MASTERARBEIT

Titel der Masterarbeit

Tumor targeting with endothelial progenitor cells

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1 Abstract

Background:

To establish a vascular network which is indispensable for malignant growth, tumors exploit several mechanisms of blood vessel formation. A growing body of evidence indicates that endothelial progenitor cells (EPCs) play a crucial role in promoting and orchestrating the process of neovascularization, however their precise function and contribution remain controversial.

Aim:

The aim of this study was to screen human prostate (DU 145, CRL-2505), lung (H460, H520, Calu-3) and breast (MDA-MB-231) cancer cells for their capability to recruit *ex vivo* expanded, peripheral blood-derived EPCs *in vitro* and *in vivo* and to define an optimal experimental setup to monitor EPC recruitment.

Methods:

Different cancer cell lines were tested for the ability to recruit *ex vivo* expanded, peripheral blood derived EPCs *in vitro*, by using transwell migration assays and *in vivo* by using subcutaneous cancer xenograft models in athymic nude mice with intraperitoneal injection of $DiIC_{18}$-labeled EPCs.

The amount of $DiIC_{18}$-labeled EPCs (1, 10, 20, 30 and 70 x 10^6) and the time point of intraperitoneal (i.p.) injection (early-stage tumors compared to established tumors) were varied to evaluate the influence of these parameters on homing efficiency.

To determine the levels of EPCs, peripheral blood, peritoneal lavage and spleen lysates were analyzed by flow cytometry and tumor lysates with fluorescence microscopy.

Results:

Of all the cell lines tested, only H460 large cell lung cancer cells significantly recruited EPCs *in vitro* ($p < 0.05$). These results were confirmed *in vivo*. When EPCs were injected i.p. after tumor development, only H460 tumors recruited EPCs. The efficiency of EPC mobilization and homing within H460 tumors was increased by improving the experimental setup concerning the amount and timepoint of EPC application. 20 x 10^6 EPCs proved optimal resulting in the highest mean blood levels of 0.25 ± 0.38 % and highest infiltration into tumors (0.008 ± 0.04%). Injection of EPCs during early stages of tumor development further improved recruitment.

Such conditions led to an intensive initial EPC mobilization from the peritoneum into the blood stream (1.45 ± 0.43 %), which decreased nearly exponentially over a period of 15 days. This enabled relative EPC amounts of 0.08 ± 0.14 % within the tumors.

In contrast, EPC injection to developed tumors showed continuously moderate levels of circulating EPCs (0.2 ± 0.04 %), which reached only a contribution to tumors of 0.008 ± 0.04 %.

Within the tumor, EPCs were distributed non-uniformly, preferentially localized near the tumor boundary.

EPCs constituted 29.45 ± 8.2 % of the intraperitoneal cells and 0.65 ± 0.74 % of the cells localized within the spleen.

Conclusion:

H460 represented the only screened cancer cell line which was able to recruit EPCs both *in vitro* and *in vivo*. The *in vivo* recruitment from the peritoneum represents a highly dose- and tumor-size dependet process, which achieved largest efficiency when injecting 20×10^6 EPC during early tumor growth. Although the levels of tumor-infiltrated EPCs are low, they might prove optimal when using genetically modified EPCs transporting suicide genes or oncolytic viral particles.

2 Zusammenfassung

Hintergrund:

Tumore nützen verschiedene Mechanismen, um ein für malignes Wachstum notwendiges Gefäßsystem aufzubauen. Es gibt immer mehr Hinweise, die darauf hindeuten, dass endotheliale Vorläuferzellen (EPCs) eine wesentliche Rolle für die Neovaskularisierung spielen, wobei jedoch ihre genaue Funktion und ihr Beitrag nach wie vor umstritten sind.

Ziel:

Das Ziel dieser Arbeit war es, humane Prostata- (DU 145, CRL-2505), Lungen- (H460, H520, Calu-3) und Brustkrebszellen (MDA-MB-231) hinsichtlich ihrer Fähigkeit, aus dem peripheren Blut stammende, *ex vivo* vermehrte EPCs *in vitro* als auch *in vivo* zu rekrutieren und weiters diejenigen experimentellen Bedingungen zu definieren, welche einen effizienten Nachweis der EPC-Rekrutierung ermöglichen.

Methoden:

Verschiedene Krebszelllinien wurden unter Verwendung von Transwell Migrations Assays auf ihre Fähigkeit hin getestet, *ex vivo* expandierte, periphere, aus dem Blut gewonnene EPCs *in vitro* zu rekrutieren. Für die entsprechenden Experimente *in vivo* wurden subcutane xenografttransplantierte Tumormodelle in athymischen Nacktmäusen verwendet, denen DilC$_{18}$-gefärbte EPCs durch eine intraperitoneale Applikation verabreicht wurden. Durch Variation der Zellzahl (1, 10, 20, 30, 70 x 10^6) und des Zeitpunktes der EPC-Injektion (frühe Tumorphase vs. spätere Tumorphase), wurde der Einfluss dieser Parameter auf die Rekrutierungseffizienz überprüft, um so die bestmöglichen experimentellen Bedingungen für eine Rekrutierung von EPCs durch den Tumor zu definieren.

Die Bestimmung der Anzahl der EPCs im peripheren Blut, in Peritoneallavagen und Milzlysaten wurde mit Hilfe von FACS (fluorescence activated cell sorter) -Analysen durchgeführt. Die Zahl der in den Tumor rekrutierten EPCs wurde fluoreszenzmikroskopisch aus Tumorlysaten ausgewertet.

Ergebnisse:

In vitro konnten alle Tumorzellen mit Ausnahme von H520 LungenkrebszellenEPCs rekrutieren, wobei jedoch nur die großzelligen H460 Lungenkrebszellen signifikante Resultate (p < 0,05) erzielten.

Diese Ergebnisse wurden durch die nachfolgenden *in vivo* Studien bestätigt, welche zeigten,

dass ausschließlich H460 Tumore in der Lage sind, intraperitoneal injizierte EPCs in den bereits etablierten Tumor anzulocken. Die Effizienz dieses EPC-Homings in den H460 Tumoren konnte durch eine Optimierung der Dosis und des Zeitpunktes der EPC-Verabreichung gesteigert werden. Dabei stellten sich 20×10^6 EPCs als ideale Dosis heraus, welche die höchsten mittleren Blutwerte (0.25 ± 0.38 %) sowie die höchste Infiltriationsrate in die Tumore (0.008 ± 0.04 %) erzielte.

Die Rekrutierung von intraperitoneal verabreichten EPCs wurde weiter verbessert, indem EPCs in einer frühen Phase der H460 Tumorentwicklung injiziert wurden. Dies resultierte in einer starken initialen EPC-Mobilisierung in den Blutstrom (1.45 ± 0.43%), welche nahezu exponentiell über einen Zeitraum von 15 Tagen abfiel und zu einem relativen Anteil im Tumor von 0.08 ± 0.14 % führten. Im Gegensatz dazu führte die intraperitoneale EPC Injektion bei bereits entwickelten Tumoren zu relativ konstanten, moderaten EPC-Mengen in der Zirkulation (0.2 ± 0,04 %), welche in der Folge nur einen Anteil von 0.008 ± 0.04 % innerhalb der Tumore erreichten.

EPCs die in Tumore aufgenommen wurden, siedelten sich bevorzugt in dessen Peripherie an.

Innerhalb der intraperitonealen Zellen machten die EPCs einen Anteil von 29.45 ± 8.2 % aus, innerhalb der Milz von 0.65 ± 0.74 %.

Zusammenfassung:

Von den untersuchten Zellen waren nur großzellige H460 Lungenkrebszellen in der Lage, EPCs sowohl *in vitro* als auch *in vivo* zu rekrutieren.

Die Untersuchung der Effektivität der EPC-Rekrutierung aus dem Peritoneum ergab eine hohe Abhängigkeit von der verwendeten Dosis sowie von der Tumorgröße, wobei die größtmögliche Effizienz bei einer Applikation von 20×10^6 Zellen bei geringer Tumorgröße erzielt wurde.

Obwohl die Tumor-infiltrierenden EPCs nur geringe Prozentzahlen erreichten, könnte durch die Verwendung von genetisch modifizierten therapeutischen EPCs, welche Selbstmordgene oder onkolytische virale Partikel in den Tumor transportieren, therapeutisches Potential entfaltet werden.

3 Tumor neovascularization

The formation of tumor-associated blood vessels is an indispensable requirement for tumors to grow and metastasise.

In order to fulfill this demand tumors make use of angiogenesis and vasculogenesis, which represent the central mechanisms of tumor neovascularization (Figure 3-1).

3.1 Angiogenic process

When tumors exceed a size of 1-2 mm^3, hypoxia and nutrient depletion trigger the require-ment for angiogenesis [1]. Angiogenesis is defined as the formation of new blood vessels from the preexisting vasculature by proliferation and migration of endothelial cells, normally highly regulated by a sensitive balance between pro- and antiangiogenic factors [2].

To overcome this barrier and to initiate angiogenesis, tumor cells undergo an angiogenic switch enabling the tumor to acquire an angiogenic phenotype [3] leading to the continuous sprouting of new vessels from the normally quiescent vasculature [4].

Tumors create an angiogenic micoenvironment that stimulates endothelial cell receptors pre-sent on the endothelial monolayer of blood vessels, leading to changes in the phenotype and function of endothelial cells (ECs). Activated ECs secrete digestive enzymes which degrade the basement membrane and the extracellular matrix (ECM) [5].

The recruitment of tumor-associated fibroblasts and platelets further support the angiogenic progress by releasing several stimulatory factors [1].

The remodeling process results in the dissolution of the endothelial cell boundary, leading to the migration and proliferation of endothelial tip cells which subsequently form new vessel sprouts [6].

However, these tumor-derived blood vessels differ in several features from vessels in normal tissues, making them both structurally and functionally abnormal.

Whereas vessel formation under physiological conditions is completed by a resolution phase that matures and stabilizes the newly formed vessels, this phase is incomplete during tumor angiogenesis leading to the development of blood vessels lacking a pericyte coverage and

even a basement membrane [6].

