CD34+/AC133+ Endothelial Progenitor Cells as Imaging Probes for Neovascularization of Tumors

20

Ali S. Arbab

Contents

Abstract

It was generally considered that blood vessel formation during postnatal life is restricted to angiogenesis only, and for decades tumor vascularization was thought to be the exclusive result of the sprouting of new vessels from the preexisting ones. However, recent studies demonstrated the existence of additional angiogenic and vasculogenic mechanisms associated with tumor growth. Tumor growth and metastasis strongly depends on neovascularization. Endothelial cells that contribute to tumor neovasculatures can originate from sprouting and co-option of neighboring preexisting vessels. However, there is emerging evidence indicating that bone-marrow derived endothelial progenitor cells (EPCs) also contribute to the vasculogenesis and growth of certain tumors. A subpopulation of CD34+ human hematopoietic stem cells (HSCs) identified by the cell-surface molecule CD133 (AC133), have been shown to be more specific for endothelial differentiation and angiogenesis. We have published the reports showing involvement of peripheral blood, cord blood and bone marrow derived EPCs into tumor neovascularization, and the EPCs' involvement in tumor neovascularization was determined by *in vivo* magnetic resonance and nuclear medicine imaging. In this communication different imaging modalities for *in vivo* tracking of EPCs to the sites of active angiogenesis/vasculogenesis are discussed, where EPCs are used as imaging probes.

A.S. Arbab (✉)
Department of Radiology, Henry Ford Health System, 1 Ford Place, 2F, mailbox 82, Detroit, MI 48202, USA
e-mail: saali@rad.hfh.edu

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Introduction

Tumor growth and metastasis strongly depends on neovascularization. The formation of blood vessels occurs by two mechanisms: vasculogenesis and angiogenesis. Vasculogenesis is the process where blood vessels are formed *de novo* by *in situ* differentiation of the primitive progenitors – i.e. angioblasts – into mature endothelial cells, which was thought to only take place during embryonic development (Risau and Flamme 1995). In contrast, angiogenesis occurs both during the embryonic development and the postnatal life, and is defined as a process that gives rise to new blood vessels by proliferation and migration of preexisting, differentiated endothelial cells (Folkman and Shing 1992). Angiogenic factors, such as vascular endothelial growth factor (VEGF), that are released by tumor cells stimulate angiogenesis by promoting activation, proliferation, sprouting and migration of endothelial cells to the tumor tissue and therefore allow for a rapid formation of new blood vessels (Ellis et al. 2001). Endothelial cells that contribute to tumor neovasculatures can originate from sprouting and co-option of neighboring pre-existing vessels (Zhang et al. 2002). However, there is emerging evidence indicating that bone-marrow derived endothelial progenitor cells (EPCs) also contribute to the vasculogenesis and growth of certain tumors (Asahara et al. 1997). A subpopulation of CD34+ human hematopoietic stem cells (HSCs) identified by the cell-surface molecule CD133 (AC133), have been shown to be more specific for endothelial differentiation and angiogenesis (Gehling et al. 2000; Janic et al. 2010). Higher expression of genes regulating angiogenesis was also observed in AC133+ cells. Additionally, our published results also showed migration and incorporation of intravenously administered CD34+/AC133+ cells in the xenoplanted tumors' neovasculatures (Arbab et al. 2006). EPCs collected from bone marrow, peripheral or cord blood have been used in different animal tumor or ischemia models to determine if these cells have the capacity to become part of the neovasculatures in tissues. When infused into immune compromised mice, EPCs were incorporated into the vasculature of xenotransplanted tumors and were correlated to tumor volume and production of vascular endothelial growth factor (VEGF) (Mancuso et al. 2003). As reported by our group and other investigators, hypoxia induced stromal cell derived factor 1α (SDF-1α) has been detected as one of the potent chemo-attractants for the migration and incorporation of bone marrow derived EPCs due to the presence of CXCR4 receptors in these cells (Ceradini et al. 2004; Arbab et al. 2008). Moreover, we have also reported the role of inflammatory cytokine RANTES, which also act as chemo attractant for these EPCs (Janic et al. 2010).

Capacity of EPCs or CD34+/AC133+ stem cells to migrate and incorporate into the tumor neovasculatures is usually demonstrated by histopathology or immunohistochemistry. Recently it was demonstrated that magnetic resonance imaging (MRI) can also monitor the migration of systemically administered magnetically (iron labeled) labeled mammalian cells to different organs, tumors, and at sites of active angiogenesis (Arbab and Frank 2008). Nuclear medicine and optical imaging techniques are also being used to determine the migration and accumulation of administered cells to the sites of lesion or sites of interest. *Ex vivo* manipulation of EPCs will allow the *in vivo* detection of the sites of homing and active neovascularization in tumors.

Characteristics of CD34+/AC133+ Endothelial Progenitor Cells (EPCs)

Characterization and phenotypical expression of EPCs is still controversial and there is still no consensus on the EPC's definition. Some investigators pointed out that EPCs are CD45 marker negative and should express CD31, KDR (VEGFR2), VE-cadherin and vWF (Ingram et al. 2004). These investigators usually collect cells from peripheral or cord blood mononuclear cells as an adherent cell population and propagate them in differentiating media containing high amount of fetal bovine serum (FBS). There are reports showing peripheral blood CD45 positive

angiogenic cells that show all the hallmarks of EPCs as well as CD45 surface marker (Duda et al. 2007).

