulanger et al., 2006) and are considered as markers of endothelial injury (Amabile et al., 2005).

Endothelial cells as well as EPCs are capable to release microparticles in response to serum deprivation or stimulation with tumour necrosis factor α (TNFα). The protein composition of MP has limited complexity, is highly enriched in membrane proteins but can vary dependent on the stimulus. Thus, the membrane protein content in MP may only be partially representative of the
plasma membrane protein profile of the parent cell. Nonetheless, EPC-derived MPs provide an opportunity to obtain sufficient membrane material for proteomic analysis, which is difficult to obtain from scarce cell populations.

1.6.1 Characterization of MPs: Size

MPs usually refer to intact vesicles formed from the plasma membrane. They have heterogeneous density and size (0.1-1.0μm) and they are released as a result of different cellular stresses. MPs can be easily separated from exosomes (VanWijk et al., 2003), apoptotic bodies and matrix vesicles by differential centrifugation (Wu et al., 1997). Apoptotic bodies are released from cells at the later stages of programmed cell death and have the largest diameter among all secretory particles (>1.5μm). These membrane-bound blebs consist of nuclear fragments as well as cytoplasm and contain surface ligands for phagocytic cell receptors (Wu et al., 1997). Exosomes are the smallest of the secretory vesicles with a diameter of 30-90nm and are released into extracellular space by exocytosis as a consequence of fusion of the late multivesicular bodies (MVBs) with the plasma membrane. MVBs are late components of the endocytic pathway which consists of early endosomes (EE), late endosomes (LE) and lysosomes (Thery et al., 2002, Johnstone, 2005). Finally, matrix vesicles bud from the plasma membrane at sites of interaction with the extracellular matrix. These vesicles are observed in the initial stages of arterial calcification and contain high levels of matrix calcium, phosphate, lipids and annexins (Wu et al., 1997).
Figure I.5. Schematic representation of extracellular secretory particles: (1) Microparticles are shed from the plasma membrane of stimulated or apoptotic cells by disruption of the membrane skeleton. (2) Apoptotic bodies are large particles released from cells at the later stages of programmed cell death. (3) Matrix vesicles are extracellular particles observed in the initial stages of arterial calcification. (4) Exosomes are the smallest of the secretory membrane particles and are secreted as a consequence of fusion of the late multivesicular bodies (MVBs) with the plasma membrane. (5) Secretory vesicles: heterogeneous population or secretory vesicles in the circulation (Pula et al., 2008b).
1.6.2 Characterization of MPs: Composition

MPs are enriched in cytoplasmic components, cell surface proteins, bioactive phospholipids (Freyssinet, 2003) derived from the plasma membrane of their parent cells and genetic material in the form of mRNA and other small RNAs such as microRNAs (miRNA). The cellular origin of MPs can be determined with antibodies directed against cell-specific markers. Importantly, the lipid and protein composition of MPs is variable and depends on the cell phenotype as well as the cellular process triggering their formation (Boulanger et al., 2006). Cells are surrounded by a phospholipid bilayer consisting of phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidylcholine (PC) and sphingomyelene (SM) (Diamant et al., 2004). In a resting cell, the membrane components tend to be asymmetrically distributed with the negatively charged PS and PE found in the inner of the membrane and the neutrally charged PC and SM exposed on the outer surface of the cell. Various enzymes maintain this asymmetry of the cell membrane, such as flippase- an inbound-directed pump specific for PS and PE, a floppase- an outward-directed pump, a lipid scramblase that stimulates a bidirectional distribution (Hugel et al., 2005) and calpain, which is activated by calcium and facilitates MP shedding by cleaving cytoskeletal filaments. During activation of the cells the membrane asymmetry collapses and the negative charged molecules are exposed on the surface of the MPs. Notably, PS plays an active role in binding coagulation factors (Hugel et al., 2005, Sims and Wiedmer, 2001, Olas et al., 2002, Abid Hussein et al., 2003, Murphy et al., 1987, Kelton et al., 1992).
Figure I.6. Microparticles in detail. MPs are shed from the cell membrane and consist of a phospholipid bilayer, cytoplasmic proteins and lipids. They also carry proteins responsible for cell adhesion, stimulation and signal transduction as well as cell type specific proteins (adapted from Zwaal & Schroit, 1997; and Hugel et al., 2005).
1.6.3 The role of MPs in cell-to-cell communication

Cells are able to communicate by a plethora of different molecules such as cytokines, secreted growth factors, adhesion molecules and small molecular mediators such as bioactive lipids and nucleotides (Lucas, Yoo et al. 2001; Levine 2004; Taback and Hoon 2004). In the past decade MPs have found to be major mediators of this communication because they are carriers of numerous proteins and lipids similar of those present in the membranes and the lipids of the cells from which they originate from. MPs have been reported to affect target cells by stimulating them directly by surface-expressed ligands (Morel, Toti et al. 2004) or by transferring surface-expressed adhesion molecules or receptors between cells (Rozmyslowicz, Majka et al. 2003) such as CD41 integrin or CXCR4 receptor as well as other particles such as prions. Furthermore, MPs are enriched in mRNA, that could be explained by the fact that mRNA molecules after translocation from the nucleus to the cytoplasm may bind to membranous organelles or vesicles to specific subcellular sites (Cohen 2005) and thus contained into the MPs. Recently, a small number of publications have also revealed that MPs might also contain and transfer small amounts of miRNAs in short distances and thus could be ideal paracrine factors (Hunter, Ismail et al. 2008) and could regulate hematopoiesis and cellular differentiation.
1.7 MicroRNAs: an emerging class of small RNAs

During development and adult life proper gene expression is of high importance in any given cell type. The control of gene expression occurs via transcriptional regulators, DNA and chromatin modifiers but also at the RNA level. The latter is taking place through alternative splicing, mRNA stability and translational control with the participation of small regulatory non-coding RNAs. These molecules include small interfering RNAs (siRNAs) and microRNAs (miRNAs) which have the capacity to regulate protein expression by directing the binding of protein complexes to specific nucleic acids (Ambros 2004; Bartel 2004). MiRNAs are an emerging class of highly conserved, non-coding single-strand small RNAs of ~22 nucleotides in length in their mature form. Moreover, miRNAs are found either as individual molecules or in arrayed clusters and are capable of regulating gene expression on post-transcriptional level by repressing the translation of protein by interacting with the 3′ untranslated regions (UTRs) of specific mRNAs or by promoting degradation of mRNA therefore silencing of gene expression (Lagos-Quintana, Rauhut et al. 2001; Altuvia, Landgraf et al. 2005; Chapman and Carrington 2007). More than 1,000 human miRNAs have been identified so far implicating the crucial role miRNAs play in various physiological and pathological processes such as oncogenesis, cardiogenesis and hematopoietic lineage differentiation (Chen, Li et al. 2004; Zhao, Samal et al. 2005; Esquela-Kerscher and Slack 2006). Indeed, specific miRNAs that regulate endothelial cell functions and angiogenesis have been identified such as miR-130a and miR-27b being positive regulators and miR-221 and miR-222 as negative regulators of endothelial cell migration and angiogenesis (Poliseno, Tuccoli et al. 2006; Suarez, Fernandez-Hernando et al. 2007; Chen and Gorski 2008).
CHAPTER II

AIMS & PROJECT DESIGN
By now, numerous studies have demonstrated that EPCs are present among PBMCs and represent a subset of circulating BMCs, which have the capacity to differentiate into endothelial cells in vivo. However, the nomenclature and the phenotype of early outgrowth EPCs are subject to on-going controversy and there are currently no specific markers, which unambiguously identify these cells. Thus, a more comprehensive approach is needed to analyse their antigenic profiles. The aim of this project was to define and characterise the early outgrowth EPCs derived from human peripheral blood mononuclear cells by using a proteomics approach. In order to perform a proteomics analysis of early outgrowth EPCs I chose to use EPC-derived MPs. MPs have the capacity to retain membrane antigens specific for the parent cell they originate from thus, represent an ideal sub-proteome for cell characterization. Mass spectrometry is the instrument of choice for this kind of research and in order to reduce sample complexity and facilitate the identification of membrane proteins by the instrument the use MPs as a more simplistic approach appeared ideal.

1) **Proteomic Analysis of EPC-derived MPs:** MPs derived from early outgrowth EPCs were subject to mass spectrometry analysis (the workflow of the proteomic analysis is illustrated in Figure II.1). A variety of membrane and other proteins were identified and validation of the proteomic findings was carried out by standard molecular techniques such as Western Blotting and RT-PCR. In addition, proteins of interest were visualized by fluorescence staining and confocal microscopy. It was also evident that other types of MPs were present among the early outgrowth EPC cultures e.g. platelet-derived MPs (PMPs) which were investigated further.

2) **Functional Changes and Identification of Mediator:** The pro-angiogenic effect of the MPs derived from EPCs was tested using the Matrigel assay. Interactions of PMPs with other cell types were investigated using the monocytic THP-1 cell line to explore whether MPs can be novel inter-cellular communicators. THP1 cells were cultured in the presence of stable isotopes (SILAC, stable isotope labelling of amino acids in culture) for 5 population doublings and
Chapter II......Aims and Project Design

incubated with PMPs. Upon incubation with PMPs, THP-1 cells were analysed further using proteomics to study protein transfer. The conditioned medium of the cells stimulated by PMPs was used for \textit{in vitro} and \textit{in vivo} Matrigel assays, wound healing, adhesion and migration assays were used to confirm the ability of PMPs to mediate paracrine effects. Additionally, the secreted factors were undergone a further proteomic analysis in order to establish the factor (CXCL7) responsible for the phenotypic and functional changes of the THP-1 cells following incubation with PMPs.

3) Paracrine Factors and miRNA Regulators: To determine whether CXCL7 mediates the functional changes appeared in the THP-1 cell line after incubation with PMPs. An antibody was used to block CXCL7 activity and the cells/conditioned medium were tested during adhesion and transmigration assays. Furthermore, the expression of miRNAs in the THP-1 cell line was assessed regarding the CXCL7 effect. Real-time PCR was employed together with 2D-DIGE proteomics, online search tools and overexpression and downregulation experiments in order to identify the secreted factor (CXCL7) responsible for the regulation of new miRNAs (miR-885-5p) as well as their targets (LCP-1). The functionality of LCP-1 was assayed using a phagocytosis experiment.
Ch[apter II…...Aims and Project Design

Figure II.1 Workflow for the proteomic analysis. PBMNCs were collected from the buffy coat after separation onto Lymphoprep™ solution and seeded on fibronectin-coated plates in endothelial basal medium containing VEGF. MPs were isolated from the conditioned medium of EPCs by differential centrifugation and resuspended in lysis buffer. The MP proteins were separated by 1D-SDS-PAGE electrophoresis and visualized by silver staining. The silver stained bands were excised and subject to in-gel tryptic digestion with a robotic digestion system (ProGest, Genomic Solutions). Subsequently, the tryptic peptides were separated by reverse-phase chromatography and identified by tandem mass spectrometry.
CHAPTER III

METHODS & MATERIALS
All general laboratory reagents and chemicals were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated.

3.1 EPC culture

EPC cultures were performed as previously described (Kalka et al., 2000, Vasa et al., 2001a, Dimmeler et al., 2001). In brief, PBMNCs from healthy volunteers (10 males and 10 females aged 25-40 years old) were isolated by Lymphoprep™ (1.077g/ml, Axis-Shield PoCAS, Oslo, Norway) gradient centrifugation. The low density fraction (<1.077 g/ml) was carefully removed from the interface and washed three times with PBS (Dulbecco’s phosphate buffered saline, Sigma-Aldrich, St Louis, MO, USA) containing 2% FBS (foetal bovine serum, heat inactivated, Gibco, Invitrogen, Carlsbad, CA, USA). Immediately after isolation, the cells were counted and 8x10^6 cells were plated on fibronectin-coated (10μg/ml Fibronectin from human plasma, Sigma-Aldrich, St Louis, MO, USA) 12-well plates containing 1ml endothelial basal medium (EBM, Cambrex Bio Science, Walkersville, MD, USA) supplemented with 20% FBS, EGM SingleQuots (10 g/ml epidermal growth factor, 3 g/ml bovine brain extract, 50 g/ml gentamicine, 50 g/ml amphotericin-B, 1 g/ml hydrocortisone, Cambrex Bio Science, Walkersville, MD, USA) and 10 ng/ml human vascular endothelial growth factor 165 (hVEGF 165, R&D Systems, Minneapolis, MN, USA). After 3 days in culture, the non-adherent cells were removed and fresh EBM medium was added. The medium was changed on day 5 and cells were kept in culture until day 7. The EPC phenotype was confirmed by marker expression and the uptake of 1, 19-dioctadecyl-3, 39-tetramethylindocarbocyanine-labeled acetyl low-density lipoprotein (DiI-Ac-LDL) and binding of ulex-lectin as described previously (Urbich et al., 2003).

3.2 Culture of endothelial cells

Human umbilical venous endothelial cells (HUVECs) were isolated from human umbilical cords and cultured in M199 medium supplemented with 1ng/ml ECGF, 3μg/ml ECGS, 10U/ml Heparin, 2.5 μg/ml Thymidine and 5% FBS. The cells were grown in T75 flasks coated with gelatine (Sigma-Aldrich, St Louis, MO, USA), incubated at 37°C in 5% CO₂-enriched atmosphere for up to 10 passages and passaged every 2 days.
3.3 Culture of THP-1 cell line

The human monocytic THP-1 cell line (American Type Culture Collection ATCC, Manassas, VA, USA) was cultured in ATCC-formulated RPMI-1640 Medium (cat: 30-2001, American Type Culture Collection, ATCC, Manassas, VA, USA) supplemented with 10% of FBS and antibiotics.

3.4 Isolation of MPs derived from EPCs

MPs were isolated following an established protocol (Leroyer et al., 2007). In brief, supernatants of EPC cultures were collected at different time points (e.g. at days 3, 5, 7 and day 8 after O/N serum deprivation) and centrifuged at 400 x g for 15min to remove floating cells and cellular nuclei, followed by a second centrifugation step at 12,500 x g for 5min to remove cell debris and apoptotic bodies. MPs were pelleted from this pre-cleared supernatant in a final centrifugation step at 20,500 x g for 150min at 4°C (Sorvall RC-6 Plus, Thermo Fisher Scientific).

3.5 Electron microscopy

Agarose-enclosed (2% low gelling agarose, 40°C) MP-pellets from the last centrifugation step (20,500 rpm) were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer pH 7.3 to 7.4, post-fixed in 1% OsO4, and embedded in Epon epoxy resin. Ultrathin sections (75-nm each) were stained with uranyl acetate and lead citrate. High-resolution transmission electron microscopic (TEM) analysis was done at 80 kV in a JEOL 1200 EX electron microscope (Jeol). Electron microscopy pictures were acquired by Dr Marianne Steiner and Dr Johannes Breuss (Center for Anatomy and Cell Biology, Medical University Vienna, Vienna, Austria; and Department of Vascular Biology and Thrombosis Research, Centre for Bio-Molecular Medicine and Pharmacology, Medical University of Vienna, Vienna, Austria).