Consequently, the blood vessels are leaky, dilated, tortuous, irregular and disorganized and therefore (although present in abnormal densities) not able to supply the tumor efficiently with oxygen and nutrients [1].

3.2 Vasculogenic process

Postnatal vasculogenesis is a further mechanism exploited by tumors to ensure sufficient blood vessel formation, [7] in which an early vascular plexus is formed by the assembly and differentiation of precursor cells, which subsequently generate a network of small blood vessels [2].

During this process precursor cells are mobilized from the bone marrow into the circulation, home to their respective sites and differentiate into ECs and assist in vascular development [8].

Considered for a long time to be restricted to embryonic development, vasculogenesis has now been shown to play a role in adult blood vessel kinetics.

Neovasculogenesis is fundamental for the revascularization of ischemic areas, wound healing and tumor progression [9].

The major cells involved in this process are endothelial progenitor cells (EPCs) [10].

EPCs not only have the ability to differentiate into ECs and thereby assisting in the formation of tumor-associated blood vessels, but also show paracrine properties, as they enhance angiogenesis by secreting proangiogenic cytokines [11].
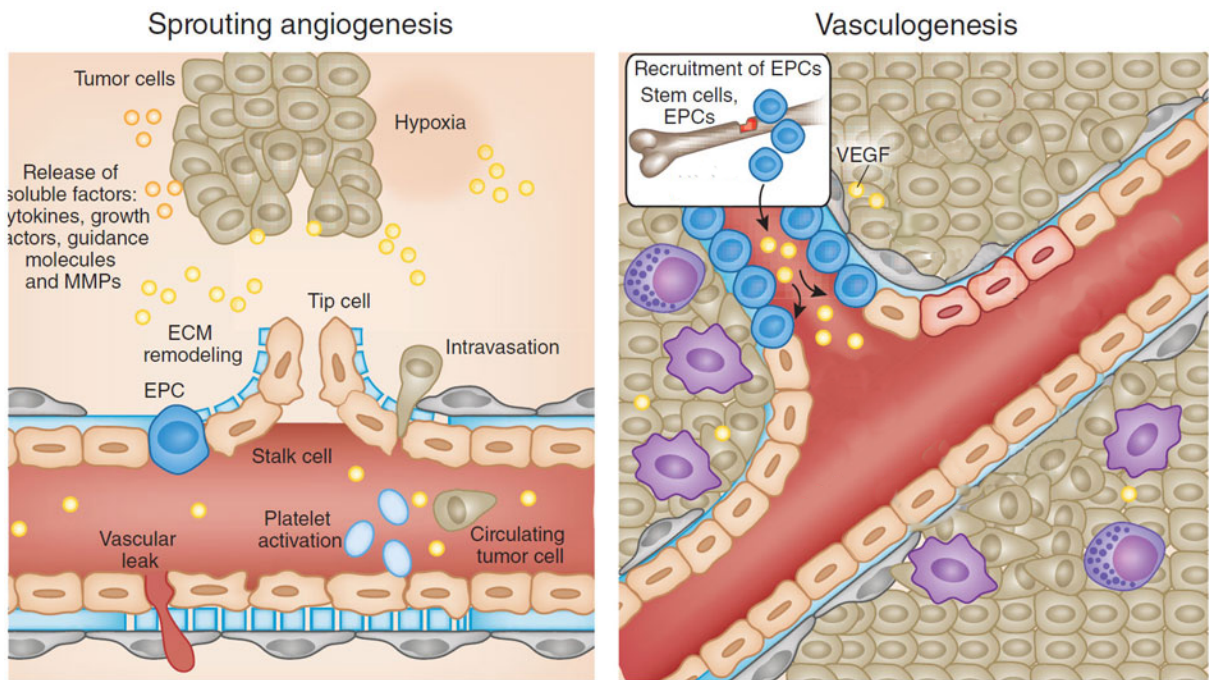
Figure 3-1 **Tumor neovascularization by sprouting angiogenesis and vasculogenesis.** *Sprouting angiogenesis is triggered by tumor-derived cytokines, growth factors, guidance molecules and metalloproteinases (MMPs). These factors initiate ECM remodeling, resulting in leaky junctions between ECs, which allows the migration and proliferation of endothelial tip cells to form new vessel sprouts. Recruitment of EPCs to sites of tumors leads to vasculogenic blood vessel formation. Modified from [1].*

4 Characterization of EPCs

EPCs comprise a heterogeneous group of stem cells with high proliferative potential, found mainly in the bone marrow (BM) but also represent a minor subpopulation of the mononuclear cell fraction in peripheral and umbilical cord blood [11].

They are characterized by the capability to contribute to vasculogenesis by proliferating, migrating and differentiating into mature ECs [12]. Hence EPCs are involved in vascular repair processe including wound healing, cardiac regeneration, atherosclerosis and cancer progression [13] [14].

Although EPCs were identified in 1997 [15], much controversy still surrounds EPC phenotypical characterization since the term EPC may not define a single cell type but rather describe several cell populations that are able to differentiate into the endothelial lineage [16] [17].

At least three potential BM-derived cell types have been suggested to represent the precursor to adult EPCs: Hemangioblasts, multipotent adult progenitor cells and myeloid cells [16].

The stage of EPC development can be defined by the expression of cell surface markers.

EPCs localized in the bone marrow typically express the early hematopoietic stem cell marker CD133, the progenitor marker CD34 and the endothelial marker vascular endothelial growth factor receptor 2 (VEGFR-2) [18].This profile changes after release into the peripheral blood (Figure 4-1) [11].

Circulating EPCs (cEPCs) seem to be more mature cells losing CD133 expression and gaining expression of CD31 (platelet endothelial cell adhesion molecule-1) and CD146 (melanoma cell adhesion molecule) [18], markers typical for the endothelial lineage, reflecting a gradual transformation into the EC lineage [19].

Terminally differentiated mature ECs show low proliferative potential and express VEFGR-2, CD146, CD31, VE-cadherin, endothelial nitric oxide synthase (eNOS) and van Willebrand factor (vWF) [11].
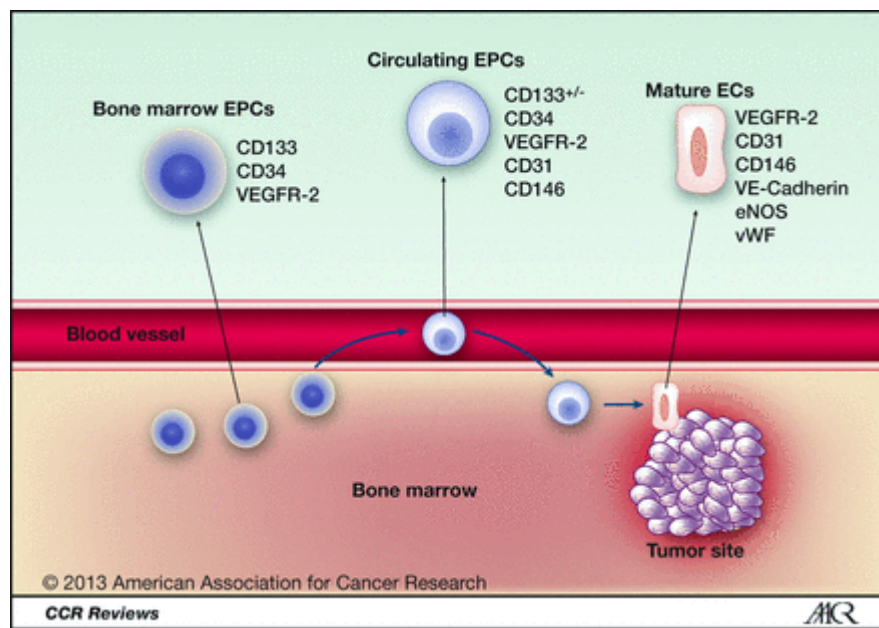
Figure 4-1 **Surface marker expression during migration and differentiation of EPCs.** *EPCs localized within the bone marrow are characterized by the expression of CD133, CD34 and VEGFR-2, while circulating EPCs lose CD133 expression but gain expression of CD31 and CD146. Terminally differentiated ECs express VEGFR-2, CD31, CD146, VE-cadherin, eNOS and vWF [11].*

5 Trafficking of EPCs in tumor neovascularization

In order to function in tumor neovascularization, EPCs migrate from the bone marrow to sites of tumors.

Therefore they have to accomplish several steps (Figure 5-1), starting with their response to chemoattractants and mobilization from the bone marrow into the peripheral blood, followed by homing to sites of tumors, where they have to invade and migrate and at least differentiate into mature ECs and/ or exert paracrine functions [20].
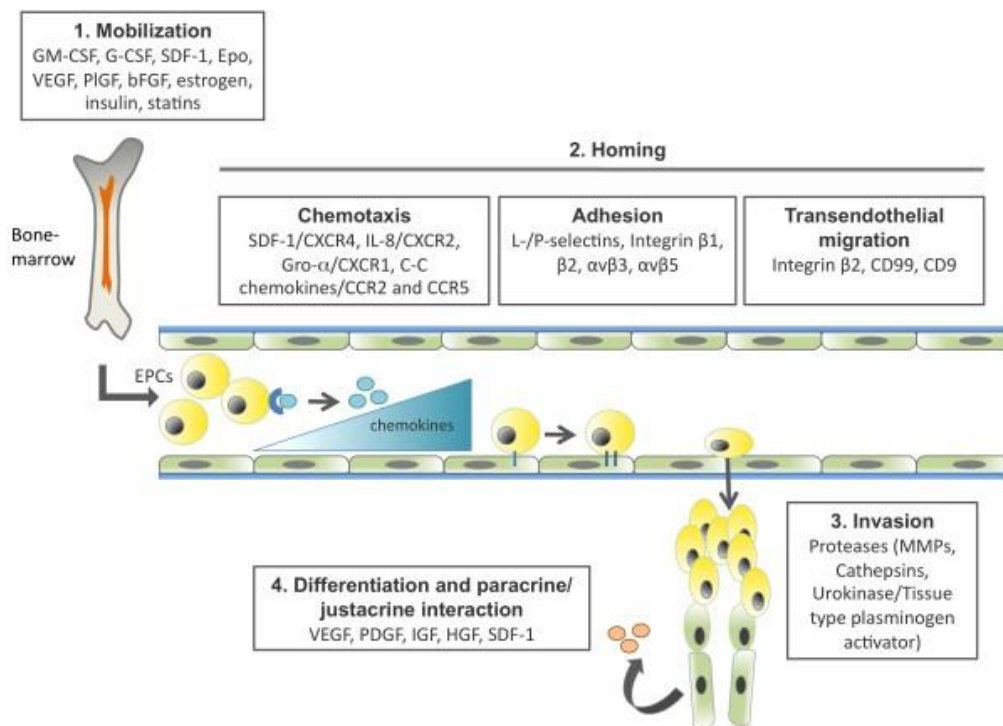
*Figure 5-1 **Trafficking of EPCs in tumor neovascularization.** The multistep process of recruiting EPCs to tumor sites includes their mobilization out of the bone marrow, chemoattraction, adhesion, transendothelial migration, invasion, differentiation and paracrine/ juxtacrine interactions [20].*

5.1.1 Mobilization from the bone marrow into the peripheral blood

Under physiological conditions, EPCs are localized in the stem cell niche, a special microenvironment in the bone marrow [21], where their maintenance and mobility is regulated by the coaction of different cells [22]. EPC release into the circulation is tightly regulated by growth factors, enzymes, ligands and surface receptors [23].