Previous studies demonstrated the existence of circulating endothelial cells (ECs) in peripheral blood in various vascular diseases (Hladovec et al. 1978). However, for a while it was unclear whether these cells or their precursors play a role in postnatal vascular growth. The breakthrough came from the work by Asahara et al. (1997) who demonstrated the presence of CD34+/vascular endothelial growth factor receptor-2 (VEGFR2)+EPCs in human peripheral blood. These cells gave rise to mature ECs in culture and were capable of incorporating into the sites of active neovascularization in animal models. This landmark work opened the possibility that in adults, endothelial stem or precursor cells may contribute to the formation of new blood vessels by vasculogenesis. Since then, researchers have been gaining significant insights into the postnatal neovascularization and the EPCs' origin, phenotype and function. However, the main factor hindering the EPCs research is the controversy on the identity of EPCs. Earlier studies defined EPCs as the cells co-expressing hematopoietic stem cell marker CD34 and endothelial marker VEGFR2. Since subsequent work showed that some mature endothelial cells also co-express CD34 and VEGFR2 and that CD34 was not an exclusive marker for hematopoietic cells, a novel CD133 glycoprotein was accepted as a more appropriate marker for immature progenitor cells (Gehling et al. 2000; Peichev et al. 2000). Glycosilated form of CD133 protein is expressed on hematopoietic stem cells but not on mature endothelial cells, and it is recognized by AC133 monoclonal antibody. Peichev et al. (2000) suggested that a subset of circulating CD34+ cells that are positive for both VEGFR-2 and AC133 represent a functional EPC population that plays a role in postnatal angiogenesis or vasculogenesis. EPCs also share many cell surface markers with ECs and with stem/progenitor cells of different tissues. However, currently it is customary to define EPCs as cells that are positive for AC133, CD34 and VEGFR2 markers, with the following distinction: AC133+/CD34+/VEGFR2+ cells represent an immature, highly proliferative EPC population localized mainly in the bone marrow, while AC133-/CD34+/VEGFR2+ cells are considered circulatory, more mature cells that are limited in their proliferative capacity (Khakoo and Finkel 2005). In addition, these more mature cells also express some of the endothelial specific antigens such as platelet endothelial cell adhesion molecule 1 (PECAM-1 or CD31), E-selectin (CD62E) and VE-cadherin (CD144), chemokine receptor CXCR-4 (CD184) and have the ability to migrate in response to the CXCR-4 ligand, SDF-1α and vascular endothelial growth factor (VEGF). It is now generally accepted that new vessels can also be formed *via* recruitment of circulating EPCs. Indeed, studies in recent years demonstrated that intravenously administered progenitors isolated from bone marrow, peripheral blood or cord blood can home to ischemic sites, including tumor sites, emphasizing the significance of the paracrine effect of tumor secreted factors. Despite of the significant amount of data available, controversy still remains on the identity and function of the putative EPC and its' functional significance and contribution to tumor vasculature and growth. Gradually, the consensus on the putative EPC phenotype is arising, nevertheless further identification and characterization of novel, more specific EPC markers is warranted.

To show the endothelial potential of cord blood CD34+/AC133+ cells, we have performed extensive *in vitro* and *in vivo* studies. Our recent publications showed the potential of long- and short-term cultured cord blood derived CD34+/AC133+ EPCs to make tube like structured both *in vitro* and *in vivo* matrigel angiogenesis studies (Janic et al. 2010). Some of our *in vivo* animals studies have already been published (Arbab et al. 2006, 2008), where we showed the incorporation of administered CD34+/AC133+ EPCs in the tumor neovasculatures.

The Role of EPCs in Tumor Neovascularization

It was generally considered that blood vessel formation during postnatal life is restricted to angiogenesis only, and for decades tumor vascularization

was thought to be the exclusive result of the sprouting of new vessels from the preexisting ones. However, recent studies demonstrated the existence of additional angiogenic and vasculogenic mechanisms associated with tumor growth, such as intussusceptive angiogenesis, vessel cooption, vasculogenic mimicry, lymphangiogenesis, and the recruitment of endothelial progenitor cells (El Hallani et al. 2010). In most cases, these mechanisms take place concomitantly and are the potential targets for novel anti-angiogenic/antitumor therapeutic strategies.

Mobilization of EPCs from the bone marrow into the circulation and their homing to the sites of neovascularization, and subsequent differentiation into mature ECs are tightly regulated processes. Investigators have tried to identify the paracrine signals associated with tissue ischemia and tumor growth that create favorable environment that attracts EPCs. The recruitment of EPCs from bone marrow (BM) is initiated by increased circulatory levels of factors such as VEGF, fibroblast growth factor (FGF), SDF-1α, granulocyte-monocytes colony stimulating factor (GM-CSF), osteopontin, etc. that are released by growing tumors. These factors activate BM microenvironment to switch from a dormant to a pro-angiogenic state and the process involves the activation of matrix metalloproteinase-9 (MMP-9) that releases bone marrow stromal cells' membrane bound c-Kit (CD117) ligand. Generated soluble form of c-Kit ligand stimulates c-Kit positive EPCs to move from BM niche to the BM vascular zone and translocate to the circulation (Heissig et al. 2002). Tissue hypoxia present in tumors and ischemic vascular diseases is considered to be central to this paracrine mechanism and this ischemic effect was shown to be mediated by marked increase in VEGF and SDF-1α circulating levels (Ceradini et al. 2004). VEGF and SDF-1α expressions are transcriptionally upregulated by tissue hypoxia induced expression and/or activation of hypoxia inducible factor-1α (HIF-1α) (Ceradini et al. 2004). In addition to the increase in their circulatory levels, VEGF and SDF-1α expressions are increased locally, within the hypoxic tissue itself that in turn stimulate recruitment of progenitor cells to the hypoxic site as well. Our recent work also indicated that the homing of EPCs into the neovessels of implanted tumor was related to HIF-1α induced SDF-1α expression (Arbab et al. 2008). Recent studies also demonstrated the correlation between VEGF and SDF-1α expression at the transcriptional and functional/effector level. VEGF was shown to up-regulate SDF-1α and CXCR-4 molecules. In addition, it was demonstrated the possible synergistic effect between two cytokines by showing that without a concurrent VEGF signal; SDF-1α was insufficient in recruiting EPCs to tumor sites (Kollet et al. 2001). In addition to VEGF and SDF-1α, numerous other factors produced by tumor and surrounding cells have been implicated to play a role in EPC recruitment to tumor sites. Tumor produced CCL2 and CCL5 were reported to mobilize EPCs from the circulation. Neurotrophins induced angiogenesis was implicated in breast tumor growth and in ischemic animal models. Adiponectin, a peptide hormone secreted by adipocytes, was also shown to promote EPC numbers, migration, and mammary tumor growth in animal model. In addition, factors that regulate physiological angiogenesis can also play a role in EPC recruitment and mobilization (Janic and Arbab 2010).

The relationship between the temporal and spatial distribution of EPCs recruited from the bone marrow and tumor growth is not completely delineated. Moreover, migration and incorporation of EPCs in relation to the expression of different angiogenic or chemo-attractant factors (such as VEGF, MMPs, PDGF, SDF-1α, HIF-1α, bFGF, $\alpha_v\beta_3$ integrin, etc.) at different stages of tumor development have not been elucidated. Noninvasive imaging methods may provide for the localization of EPCs and HSCs within the tumor, thus furthering the understanding of extracellular and stromal components required for incorporation of these cells into the neovasculatures.