3.6 Proteomics Analysis of EPC-derived MPs

For proteomics, MPs from EPC cultures of 4 healthy subjects (a total of 200ml of full blood was collected from 2 male and 2 female volunteers aged 25-40 years old) were pooled to obtain sufficient material. MPs were reconstituted in Laemmli buffer and separated by SDS–polyacrylamide gel electrophoresis.
(PAGE) gels. Large format gradient gels (4%-12%) were cast using the a2DE optimizer (NextGen Sciences). After the gels were overlaid with water-saturated butanol (2:1) and left to polymerize overnight, the stacking gel containing 4% to 5% acrylamide weakly buffered at pH 9.0 was cast over the already set resolving gel. Once samples were loaded, a constant 50-mV current was applied as proteins migrated down the stacking gel; at the stacking gel/running gel boundary the current was increased and maintained at 75mV until the dye front reached the end of the gel. After silver staining by using the Plus one silver staining kit (GE healthcare), all gel bands were excised, and were subject to in-gel tryptic digestion. For tandem mass spectrometry (MS/MS), in-gel digestion with trypsin was performed according to published methods (Shevchenko et al., 1996, Wilm et al., 1996) modified for use with an Investigator ProGest (Genomic Solutions) robotic digestion system. 10 uL of sample was injected using an autosampler (Thermo Electron Corporation, CA, USA) and loaded onto a 100 x 0.18 mm reverse-phase liquid chromatography (Hill et al.) column (BioBasic-18, particle size 5 µm, Thermo Electron Corporation, CA, USA) at 2 µl/min using an Surveyer MS pump (Thermo Electron Corporation, CA, USA) and eluted with a 90 min gradient (0.1-30% B in 35 min, 30-50% B in 10 min and 50-80% B in 5 min where A =99.9% H2O, 0.1% formic acid and B = 99.9% acetonitrile, 0.1% formic acid). The column was coupled to an electrospray source and spectra were collected from an ion-trap mass analyzer (LTQ XL, Thermo Electron Corporation, CA, USA) using full ion scan mode over the mass-to-charge (m/z) range 300-1800. MS/MS was performed on the top three ions in each MS scan using the data-dependent acquisition mode with dynamic exclusion enabled. MS/MS spectra were matched to database entries (UniProt Knowledgebase Release 7.5, consisting of: UniProtKB/Swiss-Prot Release 49.5 and UniProtKB/TrEMBL Release 32.5 of 18-Apr-2006) using TurboSEQUEST software (Bioworks 3.3, Thermo Finnigan). All peptide sequence assignments were required to result from fully tryptic cleavages of the corresponding proteins. Scaffold (version 1.0, Proteome Software Inc., Portland, OR, USA) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 99.0%
probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003, Mayr et al., 2005b, Mayr et al., 2008, Mayr et al., 2004, Mayr et al., 2005a).

3.7 RT-PCR

Total RNA was extracted from EPCs using the Trizol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. Total RNA from other cell lines was extracted with the RNeasy Mini Kit and QIAshredder (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. RNA (5μg) was converted to cDNA using the ImProm-II Promega Reverse Transcription System (Promega Corporation, Madison, WI, USA). The cDNA products were amplified by PCR using human-specific primers for CD31 and integrins (Primer sequences are shown in Section 3.34). The PCR conditions were as follows: 94°C for 4 min and then 35 cycles at 94°C for 1 min, 58°C for 1 min, 72°C for 1 min followed by 72°C for 5 min. The PCR products were detected by separation on 2% agarose gels (Ultrapure Agarose, Invitrogen, Carlsbad, CA, USA) and ethidium bromide staining.

3.8 Western Blotting

Total proteins were extracted using RIPA lysis buffer (25mM TrisHCl pH 7.6, 150mM NaCl, 1%NP-40, 1% sodium deoxycholate, 0.1% SDS with 1 tablet of Complete Mini EDTA Easypack, Roche Diagnostics, Mannheim, Germany). Protein concentrations were measured using the BCA Protein Assay Kit (Thermo Fisher Scientific) for the first part of the project and the Bradford Assay or the Nanodrop for the second part of the project. 20µg were separated by SDS-PAGE on 4-12% Tris/glycine gels (Invitrogen, Carlsbad, CA, USA) using also pre-stained molecular weight markers (SeeBlue pre-stained molecular weight markers, Novex, San Diego, CA) which were run alongside the samples to allow size determination of proteins. The gels were then run at 100V (constant voltage) for approximately 2h, using the NuPAGE MOPS-SDS running buffer, in the XCell SureLock Mini-Cell electrophoresis apparatus (Novex, San Diego, CA). After separation of the proteins the gels were transferred to nitrocellulose membranes at 100V (constant voltage) for 90min in a Trans-blot chamber (Biorad, Hercules, CA) filled with Western-blotting transfer buffer (25mM
Trizma base, 190mM glycine, and 20% (v/v) methanol). Transfer buffer was kept at 4°C by a water-circulating cooling pump. Next, the membranes were blocked for 1h with 5% (w/v) Marvel (low fat dried milk) in 1X PBS and then incubated with the primary antibody. Primary antibodies were used at 1:1000 dilution in a 5% (w/v) bovine serum albumin in 1X PBS solution. Membranes were incubated for 3h at room temperature with the primary antibody solution. The blots were washed 3 times for 10min with 1X PBS and then incubated with the appropriate species secondary horseradish peroxidise-conjugated antibodies at 1:1000 dilution in a 5% (w/v) Marvel in 1X PBS solution. Finally, the blots were washed again 3 times for 10min with 1X PBS and signal was developed by enhanced chemiluminescence (ECL), (GE Healthcare). Protein bands were visualized by autoradiography on blue-sensitive film (Fujifilm Super RX, Fujifilm, Bedford, UK).

3.9 Full Blood Count

1ml of blood was collected into BD Vacutainer EDTA tubes (4ml, K$_2$EDTA, BD Vacutainer, Becton Dickinson) and mononuclear cell preparations were obtained after Lymphoprep density gradient centrifugation. The cell preparations were analysed in duplicate using an automated cell counter (Advia 2120, Siemens Medical Solutions Diagnostic) to determine the number of white cells and platelets present by optical analysis. The different types of white cells present were distinguished by light scatter and myeloperoxidase (MPO) activity after being exposed to a myeloperoxidase enzyme substrate, which was passed through a Tungsten light source. The pattern of light scattered (size) and intensity of light absorbed (MPO activity) determined the type of white cells present. Platelets were iso-volumetrically sphered before exposure to the laser light source. The platelet count was then determined by the amount of light scattered (size) and their reflective index (activation status). The cell counter was operated by Joy Gallagher (King’s College Hospital, National Health Service (NHS) Foundation Trust, Blood Sciences Laboratory Services, London, United Kingdom).
3.10 Fluorescence Microscopy

12mm glass cover-slips were coated with 10μg/ml fibronectin for 1h at RT before PBMNCs were placed on top and cultured for 7 days in EBM complete medium. The slides were removed from the culture and fixed at different time points e.g. day 1, 3, 5 and 7 with 2% paraformaldehyde (PFA) and permeabilized with 0.05% Triton-X (Sigma-Aldrich, St Louis, MO, USA) for 10min. After blocking with 2% BSA (Sigma-Aldrich, St Louis, MO, USA) for 15 min, cells were stained with primary antibodies anti-PAR-4 (goat, Santa Cruz Biotechnology, CA, USA) and mouse anti-integrin αIIb (mouse, Chemicon International, Temecula, CA, USA) for 90 min in a 1:200 dilution followed by incubation in the dark with anti-goat TRITC (anti-goat IgG TRITC conjugate, Sigma-Aldrich, St Louis, MO, USA) and anti-mouse FITC (Biosource-International, Camarillo, CA, USA) conjugated secondary antibodies for 60 min. Finally, nuclei were counterstained with DAPI (Vectashield mounting medium with DAPI, Vector Laboratories Inc, Burlingame, CA, USA) for 3min and slides were covered with fluorescent mounting medium. Images were obtained on a Zeiss Axioplan 2 Imaging fluorescence microscope equipped with an Axiocam camera (Carl Zeiss, Jena, Germany).

3.11 Platelet preparation

Platelets were isolated from healthy individuals as previously described (Falati et al., 1999). In brief, 100ml of blood were drawn using acid citrate dextrose as anticoagulant (ACD: 120mmol/L sodium citrate, 110mmol/L glucose, 80mmol/L citric acid, 1:7 vol/vol) and centrifuged for 17min at 200 x g and 30°C in the presence of indomethacin (10μmol/L). The platelet rich plasma was then centrifuged for another 10min at 1,000 x g in the presence of prostacyclin (0.1μg/ml). The resulting platelets were resuspended in modified Tyrodes-HEPES buffer (145mmol/L NaCl, 2.9mmol/L KCl, 10mmol/L HEPES, 1mmol/L MgCl2, 5mmol/L glucose, pH7.3) at a concentration of 4x10⁸ /mL.

3.12 Preparation of platelet MPs

Platelets were activated by thrombin (0.1 U/ml, Sigma-Aldrich) and their aggregation was monitored with a turbidometric method (Chronolog 490, Chronolog Corporation, Havertown, PA, USA). Platelet MPs were harvested by
ultracentrifugation at 100,000 x g for 90 min at 4°C and carefully resuspended in complete medium (RPMI 1640 with 10% FBS). For fluorescence labelling, platelet MPs were incubated with 5 μg/mL CellMask Deep Red plasma membrane stain/lectin (Molecular Probes, Invitrogen, Carlsbad, CA, USA) at 37°C for 15 min and pelleted again at 100,000 x g in order to remove any excess dye.

3.13 Platelet MP Uptake

5 x 10^5 THP-1 cells were cultured as previously described into 12-well culture plates with 0.5 ml complete medium. Platelet MPs labelled with Deep red (D/R) lectin were added to the cultures for 2 h at 37°C. Cell suspensions were washed twice with PBS and placed on poly-D-Lysine coated cover slips (BD BioCoat Cellware, BD Biosciences, MA, USA), and fixed with 2% PFA for 10 min at 37°C. In other experiments, isolated platelet MPs were added directly to THP-1 cells and incubated for 2 days at 37°C. The cells were then centrifuged, washed twice in PBS and applied on poly-D-Lysine coated cover slips and fixed with 2% PFA as described above. The following primary antibodies were used: sheep anti-CD31 (R&D Systems, Minneapolis, MN, USA) and mouse anti-integrin αIIb (Chemicon International, CA, USA) for 90 min at a dilution of 1:200 followed by secondary antibodies anti-goat TRITC and anti-mouse FITC.

3.14 Flow Cytometry Analysis

Pelleted MPs from EPC conditioned medium were used for flow cytometry experiments. Labelling for annexin V, CD31, CD41, CD11a and CD235a was performed to determine the cellular origin of MPs in EPC cultures as reported earlier (Leroyer et al., 2007). MPs expressing Phosphatidylserine (PS) were labelled using Fluoroisothiocyanate-conjugated Annexin V (Roche Diagnostics, France) in the presence or absence (negative control) of CaCl_2 (5 mM). The MP pellet was incubated with different fluorochrome-labelled antibodies or their corresponding isotype-matched IgG controls (RT; 30 minutes in the dark). Anti-CD31-Phycoerythrin, anti-CD41-Phycoerythrin-Cyanin5 and anti CD235a-Fluoroisothiocyanate were obtained from Beckman Coulter (Villepinte, France). Anti-CD11a (LFA-1)–Phycoerythrin was purchased from BD Pharmingen. MPs were analyzed on a Coulter EPICS XL flow cytometer.
Regions corresponding to MPs were identified in forward light scatter (FCS) and side-angle light scatter (SSC) intensity dot plot representation set at logarithmic gain. The gate for MPs was defined as events with a 0.1-1 µm diameter, in comparison with calibrator beads (Megamix fluorescent beads of 0.5, 0.9 and 3µm in diameter; Biocytex, Marseille, France), and plotted on a FL/FSC fluorescence dot plot to determine the MPs labeled by specific antibodies. The staining and the FACs analysis were performed by Dr Cecile Devue and Dr Chantal M. Boulanger (Paris-Cardiovascular Research Center, Inserm U970, Hospital European Georges Pompidou, Universite’ Paris-Descartes, Paris, France).

3.15 Platelet contamination of PBMNCs-Hema Gurr Staining

PBMNCs were prepared as previously described by Lymphoprep isolation. The cells were counted using Trypan blue (solution 0.4%, Sigma-Aldrich, St Louis, MO, USA) and 1x10^5 cells (100µl volume) were subject to cytospin (Shandon Cytospin 3, Cytocentrifuge, Thermo Fisher Scientific) for 1000 rpm/3 min. The cells were fixed and stained using the Hema Gurr rapid staining set for haematology (VWR International, Poole, UK). The cell film was fixed by dipping the glass slide in Fixing solution containing methanol for 5x1 sec dips. The procedure was repeated using Solution 1 (Sodium Azide Eosin stain solution) and then Solution 2 (Methylene Blue solution) and finally rinsed with deionised water (all solutions were part of the Hema Gurr rapid staining set for haematology). The slides were drained at RT for 5 min and mounted using one drop of DPX mounting agent (Fluka Biochemika, Buchs, Switzerland). The images were taken using a Zeiss Axioplan 2 Imaging microscope (object 100x1.30 oil immersion) equipped with an Axiocam camera (Carl Zeiss, Jena, Germany).

3.16 Ulex europaeus agglutinin 1

THP-1 cells were placed on poly-D-lysine–coated coverslips (BD BioCoat Cellware; BD Biosciences) in the presence or absence of non-activated platelets and platelet MPs activated by thrombin as described in “Preparation of platelet MPs” above. The adherent cells were fixed with 3.75% PFA and incubated with UEA-1 (Lectin from Ulex europaeus FITC conjugate; Sigma-
Aldrich) for 1 hour. Staining of nuclei was performed with DAPI Nucleic Acid Stain (Molecular Probes, Invitrogen) and images were obtained at room temperature on a Leica TCS SP5 STED (Leica Microsystems) inverted confocal laser-scanning microscope.

3.17 In vitro tube formation assay

4x10^4 HUVECs were placed on Matrigel™ (10 mg/ml, Matrigel Basement Membrane Matrix, Phenol-Red Free, BD Biosciences, 50µl/well) in 8-well chamber slides. After attachment, 300µl of EPC conditioned medium was added. The formation of capillary networks was assessed after overnight incubation at 37°C. HUVECs were treated with conditioned medium derived from EPC cultures at different time points (day 3, 5 and 7). In addition, the particulate fraction was removed from the conditioned medium by filtration (0.1 µm filters) or integrin inhibitors were added to the conditioned medium of EPC cultures. The inhibitory disintegrin Echistatin (Echistatin α1 isoform, Sigma-Aldrich) is an Arg-Gly-Asp (RGD)-containing-snake-venom protein and blocks the function of integrin αVβ3/αIIbβ3 (Marcinkiewicz et al., 1997). A peptide derived from the GP IIIa molecule (GP IIIa-4) was added to inhibit the formation of the GP IIb/IIIa complex (Chiang and Zhu, 2005) (kind gift of Dr. Thomas M. Chiang). Pictures were taken on a Nikon Eclipse TS100 inverted microscope (objective 10 x /0.25). The length of the capillaries was measured by the AxioVision 3.0 Software (Carl Zeiss Vision Gmbh) and expressed as pixels^2 from 5 different reference points in duplicate wells. The experiment was repeated twice and the values of the length of the tubes are given as mean ± SD.