Tumors are able to induce EPC mobilization by releasing several EPC-mobilizing factors which subsequently switch the marrow microenvironment from a dormant to a proangiogenic and protumorigenic state [24].

In particular, the most powerful factors are vascular endothelial growth factor (VEGF), Granulocyte-Colony Stimulating Factor (G-CSF), basic fibroblast growth factor (bFGF), erythropoietin, stromal cell-derived growth factor-1 (SDF-1) and placental growth factor (PGF) which downregulate the interactions between EPCs and the bone marrow microenvironment and promote progenitor migration [17].

5.1.2 Homing to sites of tumors

After mobilization from the bone marrow to the peripheral blood, chemokine gradients, formed in tumors, guide EPCs to sites of tumors [25].

The most important chemokines and their receptors are VEGF and VEGFR-2, SDF-1 and CXCR4, interleukin-8 and CXCR2, GRO-α and CXCR1, CCR2 and CCL2 and CCR5 and CCL5 [11].

EPCs then cross the blood vessel wall to invade the underlying tumor tissue. This process is initiated through contact between EPCs and the blood vessel wall induced by interactions between the P-selectin glycoprotein ligand-1 (PSGL-1) EPC surface receptor and P- and E-selectin expressed on ECs [26].

These interactions enable the intercellular adhesion to the endothelial monolayer mediated by β1 (α4β1, α5β1, α6β1) and β2 integrins expressed on EPCs [20].

When adhering at specific homing sites, EPCs transmigrate the endothelial layer, mediated by integrin β2 and dependent on monocyte chemotactic protein-1 (MCP-1) and VEGF [27] followed by migration through the the blood vessel basement membrane and through the interstitial extracellular matrix [20].

EPCs may upregulate the production of several extracellular proteases [28] such as members of the metalloproteinase (MMP) family [29], the cathepsin family [30] and the serine protease family [31]. By degrading the matrix these proteases allow EPC migration and thereby invasion into the underlying tissue.

Additionally a VEGF gradient within the matrix may stimulate EPC migration and invasion, mediated by integrin α5β1 and α6β1 [32].

5.2 Role of EPCs in tumor neovascularization

When EPCs migrate to a tumor it is hypothesized that they have a dual function (Figure 5-2). First they provide a structural function by forming cellular clusters and differentiating into ECs that subsequently build up a new vasculature or are incorporated into preexisting vessels [39].

Secondly EPCs exert a paracrine function by secreting a variety of proangiogenic factors which enhance the angiogenic response [33].
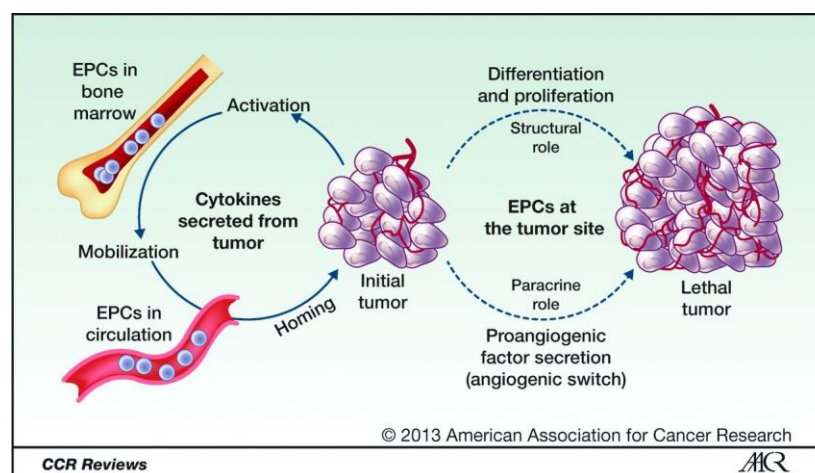
Figure 5-2 **Dual role of EPCs in tumor neovascularization.** *When EPCs arrive at sites of tumors, they support malignant growth by providing structural and paracrine functions [11].*

5.2.1 Structural function of EPCs

The structural function of EPCs comprises the formation of cellular clusters which merge into cellular networks and become incorporated into the tumor microvasculature [34]. Moreover, EPCs differentiate into ECs and incorporate into sprouting vessels [14]. The differentiation process depends on three sequential steps.

After integrin-mediated adhesion to specific ECM components, EPCs proliferate under the influence of growth factors and finally mature and acquire functional EC properties [20].

The key regulator of this process is fibronectin, an integrin ligand, localized in the extracellular matrix. Fibronectin is able to interact with EPCs by binding to integrin $\alpha5\beta1$ expressed on the surface, which promotes VEGF-induced differentiation of EPCs into ECs [20].

After differentiation, EPCs can incorporate into vessels although the relevance of this process is controversial, since the observed levels vary significantly.

The contribution ranges in preclinical studies from 50 % [35] to 5-20 % [36] or even undetectable levels [37], while clinical studies indicate an incorporation rate of 1 % among head and neck sarcoma and 12 % within lymphomas [38].

It is currently assumed that the structural contribution of EPCs is only modest and their main effects in tumor neovascularization are attributed to their instructive, paracrine functions.

5.2.2 Paracrine function of EPCs

The paracrine action of mode of EPCs seems to support tumor neovascularization by enhancing the angiogenic response. Their release of a variety of proangiogenic cytokines results in a self-amplifying loop [25].

Gene expression analysis of EPCs, purified from tumor tissues, revealed the upregulation of a variety of key proangiogenic factors, including growth factors, receptors and chemokines [39].

For instance, EPCs are able to express VEGF, which induces increased mobilization, migration and differentiation of EPCs, insulin-like growth factor 1 (IGF-1) and hepatocyte growth factor (HGF), potent antiapoptotic proteins promoting angiogenesis and SDF-1, a chemokine acting as chemoattractant to promote migration and tissue invasion of EPCs, in levels higher than from mature ECs [40].

6 EPCs as diagnostic and therapeutic tool in cancer

The clinical significance of circulating EPCs has been confirmed for malignant gliomas [41], breast [42], ovarian [43], hepatocellular [44] [45] [46] [47] and non-small lung cancer [48].

Among all these cancer types, the levels of cEPCs correlate with the stage of disease. The more advanced tumor progression, the higher are levels of cEPCs in the peripheral blood.

This leads to the conclusion, that EPCs might be useful diagnostic, prognostic and therapeutic tools.

6.1 EPCs as surrogate biomarkers

Several features indicate that cEPCs have the potential to act as diagnostic and prognostic biomarkers in tumor biology.

Due to the relationship between cEPCs and tumor progression, monitoring levels in the peripheral blood might not only give information about the tumor stage but may also identify patients who would benefit from antiangiogenic therapies [41].

When starting an anticancer or antiangiogenic intervention, changes in the levels of cEPCs might predict the response to the therapy, making them a potential surrogate biomarker for monitoring therapy efficiency [43].

Additionally they might also be useful to define the optimal biological dose of certain therapeutics [41].

Currently, counting microvascular densities and determining levels of angiogenic cytokines in serum represent the main methods to measure the angiogenic activity and efficiency of anti-angiogenic therapeutical intervention, practices which are highly invasive and not always reliable [48].

Thus assessing EPCs could offer a sensitive and less invasive alternative.

6.2 EPCs for cancer treatment

Since tumor development and progression critically depends on the formation of tumor-associated blood vessels, Folkman hypothesized in 1970 that targeting tumor vessel formation might be a promising therapeutic strategy to arrest tumor growth and metastasis [49].

Due to the need for EPCs in tumor neovascularization, their selective targeting offers a new possibility to realize this strategy and makes them a highly attractive candidate for cell-based cancer therapies.

Therefore two different approaches have been considered - blocking EPC mobilization or using EPCs as gene delivery vehicles.

6.2.1 Blocking EPC mobilization from the bone marrow

Since the relationship between high numbers of EPCs in the peripheral blood and increased angiogenesis and metastasis has been proven clinicopathologically, blocking EPC mobilization and migration from the bone marrow might impact tumor growth and metastasis [8].
The possible relevance of such a therapy is supported by Nolan et al., who showed that the ablation of EPCs in tumor-bearing mice delayed tumor growth associated with decreased vessels [50].
Blocking of EPCs might be especially of benefit in combination with chemotherapy, since preclinical and clinical data indicate that certain chemotherapeutics increase the levels of cEPCs which consequently stimulate tumor progression and metastasis [51]. Therefore blocking EPCs could abolish their negative impact and thereby enhance the efficiency of chemotherapeutics [24], a hypothesis which was indeed confirmed for selected chemotherapeutics [51] and vascular disrupting agents [52].

6.2.2 EPCs as gene delivery vehicles

In addition to blocking EPCs, their specific migration to tumors and metastases can be exploited to use them as vehicle to transport therapeutics directly into the tumor.
Beside their tumor homing properties several other features make EPCs very attractive for this purpose.

EPCs can be isolated easily from peripheral blood, show good *ex vivo* expansion properties allowing the culture of large numbers required for therapeutic applications and can be modified genetically by transduction with viruses to carry therapeutic payloads [53] [54].