It is hypothesized that once recruited to tumor sites, EPCs can have a dual role in tumor angiogenesis, i.e. they can provide structural function by incorporating into the vessels, and a supporting paracrine role where they secrete angiogenic

factors. Our initial results with cord blood derived EPCs also showed both synergistic as well as paracrine effects in the formation of tube-like structures in matrigel plate by microvascular endothelial cells (MEC) (Janic et al. 2010). Many attempts have been made in answering what is the actual contribution of EPCs on tumor vessel growth and more importantly, do EPCs incorporate into the newly form vasculature. Lyden et al. (2001) first demonstrated the contribution of EPCs to tumor neovascularization, where they showed that transplantation of circulatory EPCs restored tumor angiogenesis and growth in angiogenesis defective, Id1+/-Id3-/- host mouse and donor-derived cells were detected throughout the tumor neovessels, with 90% percent of contribution. Since then, many similar studies were done, however, the reported percentages of EPCs contributing to the tumor neovascularization varied significantly, depending on the tumor model used. On the other hand, Purhonen et al. (2008) reported that in animal model, BM derived or other EC precursors did not contribute to tumor vascular endothelium at all, and that cancer growth does not require BM derived endothelial progenitors. Since demonstrating the significant luminal incorporation of EPC within the tumor neovasculatures proved very challenging, the biological role of EPCs in tumor angiogenesis was very often questioned. On the other hand, more recent reports showed that specific ablation of BM-derived EPCs with anti-VE-cadherin antibody markedly impaired tumor growth associated with reduced vascularization (Nolan et al. 2007). This also support the idea that even with the low vessel incorporation rate, the pararcrine EPC function may be the one that is critical for tumor angiogenesis. Nolan et al. (2007) also indicated that the percentage of incorporated BM progenitors and the density of BM progenitors-derived vessels might depend on the stage and size of the tumor. Therefore, the differences in EPC incorporation in previously published reports may not only be due the diversity of tumor models/types studied, but also due to the temporal differences in tumor development at the time of study.

CD34+/AC133+ Endothelial Progenitor Cells as Imaging Probes

With more understanding of the neovascularization processes and the involvement of bone marrow derived or circulating endothelial cells in tumor neovascularization, it is now the demand of the scientific community to *in vivo* monitor the neovascularization process and to understand the involvement of EPCs. However, there has been no *in vivo* technique invented to determine the involvement of endogenous or exogenegous EPCs in tumor neovascularization unless EPCs are from transgenic source carrying reporter such as green fluorescent protein/red fluorescent protein (GFP/RFP) or luciferase or EPCs are exogenously manipulated to carry reporter gene/s or to carry contrast agents for specific imaging modalities.

Converting EPCs as Optical and Fluorescent Imaging Probes

There is growing interest in the field of optical imaging for establishing the efficacy of engineered stem cells for therapeutic applications. Organic fluorophores such as rhodamine, fluorescein, DAPI, PKH26 and alexa488, are among the most commercially available, inexpensive, widely and easily used for a variety of straightforward shorter term labeling applications in cell and developmental biology. However, organic dyes hold little promise for the current calling of long term labeling for cell tracking strategies. One of the major ways to *ex vivo* label cells is the use of reporter gene systems. The gene of interest is chosen based on the imaging modality to be used, physiological events that are to be monitored or therapeutic goals to be achieved. Even genetically encoded fluorescent proteins like GFP are subject to the limitations of generally broad emission spectra capable of generating false positive results and an overlap of emission spectra with tissue autofluorescence as well as absorption and limited resolution to a few millimeters.

Enhanced red-shifted versions of fluorescent proteins such as DsRed proved capable of giving several orders of magnitude higher signal intensity *in vivo*, as compared to bioluminescence, however the large background autofluorescence severely reduces signal-to-noise ratio. Potential improvements in brightness and photostability of *in vivo* fluorescence imaging are underway with subsequent generations of monomeric red fluorescent protein (mRFP1), of the 'mFruits' such as tdTomato and TagRFP-T. In addition methods for optimizing expression of individual components of multimodality fusion vectors are in progress, such as a thermostable variant of firefly luciferase joined with mRFP and herpes simplex virus 1 thymidine kinase gene (tk) that demonstrated superior expression from all three reporter proteins. Similarly cells can be transduced to carry bioluminescent probe such as luciferase (Arbab et al. 2009).

Involvement of exogenously administered bone marrow or peripheral blood derived or endogenous bone marrow derived EPCs in tumor neovascularization has been determined mostly by invasive or *ex vivo* methods such as immunohistochemistry from biopsy materials or by fluorescent microscope following the administration of genetically altered or organofluorophore tagged EPCs. Alternatively investigators have used transgenic animal model (usually carrying reporter protein, such as GFP or RFP) to determine the involvement of endogenous cells in tumor neovascularization (Hillen et al. 2008). Two types of models have been used; (1) animals carrying reporter protein positive cells (such as GFP+), which is universally present in all cells of the animals, (2) animals carrying promoter driven GFP+cells that can only be present in endothelial cells. The later model has been used to determine tumor angiogenesis (Hillen et al. 2008). Animals with universally GFP+cells can be used to monitor the migration and involvement of GFP+cells in implanted tumors but cannot differentiate involvement of surrounding (sprouting and co-opting) cells from bone marrow cells. Making of animal model that will allow *in vivo* tracking the involvement of endogenous bone marrow cells to tumor development and neovascularization is challenging. The following criteria should be present to make an ideal model; (1) the animal should have reporter (such as GFP or RFP) only in bone marrow cells if the target is to determine the effect of bone marrow cells, (2) all other tissues of the body except bone marrow cells should not have any reporter positive cells, (3) if vasculogenesis/angiogenesis is the target, then reporter gene should be expressed under the guidance of specific promoter, (such as Tie2 or ICAM), (4) tumors or lesion should be produced with cells that should not have similar reporter gene or protein. However, to be able to track the migration of reporter positive endogenous bone marrow cells by *in vivo* imaging, the number of promoter driven reporter positive cells should be sufficient enough or all migrated bone marrow cells should be positive for the reporter. Optical imaging (such as fluorescent or bioluminescent) and nuclear medicine imaging can be utilized to track the reporter gene positive endogenous cells to the sites of tumor or other lesions (Arbab et al. 2009). Recently a chimeric animal model has been developed in our laboratory to determine the involvement of BMPC in the tumor neovascularization (Fig. 20.1). Sub-lethally irradiated athymic mice received bone marrow cells from green fluorescent protein positive (GFP+) transgenic mice. GFP+bone marrow cells were transplanted in athymic mice 24 h following sub-lethal irradiation and the tumors were implanted after 28 days when flowcytometric analysis showed more than 70% engraftment of GFP+cells. Migration and accumulation of transplanted bone marrow cells in the implanted breast cancer and glioma were determined by optical imaging (Kodak, Carestream multi-spectral system, Carestream, USA) with proper excitation and emission profiles. Optical imaging showed gradual increase in GFP intensity in the tumors. Immunohistochemical analyses under fluorescent microscope showed multiple GFP+cells lining the blood vessels and other infrastructures of the tumors. Staining for different angiogenic factors indicated that the GFP+cell accumulated to the sites of higher expression of PDGF, SDF-1 and VEGF in the tumors. Analysis also showed that GFP+cells not only incorporated into the