3.18 Bruneck Study

Population recruitment was performed as part of the Bruneck Study (Kiechl et al., 2002). The survey area was located in the north of Italy (Bolzano Province). At the 1990 baseline evaluation, the study population was recruited as an age- and sex-stratified random sample of all inhabitants of Bruneck aged 40-79 years (125 women and 125 men in the fifth to eighth decades each). Assessments were carried out every five years and participation exceeded 90%. The current study focused on the 2005 evaluation (Xiao et al., 2007). The “outgrowth” of EPCs was assessed in a random subsample of 526 subjects by
two commonly used methods: A culture assay for early-outgrowth EPCs as described above and the formation of colony forming units (CFUs). EPC numbers were identified by double positive staining for Dil-Ac-LDL and Lectin on day 5 of culture. For CFUs, PBMNCs were suspended in Medium 199 (22340, Gibco) with 20% FBS for 48h. Non-adherent cells were re-plated at 4x10^6 cells/well on human fibronectin-coated plates kept in the same growth medium. After 7 days, CFUs were counted following strict guidelines to ensure consistency. Two trained independent senior investigators blinded to the clinical details of the subject determined the number of EPCs and CFUs. Coefficient of variation was <10% in each case. EPC number and CFUs were assessed in 526 participants. Subjects with and without EPC number and CFU assessments did not differ in age, sex and expression of cardiovascular risk factors. Statistical analysis was performed using the analysis of variance and unpaired Student t test. Results were given as means plus or minus SE. Nonparametric Spearman rank correlation coefficients were obtained using SPSS 12.0 and BMDP software (Statistical Solutions). A P value less than 0.05 was considered significant.

3.19 Isotopic Labeling in Cell Culture (SILAC)

The THP-1 cell line was cultured using the reagents and the instructions from the SILAC Protein Quantitation Kit from Thermo Scientific (Pierce). The cells were resuspended either in 50mg L-Lysine-2HCl and 50mg L-Arginine-HCl ("Light" isotopes) or in 50mg ^13C_6 L-Lysine-2HCl and 50mg ^13C_6 ^15N_4 L-Arginine-HCl ("Heavy" isotopes) for at least five population doublings at 37°C with CO_2.

3.20 2D DIGE proteomics

For cellular proteomics, THP-1 cells, THP-1+PMPs (cultured in the presence of PMPs for two days) and THP-1+PST (cultured in the presence of PST for two days) were pelleted at 1,100 x g and rinsed thoroughly with cold PBS to remove any serum components. Protein extracts were prepared from THP-1 cells using a lysis buffer (8M urea, 4% w/v CHAPS, 30mM Tris-Cl, pH 8.5) compatible with DIGE labelling (GE healthcare) and the protein concentration was determined using the method described by Bradford. The fluorescence dye labelling reaction was carried out at a dye/protein ratio of
400pmol/100μg. After incubation on ice for 30 min, the labelling reaction was stopped by scavenging non-bound dyes with 10mM lysine (L8662, Sigma) for 15 min. For two-dimensional gel electrophoresis, samples were mixed with 2x buffer (8M urea, 4% w/v CHAPS, 2% w/v DTT, 2% v/v Pharmalytes 3-10 for IEF), 50μg per sample were diluted in rehydration solution (8M urea, 0.5% w/v CHAPS, 0.2% w/v DTT, and 0.2% v/v Pharmalyte pH 3-10) and loaded on IPG strips (18cm, pH 3-10, nonlinear, GE healthcare). After rehydration overnight, strips were focused at 0.05 mA/IPG strips for 60 kVh at 20°C (Multiphor II, GE healthcare). Once IEF was complete the strips were equilibrated in 6M urea containing 30% v/v glycerol, 2% w/v SDS and 0.01% w/v Bromphenol blue, with addition of 1% w/v DTT for 15 min, followed by the same buffer without DTT, but with the addition of 4.8% w/v iodoacetamide for 15 min. SDS-PAGE was performed using 12% T (total acrylamide concentration), 2.6% C (degree of cross-linking) polyacrylamide gels without a stacking gel, using the Ettan DALT system (GE healthcare). The second dimension was terminated when the Bromophenol blue dye front had migrated off the lower end to the gels. After electrophoresis, fluorescence images were acquired using the Typhoon variable mode imager 9400 (GE healthcare). Finally, gels were fixed overnight in methanol: acetic acid: water solution (4:1:5 v/v/v). Protein profiles were visualised by silver staining using the Plus one silver staining kit (GE healthcare). For documentation, silver-stained gels were scanned in transmission scan mode using a calibrated scanner (GS-800, Bio-Rad). DIGE gels were analysed using SameSpot version 3.2 software (Nonlinear Dynamics). Spots exhibiting differential expression (p<0.05, one-way ANOVA) were excised for mass spectrometry identification.

3.21 1D Secretome analysis

For secretome proteomics, THP-1 cells were cultured as described previously in the presence of PMPs for two days. The last day of the culture the cells were rinsed thoroughly with cold PBS to remove any serum components and cultured further in plain medium without any serum additives for 12-16h. The cell supernatant was then removed and cleared of any particles by centrifugation at 1,100 x g, 10,600 x rpm and finally at 20,500 x rpm. The cleared conditioned medium was then subject to acetone precipitation. 300μl of
the supernatant were resuspended in cold acetone at a ratio of 1:5 parts and left to precipitate O/N at -20°C. The next morning the samples were pelleted at 0°C for 40 min at 13,000 x g. The pellets were dried and then resuspended in H$_2$O; the protein concentration was determined using the Nanodrop. Sufficient amounts of 4x sample buffer buffer containing 100mM Tris, pH 6.8, 40% glycerol, 0.2% SDS, 2% _-mercaptoethanol, and 0.02% bromophenol blue were added to the samples and incubated for 10 min at 96°C. 300µg of protein per sample were loaded and separated in 1D large SDS-PAGE gels. After electrophoresis, gels were stained using the PlusOne Silver staining kit (GE Healthcare). Silver staining was used for band excision to avoid cross-contamination with fainter gel bands. (Coomassie staining will predominantly stain the abundant proteins; hence, fainter gel bands could be missed.). The gel bands were excised in identical parallel positions across lanes, and no “empty” gel pieces were left behind. Subsequently, all gel bands were subjected to in-gel digestion with trypsin using an Investigator ProGest (Genomic Solutions) robotic digestion system as described previously. Tryptic peptides were separated on a nanoflow LC system (Dionex UltiMate 3000) and eluted with a 40-min gradient (10–25% B in 35 min, 25–40% B in 5 min, 90% B in 10 min, and 2% B in 30 min where A is 2% ACN, 0.1% formic acid in HPLC H2O and B is 90% ACN, 0.1% formic acid in HPLC H2O). The column (Dionex PepMap C18, 25-cm length, 75-_m internal diameter, 3-_m particle size) was coupled to a nanospray source (Picoview) using RePlay (Advion). After the direct LC-MS run, the flow was switched, and the portion stored in the capillary of the RePlay device was reanalyzed (“replay run”). Spectra were collected from an ion trap mass analyzer (LTQ-Orbitrap XL, Thermo Fisher Scientific) using full ion scan mode over the mass-to-charge (m/z) range 450–1600. MS/MS was performed on the top six ions in each MS scan using the data-dependent acquisition mode with dynamic exclusion enabled. MS/MS peak lists were generated by extract_msn.exe and matched to a trypsin_human_bovine_SILAC database (UniProtKB/ Swiss-Prot Release 14.6, 20,333 protein entries) using SEQUEST version 28 (revision 13) (Bioworks Browser 3.3.1 SP1, Thermo Fisher Scientific) and X! Tandem (version 2007.01.01.2). Two missed cleavages were allowed. Scaffold (version 2.0.5, Proteome Software Inc., Portland, OR) was used to calculate the spectral counts and to validate MS/MS-based peptide and protein identifications.
3.22 Matrigel plugs

50μl of Matrigel were mixed with 100μl of a) conditioned medium from THP-1 cells-serum starved O/N, b) conditioned medium from THP-1+PMPs-serum starved O/N, c) PMPs, and then injected subcutaneously into the back or flank of NOD/SKID mice. Two injections were performed for each group. The mice were killed on day 30, and the plugs were harvested and frozen immediately in liquid nitrogen, followed by embedding with OCT, sectioning and haematoxylin-staining. The sections were visualized by phase contrast microscopy (objective 4X/0.10, microscope Nikon Eclipse TS100, connected to a Nikon Digital Sight imaging system).

3.23 Wound healing assay

HUVECs were cultured in 6 well-plates to confluence and stained by using the green cell tracker as described previously. The day of the experiment, the monolayer was injured manually using a pipette tip; following a brief wash with PBS the conditioned medium from THP-1 cells, THP-1+PMPs and plain RPMI 1640 was added. The healing of the injury was followed at time 0, 2, 6, 8 and 20 hours by fluorescent microscopy (objective 10X/0.10, microscope Nikon Eclipse TS100, connected to a Nikon Digital Sight imaging system).

3.24 Parallel plate flow chamber assay

HUVECs were grown to confluence on collagen I-coated Nunc Slide Flaskettes (4 cm²; Nalge Nunc International). The day of the experiment HUVECs were activated using TNF-α (10ng/ml) for 4h; the flasks were then disassembled to use the slide for rolling assays. Slides were washed twice with PBS and mounted in a chamber slide-base parallel plate flow chamber (channel height 0.15 cm; custom made by Imperial College London). The THP-1 cells (control or THP-1+PMPs; stained with green or orange cell tracker respectively) were resuspended at a density of 3x10⁵/mL in RPMI 1640 medium and perfused at 37°C over the HUVEC-coated slides at a fixed shear stress of 2.5 dyn/cm² with added flow. Rolling cells were defined as those with a mean velocity between 3 and 200m/s; adherent cells were specified as those moving less than 3m within a 10-second time period. Experiments were visualized using a Diaphot 300.
inverted fluorescence microscope (Nikon) connected to a JVC TK-C1360B colour video camera and recorded on a Panasonic AG-6730 S-VHS video recorder (Microscope Service and Sales). Images were acquired into a video file (In Video PCI; Focus Enhancements, Campbell, CA) for 9min and 40sec and numbers of cells undergoing arrest and transmigration of the population were calculated using fluorescence microscopy with a × 10 objective (800 × 600 mm²).

3.25 Migration assay
The method was adapted from previous studies. For THP-1 migration assays, 10⁵ cells tracked with either green or orange cell tracker (CellTracker Probes for Long-Term Tracing of Living Cells, CellTracker Green CMFDA and CellTracker Orange CMRA, all from Invitrogen) were added to the top inserts of 24 well-transmigration microplates (pore size 5 μm, Corning). The bottom chamber of the transmigration microplate was either empty or seeded with 2x10⁵ HUVECs (either untreated or TNF-α activated (10ng/ml)), following coating with fibronectin (20μg/ml, Sigma). Transmigration was quantified 8 hours after assembly of the chemotaxis chamber by fluorescent microscopy (objective 10X/0.10, microscope Nikon Eclipse TS100, connected to a Nikon Digital Sight imaging system).

3.26 Adhesion assay
HUVECs were grown to confluence on collagen I-coated 24-well plates. The day of the experiment, HUVECs were activated with TNFα (10ng/ml) for 4h or left untreated. THP-1 cells (control or THP-1+PMPs; stained with green or orange cell tracker respectively) (CellTracker Probes for Long-Term Tracing of Living Cells, CellTracker Green CMFDA and CellTracker Orange CMRA, all from Invitrogen) were added to the wells at a density of 5x10⁴ cells per well per condition. The cells were left to adhere for 1h, the medium with the non-adherent cells was aspirated and the wells were thoroughly washed with cold PBS. The cells were fixed using 3.75%PFA and the adherent cells were measured using fluorescence microscopy as previously described.
3.27 Transmigration assay

For THP-1 transmigration assays, $10^5$ HUVECs were placed on the top inserts of a 24 well-transmigration microplates (pore size 5 μm, Corning) coated with collagen (20μg/ml, Sigma) and left to grow to confluence. When HUVECs created a monolayer the same amount of THP-1 cells (conditioned or not) tracked with either green or orange cell tracker (CellTracker Probes for Long-Term Tracing of Living Cells, CellTracker Green CMFDA and CellTracker Orange CMRA, all from Invitrogen) added to the top inserts of the microplates. The bottom chamber of the transmigration microplate was either filled with RPMI 1640 plain medium or conditioned medium derived from THP-1+PMPs cells. Transmigration was quantified 2 hours after assembly of the chemotaxis chamber by fluorescent microscopy (objective 10X/0.10, microscope Nikon Eclipse TS100, connected to a Nikon Digital Sight imaging system).

3.28 TaqMan Assay

RNA extraction was performed using the miRNeasy kit (Qiagen) from PLTs, PMPs and THP-1 treated and untreated cells. MiRNAs were reverse-transcribed using the Megaplex Primer Pools (Human Pools A v2.1 and B v2.0), and expression was screened using TaqMan miRNA Arrays A (all from Applied Biosystems). TaqMan miRNA assays were used to determine the expression of individual miRNAs.

Reverse transcription and pre-amplification.

To assess levels of specific miRNAs in individual samples a fixed concentration of 23 ng of total RNA solution from the 25μl eluate was used as input in each reverse transcription (RT) reaction. An RT reaction and pre-amplification step were set up according to the company’s recommendations. RT-PCR and pre-amplification products were stored at -20°C. MiRNAs were reverse transcribed using the Megaplex Primer Pools (Human Pools A v2.1) from Applied Biosystems. Pool A enables quantitation of 377 human miRNAs and in each array, three endogenous controls and a negative control were included for data normalization. RT reaction was performed according to the company’s recommendations (0.8μl of Pooled Primers were combined with 0.2μl of
100mmol/L dNTPs with dTTP, 0.8μl of 10x Reverse-Transcription Buffer, 0.9μl of MgCl2 (25mmol/L), 1.5 μl of Multiscribe Reverse-Transcriptase and 0.1μl of RNAsin (20U/μl) to a final volume of 7.5μl. The RT-PCR reaction was set as follows: 16°C for 2 min, 42°C for 1 min and 50°C for 1 sec for 40 cycles and then incubation at 85°C for 5 min using a Veriti thermocycler (Applied Biosystems). The RT reaction products were further amplified using the Megaplex PreAmp Primers (Primers A v2.1). A 2.5μl aliquot of the RT product was combined with 12.5μl of Pre-amplification Mastermix (2x) and 2.5μl of Megaplex PreAmp Primers (10x) to a final volume of 25μl. The pre-amplification reaction was performed by heating the samples at 95°C for 10 min, followed by 12 cycles of 95°C for 15 sec and 60°C for 4 min. Finally, samples were heated at 95°C for 10 min to ensure enzyme inactivation. Pre-amplification reaction products were diluted to a final volume of 100μl and stored at -20°C.