To confer EPCs with therapeutic properties, different payloads are under consideration such as arming EPCs with suicide, antiangiogenic and immune-boosting genes or using them as packaging cells for oncolytic viruses or other toxins.

Several preclinical studies demonstrated benefit effects of such therapeutic approaches on tumor growth and animal survival [55].

However, using EPCs as gene delivery vehicle also includes possible risks, since EPC can migrate into other ischemic areas present in the bone marrow or during the female reproductive cycle as well [56]. Therefore, the payloads have to be carefully chosen to avoid damage to other sites.

7 Aims of the study

In the last two decades, EPCs have been ascribed a growing importance in the progression of tumors. In response to tumor-derived signals, EPCs migrate from the bone marrow to sites of tumors where they support neovascularization and trigger malignant growth. These homing properties offer new opportunities for cell-based cancer therapies.

However, the homing efficiency of EPCs seems to vary among different tumor types and tumor stages, since every tumor shows unique features and patterns of neovascularization. To date, only a few tumor cell lines have been tested for the capability to recruit EPC.

Therefore the aim of this study was:

1. To screen human breast (MDA-MB-231), prostate (DU 145, CRL-2505) and lung (H460, H520, Calu-3) cancers cells for the ability to attract *ex vivo* expanded, peripheral blood derived EPCs *in vitro* and *in vivo*. While *in vitro* studies were performed with transwell migration assays, during *in vivo* studies tumor cells were implanted subcutaneously into athymic nude (*nu/nu)* mice and were tested for the capability to attract EPCs from the peritoneal cavity.

2. To gain insight into the interperitoneal route of EPC delivery, which represents a new administrative approach.

3. To define the optimal dose of EPCs and optimal timepoint of injection to develop an efficient experimental setup.

8 Material and Methods

8.1 Cell lines and standard growth conditions

All cell lines were cultured on 15 cm tissue culture dished in 20 ml appropriate medium in an incubator (Bender) in a fully humidified air atmosphere containing 5 % CO_2 at 37 °C.

8.1.1 Tumor cell lines

All used tumor cell lines (summarized in Table 8-1) were cultured in Dulbecco's modified Eagle's medium (DMEM, PAA, Pasching, Austria) supplemented with 10 % fetal calf serum (FCS; PAA), 0.1 M nonessential amino acids, 100 U/ml penicillin and 100 µ/ml streptomycin.

Table 8-1 Used tumor cell lines.

Name	ATCC number	Type
CRL-2505	CRL-2505	Human prostate carcinoma
DU 145	HTB-81	Human prostate carcinoma
H520	HTB-182	Human squamous cell bronchus carcinoma
H460	HTB-177	Human large cell lung carcinoma
Calu-3	HTB-55	Human bronchus adenocarcinoma
MDA-MB-231	HTB-26	Human breast adenocarcinoma

8.1.2 Endothelial progenitor cells

Human circulating endothelial progenitor cells were isolated from peripheral blood mononuclear cells from healthy donors on type I collagen (Sigma) and expanded in EGM-2 medium (Lonza, Basel, Switzerland) for at least 10 population doublings before commencement of experiments [54]. They are characterized by expressing CD146, CD141, CD31 and VEGFR-2 while lacking CD45 and CD41 cell makers [54].

8.2 Subcultivation

For subcultivation of adherent cells, the medium was aspirated, the cells washed once with 15 ml PBS (Dulbecco's PBS, PAA, Pasching, Austria) and incubated with 1 ml trypsin/ EDTA for approximately 5 min, at 37 °C and 5 % CO_2. Trypsin was inactivated by adding full medium and detached cells were transferred into a falcon tube and centrifuged for 5 min at 1500 g (Heraeus Labofuge 400R). The remaining pellet was resuspended in fresh medium and recultivated at the desired ratio.

8.3 Cell counting

To adjust cells to a specific cell number, cells were counted with a cell counter (Millipore, Darmstadt, Germany). Cells were first detached from culture dishes with trypsin, centrifuged and resuspended in 1 ml of appropriate medium. The cells were counted after diluting the suspension in PBS (1:100).

8.4 Migration assay

To compare the capability of different cancer cell lines to recruit EPCs, a transwell migration assay was performed.

The assay relies on a permeable layer of support which is positioned between two compartments. Cells on the upper side of the membrane can migrate in response to chemoattractants through micropores and be quantified.

EPCs (1×10^5 in 0.5 ml DMEM without FCS) were placed on the upper layer of an 8 µm permeable support (BD Falcon) and positioned into a well of a 24-well plate, containing either 5×10^5 cancer cells in 2 ml DMEM or DMEM alone as a control.

After 24 h, the medium was removed and the membranes were washed twice with PBS. Cells from the upper side of the membrane were removed with a cotton swab. The membranes were cut out of the supports using a scalpel, inverted, washed in PBS and fixed in methanol for 10 min at -20 °C. After washing in PBS, membranes were stained with 0.1 µg/ ml 4'-6-Diamidino-2-phenylindole (DAPI) in PBS for 10 minutes at room temperature and

washed again with PBS. Membranes were then embedded in Cityflour (Cityflour, Leicester, UK) on glass slides and representative sectors of migrated cells were counted under a fluorescence microscope.

8.5 EPC staining

EPCs were labeled with the lipophilic tracer $DilC_{18}$.

10 µl $DilC_{18}$ stock solution (Invitrogen, Carlsbad, USA) were added to 2 ml cell suspensions in PBS, incubated for 5 min at 4 °C, followed by 30 min incubation at 37 °C and 5 % CO_2. The cells were centrifuged (1500 g, 5 min) and the pellet washed in PBS. The staining procedure was performed twice.

8.6 Subcutaneous cancer xenograft model

The experiments performed in this study were approved by the Institutional Animal Care and Use Committee at the Vienna Medical University with pathogen-free, male 4 week-old athymic *nu/nu* mice (Charles River, Sulzfeld, Germany).

The animals were housed under sterile conditions at the Department of Biomedical Research at the Medical University of Vienna.

Four mice experiments were performed which only differed in regard to the amount and timepoint of EPC application.

The experiments were performed as subcutaneous cancer xenograft model, where tumor cells were injected subcutaneously through a 27-gauge needle into the left flank region, and $DilC_{18}$-labeled EPCs were injected into the peritoneal cavity (i.p.).

Tumor size was monitored every two to three days with calipers to calculate tumor volumes using the formula $V = \pi / 6 * (length * width * width)$.

To measure the number of circulating EPCs, peripheral blood was collected by tail vain puncture every one to three days and analyzed with fluorescence activated cell sorting (FACS).

Mice were sacrificed dependent on tumor sizes or experimental progress by cervical dislocation.

Subsequently, peritoneal lavages were performed and analyzed with FACS.

The tumors were removed, weighed and cut into three pieces; one part was frozen in liquid nitrogen and stored at -70 °C, one part used for flow cytometry and the third fixed in formalin und embedded in paraffin.

In addition, lung, liver, spleen, brain, heart, muscle and kidney were isolated and either frozen in liquid nitrogen or fixed in formalin.

The experimental details of each experiment are summarized in table 8-2.

Table 8-2 Experimental setup of the in vivo experiments.

Experiment	Tumor type	Amount of tumor cells	Timepoint of EPC injection	Amount of EPCs	Number of mice
1	CRL-2505	15×10^6	After tumor development	1×10^6	1
				10×10^6	1
	DU145	10×10^6	After tumor development	1×10^6	1
				10×10^6	1
	H520	10×10^6	After tumor development	1×10^6	1
				10×10^6	1
	H460	5×10^6	After tumor development	1×10^6	1
				10×10^6	1
	Calu-3	10×10^6	After tumor development	1×10^6	1
				10×10^6	1
	MDA-MB-231	10×10^6	After tumor development	1×10^6	1
				10×10^6	1
2	H460	5×10^6	After tumor development	10×10^6	2
				30×10^6	2
				70×10^6	2
3	H460	5×10^6	Before tumor development	20×10^6	2
			After tumor development	20×10^6	2
4	H460	5×10^6	Before tumor development	20×10^6	6

8.7 Flow cytometry

Fluorescence activated cell sorting allowed the determination of EPC levels in the peripheral blood, peritoneal lavage and spleen lysates. Since EPCs were DilC$_{18}$-labeled before starting experiments, no further staining was necessary.

Erythrocytes within the peripheral mouse blood were lysed in 155 mM NH$_4$Cl, 10 mM KHCO$_3$ and 0.1 mM EDTA pH 8 (for 12 min at 4 °C). Cells were then washed in PBS, centrifuged for 6 min at 540 g and resuspended in PBS.

Tumor and spleen sections were first rinsed in PBS and passed through a 100 µm cell strainer (BD Biosciences, Franklin Lakes, USA) before lysis of red blood cells.

Viable events (20^4) were analyzed with a FACscan flow cytometer (BD Biosciences) with an argon laser tuned to 488 nm.

8.8 Immunohistochemistry and microscopial analysis

By using immunohistochemistry and fluorescence microscopy localization of EPCs in tumors and tissues can be determined.

To distinguish between EPCs and erythrocytes, which both fluoresce in the same spectral area, the slides were first DAPI stained to stain the cell nucleus blue. This allowed the detection of EPCs by colocalization of the blue colored nucleus with the red DilC$_{18}$-stained cytoplasm. After detection of EPCs, digital photographs were taken and the corresponding coordinates noted. In the next step, the slides were HE stained and photographed again with the appropriate coordinates to show EPCs together with the microanatomy of the tissues.

8.8.1 Fixing tissues and paraffin embedding

The extracted tumors and organs from the xenograft experiments were fixed in a 4 % formaldehyde solution for one week at 4 °C, followed by embedding in paraffin and cutting into 2.5 µm sections.

8.8.2 DAPI staining

Following the preparation of paraffin sections, the slides were deparaffinated by incubating in xylene, rehydrated by passing them through graded alcohols and rinsed in PBS. Nuclei were stained with 0.1 µg/ ml 4'-6-Diamidino-2-phenylindole (DAPI) in PBS for 10 min at room temperature and embedded in Cityfluor.