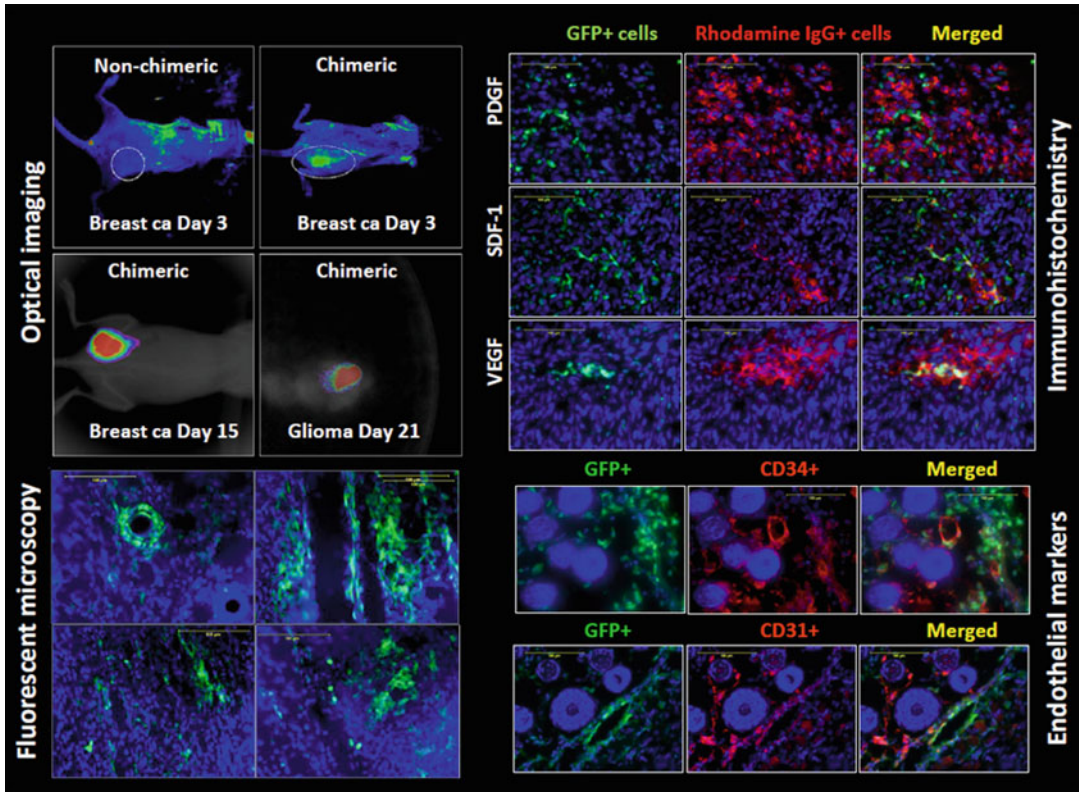

Fig. 20.1 Tracking of endogenous bone marrow progenitor cells (BMPC) to tumors. Chimeric mouse was developed by transplanting GFP+bone marrow cells from donor mouse in an athymic sub-lethally irradiated mouse. Tumors (breast cancer or glioma) were implanted when 70–80% engraftment efficiency achieved. Optical imaging shows (*left upper panel*) accumulation of GFP+cells (fluorescent signals) in the tumors. Accumulation of BMPC was observed as early as 3 days following implantation of tumors. Non-chimeric athymic mouse does not show any fluorescent intensity in the tumor. Fluorescent microscopy (*left lower panel*) shows accumulation of GFP+cells all over the tumor tissues including blood vessels. Immunohistochemistry shows higher expression of PDGF, SDF-1α and VEGF (*right upper panel*) at the sites of accumulation of GFP+cells in tumors. Some of the GFP+cells that are incorporated into tumor blood vessels also show endothelial cell markers, CD31 and CD34 (*right lower panel*)

neovasculatures but majority of the accumulated GFP+cells also remained at the peri-vascular regions. The GFP+cells that incorporated into neo-vessels also showed endothelial cell markers (CD34 and CD31). However, there were also GFP+cells in the tumors which were not positive for endothelial cell markers. These findings are in line of our previously reported results where we showed that cord blood derived EPCs not only incorporated into *in vitro* angiogenesis (matrigel studies) but also helped human dermal microvascular endothelial cells (HDMEC) to form tube like structures (Janic et al. 2010) indicating their paracrine effects. One of the disadvantages of making chimeric animal using universally GFP+bone marrow cells is that there is no way to determine the precise involvement of bone marrow derived EPCs in tumor neovascularization. To know the precise involvement of EPCs in the formation of tumor neovessels we need to make chimeric animal where reporter gene will be expressed only by conditional promoter such as Tie2 in endothelial cells.