**TaqMan miRNA array.**

The expression profile of miRNAs in all samples was determined using the Human TaqMan miRNA Array A (Applied Biosystems). PCR reactions were performed using 450μl of the Taqman Universal PCR Master Mix No AmpErase UNG (2x) and 9μl of the diluted pre-amplification product to a final volume of 900 μl. 100μl of the PCR mix was dispensed to each port of the TaqMan miRNA Array. The fluidic card was then centrifuged and mechanically sealed. Q-PCR was carried out on an Applied Biosystems 7900HT thermal cycler using the manufacturer’s recommended programme. Detailed analysis of the results was performed using the Real-Time Statminer Software (Integromics).

**TaqMan q-PCR assay.**

TaqMan miRNA assays were used to assess the expression of individual miRNAs. 0.5μl of the diluted pre-amplification product were combined with 0.25 μl of Taqman miRNA Assay (20x) (Applied Biosystems) and 2.5μl of the TaqMan Universal PCR Master Mix No AmpErase UNG (2x) to a final volume of 5μl. Q-PCR was performed on an Applied Biosystems 7900HT thermal cycler at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. All samples were run in triplicates and standardized to RNU48 using SDS2.2 (Applied Biosystems) software.
3.29 Search for putative targets

The microRNA databases and target prediction tools such as the miRBase (http://microrna.sanger.ac.uk/), the PicTar (http://pictar.mdc-berlin.de/), the DIANA (http://diana.cslab.ece.ntua.gr/microT/) and the TargetScan (http://www.targetscan.org/index.html) were used to identify potential microRNA targets.

3.30 Transfection Assay for miRNAs

THP-1 cells were transfected with precursor and inhibitors of miR-885-5p (100nM, Ambion) using Lipofectamin 2000 (Invitrogen) as described by the manufacturers. In short, 387.5μl of RPMI 1640 medium was mixed with 12.5μl of precursor/inhibitor and negative controls (Ambion) as Mastermix A. In Mastermix B 400μl of RPMI 1640 medium was combined with 25μl of Lipofectamin RNAiMAX (Invitrogen). The mastermixes were combined and incubated for 10min at RT. The precursor/inhibitor/negative control duplex-lipofectamine was then added to the THP-1 cells and mixed gently. 5h following addition of the duplex RPMI 1640 supplemented with 10%FBS but without antibiotics was added to the cells. The next morning fresh complete medium was supplemented the cells, which left to incubate for 48h at 37°C in a CO₂ incubator or until ready to assess gene expression.

3.31 Phagocytosis assay

Transfected THP-1 cells with pre-miR, anti-miR and negative controls for the miR-885-5p were assayed for their ability to phagocytosed pHrodo™ E.coli bioparticles conjugates for phagocytosis (Molecular Probes, Invitrogen). The assay was performed following the manufacturers protocol. In brief, 100μl of the transfected cells (100,000 cells/well) were either attached to fibronectin coated 96-well plates or left unattached. 100μl of the pHrodo™ E.coli bioparticles were then placed to the THP-1 cells and left to incubate for 2-3h at 37°C. All experimental, control, and no-cell control wells of the 96-microplate were scanned using fluorescence plate reader (TECAN) at ~550 nm excitation, ~600 nm emission. The net phagocytosis and the response to the phagocytosis effector agent (pre/anti-miR-885-5p) were calculated by subtracting the average fluorescence intensity of the no-cell negative-control wells from all positive-
control and experimental wells. The mean and standard deviation of the net positive control and net experimental wells was then calculated. The phagocytosis response to the experimental effector (% Effect) is the fraction of the net positive control phagocytosis: % Effect = Net experimental phagocytosis × 100% / Net positive control phagocytosis.

3.32 Source of antibodies

PECAM-1 (CD31) (R&D Systems, Minneapolis, MN, USA), Integrin beta3 (R&D Systems, Minneapolis, MN, USA), Integrin alphaIIb (Chemicon International, Temecula, CA, USA), VASP (Alexis Biochemicals, San Diego, CA, USA), Talin (Upstate Biotechnology, Lake Placid, NY, USA), CXCL7 (R&D Systems, Minneapolis, MN, USA), LCP-1 (Abcam, Cambridge, UK), Mouse IgG HRP (DAKO Cytomation, A/S, Denmark), Goat IgG HRP (DAKO Cytomation, A/S, Denmark), Rabbit IgG HRP (DAKO Cytomation, A/S, Denmark) and β-actin (Abcam, Cambridge, UK).

3.33 Chemokines

Recombinant human NAP-2/CXCL7 (R&D Systems, Minneapolis, MN, USA) and recombinant human TNF-α/TNFSF1A (R&D Systems, Minneapolis, MN, USA).

3.34 Primers for RT-PCR

CHAPTER IV

RESULTS

PROTEOMIC ANALYSIS OF ENDOTHELIAL PROGENITOR CELL-DERIVED MICROPARTICLES

- 79 -
4.1 Isolation and visualization of MPs derived from EPC cultures

MPs were collected from the conditioned medium of early-outgrowth EPC cultures (Figure IV.1A and B). The EPCs were derived from PBMNCs isolations of 4 healthy donors pooled together, in detail from 200ml of full blood a total of 1-1.5x10⁸ PBMNCs were cultured on fibronectin-coated dishes and supplemented with a variety of growth factors eg. hVEGF 165. The washed PBMNCs were cultured for a period of 7 days according to the method described in section 3.1. Non-adherent cells were removed from the culture at day 3 and 5. The adherent cells were subject to RT-PCR and immunostaining using a panel of marker proteins (Figure IV.1C and D). To investigate their proteomic profile, the EPC cultures were subject to O/N serum deprivation at day 3 (early time point) and at day 7 (late time point). The supernatants were collected and stored at -80°C until use. The MPs were harvested from the supernatants using differential centrifugation to deplete floating cells, cell debris, and apoptotic bodies. The MPs were pelleted from the pre-cleared supernatant in a final centrifugation step at 20,500xg. The resulting MPs were subject to high-resolution transmission electron microscopy (TEM) (Figure IV.1E). As expected, the MPs varied in size but were less than 1 µm in diameter.
Figure IV.1 EPC characterization and MPs visualization. PBMNCs were cultured under endothelial cell conditions for 7 days. (A) Day 0 of the culture. (B) Day 7 of the culture, spindle-like shaped cells (10x Bright field using a Nikon Eclipse TS100 inverted microscope equipped with a Nikon camera.). (C) EPC cultures endocytose acLDL and bind lectin *Ulex europaeus* agglutinin (arrows indicate double positive cells), (D) mRNA levels for monocytes, HUVECs and EPCs- assessment of EPC markers (E) Image of MPs harvested from the conditioned medium of EPC cultures. MPs were heterogeneous in size but rich in membrane material (EM image courtesy of Steiner M.)
4.2 Proteomic analysis of MPs obtained from EPC cultures

MPs derived from the EPC cultures of 4 healthy subjects were pooled together to obtain sufficient material. Two different conditions were compared; MPs isolated from early outgrowth EPC cultures at day 3 and at day 7 of the culture following an O/N serum deprivation. MPs were separated by 1D SDS-PAGE on large format gels. The proteins were visualized using silver staining and the gel bands were excised and subject to in-gel tryptic digestion. After enzymatic degradation the peptides were separated by high-performance liquid chromatography system and analysed by an LTQ XL ion-trap tandem mass spectrometer (MS/MS). The resulting spectra were searched against a human/bovine UniProt/SPROT database using the SEQUEST algorithm. Scaffold software was used to validate MS/MS-based peptide and protein identifications. The proteomic analysis revealed the presence of over 331 proteins in the first sample (early time point) and a total of 552 proteins in the second sample (late time point). A comprehensive list containing 618 proteins is provided in Supplemental Table 7.1 (merged protein results from biological samples obtained at both time-points). The most abundant proteins were of membrane and cytoskeletal origin. For instance, Talin-1, Vinculin, and Filamin A link integrins to the actin cytoskeleton. Additionally, other proteins such as surface receptors were identified; examples include the CD45 leukocyte common antigen and the CD47 leukocyte surface antigen - proteins of monocytic origin. Other important molecules identified were the intercellular adhesion molecule (ICAM3) that mediates cell-cell interactions through its ligand LFA-1 and the fibroblast growth factor receptor 2 (FGFR-2), which interacts with the angiogenic mediator FGF-1. A table is provided (Table 4.1) showing the most abundant proteins found in MPs. When all the proteins identified by MS/MS analysis were classified based on their Gene Ontology (GO) annotation, integrin signalling was returned as the top canonical pathway (p<0.001), followed by the actin cytoskeleton signalling and the leukocyte extravasation cascade.
Figure IV.2. Proteomic Analysis of EPC-derived MPs. (A) Silver stained gel containing the two samples (Sample A= Day 3, Sample B= Day 7). The gel bands were cut into 52 bands and processed further to be analysed on a mass spectrometer. (B) Venn Diagram showing the overlap of protein identifications in MPs derived from day 3 and day 7 of the EPC culture. (C) Pie chart of proteins identified in the MPs fractions.
Table 4.1 Membrane Proteins Identified in Microparticles Derived from EPC Cultures

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>SWISS PROT Accession Name</th>
<th>MW (kDa)</th>
<th>Spectra (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Integrins, alpha chain</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Integrin alpha-IIb precursor, CD41 antigen</td>
<td>ITA2B_HUMAN</td>
<td>113</td>
<td>156</td>
</tr>
<tr>
<td>Integrin alpha-6 precursor, CD49f antigen</td>
<td>ITA6_HUMAN</td>
<td>127</td>
<td>21</td>
</tr>
<tr>
<td>Integrin alpha-M precursor, CD11b antigen</td>
<td>ITAM_HUMAN</td>
<td>127</td>
<td>13</td>
</tr>
<tr>
<td>Integrin alpha-2 precursor, CD49b antigen</td>
<td>ITA2_HUMAN</td>
<td>129</td>
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<td>Integrin alpha-X precursor, CD11c antigen</td>
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<tr>
<td><strong>Integrins, beta chain</strong></td>
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<td></td>
</tr>
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<td>Integrin beta-3 precursor, CD61 antigen</td>
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<td>Integrin beta-2 precursor, CD18 antigen</td>
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<tr>
<td>Integrin beta-1 precursor, CD29 antigen</td>
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</tr>
<tr>
<td><strong>Other surface receptors</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Platelet glycoprotein lb alpha chain CD42b-alpha/CD42b antigen</td>
<td>GP1BA_HUMAN</td>
<td>69</td>
<td>21</td>
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<tr>
<td>Leukocyte antigen MIC3, CD9 antigen</td>
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<td>18</td>
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<tr>
<td>Platelet glycoprotein lb beta chain CD42b-beta/CD42c antigen</td>
<td>GP1BB_HUMAN</td>
<td>22</td>
<td>15</td>
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<td>FGFR2_HUMAN</td>
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<td>13</td>
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<td>Regulating synaptic membrane exocytosis protein 1</td>
<td>RIMS1_HUMAN</td>
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<tr>
<td>Nck-associated protein 1-like</td>
<td>NCKPL_HUMAN</td>
<td>128</td>
<td>3</td>
</tr>
<tr>
<td><strong>Receptor-associated proteins</strong></td>
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<td></td>
</tr>
<tr>
<td>Protein tyrosine phosphatase receptor type C-associated protein</td>
<td>PTCA_HUMAN</td>
<td>21</td>
<td>6</td>
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<tr>
<td>Tyrosine-protein phosphatase non-receptor type 6</td>
<td>PTN6_HUMAN</td>
<td>68</td>
<td>5</td>
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<tr>
<td>Growth factor receptor-bound protein 2</td>
<td>GRB2_HUMAN</td>
<td>25</td>
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<td>Adipocyte-derived leucine aminopeptidase</td>
<td>ERAP1_HUMAN</td>
<td>106</td>
<td>3</td>
</tr>
<tr>
<td>Receptor-type tyrosine-protein phosphatase eta</td>
<td>PTPRJ_HUMAN</td>
<td>146</td>
<td>2</td>
</tr>
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</table>
4.3 Proteins of platelet origin were identified in EPC cultures

The integrins with the highest number of identified spectra were the integrins αIIb, β3, β2 and α6 (Table 4.1). Surprisingly, the two most abundant integrins (αIIb and β3) are also known as platelet glycoprotein IIb-IIIa (GPIIb-IIIa, CD 41/CD61) and integrin αIIb is platelet specific. Representative product ion spectra are shown in Figure IV.3. Validation of the proteomic data by immunoblotting confirmed that GPIIb-IIIa was not only present in EPC-derived MPs but also in EPCs (Figure IV.4A). Similarly, the presence of PECAM, talin and VASP was verified by western blotting. Despite its abundance at the protein level, GPIIb-IIIa mRNA was not detectable in EPCs (Figure IV.4A). Similarly, integrin α6 alpha, another platelet protein, was present in MPs, but no mRNA expression was detectable in EPCs (Figure IV.4B). Notably, these proteins of platelet origin were also detectable in the monocytic fractions. GPIIb-IIIa is not expressed in monocytes, yet is present in abundance in PBMNC preparations (Figure IV.4A). Thus, our proteomic analysis of EPC-derived MPs unravelled the presence of platelet proteins among early-outgrowth EPCs, a culture method that has been fundamental to many studies published on EPCs to date.
Figure IV.3. Product ion spectra of doubly charged tryptic peptides identified as the platelet integrin alpha-IIb (GQVLFGLQSEQLR) and integrin beta-III (SILYVVEEPECPK).
Figure IV.4. Presence of platelet proteins in MPs of EPCs cultures. (A) Confirmation by immunoblotting. Note that the platelet integrin αIIbβ3 was enriched in the MP fraction of EPC cultures. Beside integrins, MPs also contain CD31 (PECAM-1), although at lower abundance than integrin αIIbβ3, consistent with the spectral counts presented in Table 4.1. Talin-1 and VASP, two downstream mediators of integrin signalling, showed a molecular weight shift indicative of phosphorylation. Actin was used as loading control. (B) Integrin expression in PBMNCs, HUVECs and EPCs. Expression of GPIIb-IIIa was not detectable in EPC cultures. A similar result was obtained for the platelet integrin α6. The less abundant integrins (according to the spectral counts in Table 4.1) were expressed at the mRNA level. The rt-PCRs are representative of at least three independent experiments.
Section 4.4: The presence of platelets in EPC cultures

Additional experiments were performed to demonstrate that the platelet contamination originates from the first step of the EPC culture - the isolation of PBMNCs by density barrier centrifugation. An automated cell counter revealed that despite extensive washing with PBS, platelets were not entirely removed by density gradient centrifugation. For example, blood was taken from 3 healthy individuals and the cellular composition of the samples was compared before and after Lymphoprep isolation. The cell count revealed that there were more platelets than monocytes in freshly isolated buffy coats (Table 4.2). The presence of platelets among PBMNCs was further confirmed by using Hema-Gurr staining. The PBMNCs were fixed, stained and visualized under an optical microscope. Monocytes as well as other components of the white blood cells were identified (Figure IV.5/purple cells). Platelets and platelet aggregates were clearly visible among PBMNCs (Figure IV.5). Next, platelets were visualized among PBMNCs by immunostaining for PAR-4 and integrin αIIb (CD41), both proteins of platelet origin (Figure IV.6/Day 1). Platelets have no nucleus and are readily distinguished from PBMNCs using DAPI staining. During the 7 days of the early EPC culture, platelets and platelet aggregates disintegrated into platelet MPs (PMPs) (Day 3). These PMPs were subsequently taken up by the mononuclear cell population (Day 5; insets to Figure IV.6). At day 7, most of the adherent cells stained positive for platelet proteins.