8.8.3 HE staining

HE provides a comprehensive picture of the microanatomy of organs and tissues. Haematoxylin stains nuclear components with a blue-purple hue, whereas eosin colors cytoplasmic components bright pink.

The DAPI pre-stained slides were cleaned with xylol and rehydrated in graded alcohols. After rinsing in water, the slides were applied with stained with hematoxylin for two min and a blue colour was developed by rinsing in running water for two minutes. The nuclear staining was followed by counterstaining with eosin for three minutes. After rinsing in water for one minute, the slides were dehydrated in graded alcohols, cleared in xylol and mounted in DPX (Fluka, St. Luis, USA).

9 Results

9.1 Capability of different cancer cell lines to recruit EPCs

Initially six different human cancer cell lines were screened for the ability to recruit EPCs to sites of tumors by using *in vitro* and *in vivo* experiments. The analyzed cells comprised breast (MDA-MB-231), lung (H460, H520, Calu-3) and prostate cancer cells (DU 145, CRL-2505).

9.1.1 EPC recruitment *in vitro*

To determine the capability of the different tumor cell lines to recruit EPCs *in vitro*, a transwell migration assay was performed, which relies on a microsporus membrane which enables the migration of EPCs in response to tumor cell-released chemoattractants.

With the exception of H520, all cell lines were able to recruit EPCs, whereas H460 tumor cells promoted EPC migration to the highest extent (Figure 9-1). Migration assays were repeated three times with H460 cells and showed significant EPC recruitment (p < 0.05). (Figure 9-1, Figure 9-2)

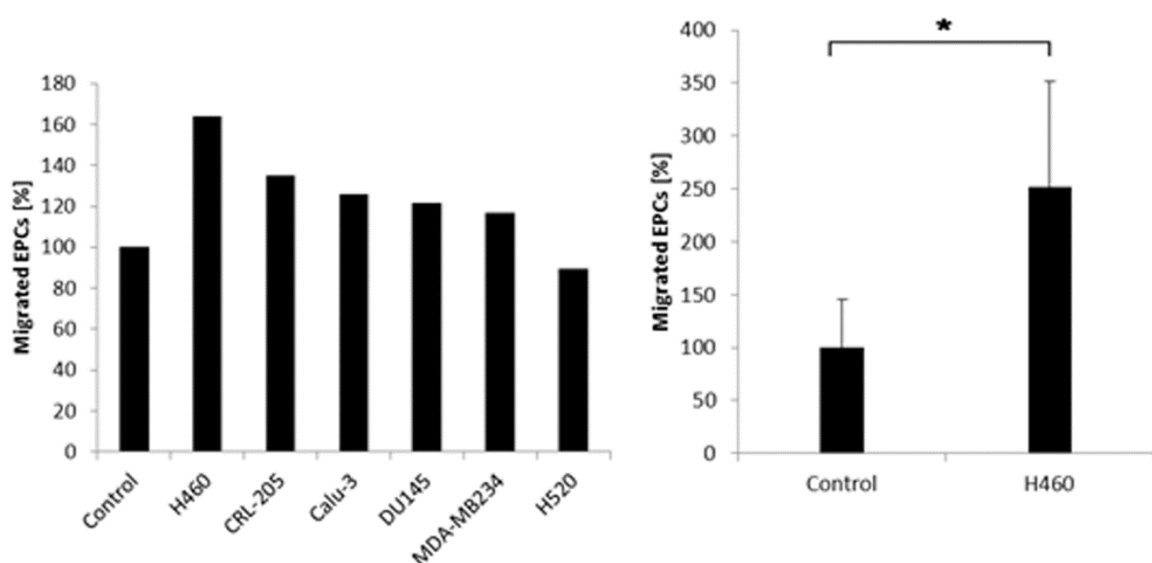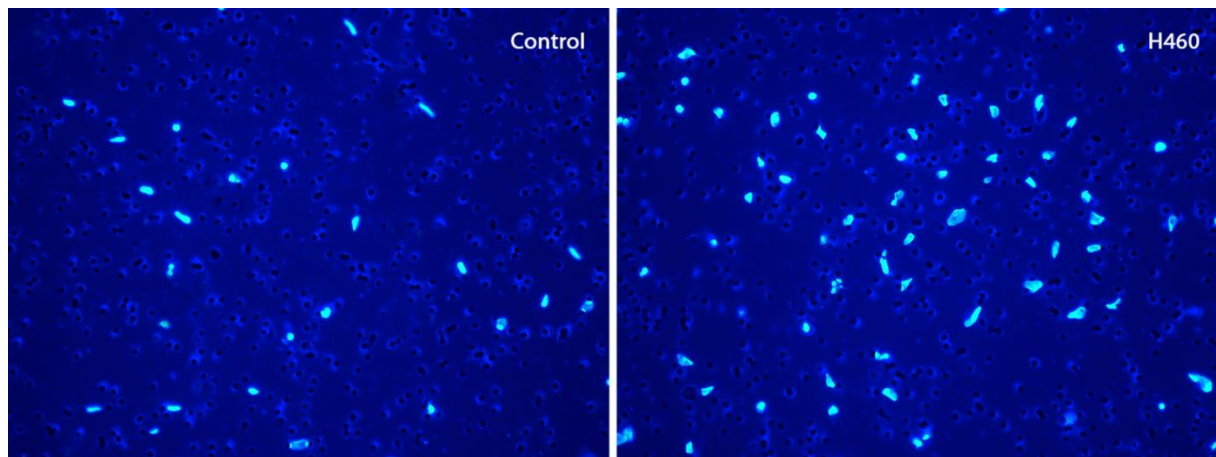*Figure 9-1* **EPC migration in vitro.** *Left: Relative EPC migration is dependent on the tumor cell line. Right: Relative amount of H460-recruited EPCs. Data incorporate the standard deviation of three experiments (*, p < 0.05).*

*Figure 9-2 **H460 large cell lung cancer cells promote EPC migration in vitro.** H460 cells promoted a significant increase in the number of migrated EPCs, compared to control (p < 0.05) (Magnification, x200).*

9.1.2   EPC recruitment *in vivo*

To investigate whether EPCs can be mobilized from the peritoneum into the blood circulation and migrate subsequently into the tumor bed, subcutaneous tumors were implanted in immunocompromised athymic *nu/nu* mice for 15 days, followed by intraperitoneal EPC injection. In each group of tumor cell lines, mice received either 10^6 or 10^7 DilC$_{18}$-labeled EPCs.

9.1.2.1 EPC mobilization into the peripheral blood

Blood analysis with a fluorescence activated cell sorter showed that EPCs were mobilized from the peritoneum into the circulation in all cancer lines, but at very low levels with some cell lines, especially when receiving a dose of 1 x 10^6 EPCs (Figure 9-3).
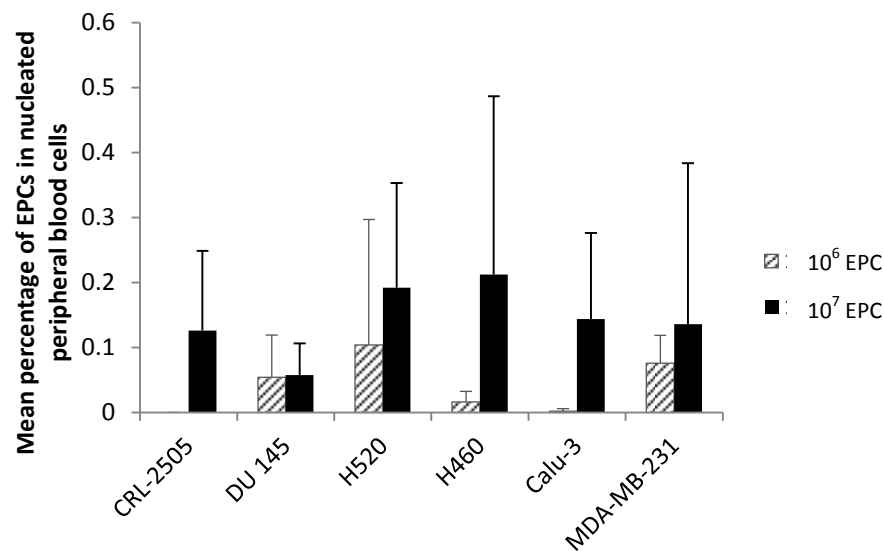
Figure 9-3 **Levels of circulating EPCs are dependent on the tumor cell line and amount of EPC injected.** *EPC levels were generally low and varied extensively within and between each group, whereby no correlation was observed.*

9.1.2.2 EPC recruitment into tumors

EPC homing to sites of tumors was only observed in H460 tumors and also only in the mouse receiving 10^7 EPCs. The ability of H460 to attract EPCs might be related to tumor growth characteristics as H460 had the highest tumor volumes compared to the other cell lines, as shown in figure 9-4.
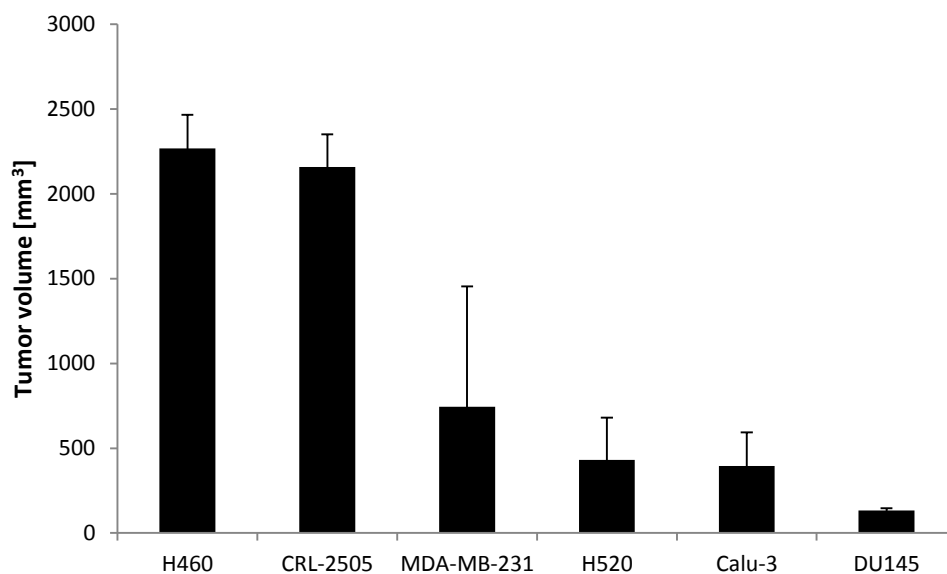
Figure 9-4 **Tumor volumes of the different cancer cell lines on day 28**. *H460 large cell lung cancer cells resulted in the highest tumor volumes (2269 ± 197 mm^3).*

9.2   Optimizing the EPC recruitment of H460

Since the initial screening revealed that only H460 tumor cells are able to recruit EPCs *in vitro* and *in vivo*, the following experiments were focused on increasing the efficiency of EPC recruitment.