Making EPCs as Nuclear Medicine Imaging Probes

Different radioisotopes and radiopharmaceuticals have been used to label cells *in vitro* and track the

administered cells *in vivo* by gamma camera and or single photon emission computerized tomography (SPECT) imaging. Indium-111 (In-111) oxine is one of the Foods and Drug Administration (FDA) approved agents that is readily available and used for cell labeling (Read et al. 1990). The early biodistribution and homing of free/dissociated In-111 in the bone marrow may not reflect the location of labeled cells due to presence of unbound free In-111. Therefore, to determine the homing of In-111 oxine labeled cells it is necessary to wait until the clearance of unlabeled radioactivity from the circulation. Post-administration scanning is usually performed 24–72 h after injection of labeled cells to determine the specific distribution at the sites of interests. Due to short half-life, In-111 labeled stem cells cannot be tracked for more than 7 days (Read et al. 1990). However, it is also important to balance between the added radioactivity and number of cells during labeling. Added radioactivity more 4–5 Bq per cells showed impairment of functions (Yoon et al. 2010). Mesenchymal stem cells (MSC) have been labeled with In-111-oxine to track the migration of the cells in myocardial infarction. However, high resolution computed tomography (CT) images were used to co-localize the migrated cells in the heart. SPECT images with limited spatial resolution are commonly superimposed on high-resolution imaging such as MRI or CT for tracking cell-based therapies and/or cellular probes. We have used In-111-oxine labeled cord blood EPCs to monitor the migration and accumulation to the sites of glioma and confirmed that these cells incorporated in tumor neovessels (Fig. 20.2a). Cord blood derived EPCs were labeled with In-111-oxine on the day of IV administration in human glioma U251 bearing rats. SPECT images were obtained on days 0, 1 and 3. The whole body biodistribution and accumulation of administered EPCs in glioma was also calculated. It was determined that about 2–2.25% administered EPCs accumulated in the tumors in 24 h. Concomitant injection of magnetically labeled EPCs confirmed the accumulation and incorporation into glioma neovessels on histochemistry. *Ex vivo* labeling of cells with radioactive agents is very advantageous when whole body biodistribution and number of accumulated cells to the sites of interests need to be calculated. Precise calculation of the distribution of administered cells is possible by determining the doses of administered cells (both the number and the radioactivity at the time of administration). The easiest way to calculate the number of accumulated cells is to normalize it with the whole body activity on day zero (considered as administered dose).

SPECT can also be used to monitor the migration and homing of genetically modified stem cells (Arbab et al. 2009). The most effective reporter gene that can be inserted into stem cells is sodium iodide symporter (NIS) using viral vectors. Injection of radioactive iodine (such as I-123 or I-131) or technetium-99m-pertechnetate (Tc-99m) will result in the uptake by stem cells carrying NIS and these cells can subsequently be tracked by gamma camera or SPECT scanners. Tracking of these genetically tagged cells can be performed repeatedly as long as the cells express the gene, however, the long term effects of radiation to these cells following repeated exposures needs to be determined. Herpes simplex virus thymidine kinase gene carrying cells can be tracked by using iodinated 2′-fluoro-2′-deoxy-1-ß-D-arabinofuranosyl-5-iodouracil (FIAU) or 1-(2-Deoxy-2-fluoro-ß-L-arabinofuranosyl)-5-methyluracil (FMAU). Most favorable isotope is I-123, which would be expensive for repeated scanning procedures. We have successfully transduced cord blood derived EPCs by both adeno and lenti viral vectors and showed the migration and incorporation of the IV administered transgenic EPCs carrying NIS to the sites of tumor neovessels both in breast cancer and glioma studies (Rad et al. 2009; Varma et al. 2012) (Fig. 20.2b). Both locally or systemically administered transgenic EPCs showed incorporation of EPCs into tumor neovessels and expression of transgene products, which was detected by Tc-99 m SPECT. To determine the number of administered cells that are migrated and incorporated into tumor neovessels, one has to confirm that 100% administered cells should be traduced and should express gene products, and the expression should be sufficient enough to accumulate administered radioactive agent to be detected by SPECT. Based on our In-111-labeled EPCs studies in glioma, we concluded that at least 100,000 NIS positive EPCs need to be accumulated in the tumors to be detected by Tc-99 m-SPECT (Varma et al. 2012).