Table 4.2. Cellular Composition Before and After Lymphoprep Isolation

<table>
<thead>
<tr>
<th>Cell type, cells/mL</th>
<th>Full blood</th>
<th>PBMNCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte count</td>
<td>5.85 × 10⁶ (± 0.464)</td>
<td>2.56 × 10⁶ (± 0.251)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>3.31 × 10⁶ (± 0.358)</td>
<td>0.04 × 10⁶ (± 0.002)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.84 × 10⁶ (± 0.029)</td>
<td>1.56 × 10⁶ (± 0.385)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.36 × 10⁶ (± 0.061)</td>
<td>0.69 × 10⁶ (± 0.052)</td>
</tr>
<tr>
<td>Erythrocyte count</td>
<td>5.43 × 10⁶ (± 0.052)</td>
<td>0.01 × 10⁶ (± 0.001)</td>
</tr>
<tr>
<td>Platelet count</td>
<td>268.33 × 10⁶ (± 15.632)</td>
<td>15.83 × 10⁶ (± 2.732)</td>
</tr>
</tbody>
</table>

Values are means of 3 independent experiments (± SEM), each performed in duplicate. Bold numbers highlight the abundance of platelets compared to monocytes.
Figure IV.5. Platelet contamination of PBMNCs. PBMNCs were stained using the Hema-Gurr rapid staining for hematology. Platelets and platelet aggregates were present among the washed PBMNCs. Note that platelet aggregates have being trapped between 3 monocytes. PLT denotes platelets; M, monocytes; L, lymphocytes.
Figure IV.6. Platelets contaminate PBMNCs isolations and the cellular uptake of platelet MPs. Intact platelets stained positive for PAR-4 and Integrin αIIb (CD41) among the PBMNCs counterstained with DAPI (Day 1). Over time, the platelets disintegrated, but platelet proteins remained detectable in EPC cultures (Day 3) and were taken up by the adherent cell population (Day 5, inset). By Day 7, most cells stained positive for platelet markers (insets: scale bar represents 25 μm).
4.5 PMP-mediated Transfer of “Endothelial” Markers

When the conditioned medium of EPC cultures was analysed by flow cytometry, the presence of MPs was confirmed by Annexin-V binding; measuring the surface exposure of phosphatidylserine (Combes et al., 1999). To validate the hypothesis that platelet MPs (PMPs) are present in these cultures, markers for platelets and endothelial cells were used (antibodies for CD41 and CD31, respectively). An abundance of PMPs (CD31⁺CD41⁺), but a scarcity of endothelial MPs (CD31⁺CD41⁻) was identified in EPC cultures (Figure IV.7A). Consistent with the observed uptake, the number of PMPs decreased over time (Figure IV.7B). To explore the contribution of PMPs to the positivity for “endothelial” markers in EPC cultures, the cells were costained for CD31 and CD41. On day 1, platelets were in close proximity, but separate from the CD31 negative mononuclear cell population (Figure IV.8). In contrast, by day 7, the cells stained positive for CD31 and the platelet-specific integrin αIIb (CD41), suggesting an uptake of platelet proteins via PMPs.
Figure IV.7. Flow cytometric analysis for MPs present in EPC cultures. MPs were harvested from the conditioned medium of early outgrowth EPCs at different time points (day 1, 2, 3, 5, and 7) of the culture and analysed by flow cytometry. (A) MPs were gated as events of 100nm to 1μm in diameter when compared with calibrator beads. Note the scarcity of the endothelial MPs (CD31+CD41-) but the abundance of platelet MPs (CD31+CD41+) in the EPC cultures. (B) Phosphatidylserine positive MPs were identified with Annexin V-FITC labeling. EPC-derived MPs (CD31+CD41-) numbers decline in the conditioned medium corresponding to the observed uptake by the mononuclear cell population (Data image courtesy of Boulanger C).
Figure IV.8. Fluorescence microscopy of EPC cultures stained for the endothelial marker CD31 and the platelet marker CD41. On day 1 of the culture, CD31 was confined to platelets. By day 7, all the cells were stained positive for CD31, however they were also positive for the platelet marker CD41 (integrin αIIb).
4.6 Confirmation of PMP Uptake in THP-1 cells

To investigate to what extent marker proteins can be exchanged between cell types via MPs, we used the monocytic THP-1 cell line. PMPs were generated by activating platelets with thrombin. PMPs were washed carefully and labelled with a fluorescence-conjugated lectin, staining cell membranes. Following extensive washing, the fluorescent PMPs were incubated with the THP-1 monocytic cell line. Within 1 to 2 h, labelled PMPs were taken up by 5-10% of the THP-1 cells (Figure IV.9A). Notably, platelets also stained positive for *Ulex europaeus* I agglutinin (UEA-1), a lectin that has been widely used to demonstrate the endothelial potential of EPCs. For that reason, platelets or PMPs were incubated with THP-1 cells and tested for their binding to UEA-1. After co-culture for 2 days most THP-1 cells bind to UEA-1 (Figure IV.9B). In addition, THP-1 cells were positive for other commonly used EPC markers such as CD31 (Figure IV.10A) and vWF (Figure IV.10B), but the staining co-localized with integrin α IIb, replicating the observed phenotype in EPC cultures.
Figure IV.9. PMPs and endothelial characteristics (A) PMPs were generated by activating platelets with thrombin and labeled using a red fluorescent-conjugated lectin. An uptake of PMPs by the THP-1 cells was observed within 1-2 hours (B) THP-1 cells bound UEA-1 after co-incubation with intact platelets or PMPs. PLT denotes platelets; arrows depict platelets among THP-1 cells; scale represents 25μm.
Figure IV.10. PMPs transfer endothelial characteristics in THP-1 cells. (A) THP-1 cells were co-incubated with PMPs for 2 days and stained positive for CD31, box area represents enlarged images of single cells (B) and vWF but also for integrin αIIb (CD41). Scale represents 25μm.
4.7 SILAC labelling confirms protein transfer by PMPs

THP-1 monocytes were SILAC-labelled for 5 population doublings until virtually all proteins had a “heavy” arginine or lysine. Then, they were incubated with freshly isolated PMPs (non-labelled) for 48h before their cellular proteome was separated by 2-DE and compared to untreated THP-1 cells (Control). Platelet supernatant depleted of MPs (non-labelled (PST)) was used as additional negative control to ensure that the observed effects in THP-1 cells are due to the MP fraction and not due to soluble factors. The three biological samples (Control, +PMPs and +PST) were compared by difference in-gel electrophoresis (DIGE). Samples were labelled with Cy3 and Cy5 and run on the same IPG strip and 2-DE gel, offering the opportunity for direct comparison and avoiding gel-to-gel variations inherent to comparative gel analysis. The use of an internal standard (a pool of all three samples) labelled with Cy2 improved the accuracy of quantitative comparisons in differential display experiments. The gels were stained using silver staining and images were acquired using the DIGE Imager (Figure IV.11). Spots showing a statistically significant difference in intensity were excised for in-gel tryptic digestion and analysed by MS/MS as previously described with the exception that the heavy amino acids were chosen as a variable modification in the database search. Any non-labelled / “light” peptides in the cellular proteome of THP-1 cells treated with PMPs should be platelet-derived. The proteomic analysis revealed the presence of 25 unmodified proteins - denoting platelet origin (Table 7.2-Supplement). For example, the LC-MS/MS analysis of a 2-DE spot containing annexin A5 returned 35 spectra in total (Figure IV.12A), 5 of which had a ‘light’ peptide confirming a substantial uptake of platelet MP proteins (Figure IV.12B). Annexin V, is a protein of high specificity for aminophospholipids and currently used in flow cytometry as a rapid method to assess the loss of phospholipid asymmetry in cell membranes and platelet cell vesiculation (Dachary-Prigent et al., 1993). Thus, it is possible to discern protein exchange from protein expression and determine the cellular origin of proteins in co-cultures by using metabolic labeling.
Figure IV.11 (A) 2D DIGE Silver-stained Gel. Differentially expressed proteins (P<0.05; 1-way ANOVA, SameSpot software, Nonlinear Dynamics) in samples containing PMPs vs Control (numbered from 1-32). The red arrow denotes the Annexin V spot. (B) Samples containing PST vs Control (numbered from 33-112).
Figure IV.12 SILAC for Protein Transfer. THP-1 monocytes labelled with ‘heavy’ arginine and lysine were incubated with unlabeled ‘light’ platelet MPs (PMP). Untreated THP-1 cells and THP-1 cells treated with PMP-free platelet supernatant served as controls. Despite a similar number of total spectra assigned to annexin A5 (A), unmodified (‘light’) peptides were only identified in THP-1 cells treated with PMP (B). In the cellular proteome of THP-1 monocytes, 5 out of 35 spectra for annexin A5 were platelet-derived confirming a substantial uptake of PMP proteins. Spectra of a ‘heavy’ (Arg R+10) and a ‘light’ peptide (Arg, R) of annexin A5 as identified by LC-MS/MS are shown in the bottom half of panel A and B, respectively.
4.8 Correlation between platelet counts and EPC numbers in the Bruneck study

We explored whether the platelet counts correlate with the numbers of colony-forming units (CFUs) or early outgrowth EPCs (double positive for diI-Ac-LDL and lectin) in a large population-based study (n=526). Among leukocytes, the strongest correlation was observed for monocytes (expressed as percentage of total leukocytes, nonparametric Spearman rank correlation coefficient \( r=0.215 \), \( P<0.01 \)). Apart from monocytes, only the platelet count (\( r = 0.092 \) and \( r = 0.089 \) for CFUs and EPCs respectively, \( p<0.05 \)) and the product of the platelet count and the mean platelet volume (MPV; \( r = 0.110 \) and \( r = 0.137 \) for CFUs and EPCs [\( P < .05 \) and \( P < .01 \)], respectively) emerged as a significant predictor for EPC numbers and CFUs in the general population (Table 4.3).

<table>
<thead>
<tr>
<th></th>
<th>CFU</th>
<th>EPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leukocyte count</td>
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</tr>
<tr>
<td>Neutrophils, %</td>
<td>0.321†</td>
<td>0.258†</td>
</tr>
<tr>
<td>Lymphocytes, %</td>
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<td>Monocytes, %</td>
<td>0.215†</td>
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</tr>
<tr>
<td>Erythrocyte count</td>
<td>0.092*</td>
<td>0.089*</td>
</tr>
<tr>
<td>Platelet count</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Platelet count* MPV</td>
<td>0.110*</td>
<td>0.137†</td>
</tr>
</tbody>
</table>

Values presented are nonparametric Spearman rank correlation coefficients; correlation coefficients in bold are significant at \( P < .05 \) (\( * \)) or \( P < .01 \) (\( † \)). CFU indicates colony forming unit; EPC, endothelial progenitor cell; and MPV, mean platelet volume.
4.9 The pro-angiogenic effect of PMPs

To test the ability of PMPs to influence the angiogenic activity of EPCs (Brill et al., 2005, Deregibus et al., 2007), conditioned medium of EPC cultures was collected at different time points (Day 3, 5 and 7) and applied to HUVEC monolayers embedded in Matrigel matrix. The addition of conditioned medium containing PMPs stimulated endothelial tube formation. This pro-angiogenic effect in the Matrigel assay was more pronounced on day 3 than on day 5 and 7 arguing against an outgrowth of more potent vascular progenitor cells in EPC cultures (Figure IV.13A). Instead, removing the PMPs from the conditioned medium by filtration or centrifugation markedly attenuated capillary network formation (Figure IV.13B). Moreover, addition of the generic disintegrin, echistatin, reduced the angiogenic effect. Additionally, the GPIIIa-4 peptide, a specific inhibitor against integrin αIIbβ3 (a kind gift by (Chiang and Zhu, 2005)), significantly attenuated the effect of MPs on endothelial tube formation (Figure IV.13C) Thus, PMPs contribute to the pro-angiogenic effect of the conditioned medium from EPC cultures.

Next, PMPs and conditioned medium from THP-1 cells treated with PMPs were compared in the Matrigel assay. Overnight incubation demonstrated a more pronounced pro-angiogenic effect upon the addition of conditioned medium from THP-1 cells treated with PMPs (Figure IV.14 A & B). The same principle was followed in an in vivo experiment by injecting Matrigel plugs into the back or flank of NOD/SCID mice. 150μl of Matrigel was mixed with either RPMI, PMPs, THP-1 conditioned medium and THP-1+PMPs conditioned medium. Two injections were performed for each condition and 30 images were taken across sections to quantify the number of neovessels. PMPs were shown to promote vasculogenesis (Figure IV.13C).

The contribution of CM to the endothelial motility and repair after injury was further investigated in a wound-healing assay. HUVEC monolayers were stained using a green cell tracker and the surfaces were scratched with a pipette tip. The wells were washed carefully in order to remove detached cells and fresh medium or conditioned medium (THP-1+CM) was placed slowly on the top of the cells (Figure IV.15A). When conditioned medium derived from the THP-1 cell cultures pre-treated with PMPs (THP-1+PMPs CM) was added to the injured
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HUVEC monolayer for 24h, the migratory response of HUVECs was accelerated compared to conditioned medium of THP-1 cells alone (Figure IV.15B).
Figure IV.13. PMPs mediate the pro-angiogenic effect of EPCs. (A) The pro-angiogenic effect of the conditioned medium of the EPC cultures decreased over time, arguing against an outgrowth of a progenitor cell population. (B) The depletion of MPs from the conditioned medium (C/M-MPs) of the EPC cultures reduced endothelial tube formation in the Matrigel assay. (C) The addition of the disintegrin echistatin and inhibitory peptide GPIIIa-4 against the platelet integrin αIIbβ3 attenuated the pro-angiogenic effect of the conditioned medium in the presence of PMPs (C/M+MPs). *P<.05 level, **P<.01. Results were obtained in 3 independent experiments (3 different populations of early outgrowth EPCs).
Figure IV.14. The pro-angiogenic effect of PMPs. (A) The pro-angiogenic effect of PMPs and the secreted factors release by THP-1 cells after pre-treatment with PMPs was tested in a matrigel assay. HUVECs were labeled using a green stain and placed on a matrigel together with plain medium (RPMI), PMPs, THP-1 secretome (THP-1 CM) and THP-1+PMPs secretome (THP-1+PMPs CM). (B) A significant increase in the ability of HUVECs to form vascular tubes was observed after the addition of the THP-1+PMPs CM. (C) In vivo, PMPs and conditioned medium from THP-1 cells treated with PMPs had an angiogenic effect.
Figure IV.15. Wound healing assay. (A) HUVEC monolayers were scratched and closure of the wound was measured after addition of RPMI control medium, THP-1 conditioned medium and THP-1+PMPs conditioned medium. (B) Graphical representation of wound healing over time.