9.2.1   Defining the most effective dose of EPCs

The extent of EPC mobilization and migration might depend on availability and thereby on the injected dose.

To assess this hypothesis, different amounts of EPCs (1, 10, 20, 30 and 70 x 10^6) were administered i.p. after the development of H460 tumors and levels in blood and tumors were determined to define the most efficient EPC dose.

Figure 9-5 summarizes the levels of EPCs found in nucleated peripheral blood cells which are dependent on the amount injected.
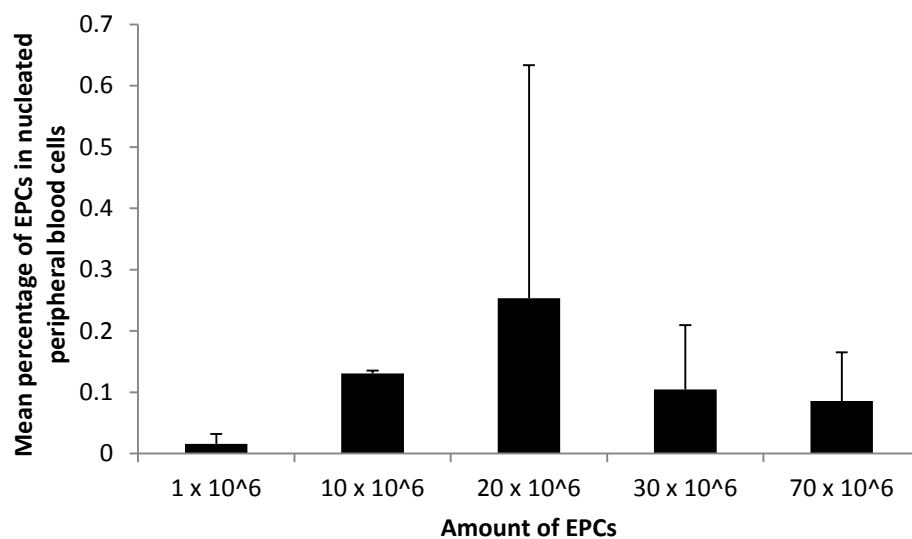
Figure 9-5 **Mean levels of circulating EPCs are dependent on the amount injected.** *Injecting an amount of 20 x 10^6 is the most efficient dose, resulting in the highest mean blood levels of EPCs.*

A dose of 1 x 10^6 EPCs resulted in low mean blood levels (0.016 ± 0.016 %) and no infiltration into tumors.

Increasing the number to 10 x 10^6 cells increased mean circulating levels to 0.13 ± 0.005 % and resulted in the homing of a few cells into the tumor. By increasing the number of EPCs to 20 x 10^6, results were further improved, leading to higher mean blood levels (0.25 ± 0.38 %)

and a contribution to tumors of 0.008 ± 0.04 %.

Raising the applied levels to 30 or 70 x 10^6 reduced peripheral levels and tumor infiltration. These amounts were too high since EPCs agglutinated within the peritoneum.

Accordingly, 20 x 10^6 were determined to be the most efficient dose, resulting in relative high numbers of circulating EPCs and appropriate infiltration into tumors.

9.2.2 Defining the most efficient timepoint of EPC injection

Beside the amount of EPCs, a growing body of evidence indicates that also the timepoint of application with reference to tumor development influences the degree of EPC infiltration into tumors.

To investigate a possible stage-dependent influence on the kinetics of circulation and homing of EPCs, this study then compared early and developed H460 tumors.

20 x 10^6 EPC were injected i.p. either immediately after tumor implantation or after tumor development.

Assessing the levels of DilC$_{18}$-labeled circulating EPCs demonstrated that early tumors are characterized by an intensive initial recruitment of EPCs from the peritoneum into the blood stream (1.45 ± 0.23 %), which decreased nearly exponentially, while late tumors showed continuously moderate levels (0.2 ± 0.04 %) of circulating EPCs (Figure 9-6).
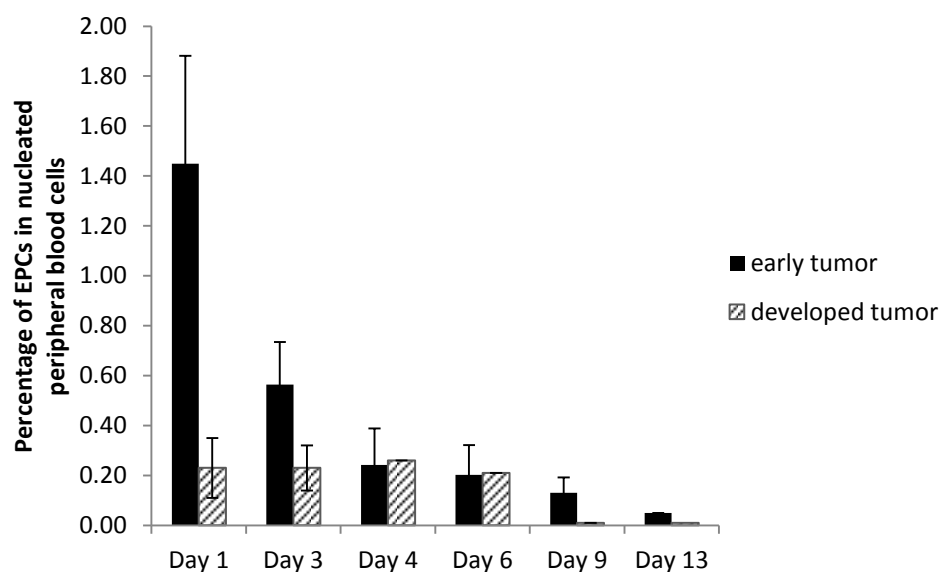
Figure 9-6 **Comparison of circulating EPC levels between early and developed H460 tumors.** *While intra-peritoneal injection of EPCs in mice bearing early-stage tumors (day 1) led to a strong initial EPC mobilization into the peripheral blood, which declined nearly exponentially, EPC injection into mice bearing developed tumors (day 5) resulted in constant, moderate blood levels.*

Fluorescence microscopical analysis of tumor lysates revealed that EPC homing to tumors is more efficient, when injecting the cells during an early phase of tumor development, indicating that the recruitment is an early event in tumor development (Figure 9-7).

Those tumors contained 0.08 ± 0.14 % EPCs, while late-stage application achieved values of only 0.008 ± 0.04 %.
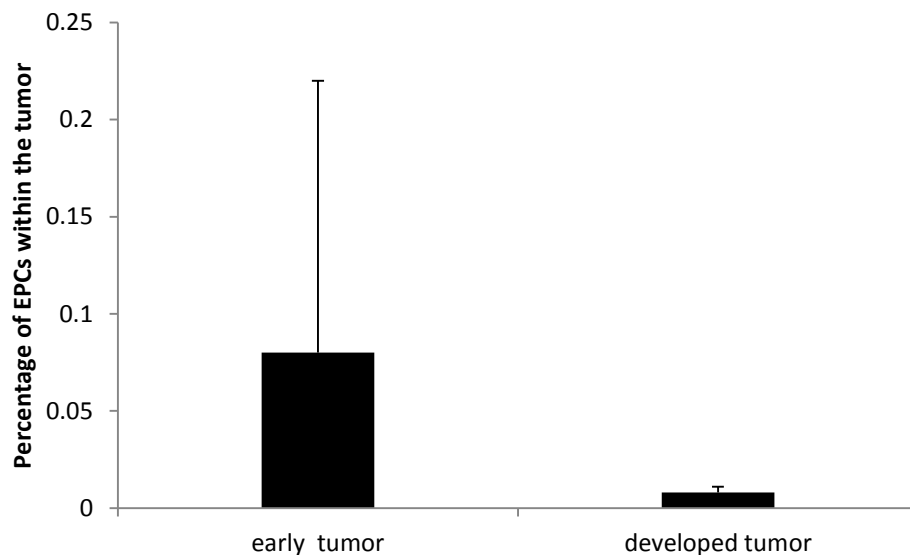
*Figure 9-7 **EPC contribution to early and developed H460 tumors.** Early-stage H460 tumors were infiltrated by 10-fold more EPCs (0.08 ± 0.14 %) than developed tumours (0.008 ± 0.04 %).*

In summary, the ideal conditions to achieve infiltration of EPCs into H460 tumors require the injection of 20×10^6 EPCs during the early stages of tumor development.

9.3 Distribution of EPCs

To gain insight into the interperitoneal route of EPCs, peritoneal lavages, blood, spleen tissue and tumors of mice receiving 20×10^6 EPCs prior to the development of H460 tumors, were analyzed.