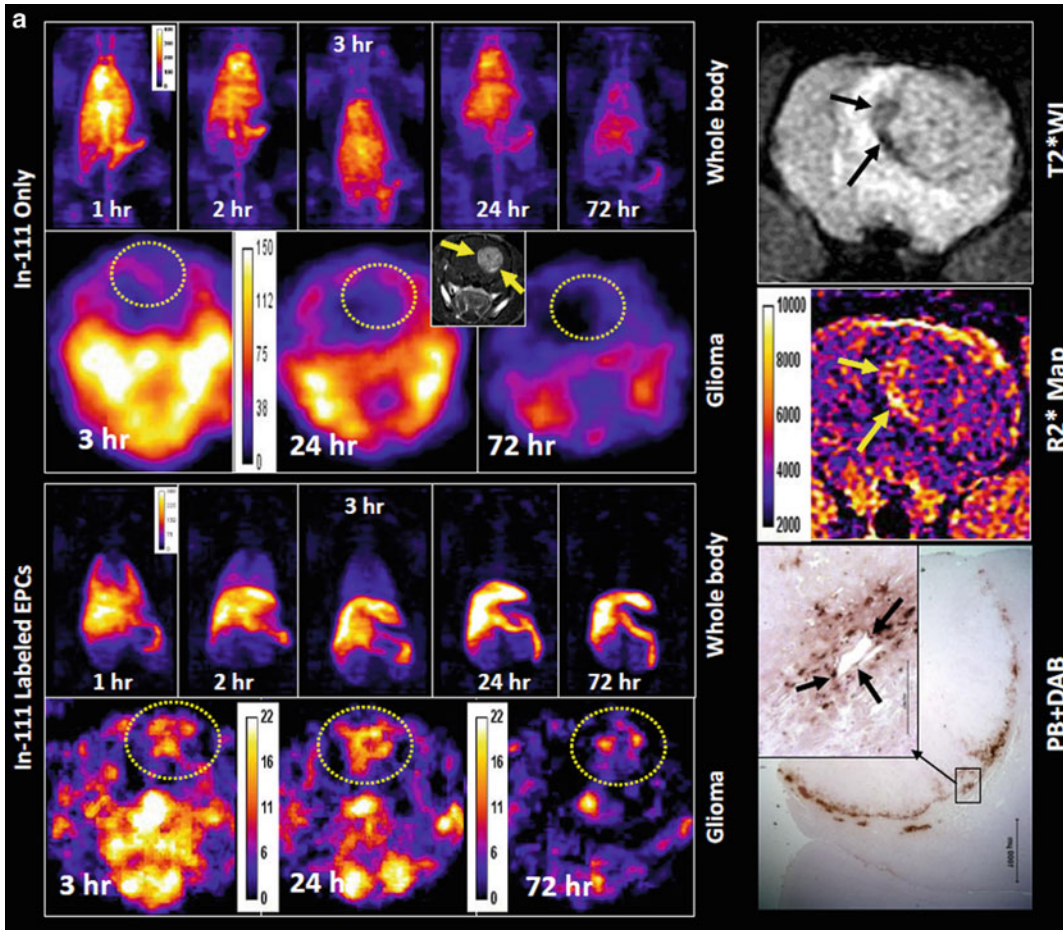

Fig. 20.2 Tracking of EPCs by SPECT and MRI. (**a**) Tracking of labeled EPCs: EPCs were labeled either with In-111-oxine or iron oxides and the migration and accumulation of administered cells were determined by SPECT or MRI, respectively. SPECT images show the biodistribution of administered cells in different organs as well as in the tumor (*left lower panel*, *yellow circles*). The accumulation of In-111 labeled cells was highest after 24 h. Biodistribution of cell associated radioactivity is different from that of administered In-111 only (*left upper panel*). Note that injected In-111 does not accumulate in the tumor (*yellow circle left upper panel*). MR images detect the iron oxides labeled EPCs in the tumor (*right panel*) which is also confirmed by DAB enhanced Prussian blue staining. Both In-111 and iron oxides labeled EPCs were injected into the same animals. Note the incorporation of administered EPCs in tumor vessels. (**b**) Tracking of transgenic EPCs: SPECT and MRI have detected the transgenic and iron oxides labeled EPCs to the sites of glioma (*upper panel*) and breast cancer (*lower panel*). EPCs were genetically transformed to carry NIS and then magnetically labeled with ironoxides. Post EPC administration MR images show low signal intensity in the tumor (circle in glioma and arrows in breast cancer). Tc-99m-SPECT show increased activity of Tc-99m in the tumors indicating functional expression of transgene (NIS). Immunohistochemistry show the presence of iron positive cells in the tumors as well as endothelial cells which are also positive for NIS

There has been limited success in the *in vitro* labeling of stem cells with positron emitting radioisotopes because of acute radiation injury causing death of the labeled cells (Arbab et al. 2009). Moreover, the half lives of available positron emitters are short, making this technique impractical for long-term follow up beyond 2–3 days. Transfecting cells with the reporter genes such as thymidine kinase has allowed for the tracking of cells in experimental and clinical studies by PET when combined with intravenous injection of positron emitters (i.e., 18 F-FHPG) or SPECT agents FIAU. PET reporter gene approaches assume that the primary stem cells would be stably transfected

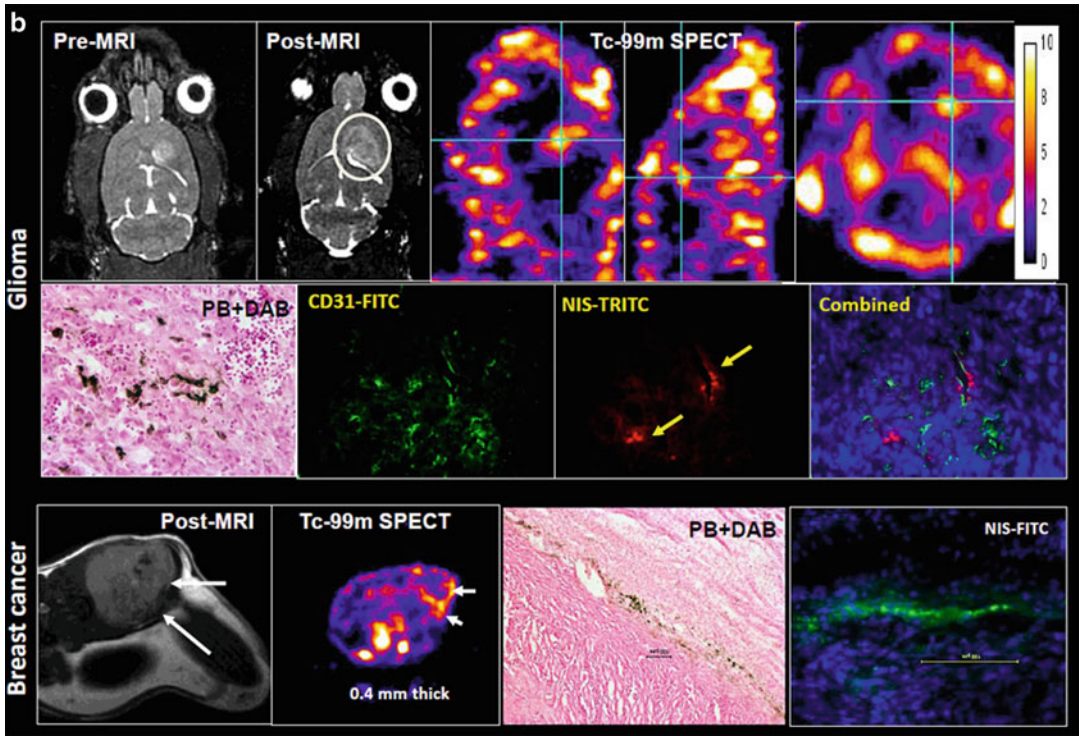

Fig. 20.2 (continued)

and these cells can be tracked following repeated injection of positron emitters. Although PET scans have higher sensitivity (i.e., contrast to noise because of lack of inherent background signals) they have an inherent low spatial resolution as compared to MRI and CT. Therefore similar to SPECT, PET scans usually require high resolution anatomical image to locate the activity *in vivo*. The potential loss of expression of reporter genes over time makes PET impractical for longitudinal imaging studies. Investigators have also used genetically engineered cells for multimodality scanning, however, image resolution and localization of the signal from cells is limited. Tamura et al. (2004) reported the recruitment of bone marrow EPCs to implanted tumor by PET scanning where EPCs were *ex vivo* labeled with [2-(18)F] 2-fluoro-2-deoxy-D-glucose (FDG).