*Denotes P < 0.05 RPMI vs THP-1 CM; ** denotes P < 0.02 RPMI vs THP-1+PMPs CM, n=2
4.10 PMPs promote THP-1 cell extravasation

Next, the effect of PMPs on monocytes was investigated:

1) A migration assay under static conditions was used to study the capture and the rolling of the monocytes. The bottom chamber of a modified Boyden chamber with a 5μm pore size was filled with plain medium (RPMI-Plastic) or covered by a HUVEC monolayer with or without pre-treatment by TNF-α. In parallel, the THP-1 cell culture was supplemented either with PMPs (THP-1+PMPs) or with the secretome of the THP-1+PMPs (THP-1+CM) for two days. The cells were labelled by using a green and an orange cell tracker. The same number of control and treated cells (THP-1+PMPs or THP-1+CM) were placed together in the upper section of the Boyden chamber and left to migrate for two hours. The treated cells (THP-1+PMPs and THP-1+CM) were compared. Whenever a HUVEC monolayer was present, increased migration was observed in response to PMPs (Figure IV.16A). However, the effect was more pronounced on activated HUVECs (activated by using 10ng/ml of TNF-α). Similarly, the effect of PMPs on adhesion and arrest was confirmed. When the two different conditions (THP-1+PMPs and THP-1+CM) were compared, both exerted significant effects compared to controls (Figure IV.16B).

2) In a modified transmigration assay, PMPs also promoted endothelial cell transmigration of THP-1 cells. HUVECs were placed on the top chamber of a modified Boyden chamber and cultured until confluence to create a monolayer on top of the filter. Conditioned medium of THP-1+PMPs was added to the bottom chamber. In both experiments, the effect of either PMPs or the CM was significant (Figure IV.16C).

3) The capacity of THP-1 cells to transmigrate through an endothelial monolayer before and after treatment with PMPs was evaluated in a parallel plate flow assay (Burns et al., 2010). HUVECs were grown in monolayers and stimulated with TNF-α. THP-1 cells were perfused and tracked over time by timelapse brightfield microscopy. Control THP-1 cells were labelled by using green cell tracker while THP-1+PMPs cells were labelled using an orange cell tracker. The potential of THP-1 cells to adhere, role and transmigrate through the endothelial monolayer was directly compared between the two conditions. The
same number of cells (Figure IV.17A) was perfused over activated HUVECs monolayers at 2.5dyn/cm² as previously described (Burns et al., 2010). The number of adherent and transmigrating cells was determined by counting the cells under a fluorescence microscope. The assay revealed that the addition of PMPs to THP-1 cells promotes their rolling and adhesion (Figure IV.17B). THP-1 cells pre-treated with PMPs also transmigrated through the endothelial monolayer (Figure IV.17B & C and Figure IV.18).
Figure IV.16. (A) Migration Assay. THP-1 cells were treated with PMPs or THP-1+PMPs conditioned medium and tested for their ability to migrate towards plastic surfaces, HUVECs and HUVECs pretreated with TNF-α. The biggest increase in the number of migrated cells was observed for the HUVEC monolayer activated by TNF-α and for THP-1 cells pre-treated with PMPs. (B) Adhesion Assay. Graphical representation of the number of adherent cells. (C) Transmigration Assay. Plain medium (RPMI) or the THP-1+PMPs CM was placed in the bottom chamber. The cells were left to migrate for 2 hours before they were counted by using fluorescent microscopy. Both treatments had a significant effect in the capacity of THP-1 cells to migrate through endothelial cells.
Figure IV.17. THP-1 cell transmigration through activated HUVECs monolayer. (A) Representation of THP-1 cells +/- PMPs labeled using green and orange cell trackers. (B) Cell counts of adherent and transmigrating cells in an over flow assay. (C) Brightfield and fluorescence images showing adherent (phase bright) and transmigrating cells (phase dark).
Figure IV.18. Magnification of THP-1+PMP cells transmigrating through a TNF-α-activated HUVEC monolayer. Yellow arrows show a THP-1+PMPs cell to attach (Panel 1-3), transmigrate through an endothelial monolayer (Panel 4-6) and hide completely under the activated HUVECs (Panel 7-9). In the initial panels the monocyte is visible as “phase bright” while after transmigration is “phase dark” and hidden beneath the endothelial cell. The time course of the experiment is 6min.
4.11 Secretome analysis of THP-1 cells in the presence of PMPs

To clarify the molecular mechanism involved in the above functional changes, a proteomic analysis of the secretome of THP-1 cells treated with PMPs was performed. Proteins in the conditioned medium were separated by SDS-PAGE and analysed by mass spectrometry (Figure IV.19A). To minimize cross-contamination with bovine proteins, the cells have to be washed extensively with plain medium before the secreted factors are sampled in serum-free medium. Despite these efforts to ensure that the collected conditioned medium contains no other extraneous proteins, except for the secreted or shed proteins, there was cross-contamination with bovine proteins. The gel was silver stained and the bands were excised and digested by trypsin. The proteomic analysis revealed a total of 129 proteins in the control sample and 205 in the PMP treated sample (Figure IV.19B). The most abundant proteins found in the treated samples were of platelet origin and related to PMPs. For example, filamin A, talin-1 and vinculin are related to platelet cytoskeleton, while secreted proteins such as CXCL7, PF4, vWF and P-selectin are proteins associated to cell-cell interactions and cell adhesion (Table 7.3- Supplement) (Figure IV.20). As expected, the most common platelet integrin αIIbβ3 was highly abundant in the THP-1+PMPs sample. Based on their Gene Ontology (GO) annotation integrin, leukocyte extravasation and VEGF signaling were among the top scoring pathways. CXCL7 was one of the differentially expressed proteins identified. CXCL7 is of platelet origin and plays a major role in neutrophil transmigration through the endothelium.
Figure IV.19. (A) SDS-PAGE. The silver stained gel shows two samples (THP-1 CM; Control and +PMPs CM; treated) in two technical replicates (A and B). The gel bands were excised, digested with trypsin and analysed by mass spectrometry. (B) Venn diagram displaying the overlap of protein identifications in the control (THP-1 CM) and the treated condition (+PMPs CM). (C) Pie chart depicting the cellular composition of the identified proteins.
Figure IV.20. Schematic representation of secreted proteins in the conditioned medium of THP-1 cells treated with PMPs (THP-1+PMPs). The two conditions (control: THP-1 CM and treated: THP-1+PMPs CM) were compared and fold change values were plotted against control. Among the upregulated proteins were the platelet integrin αIIbβ3 and the major platelet chemokines CXCL7 and platelet factor 4 (highlighted in red).
4.12 CXCL7 mediates the extravasation of THP-1 cells

The proteomic analysis revealed increased secretion of CXCL7 following treatment with PMPs. Therefore, THP-1 cells were treated with recombinant CXCL-7 and a blocking antibody for CXCL7. Three conditions were compared: a) THP-1+ CXCL7, b) THP-1+PMPs and c) THP-1+PMPs/ab (ab=CXCL7 blocking antibody).

As far as the cell adhesion is concerned, CXCL7 had a modest effect on the number of adherent THP-1 cells compared to PMP treatment. Blockade of CXCL7 did not show a significant reduction (Figure IV.21A). In the transmigration assay, the impact of CXCL7 was more pronounced (Figure IV.22A). Therefore, the next experiments include the comparison of the THP-1 control to a) Tyrode’s buffer (TB used as negative control-contained in the PST sample), b) THP-1+PST and c) THP-1+PST/ab. For the adhesion assay the THP-1+PST sample showed a significant improvement in the adhesion of the cells and the CXCL7 blocking antibody attenuated this effect almost (Figure IV.21B) supporting the notion that CXCL7 is one of the major mediators of cell adhesion to the endothelium (Figure IV.21B). In contrast, transmigration could not be attributed to CXCL7 (Figure IV.22B).

Finally, the last group of conditions investigated included the THP-1 control compared to a) CM (plain conditioned medium derived from THP-1 cultures), b) CM/ab (conditioned medium with CXCL7 blocking antibody) c) CM/PMP (conditioned medium of THP-1+PMPs cultures) and d) CM/PMP/ab (conditioned medium of THP-1+PMPs cultures enriched with CXCL7 blocking antibody). Only the CM/PMP showed a significant increase in the adhesion of the cells and the CXCL7 blocking antibody showed a 50% inhibition (Figure IV.21C). In the transmigration assay the result was similar but the antibody entirely blocked the effect (Figure IV.22C).

Treatment with recombinant CXCL7 led to increased adhesion and transmigration of the THP-1 cells. In line with these findings pre-treatment of the PMPs, PST, and CM/PMPs with a neutralizing antibody to CXCL7 could effectively inhibit this response. These findings suggest that the increase in adhesion and transmigration that is observed in THP-1 cells treated with PMPs is at least partially mediated by CXCL7.
Figure IV.21. Adhesion Assay. Anti-CXCL7 blocked increased adhesion of THP-1 cells to endothelial cells in response to PST but not PMP. The addition of the blocking antibody had also an effect in the CM/PMP condition.
**Figure IV.22. Transmigration Assay.** CXCL7 plays an important role in the transmigration of the monocytes through an EC monolayer. The addition of the blocking antibody blocked the effect of PST as well as the CM/PMP.
4.13 CXCL7 induces the expression of miR-885-5p

Recently it was shown that platelets also contain microRNAs (miRNAs) (Landry et al., 2009). MiRNAs are small non-coding RNAs that regulate gene expression at the post-transcriptional level. A miRNA screening was performed using Human TaqMan miRNA arrays (Pool A covering 381 of the most studied miRNAs) to explore potential downstream targets of CXCL7. An initial screening was performed in 2 pooled samples consisting of 4 preparations of platelets and PMPs derived from thrombin activated platelets (Figure IV.23A & B). Because platelets contain ~12,500-fold less mRNA than a nucleate cell, a pre-amplification step was included in the miRNA analysis. Real-time PCR was performed to examine the expression of miRNAs using 3 internal controls. A normalization correction factor, RNU48, was selected on the basis that it showed the smallest variation in ΔCt values. A distinct expression profile of 138 miRNAs was identified in the PMPs sample with a Ct value of <36 (Figure IV.23C). When PMPs were compared with platelets (PLTs), PLTs contained more miRNAs (Figure IV.23C), but the pattern of miRNA expression was similar in both samples (Figure IV.23D & IV.24). In agreement with the findings by Landry et al (Landry et al., 2009), miR-223, -191, -17, -126, -24, -320, let-7e and -142-3p were among the most abundant miRNAs in PLTs (Figure IV.24) but also in PMPs. Further screening of THP-1 cells treated with PMPs showed that miR-885-5p is highly upregulated. Interestingly, neither THP-1 cells nor PLTs express miR-885-5p suggesting that this is a de novo induction in response to activation by PMPs.

THP-1 cells co-cultured with thrombin-derived PMPs for 2 and 5 days were included in the miRNA analysis, including a pre-amplification step. The experiment was repeated twice using the same batch of the THP-1 cell line but at different passages. Similar patterns of miRNA expression were obtained in both experiments (Table 7.4-Supplement) - 210 miRNAs in the first array compared to 227 in the second (Figure IV.25A). The incubation of THP-1 cells with PMPs did alter the expression of certain miRNAs (Figure IV.25B). The most significant differences were identified for miR-200, -545, -330-5p, -127-3p, -486-3p, -885-5p and -874 (Figure IV.26). The RQ values from the two experiments were merged and normalized using the RNU48 normalizer and compared to THP-1
controls. Five of the most upregulated miRNAs were selected for further validation by qPCR. MiR-127-3p, 200a, 330-5p, 545 and 885-5p were tested at days 2 and 5. While expression changes of miR-127-3p and -885-5p were confirmed in the TaqMan array, miR-330-5p and 200a showed an increase but not to the same extent as in the array. (Figure IV.27). MiR-545 was not confirmed. For most miRNAs there was a gradual increase in the expression starting from day 2 to day 5. MiR-885-5p was the only one already present in the control THP-1 cell line but at very low levels (ΔCt ≥34cycles); in contrast, miR-885-5p was absent from both PLTs and PMPs. Thus, a de novo synthesis of miR-885-5p in response to PMPs seems most likely (Figure IV.26 insert).

The initial screening of the miRNA pool of THP-1 cells treated with PMPs showed that miR-885-5p is highly upregulated. To determine whether a secreted factor is responsible for the regulation of miR-885-5p expression, we treated THP-1 cells with recombinant CXCL7 and assessed miR-885-5p levels using qPCR. 50nM of the CXCL7 chemokine was added to the culture medium of the THP-1 cell line for 2 days. Expression levels of miR-885-5p (Figure IV.28A) were compared with THP-1 cells incubated with PMPs, PST and CM/PMPs (THP-1+PMPs CM). In all conditions increased expression of miR-885-5p was detected (Figure IV.28B, C & D). When PMPs, PST and CM/PMPs were pretreated with the CXCL7 blocking antibody for 2h at 37°C, the CXCL7 blocking antibody did not attenuate the effect of PMPs (Figure IV.28B), but attenuated miR-885-5p expression in response to PST (Figure IV.28C) and CM from THP-1 cells stimulated with PMPs (Figure IV.28D). Thus, expression levels of miR-885-5p in the THP-1 cells are at least partially regulated via CXCL7.
Figure IV.23. Representation of miRNA expression of platelets (A) and platelet microparticles (B) in the Human TaqMan miRNA Arrays. (C) Comparison of miRNA expression between PLTs and PMPs. The blue columns represent the detected miRNA transcripts. (D) Hierarchical Clustering. Correlation of miRNAs between PLTs and PMPs.
Figure IV.24. Graphical representation of the 50 most highly expressed miRNAs in PLTs and PMPs. MiRNAs were ranked based on their expression profiles (Ct values).
Figure IV.25. Comparison of miRNAs expression in THP-1 cells with or without addition of PMPs. 

(A) Failing detectors by sample. MiRNA expression profiles were compared between three different conditions (Control=THP-1 cells; Day 2= THP-1+PMPs at Day 2; Day 5= THP-1+PMPs at Day 5) in two different experiments (I= 1st experiment; II= 2nd experiment). In red colour the number of failing miRNAs is shown while the blue columns represent the identified miRNA transcripts. 