9.3.1 EPCs in the peritoneum

After sacrifice, peritoneal lavages were performed to determine the amount of EPCs which weren't mobilized and were resident in the peritoneum. FACS analysis demonstrated that EPCs represented 29.45 ± 8.2 % of the peritoneal cells (Fig. 9-8).
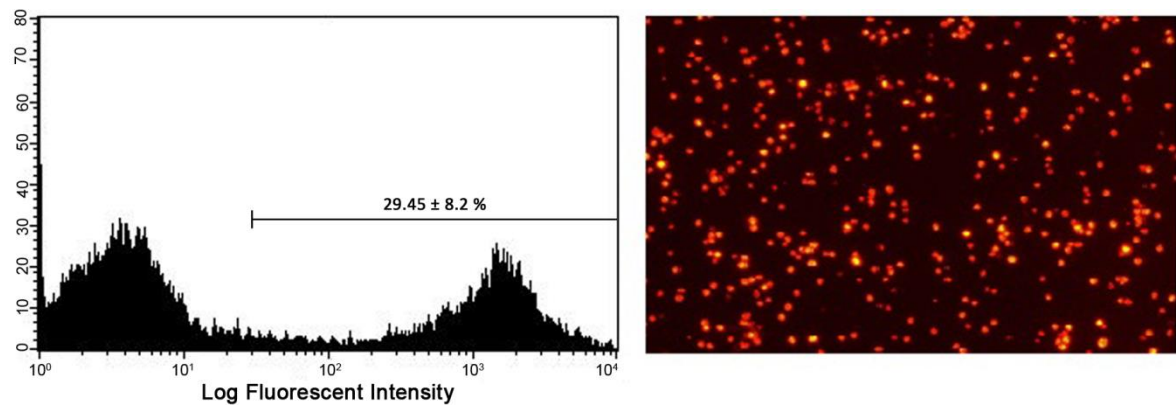
Figure 9-8 **EPCs within the peritoneum.** *Left: Representative fluorescent activated cell sorter histogram showing the levels of DilC$_{18}$-stained EPCs in the peritoneum, Right: Representative fluorescence microscopical image of DilC$_{18}$-stained EPCs within the peritoneum (Magnification, x100).*

9.3.2 EPCs in the peripheral blood

Those cells, which were mobilized out of the peritoneum into the peripheral blood, were assessed by regularly measuring the levels of circulating EPCs.

Figure 9-9 shows a typical blood scatter plot.
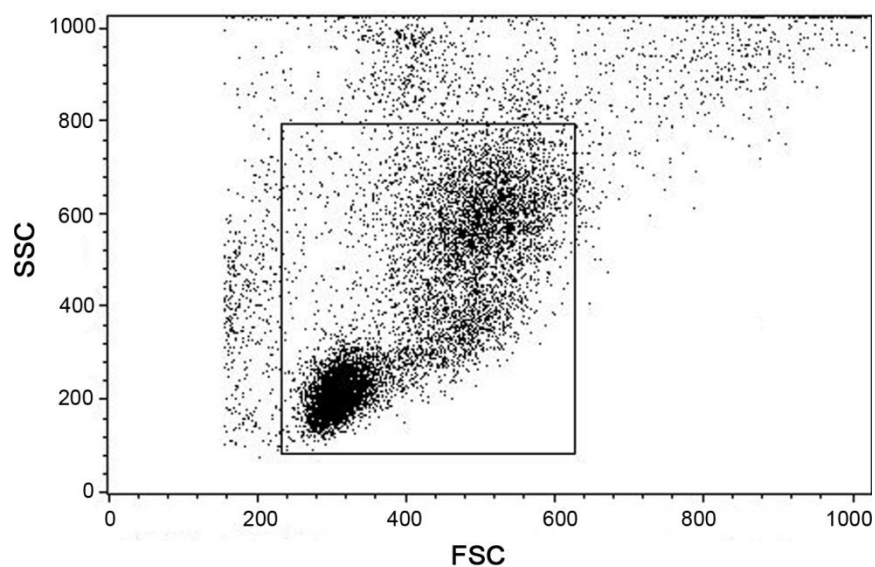
Figure 9-9 **Representative lysed peripheral blood scatter.**

By setting an appropriate gate the cells of interest were selected and quantified with a for-ward scatter plot. (Fig. 9-10)
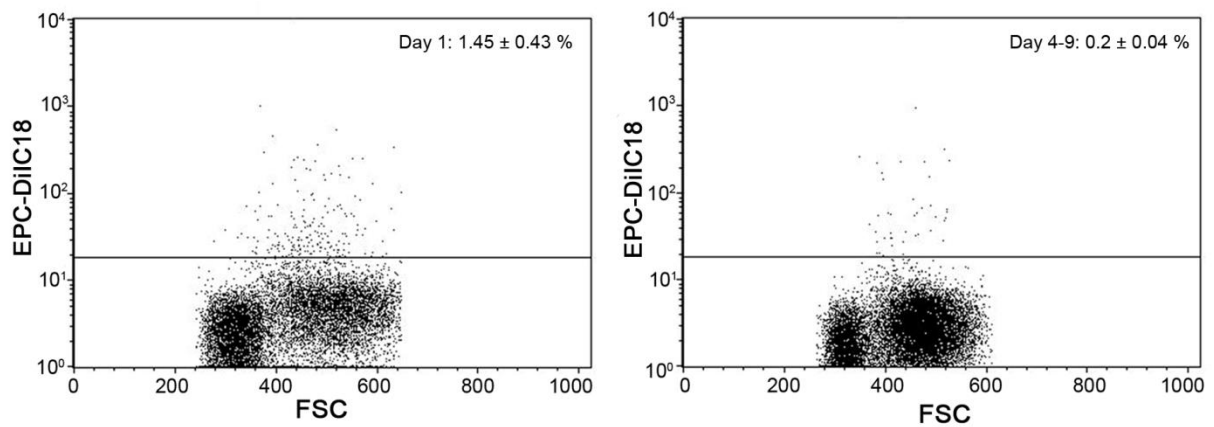
*Figure 9-10 **Representative forward scatter plots.** DilC$_{18}$-stained EPCs constituted 1.45 ± 0.43 % of nucleated peripheral blood cells on Day 1 (left), which declined and stabilized to levels of 0.2 ± 0.04 % between day 4 and 9 (right).*

Monitoring EPC levels in the peripheral blood demonstrated a strong initial mobilization from the peritoneum (1.45 ± 0.42 %) which stabilized at 0.2 ± 0.04 % (Figure 9-10) finally declining to 0.03 ± 0.02 %. The entire course of EPC blood levels resembled an exponential drop over a period of 15 days, as shown in figure 9-11.

These EPC blood values seem to create favorable conditions to permit subsequent infiltra-tion into tumors (0.08 ± 0.14 %).
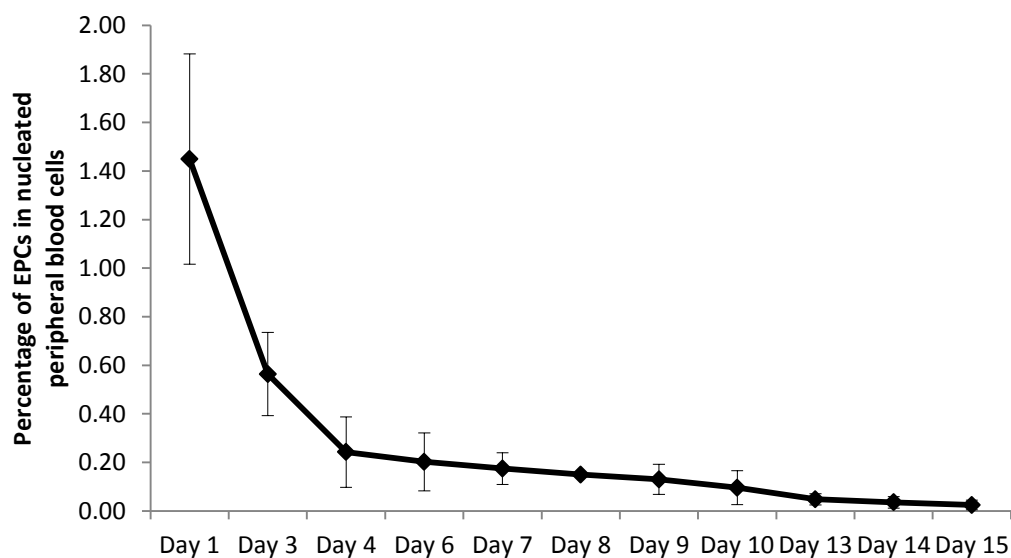
*Figure 9-11 **Blood levels following injection of 20 x 10^6 EPC in early-stage H460 tumor-bearing mice.** EPC mobilization from the peritoneum is characterized by a strong initial mobilization which decreased nearly exponen-tially over a period of 15 days.*

9.3.3 EPCs in the spleen

FACS analysis of spleen lysates showed that this tissue contained 0.65 ± 0.74 % EPCs after an experimental duration of 15 days.

9.3.4 EPCs within the tumor

Next to the relative amount of tumor-infiltrated EPCs, their localization within the tumors was of interest too.

Therefore histological tumor sections were stained with DAPI and analyzed with fluorescence microscopy followed by HE staining to gain insight into the surrounding microanatomy.