Making EPCs as MR Imaging Probes

Superparamagnetic iron oxides nanoparticles (SPIO) are a class of MRI contrast agents that are being used to magnetically label to track these cell using T2W or T2*W images (Arbab and Frank 2008). Although several approaches for labeling cells with SPIO nanoparticles have been explored, most of the agents used were proprietary compounds, involved unique or complex synthesis, or biochemical modification of the dextran coat of the SPIO nanoparticles to stimulate endocytosis by cells. In 2002, a straightforward approach was developed combining (U)SPIO nanoparticles (e.g., ferumoxtran and ferumoxides) with commonly available polycationic transfection agents to effectively label cells (Frank et al. 2003). Arbab et al. (2004) showed that by mixing two FDA approved agents, ferumoxides (Feridex IV, Berlex, NJ) and protamine sulfate together to form a complex efficiently and effectively labeling stem cells. Ferumoxides is a dextran-coated colloidal SPIO nanoparticles that magnetically saturates at low fields and has an extremely high NMR T2 relativity. Changes in R2 (R2 = 1/T2) are linear with respect to iron concentration. Protamine sulfate is a drug that contains >60% arginine and is used for treatment of heparin anticoagulation

overdose. Both agents are being used off-labeled for magnetic labeling of cells. Cells are labeled with the ferumoxides-protamine sulfate (FePro) complex via macropinocytosis and can be imaged at clinically relevant MRI fields using standard imaging techniques. Unlabeled stem cells usually contain less that 0.1 picograms of iron per cell whereas labeled cells grown in suspension (i.e., HSC, T-cells) contain 1–5 picograms iron per cells and cells that adhere to culture dish (i.e., MSC, human cervical cancer cells, macrophages) can take up from 5 to >20 picograms iron per cell. Labeling embryonic or adult stem cells with ferumoxides-protamine sulfate does not alter the viability and functional capability of cells or the differential capacity of the stem cells (Arbab et al. 2004). Recent studies have shown that cells can safely handle the iron load following FePro labeling (Pawelczyk et al. 2006). In the cell, SPIO nanoparticles are in endosomes that with time fuse with lysosomes and start to dissolve over 3–5 days. Cellular metabolism of the increased iron load results in a transient decrease in transferrin receptor mRNA and protein levels and a corresponding increase in ferritin gene and protein expression that was maintained for at least 1 month in MSCs (Pawelczyk et al. 2006). Previously we have shown that retention of iron in the labeled cells depends on the rate of division and metabolic activity of cells (Arbab et al. 2003). In rapidly growing cells the intracellular iron completely disappeared by 5–8 divisions. On the other hand, the intracellular iron was observed after 6 weeks in cells, where cell division was almost inhibited. Janic et al. (2009) has further improved the labeling procedures using ferumoxides-protamine sulfate and used extensively to label EPCs and track them in neovascularization both in the tumor and stroke.

Anderson et al.(2005) first reported the tracking of IV administered mouse EPCs to the sites of implanted glioma (orthotopic) and tumor neovascularization by *in vivo* MRI and confirmed it with *ex vivo* very high resolution MRI followed by immunohistochemistry. Endothelial lining of new blood vessels showed positive markers for endothelial cells (CD31) and some of the CD31+ cells also positive for iron (Prussian blue staining). In this proof of principal study the authors have not investigated the relation of tumor size and timing of injection of EPCs for incorporation into tumor neovessels. In the next report Arbab et al. (2006) from the same group showed that the migration and incorporation of IV administered human peripheral blood derived AC133+ EPCs were related to the size of the tumor (subcutaneously implanted glioma). The investigators had administered magnetically labeled EPCs in groups of animals on the day of tumor implantation and on day 3 (when tumor was at 0.2 cm size) and the MRI were obtained when the tumor grew to 0.5, 1.0, and 1.5 cm in size. MRI was able to detect the migration and accumulation of iron positive EPCs to the sites of tumors as early as 3 days following IV administration in tumors that received EPCs on day 3 of implantation. However, MRI showed higher amount of low signal intensities on T2*-weighted images (T2*WI) in tumors when it grew to 1.0 cm in size in both groups of animals (IV injected on day 0 or day 3 of tumor implantation) indicating tumor size dependent accumulation and incorporation of EPCs into neovessels. The goal of this study was to detect the migration of labeled cells into growing tumors. Once the tumors were approximately 1 cm in size, there was no apparent difference on MRI or histopathology between group that received IV EPCs on the day of tumor implantation and group that received IV EPCs on day 3 of tumor implantation (i.e., the timing of labeled cell administration did not matter). At the early stages of tumor growth (0.5–1 cm), most of the labeled cells were found along the tumor margins and in between tumor and surrounding muscles or connective tissues. When tumors grew to 1.5 cm, the areas of low signal intensity detected on MRI as well as iron positive cells on histology were no longer at the periphery of the tumor. This might be a result of the lack of availability of magnetically labeled AC133 cells for migration and homing from the bone marrow, lung, liver, or spleen to the tumor due to apoptosis. Alternatively, the labeled cells may have undergone several cell divisions, thereby diluting the intracellular iron label to levels that could not be visualized

on MRI or histopathology. The importance of the size of the tumor for the accumulation and incorporation of administered EPCs might be related to activation and release of angiogenic growth factors or cytokines. It has been proven that release of VEGF, a strong angiogenic factor, is related to tumor hypoxia, which is related to tumor size and distance from existing vessels. This study also clearly showed the involvement of exogenously administered EPCs in tumor neovascularization. There is an argument that host macrophage will take up the dead iron-labeled cells after homing and incorporation into target tissues, and these macrophages along with dead cells will produce misleading low signal intensity on MRI or can show iron positive cells on Prussian blue staining. With this report and with subsequent publications Arbab et al. have shown that host macrophages (mouse) did not show any iron on Prussian blue staining, where either rat glioma or human tumors were implanted subcutaneously in mouse and magnetically labeled human EPCs were administered intravenously. Mouse macrophages were seen mostly at the site of necrosis in the tumor whereas iron positive human EPCs were seen along the peripheral part of the tumors (Arbab et al. 2006, 2008).