(B) Hierarchical Clustering. A general overview of the miRNA correlation between all conditions based on expression profiles is shown (red represents failing detectors while green represents the presence of miRNAs).
Figure IV.26. Upregulated miRNAs in THP-1 cells pretreated with PMPs. Mean RQ values of day 2 and 5 calibrated to THP-1 control. Comparison of specific miRNAs between PLTs, PMPs and THP-1 cells at baseline and 2 and 5 days after incubation with PMPs. Failing detectors by biological groups. Valid or flagged values in blue and red respectively (insert).
Figure IV.27. Validation by qPCR. The five selected targets were validated further by real time PCR. From the results only two miRNAs showed a strong correlation to the results obtained by using the Human TaqMan Megaplex Array.
Figure IV.28. The effect of CXCL7 and platelet derivatives in the miRNA levels of miR-885-5p. (A) miR-885-5p levels of the THP-1 cells in the presence of CXCL7 chemokine, (B) miR-885-5p levels of the THP-1 cells after addition of PMPs and PMPs/ab, (C) miR-885-5p levels of the THP-1 cells after addition of TB (Tyrode’s buffer=control) PST and PST/ab, (D) miR-885-5p levels of the THP-1 cells after addition of CM (control), CM/PMPs and CM/PMPs/ab.
4.14 LCP-1 is a target of MiR-885-5p in THP-1 cells

Changes in miR-885-5p expression are likely to induce protein changes in the THP-1 cells. Using online search tools such as DIANA, TargetScan and MiRanda, we searched for miR-885-5p targets among the differentially expressed proteins in THP-1 cells after the addition of PMPs. A total of 31 differentially expressed proteins were identified by proteomics (Figure IV.29A). Comparative analysis of the predicted targets revealed that Plastin-2 (L-plastin or Lymphocyte cytosolic protein 1 (LCP-1)) ranked among the top putative targets of miR-885-5p (Figure IV.30A). Consistent with the induction of miR-885-5p, LCP-1 was downregulated in THP-1 cells upon incubation with PMPs and PST (Figure IV.29B). To establish a mechanistic link, expression levels of miR-885-5p were altered by transfecting THP-1 cells with miR-885-5p inhibitors and precursors. Anti-miR-885-5p is a chemically modified, single stranded nucleic acid designed to specifically bind to and inhibit the endogenous miRNA. PremiR-885-5p mimics the endogenous mature miR-885-5p. The overexpression (pre-miR) and the inhibition (anti-miR) of miR-885-5p were validated by qPCR (Figure IV.31A & B). Western blots showed that overexpression of miR-885-5p led to a downregulation of LCP-1 (Figure IV.31). Inhibition of miR-885-5p did not result in an increase of LCP-1 (Figure IV.31 C). THP-1 cells treated with PMPs were included for comparison (Figure IV.31C). Treatment with platelet supernatant or recombinant CXCL7 reproduced the decrease in LCP-1 protein levels. Addition of a CXCL7 blocking antibody (ab) antagonized this effect (Figure IV.31D). LCP-1 is an actin-bundling protein localized at structures involved in cell adhesion, motility, invasion and also in phagocytosis (Delanote et al., 2005, Evans et al., 2003, Janji et al., 2006). When THP-1 cells were transfected either with the precursor miRNA or the anti-miRNA for 885-5p, their ability to phagocytise labelled E.coli particles was affected on day 3 in both non-adherent and fibronectin-adherent conditions (Figure IV.32A & B). Thus, miR-885-5p expression inhibits LCP-1 expression and alters THP-1 cell function.
Figure IV.29 (A) The 2D-DIGE gel of the THP-1 cells in the presence of the PMPs. The blue arrow targets the spot No 2. (B) The spot No 2 was verified as protein LCP-1, highly downregulated by the addition of the PMPs in the THP-1 cells.
Figure IV.30. (A) An online search using the DIANA search tool revealed Plastin-2 as potential target of miR-885-5p. (B) MiR-885-5p expression in THP-1 cells in the presence of PMPs at different time points (the expression was validated by using the TaqMan miRNA Megaplex Assay and qPCR). (C) Expression of the LCP-1 levels in the whole cell lysates validated by 2D DIGE.
Figure IV.31. MiR-885-5p targets the expression of LCP-1 in the monocytic cell line THP-1. (A) Overexpression of miR-885-5p using synthetic pre-miRs, (B) downregulation of miR-885-5p using anti-miRs, (C) immunoblotting for LCP-1 (D) LCP-1 protein levels after the addition of PMPs, PMP/ab, PST, PST/ab, CM/PMPs and the addition of CXCL7.
Figure IV.32. Phagocytosis assay after overexpression and downregulation of miR-885-5p. (A) In non-adherent monocytes, (B) in adherent monocytes.
CHAPTER IV

DISCUSSION
5.1 The controversy of EPCs: Proteomics provides new insights

EPCs were first described in 1997 by Asahara and colleagues who showed that purified CD34⁺ / KDR (VEGFR2)⁺ mononuclear cells from adults can differentiate ex vivo to an endothelial phenotype (Asahara et al., 1997). This seminal paper did not directly demonstrate whether these cells also have in vivo vessel forming ability, but subsequent studies showed that EPCs contribute to the recovery of the ischemic cardiac tissue and were proposed as a therapeutic option to rescue tissue after ischemia (Rafii and Lyden, 2003). By now, a Pubmed search on EPCs returns thousands of publications (Deb and Patterson, 2010).

Despite the immense explosion of interest in this area, there is neither a standard definition nor an accepted methodology for their enumeration (Povsic et al., 2009). In flow cytometry analysis, CD34, CD133 and KDR are commonly used, but there is conflicting evidence whether CD34⁺, CD133⁺ and KDR⁺ cells represent endothelial precursors or are primitive hematopoietic progenitors (Peichev et al., 2000, Case et al., 2007). Also, circulating EPCs defined by these criteria are extremely rare and difficult to quantify as highlighted by a methodological comparison of different flow cytometry approaches (Van Craenenbroeck et al., 2008). In vitro culture methods were introduced as alternative approach: the endothelial CFU assay was proposed as a surrogate measurement for the number of circulating EPCs in clinical studies and the culture of early outgrowth EPCs was used as an in vitro expansion method to obtain sufficient cell numbers for mechanistic experiments (Tunica et al., 2009, Kalka et al., 2000). In both methods, EPCs were isolated from mononuclear cells by density barrier centrifugation (Lymphoprep, Ficoll, etc) because it is a fast and “stress”-free equilibrium method while the cell-sorting step for CD34⁺ KDR⁺ cells was eliminated.

Thus, different cell types were assessed in vitro and in vivo (Segers and Lee, 2008, Timmermans et al., 2009, Timmermans et al., 2007) and at present, there are no specific markers, which unambiguously identify EPCs (Urbich and Dimmeler, 2004, Horrevoets, 2009, Kim et al., 2009). Other studies challenged the assertion that CFUs and early outgrowth EPCs are bona fide EPCs. Clonal
analysis performed by Yoder et al revealed that CFUs are derived from the hematopoietic system, possess myeloid progenitor cell activity, and differentiate into phagocytic macrophages (Yoder et al., 2007). Rohde et al demonstrated that CFUs formed as a result of a functional cross between T cells and monocytes (Rohde et al., 2007). By now, findings from different groups converged showing that early outgrowth EPCs fail to incorporate in the vasculature and do not differentiate to endothelial cells (as reviewed in (Richardson and Yoder, 2011)), but that blood monocytes mimic EPCs and mediate an angiogenic effect in a paracrine manner (Rohde et al., 2006, Rehman et al., 2003). Yet, it remained unclear how these cells acquire endothelial characteristics and promote angiogenesis.

Proteomics helped to shed new light on the caveats of this common stem cell assay in cardiovascular research: in our proteomics analysis of MPs in the conditioned medium of early outgrowth EPCs, the platelet-specific integrin alpha IIb emerged as the most abundant integrin (Dormeyer et al., 2008). Conventional methods for isolating mononuclear leukocytes (lymphocytes, monocytes, and natural killer cells) using density barrier centrifugation deplete erythrocytes and granulocytes (mainly neutrophils), but a platelet contamination is commonplace (Dormeyer et al., 2008, McFarland et al., 2006) and can vary depending on the stringency of the washing steps. Most investigators are either unaware of the presence of platelets in EPC cultures or assume that the platelet contamination is of minor importance as the platelets disappear within few days of culture. The platelets, however, just disintegrate into smaller PMPs, which are subsequently incorporated by the adherent leukocyte population.

Generally, platelets are not considered while staining or performing actual phenotypic analysis of EPCs. Platelets and PMPs bind *Ulex europaeus* agglutinin-1 (UEA-1) and the uptake of PMPs by the adherent mononuclear cell population can result in a transfer of the “endothelial” markers CD31 and von Willebrand factor (vWF). As macrophages also incorporate acetylated low density lipoprotein (LDL) (Bel et al., 2003), studies evaluating EPCs based on acetylated LDL uptake and UEA-1 binding are not reliable. Similarly, cells staining double positive for haematopoietic and endothelial markers may not be EPCs (Yoder, 2009). Although PMP-induced transfer of marker proteins is
unlikely to permanently change a marker expression profile, a PMP uptake was clearly noticeable between day 3 and day 7 of culture, when most investigators are evaluating the outgrowth of EPCs by immunostaining. Addition of an immunophenotypic marker for platelet proteins would be a prudent measure to avoid misinterpretations of immunolabelling for CD31 and vWF. Co-incubation of platelets and peripheral blood mononuclear cells dose-dependently increased the number of adherent EPC (Dernbach et al., 2008) and in a large population-based study (Bruneck), platelet and monocyte counts emerged as a positive predictor for the number of CFUs and early-outgrowth EPCs (Dormeyer et al., 2008). These findings constitute a paradigm shift from the original definition of an EPC phenotype (Yoder, 2009) and provide an explanation for the misinterpretation of their cellular progeny.

Furthermore, in many studies early outgrowth EPCs and their conditioned medium were used for functional experiments, but saline injections or plain medium served as controls. PMPs may, at least partially, be responsible for their angiogenic effects. Soluble factors released by EPCs have been previously analysed using microarrays (Urbich et al., 2005a), but of course a contamination with platelet proteins would have gone unnoticed, demonstrating the advantages of a proteomics approach. It is well established that platelets and PMPs bind to monocytes/macrophages (Silverstein et al., 1989) and increase their adhesiveness. In this respect, it is not surprising that platelets and PMPs have a similar effect on EPCs (Abou-Saleh et al., 2009, Mause et al., 2010). Platelets also contain a range of pro-angiogenic growth factors, including VEGF, and PMPs are potent inducers of angiogenesis (Boulanger and Tedgui, 2005).

Meanwhile, it has been shown that MPs contribute to the activation of an angiogenic program in EPCs (Deregibus et al., 2007), that the depletion of the MPs reduces the angiogenic activity of their conditioned medium (Dormeyer et al., 2008) and that PMPs enhance the potential of EPCs to restore endothelial integrity after vascular injury (Mause et al., 2010). In the latter experiment, the effect of PMPs alone was not evaluated in vivo (Mause et al., 2010). In a rat model of chronic myocardial ischemia, however, injections of platelet MPs were sufficient to stimulate post-ischemic revascularization in the myocardium (Brill et al., 2005). In hindsight, functional improvements should not have been
attributed to EPCs without an in-depth analysis of the protein content in their conditioned medium. The question whether EPCs are “stem cells of monocytic origin” or “angiogenic macrophages” is not solely semantic and it has been proposed that the term “progenitor” should be retired for early outgrowth EPCs without clonal proliferation and differentiation potential (Richardson and Yoder, 2010, Hirschi et al., 2008). New names, such as circulating angiogenic cells, early angiogenic cells, early outgrowth cells, etc. are currently being introduced but cannot overcome the known limitations of this co-culture assay.

As outlined by Hirschi et al (Hirschi et al., 2008), more stringent criteria are needed for EPC studies and have already been implemented by an American Heart Association Journal. There is no general consensus on how to define macrophage phenotypes and the distinction between M1 versus M2 macrophages is an overly simplistic representation of a very complex area of biology. In this respect, the recent findings demonstrating the transfer of mRNA (Deregibus et al., 2007) and proteins (Mause et al., 2010) from PMPs to mononuclear cells, including the chemokine receptor CXCR4, open exciting new possibilities how platelets may alter macrophage function or possibly influence their angiogenic activity (Kim et al., 2009, Horrevoets, 2009, Grunewald et al., 2006).

Of course, a protein transfer between cell types also represents an analytical challenge for proteomics. If protein containing-vesicles are taken up by recipient cells, the transferred proteins will not be distinguishable from the endogenous proteins as long as the proteins are expressed by both cells types. This can be addressed by adopting a SILAC approach: THP-1 monocytes were SILAC-labelled for 5 population doublings until virtually all proteins had a “heavy” arginine or lysine. Then, they were incubated with freshly isolated PMPs for 48h before their cellular proteome was separated by 2-DE and compared to untreated THP-1 cells. Platelet supernatant depleted of MPs was used as additional negative control to ensure that the observed effects in THP-1 cells are due to the MP fraction and not due to soluble factors. Differentially expressed proteins were identified by LC-MS/MS. In this case, any non-labelled / “light” peptides in the cellular proteome of THP-1 cells treated with platelet MPs should be platelet-derived. Indeed, the LC-MS/MS analysis of a 2-DE spot containing annexin A5 returned 35 spectra in total, 5 of which had a ‘light’
peptide confirming a substantial uptake of platelet MP proteins. Thus, it is possible to discern protein exchange from protein expression and determine the cellular origin of proteins in co-cultures by using metabolic labelling. Such insights can only be obtained by proteomics and not with conventional antibody-based techniques.

5.2 The Role of PMPs in Vascular Repair

Angiogenic monocytes (also reported as EPCs) have a beneficial effect on neovascularization either directly by having a structural role, or indirectly by secreting growth factors and cytokines that promote the proliferation and migration of local ECs (Dai et al., 2008). The conditioned medium of the EPC cultures stimulated the endothelial tube formation in the Matrigel assay; since EPC cultures are heavily contaminated by PMPs, this “angiogenic” capacity may be either attributed to secreted factors from platelets or PBMNCs that were activated by PMPs. Therefore, in the last part of this thesis the interaction of monocytes with PMPs and their functional significance in vascular repair was further explored. Experiments using the monocytic cell line THP-1 showed that the conditioned medium of THP-1 cells treated with PMPs can enhance the wound healing capacity of ECs and increase their angiogenic ability. Importantly, treatment of THP-1 cells with PMPs also induced functional changes in the monocytic cells such as an increased chemotaxis, adhesion and transmigration towards ECs.

In an attempt to identify the molecular mechanism involved, a proteomic analysis in both the cellular proteome and the secretome of THP-1 cells treated with PMPs was performed. This proteomic analysis revealed that following treatment with PMPs the chemokine CXCL7 expression is significantly increased in the conditioned medium of THP-1 cells. Treatment of THP-1 cells with recombinant CXCL7 led to increased adhesion and transmigration towards ECs. In line with these findings pre-treatment of the PMP/CM with a neutralizing antibody to CXCL7 could effectively inhibit this response. These findings suggest that increased adhesion and transmigration of THP-1 cells treated with PMPs is at least partially mediated by CXCL7. CXCL7 or neutrophil activating peptide 2 (NAP-2) is the truncated product of the connective tissue-activating
peptide III (CTAP-III). CTAP-III and beta-thrombogulin (beta-TG) are proteolytically processed carboxy-terminal fragments of the platelet basic protein (PBP), which is found in the alpha-granules of human platelets. CXCL7 is a member of the CXC chemokines and similar to other ELR domain containing CXC chemokines, such as IL-8 and the GRO proteins, binds on its ligand CXCR2 and the adhesion molecule integrin macrophage-1 Ag (MAC1 (CD11b/CD18)). It chemoattracts, activates and stimulates adhesion of neutrophils on the endothelium (Schenk et al., 2002).