As shown in figure 9-12, EPCs preferentially localized near the tumor periphery, which presents the actively growing region.
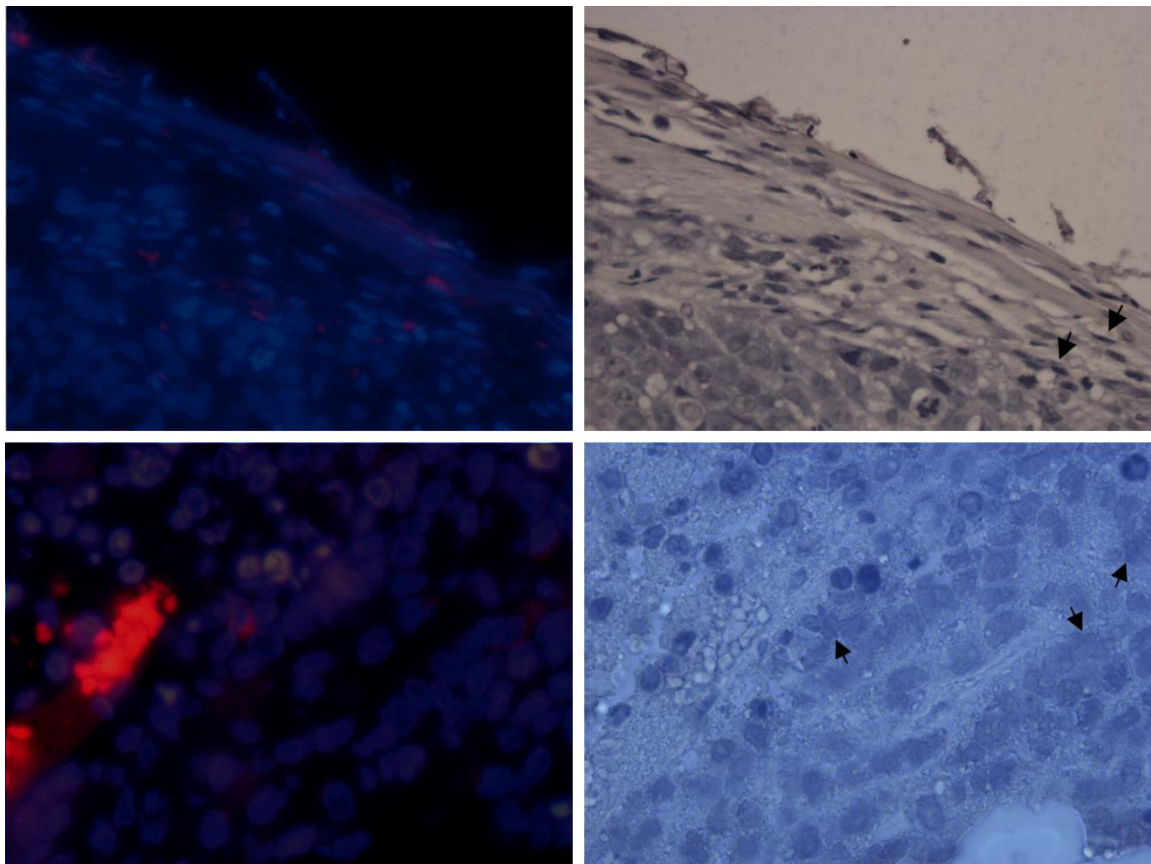
Figure 9-12 **EPC localization within H460 tumors.** *EPCs localized preferentially near the tumor boundary, shown by colocalization of DAPI-stained blue nuclei with DilC$_{18}$-stained red cytoplasm in fluorescence microscopical images (left side) and by arrows in the corresponding HE-stained images (right side).*

10 Discussion

Neovascularization is an indispensable requirement for malignant tumor growth. Since Lyden et al. demonstrated in 2001 the involvement of EPCs in the vascularization process, these cells became a main focus of research [57] which was heightened further when EPC homing to tumors was discovered.

This feature makes them together with their ease of isolation, self-renewal *in vitro* and amenability to infection with viruses [54] highly attractive for the use as gene delivery vehicles and offers new possibilities for cell-based cancer therapies.

As EPCs not only home to primary tumors but also to distinct even undetectable metastases, they might be especially of benefit for late-stage cancer patients, where curative therapy is often not possible [55].

However their precise function and the importance of these functions in the whole process of neovascularization remains controversial.

A hindering factor for systematic analysis of EPC is the missing unequivocal phenotypical characterization, which is associated with the lack of definitive methods to identify EPCs reliably within tumors.

Furthermore tumor neovascularization has a high complexity, involving not only a variety of cellular players but also several mechanisms, whereby newly discovered processes like vascular mimicry and mosaic vessel formation [7] are gaining growing importance. Therefore it proves challenging to identify the interplay of all these interrelated processes and even more to define the role of single cells like EPCs, which may additionally differ among different tumors.

In general, tumors are able to recruit EPCs from the bone marrow by a multi-stage process which involves mobilization into the peripheral blood and homing to sites of tumors where they have to transmigrate the blood vessel wall to invade into tumor tissues [20].

Once within the tumor, it is hypothesized that EPCs support progression by stimulating tumor neovascularization by structural and a paracrine mechanisms [34].

To provide a structural function, EPCs form cellular clusters in the proximity of vascu-

lar sprouts and incorporate into blood vessels. However the actual incorporation rate is a devisive topic, since the observed preclinical levels vary extensively from 50 % to undetectable amounts [50]. In clinical trials including several human cancer types, mean contributions of 5 % to tumor endothelium have been found [38].

At present, it is considered that EPCs provide only modest structural function in the formation of tumor-associated blood vessels and the pivotal effect on neovascularization stems from their instructive, paracrine function. By releasing a variety of proangiogenic factors, EPCs seem to guide nascent vessels and enhance the angiogenic response.

Meanwhile it has been suggested that the contribution of EPCs to tumor neovascularization represents a highly tumor type- and stage-dependent process [50], theories which are also confirmed by the present study.

Six different cancer cell lines (CRL-2505, DU 145, H460, H520, Calu-3, MDA-MB-231) were screened for the capability to recruit *ex-vivo* expanded, peripheral blood-derived EPCs *in vitro,* by using transwell migration assays and *in vivo*, by using subcutaneous cancer xenograft models with intraperitoneal EPC injection after tumor development.

Both *in vitro* and *in vivo* experiments demonstrated that from these six cell lines, only H460 human large cell lung cancer cells were able to recruit EPCs.

The most obvious reason to explain this discrepancy might lay in the individual characteristics of different tumors.

Tumors and even tumor stages within a single tumor differ in several features including the extent of ischemia, vascular injury, stromal composition and the local chemo- and cytokine milieu [25].

All these parameters result in different patterns of growth and neovascularization, ranging from avascular to highly vascular [4] and may therefore also influence the degree of EPC recruitment into tumors.

H460, which are characterized by a short doubling period of 24h *in vitro*, represented the tumor with the fastest and largest growth analysed.

These growth properties might eventually trigger the recruitment of EPCs to support the establishment of a tumor-associated vascular network which might enable further

growth and metastasis.

In addition to dependency on the tumor type, a growing body of evidence suggests tumor stage is of importance in EPC recruitment. This study also compared the homing efficiency of EPCs between early and developed H460 tumors.

EPC recruitment in H460 tumors was identified as a stage-dependent event, which preferentially takes place in early-stage tumors as EPC infiltration in these tumors was 10-fold higher compared to developed tumors.

These results are in accordance with the kinetic analysis of Nolan et al., who demonstrated that EPCs are predominantly recruited to early lewis lung carcinomas, even prior to vessel formation [50].

Furthermore, EPCs have been shown to contribute to the angiogenic switch in lung metastasis [39], which may occur in early stages of tumor growth.

All these findings indicate a fundamental role of EPCs in triggering the initial phase of neovascularization.

In contrast, Spring et al. demonstrated, that only advanced tumors recruit and incorporate EPCs into neovessels, possibly to further escalate the neovasculogenic process [25].

This shows that the stage-specific contribution of EPCs depends on the tumor type, underscoring the complex and multifactor-dependent nature of tumor-associated blood vessel formation.

These insights might also offer explanations for the lack of recruitment to the remaining screened cancer cell lines. It is possible that homing may occur at other application timepoints.

In addition to the tumor stage, the amount of applied EPC also influenced the degree of EPC homing. By examining different amounts of EPCs, a number of 20×10^6 emerged as the most efficient dose, resulting in the highest levels of circulating EPCs and pronounced homing to H460 tumors.

In light of the results outlined here, H460 large cell lung cancer cells recruit EPCs most efficiently into the peripheral blood and to sites of tumors, when applying 20×10^6 EPCs i.p. in early stages of tumor development, representing thereby the optimal

experimental setup for these cells.

Tumor-infiltrated EPCs preferentially localized near the tumor periphery, which is the actively growing region, normally characterized by the highest vascular densities [58]. However the vascularity within the analyzed H460 tumors was very immature, observations which are in line with the described early recruitment of EPCs and support thereby the assumpation that EPCs are especially necessary in the initial phase of neovascularization to boost the formation of tumor-associated blood vessels.

The contribution of 0.08 ± 0.14 % to tumors might seem thoroughly low compared to the achieved levels when injecting EPCs systematically [50] [57].

However, whereas a systemic injection in mice is mostly performed as a single shot and may result in abruptly unphysiological high amounts of circulating EPCs, intraperitoneal injected EPCs have first to be mobilized into the peripheral blood, resulting in lower and nearly physiological levels of circulting EPCs which are maintained over a prolonged period. Therefore this application method (to my knowledge the first time described) might predict the homing efficiency when administering physiological levels of therapeutic EPCs via an infusion therapy in human cancer patients.

This investigation demonstrates that the amount of tumor-infiltrated EPCs would be sufficient to achieve therapeutic effects when using genetically modified EPCs delivering oncolytic viruses or suicide genes.

Especially when exploiting EPCs as packaging cells for viral vectors a low infiltration rate compared to tumor masses would be favorable, since the numerous releases of viral particles from every cell which homes into the tumor exaggerates the therapeutic effect.

11 Summary

This study showed that *ex vivo* expanded peripheral blood-derived EPCs home to H460 large cell lung cancer cells *in vitro* and *in vivo* as well. The *in vivo* migration out of the perito-neum into tumor tissues represents a highly dose- and tumor-stage dependent process, which achieved largest efficiency when using a setup including the injection of 20 x 10^6 EPC before tumor development. The reached infiltrated levels are actually low, but might emerge as optimal, when using genetically manipulated, therapeutic EPCs delivering an oncolytic virus.

12 Abbrevations

BM	Bone marrow
Calu-3	Human bronchus adenocarcinoma
CD31	Platelet endothelial cell adhesion molecule-1
CD146	Melanoma cell adhesion molecule
CRL-2505	Human prostate carcinoma
cEPC	Circulating endothelial progenitor cell
DAPI	4'-6-Diamidino-2-phenylindole
DU 145	Human prostate carcinoma
EC	Endothelial cell
ECM	Extracellular matrix
eNOS	Endothelial nitric oxid synthase
EPC	Endothelial progenitor cell
FACS	Fluorescence activated cell sorter
G-CSF	Granulocyte-Colony Stimulating Factor
HGF	Hepatocyte growth factor
H460	Human large cell lung carcinoma
H520	Human squamous cell bronchus carcinoma
IGF-1	Insulin-like growth factor 1
i.p.	Intraperitoneal
MDA-MB-231	Human breast carcinoma
MCP-1	Monocyte chemotactic protein-1
MMP	Metalloproteinase
Nu/nu	Nude
PB	Pheripheral blood

PGF   Placental growth factor

PSGL-1   P-selectin glycoprotein ligand-1

SDF-1   Stromal cell-derived factor 1

VEGF   Vascular endothelial growth factor

VEGFR-2   Vascular endothelial growth factor receptor 2

vWF   Van Willebrand factor

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14 Curriculum vitae

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