In a subsequent investigation Arbab et al. (2008) showed the involvement of magnetically labeled peripheral and cord blood derived EPCs in the tumor neovascularization following local administration within the tumors. Using rat glioma and melanoma models the migration of locally implanted, magnetically labeled peripheral and cord blood derived CD34+/AC133+ EPCs was monitored by MRI. After completely mixing two populations of cells (CD34+/AC133+ and tumor cells) and implanting them as a mixture, one would expect a homogenous distribution of human CD34+/AC133+ cells throughout the growing tumor mass. The objectives of this study were to determine (1) whether cellular MRI can detect the migration of magnetically labeled AC133+ cells in relation to tumor growth when co-implanted with tumor cells, and (2) whether the migration of AC133+ cells is related to the expression of angiogenic and other tumor or surrounding tissue micro environmental factors. It was hypothesized that if hypoxia is the sole factor for stimulating angiogenesis, the co-implanted CD34+/AC133+ EPCs will remain at the center of the tumor to support neovascularization. Alternatively, if angiogenic factors from within the growing tumor or at the tumor-tissue interface are expressed in sufficient amount, EPCs will migrate towards those areas. Visualizing implanted EPCs' differential migration by MRI at different stages of tumor growth or development can provide insight into some of molecular mechanisms involved in *in vivo* tumor angiogenesis. However, after implantation, CD34+/AC133+ cells re-distributed, and when tumor grew to 1–1.5 cm in size MRI and histological analysis revealed that most of the cells migrated towards the peripheral parts of the tumor that co-localized with the strong expression of HIF-1α and SDF-1α that indicated the more hypoxic microenvironment. The same areas also expressed high levels of PDGF and MMP-2 that usually reflects high angiogenic activity. Western blot analysis also indicated higher expression of SDF-1α, PDGF, and MMP-2 at the peripheral part of the tumors. It was also shown by immunofluorescent staining that human CD34+/AC133+ cells that were detected at the periphery incorporated into the tumor neovasculature (Arbab et al. 2008). In addition to shedding more lights into the biological mechanisms of EPC migration within the tumor environment, this work also introduced a novel non-invasive MRI method for *in vivo* stem cell tracking that may facilitate the development of novel diagnostic and treatment strategies (Fig. 20.3). This study also showed the tumor size dependent migration of locally implanted EPCs towards the periphery of the tumor and incorporation into tumor neovascularization. The phenomenon was unrelated to tumor types. Both glioma and melanoma showed similar migratory patterns of locally implanted EPCs and the migration was clearly observed by MRI. Moreover, iron labeled EPCs also worked as histological marker, which was easily detected by Prussian blue. Surprisingly, host macrophage did not show any iron positivity even with local implantation of magnetically labeled EPCs.

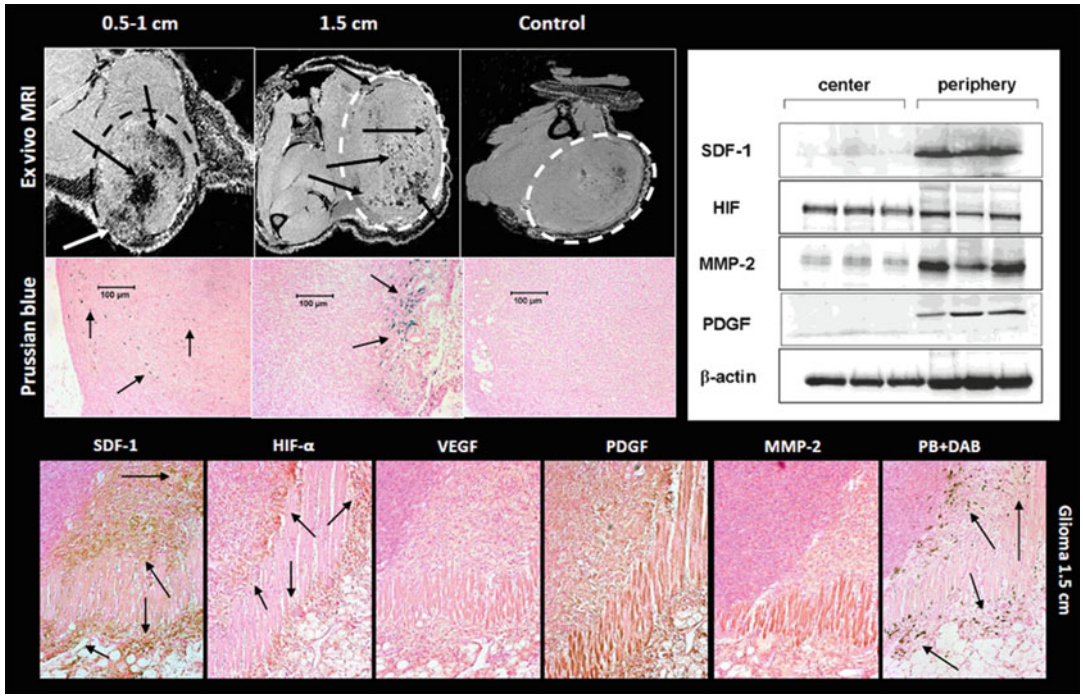

Fig. 20.3 Relation of EPCs' migration and expression of angiogenic factors. MRI images show the migration of locally implanted magnetically labeled EPCs towards the periphery of the tumors (*left upper panel*). The low signal intensity areas seen on MRI due to iron oxides labeled EPCs are different from the low signal intensity areas seen in control tumor (due to necrosis). Presence of iron positive cells is seen at the periphery of the tumors that received EPCs. Control tumor does not show any iron positive cell. Immunohistochemistry (*lower panel*) show the expression of different angiogenic factors at the peripheral part of the tumor, where abundant iron positive cells are observed. Western blot analysis of the tumor lysate show higher amount of SDF-1, MMP-2 and PDGF at the peripheral part of the tumor (*right upper panel*)

Using EPCs as Multimodal Imaging Probes

Very recently our group also reported the use of EPCs as gene carrier and MRI imaging probes to target tumor neovascularization (Varma et al. 2012). The gene that was used also acted as imaging probes for nuclear medicines techniques, such as SPECT. The purposes of this study were to determine whether (1) intravenously administered genetically transformed cord blood derived EPC can carry hNIS to the sites of tumors in rat orthotopic model of human glioma and express transgene products, and (2) whether accumulation of these administered EPC can be tracked by *in vivo* MRI and the expression of hNIS can be determined by *in vivo* Tc-99m SPECT. Ultimate goal was to determine whether in future, EPC could be used as gene carrier/delivery system for glioma therapy. This was the first study to report the use of cord blood derived EPCs to carry a gene (hNIS) to the sites of glioma, and the migration and the expression of gene products were determined by *in vivo* MRI and SPECT studies, respectively. These EPCs were used both as gene carrier and imaging probes. EPCs can be used to deliver therapeutic genes to the sites of lesions. The use of EPCs to carry therapeutic gene to the sites of neovascularization in different lesions are underway in our laboratory. Ultimate goal is to use EPCs as therapeutic agent either to enhance or reduce neovascularization.

In conclusion EPCs can effectively separate from peripheral blood, bone marrow and cord blood. With the established culture technique developed by our group, one can propagate the

separated EPCs to many folds, which then can be manipulated *ex vivo* to carry contrast agents or reporter gene. Following systemic administration these cells can be tracked by different imaging modalities to the sites of active angiogenesis/vasculogenesis in tumors. EPCs can be used as probes for real time detection of tumor neovascularization.

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