Besides, cell-derived microvesicles have the capacity to alter the phenotype and the fate of other cell populations. For example tumour cell-derived MPs target monocytes and T-lymphocyte MPs target fibroblasts (Baj-Krzyworzeka et al., 2006, Hakelien et al., 2002). The phenotypic alteration of the recipient cell may be accomplished either by the transfer of membrane receptors, growth factors and bioactive lipids and/or epigenetic reprogramming based on the transfer of genetic material such as mRNA, miRNAs and protein-based transcription factors (reviewed by (Quesenberry and Aliotta, 2010)). Following platelet activation, PMP shedding starts within minutes and a myriad of PMP accumulates at sites of tissue injury and leaks into the circulation. In this project, we demonstrated that PMPs are not only taken up by mononuclear cells and transfer membrane receptors such as the platelet integrin αIIb, but also alter their phenotype by inducing miRNA alterations.

MiRNAs are an emerging class of highly conserved, non-coding single-strand small RNAs of ~22 nucleotides in length in their mature form, found either as individual genes or in arrayed clusters. They are capable of regulating gene expression on post-transcriptional level by repressing the translation of protein by interacting with the 3΄ untranslated regions (UTRs) of specific mRNAs or by promoting degradation of mRNA therefore silencing of gene expression (Lagos-Quintana et al., 2001, Chapman and Carrington, 2007, Altuvia et al., 2005). 29. The human genome has been estimated to encode up to 1000 miRNAs, which are predicted to regulate between 30% and 92% of all genes and to control numerous biological processes, including megakaryocytic differentiation of CD34+ hematopoietic progenitor cells and angiogenesis. MiRNAs target translational repression by lowering the intrinsic rate of initiation. More than 1,000 human miRNAs have been identified so far
implicating the crucial role miRNAs play in various physiological and pathological processes such as oncogenesis, cardiogenesis and hematopoietic lineage differentiation (Esquela-Kerscher and Slack, 2006, Zhao et al., 2005, Chen et al., 2004a). Indeed, specific miRNAs that regulate endothelial cell function and angiogenesis have been identified such as miR-130a and miR-27b being positive regulators and miR-221 and miR-222 as negative regulators of endothelial cell migration and angiogenesis (Chen and Gorski, 2008, Poliseno et al., 2006, Suarez et al., 2007).

Few studies have addressed the role of miRNAs in platelets. Even though platelets are anucleate, they harbour a small, but diverse, transcriptome, and have recently been shown to contain abundant quantities of small non-coding regulatory RNAs, including miRNAs. In fact, the relative abundance of platelet miRNAs translates into a markedly increased miRNA/mRNA ratio versus nucleated cells. Besides, platelets also contain the Dicer and Argonaute 2 complexes, which function in the processing of exogenous miRNA precursors and the control of specific reporter transcripts, respectively (Landry et al., 2009). Therefore, the possibility of PMPs to transfer or induce miRNAs in other cell types such as monocytes was investigated. Based on miRNA array analysis, it was evident that PMPs carry platelet miRNAs and that some of these miRNAs are transferred to other cells. An initial screening of the miRNA pool of THP-1 cells treated with PMPs showed that the miR-885-5p is induced. Interestingly, neither PMPs nor PLTs express miR-885-5p suggesting that this is a de novo induction occurring in response to PMPs. To determine whether CXCL7 could regulate miR-885-5p expression THP-1 cells were treated with recombinant CXCL7. Levels of miR-885-5p increased upon incubation with CXCL7. Bioinformatics analysis of the putative targets of miR-885-5p identified LCP-1. Indeed, overexpression of miR-885-5p led to downregulation of LCP-1 protein whereas treatment with recombinant CXCL7 decreased LCP-1. Thus, PMP-derived CXCL7 might enhance the recruitment of THP-1 cells in areas of inflammation by inducing the expression of miR-885-5p and downregulating LCP-1. Reduced levels of this actin-bundling protein facilitate increased cell adhesion and migration. Besides being an important determinant of plaque thrombogenicity, PMP might play a previously unrecognized role in modulating monocyte function.
Figure V.1. Schematic representation of findings. The release of platelet microparticles (PMPs) and their uptake by PBMNCs results in an EPC-like phenotype. These cells show phenotypic and functional characteristics replicates the early outgrowth EPCs. On the other hand in the in vitro experiments using the THP-1 cell line it was revealed that treatment of the cells with PMPs releases in the conditioned medium amounts of the cytokine CXCL7. CXCL7 is uptaken by THP-1 cells existing in the surroundings and upregulates the miR-885-5p which suppresses the production of the LCP-1 protein responsible for the phagocytic function of the monocytes.
5.3 Lessons to be learnt for stem cell research

The bulk of the cardiovascular stem cell literature is based on immunolabelling for marker proteins combined with functional improvements in animal models, but the detailed mechanisms of these effects remain elusive (Marban and Cheng, 2010). Frequently, the correct interpretation of the findings relies on the validity of the following assumptions:

5.3.1 The concept of marker proteins – is co-staining equivalent to co-expression?

Stem cells in the cardiovascular system are classically assessed by co-staining for a progenitor and a cardiac or vascular differentiation marker. In vitro, marker expression is confirmed at the transcript level, but in vivo studies predominantly rely on immunolabelling. The widely held view is that positive staining for marker proteins is consistent with gene expression, but there might be notable exceptions: In areas of tissue injury where cell death, platelet activation and inflammatory cell infiltration occur, the possibility of a temporary exchange of antigens between cell types should be taken into consideration. Under these circumstances, the concept of co-staining for marker proteins may not be reliable. If we re-evaluate the stem cell literature bearing in mind that staining might occur without concurrent gene expression, it is evident that the expression of differentiation markers should be under increased scrutiny. For example, positive staining for CD31 or PECAM-1 (platelet endothelial cell adhesion molecule-1) is widely used to proof a conversion of hematopoietic stem cells into endothelial cells. However, CD31 is not specific for endothelial cells, but also present on platelets and to different degrees on most leukocyte sub-types (Kim et al., 2009, Woodfin et al., 2007). If injected stem cells incorporate platelet material, they could be masquerading as “stem-cell derived” endothelial cells. Similarly, the tie2 promoter has been used extensively to follow the fate of EPC, but it is not a specific marker and is expressed by different non-endothelial cell types, including a monocytic/macrophage cell fraction (Deb and Patterson, 2010). With respect to vascular smooth muscle, staining for smooth muscle actin is insufficient evidence for the differentiation of progenitor cells towards the smooth muscle lineage (Sata et al., 2002). The reliance on such non-specific
markers results in an overestimation of bone marrow-derived cells (Sata et al., 2003). In fact, a recent time-course analysis in a mouse model of femoral artery injury suggested that the contribution of bone-marrow derived cells to neointima formation is limited to a transient period of the inflammatory response (Daniel et al., 2010). There was also little evidence for a direct contribution of circulating EPCs to plaque endothelium in apoE-deficient mice (Hagensen et al., 2010). Other studies used co-cultures with neonatal rat cardiomyocytes to demonstrate differentiation of bone marrow stromal cells (Xu et al., 2004) and EPCs into cardiac phenotypes (Badorff et al., 2003). The conclusion that human EPCs transdifferentiate into functional active cardiomyocytes was based on immunostaining for cardiomyocyte markers and the recording of cardiac action potentials (Badorff et al., 2003). The alternative explanation is that EPCs have incorporated cardiomyocyte markers and the action potentials were inadvertently recorded from neighbouring cardiomyocytes or that cardiomyocytes have incorporated cell material from EPCs. Indeed, several studies failed to detect permanent engraftment and transdifferentiation of transplanted bone marrow-derived hematopoietic stem cells (Murry et al., 2004, Balsam et al., 2004). Cell fusion of bone marrow-derived donor cells with recipient cardiomyocytes has been suggested as a potential mechanism (Nygren et al., 2004, Alvarez-Dolado et al., 2003), but this is contested by others (Kajstura et al., 2005). Notably, membrane vesicles could contribute to an exchange of marker proteins without classical cell fusion events. Thus, caution should be exercised in the interpretation of immunolabelling, particularly in areas with tissue injury and in co-culture systems.

5.3.2 Paracrine effects - can the functional improvements be attributed to stem cells?

Progenitor cells have repeatedly been implicated in cardiovascular tissue repair, but the mechanisms by which they act remain unsettled. In clinical trials, the percentage of retained cells is small and the number of cells that can be delivered via the intracoronary route is limited because of the risk of microinfarction, aggravating rather than repairing the injury. Moreover, some methods of cell labelling for imaging, i.e. iron particle-based magnetic resonance
imaging, have substantial limitations, i.e. upon death of the delivered cells, the particles can accumulate in macrophages and may not reflect stem cell fate (Kajstura et al., 2005). In view of the poor engraftment and survival rates for injected stem cells, the observed improvements in cardiac function after cell therapy must be explained by mechanisms other than stem cell differentiation. The pendulum was swinging to indirect effects on angiogenesis and functional regeneration of the heart (Mirotsou et al., 2011). Paracrine effects are a plausible explanation, but the question arises whether the observed improvements can be attributed to stem cells, if the cell preparation is a heterogeneous population? Arguably, the choice of appropriate controls for stem cell therapies is not trivial. Stem cells may need other cell types and a mixture of cells could be a more potent “biofactory” of paracrine factors than a purified stem cell population. Nonetheless, saline or plain medium are inadequate controls to establish whether the other cells in the mixture actually require the presence of stem cells and whether the paracrine factors are stem cell-derived. By now, positive effects have been reported with many different cell preparations. Clearly, it is the major challenge facing cardiovascular cell therapy to identify the most suitable stem / progenitor cell type for transplantation (Wollert and Drexler, 2005), but other cell types in unpurified cell preparations must not be ignored.
Figure V.2. Revised working model for early outgrowth EPCs. Early outgrowth EPCs were supposed to be a type of stem cell that differentiates into endothelial cells and incorporates in the lining of blood vessels. Based on our proteomics analysis of EPC cultures and findings by many other investigators, a tentative model, where vascular repair may be initiated by PMPs that recruit monocytes (MNC) to areas of vascular injury, seems more likely. A PMP-mediated protein and mRNA exchange could alter monocyte function and/or promote the transition to a proangiogenic macrophage phenotype. Together, PMPs and angiogenic macrophages may facilitate the recruitment of neighboring endothelial cells. For example, thymidine phosphorylase (previously referred to as platelet-derived endothelial growth factor) was identified to be among the proangiogenic factors in EPC cultures. Thymidine phosphorylase produces 2-deoxy-D-ribose-phosphate (dRP), an angiogenic metabolite. The expression of thymidine phosphorylase by EPCs may promote endothelial migration to occur along a gradient toward the injury and stimulate wound healing. This alternative concept would help to reconcile the literature documenting beneficial effects of EPCs on cardiovascular function with the recent finding that early outgrowth EPCs are not genuine endothelial precursors and do not incorporate in the vasculature.
5.4 Concluding remarks

The fascination with stem cells stands from their unique capacity for self-renewal and capability of forming at least one, and sometimes many, specific cell types. The fundamental property of stem cells is that they can regenerate the functional capacity of organs by replacing degenerative or dying cells. The key deliverable of cardiac stem cell therapy is the generation of cardiac muscle to repair chronic scars. Long-term success is less likely, if the cells used for therapy have no cardiomyogenic potential but induce angiogenesis while the underlying condition remains a scarred ventricle. The initial concept of delivering bone marrow cells to the injured myocardium was, at least partially, based on the assumption that subpopulations, such as EPCs, can differentiate into cardiomyocytes and functionally regenerate the heart. Meanwhile, the consensus seems to emerge that the functional benefit of bone marrow cell therapy involves stem cell–mediated angiogenesis but not cardiomyogenesis (Abou-Saleh et al., 2009, Marban and Cheng, 2010). Reports that some previous definitions of EPCs were not reliable and that platelet MPs contributed to their angiogenic activity, further challenge the concept of using unpurified bone marrow mononuclear cell preparations for therapy. Future studies will need to explore whether variations in platelets and especially in platelet MPs (which tend to get overlooked because of their small size (Assmus et al., 2010)) can help to explain the inconsistent results in clinical trials (Lunde et al., 2006, Schachinger et al., 2006a). After all, progenitor cell-based regenerative therapeutics are now commercially available to treat patients. If platelet MPs recruit or convert angiogenic monocytes, then the identification and administration of these active components in platelet MP may overcome the need for a bone marrow cell-based therapy and ultimately result in novel cell-free therapeutic strategies. In an ironic twist, the very feature of platelet activation, the formation of platelet MP that may allow for an enhanced vasoregeneration of EPCs (Mause et al., 2010), is inhibited by anti-platelet drugs and one might consider if a more tailored anti-platelet therapy could preserve some of these beneficial effects of platelet MPs in promoting tissue repair. Regardless, EPCs and cell therapy are likely to be subject to ongoing controversy in cardiovascular research (Schwartz, 2006). While proteomics cannot be the method of choice for routine quality control, especially
given the inherent problems of low stem cell numbers in clinical samples, proteomic technologies are an important research tool that can help to solve some of the fundamental problems that are plaguing the stem cell therapy field. By identifying surface proteins and defining cell specific markers and secreted factors, proteomics may have a clinical impact for developing new methods for better cell sorting and cell characterization. Ultimately, routine testing of stem cell functionality needs to be done with an easy and inexpensive method that can be performed with very low cell numbers.
CHAPTER VI

FUTURE WORK
This study provides evidence that the cell type consistent with current definitions of an EPC phenotype may arise from an uptake of platelet antigens by mononuclear cells. This finding has important implications for clinical trials using EPCs or unpurified BMCs for cell therapy. EPC cultures are heavily contaminated by PMPs and the interaction of monocytes with PMPs may play a functional role in vascular repair. Thus far, it has not been unambiguously shown that stem cells mediate the beneficial effects after cardiovascular cell therapy. Future experiments must address to what extent contaminations with PMPs or other microvesicles, i.e. exosomes (30-90nm in diameter) and larger apoptotic bodies (>1.5μm diameter), may contribute to the observed effects in neovascularization and/or vascular repair after injecting unpurified bone marrow mononuclear cell preparations. Moreover, further experiments are needed to explore the role of miR-885-5p in functional assays such as monocyte migration, adhesion and transmigration. Moreover, the signalling pathways that are activated by the chemokine CXCL7 and lead to the induction of miR-885-5p need to be determined.

In the present study, we performed the first systematic analysis of PMP-induced miRNA changes in monocytes and demonstrate that PMP carry an intravesicular miRNA load and alter protein expression as well as function of monocytes. A comprehensive analysis of their miRNA content provides an opportunity to bridge the gap between cellular plasticity and our current inability to unambiguously characterize monocyte/macrophage phenotypes. Considering that activated platelets release PMP, it is tempting to speculate that PMP may act as delivery system for miRNAs within the cardiovascular system. By identifying miRNAs changes resulting from platelet-monocyte interactions, one might find new biomarkers that provide an association with cardiovascular outcome or open potential avenues for miRNA-based therapies.
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All supplemental material regarding proteomics and miRNAomics work could be found in an electronic form in the attached CD (CD pocket) at the back of this thesis.
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