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Vascular resident endothelial progenitor cells and their use in neovascularization research

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This thesis was written without any further help from others and no other than all the stated auxiliaries has been used.

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I didn't participate in any other doctoral procedure in the University of Lübeck.

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Contents

List of abbreviations

BrdU	5-bromo-2-deoxyuridine
BSA	Bovine Serum Albumin
CD	Cluster of Differentiation
CT	Computer Tomography
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPCs	Endothelial Progenitor Cells
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
Flk	Fetal liver kinase
Flt	Fms-related tyrosine kinase
GFP	Green Fluorescence Protein
IgG	Immunoglobulin G
PBS	Phosphate Buffer Saline
VREPCs	Vascular Resident Endothelial Progenitor Cells
vWF	Von Willebrand factor
3D	Three dimensions

List of figures

1. Introduction

1.1 Chronic foot and leg ulcer

Chronic foot and leg ulcer is a common disease in plastic and hand surgery department, this kind of ulcer mainly affects the elderly. It is estimated that in European people aged 65 or higher, the prevalence of foot and leg ulcer is about 1.0 to 3.3 percent (Baker et al. 1992; O'Brien et al. 2000).The patients are always suffering from the pain and discomfort in their everyday life. When the chronic ulcer is associated with secondary infections, some patients even need amputations to save their lives. There are a lot of causes of chronic foot and leg ulcer, such as poor blood circulation, injuries, diabetes, tumors and infections. The most common cause of the chronic leg ulcer is poor blood circulation. As a result, in clinic level, chronic leg and foot ulcer is usually treated by treating the original vessel diseases to improve the blood circulation in the ulcer area. Until now, there are still quite few effective ways to treat chronic leg and foot ulcer. But cell therapy is now drawing more and more attention. Since the discovery of endothelial progenitor cells, endothelial progenitor cell therapy is used more and more in the treatment of circulation disease.

1.2 Endothelial progenitor cells

Endothelial progenitor cells (EPCs) are a group of immature cells that are considered to be the best target cell for blood circulation research so far. They can

form new vessels, which can help ischemic tissue to restore more blood supply, both in vitro and in vivo (Asahara et al. 1997; Ingram et al. 2004; Ingram et al. 2005; Zengin et al. 2006; Aicher et al. 2007).They have the potential of differentiating into endothelial cells under certain circumstances. However, since the finding of EPCs in 1997(Asahara et al. 1997), the concept and the exact location of EPCs is still under debate. Primarily, EPCs are defined by specific antigen expression patterns (e.g. CD133+, Flk1+ and Flt1+) and the ability to differentiate to endothelial cells (Leri and Kajstura 2005). And they are obtained usually from adult peripheral blood and bone marrow. Their homing to the ischemia region is considered to be the initial force for postnatal neovascularization.

1.3 Vascular resident endothelial progenitor cells

The two major sources of EPCs are peripheral blood and bone marrow. Few researches have focused on tissue resident endothelial progenitor cells. (Alessandri et al. 2001) proved for the first time that local endothelial progenitors are involved in the formation of human microvessels. They found a progenitor cell layer in the external layer of the embryonic aorta wall by aortic ring assay. Later, we also found a progenitor layer in adult human arteries. Thus, whether the homing progenitor cells from periphery blood and bone marrow or the local resident cells are the major source of the neovascularization is an intriguing question. In the present study, we identified a group of endothelial progenitor cells from a group of self immortalized vessel wall endothelial cells. This group of cells was introduced in

1996 as a spontaneously immortalized micro-vasculature derived endothelial cell line (Derhaag et al. 1996). The cells have high proliferation ability and can be passed for more than 100 population doublings. The self immortalization ability remained an enigma since the discovery of the cell. One reliable explanation is that it contains a group of complete outgrowth EPCs. To prove our hypothesis, we identified a subpopulation from the original cell group based on single cell clonogenic assay and further characterized them in vivo.

1.4 Angiogenesis and arteriogenesis

Angiogenesis is the formation of new capillary network by sprouting of the new vessels from the pre-existing vessels. It is a phenomenon that can be detected in many physiological or pathologic processes, such as myocardial infarction, peripheral artery disease and cancer(Carmeliet 2000). It was first used by a British surgeon Dr. John Hunter to describe blood vessels growing in the reindeer antler.

Arteriogenesis, on the other hand, refers to the growth of preexisting collateral arterioles after the occlusion of the main stem artery. With the contribution of shear force, monocytes and several cytokines, these vessels will become corkscrew like, the diameter of the vessels will be much larger, the vessel walls will become thicker(Ito 2003).

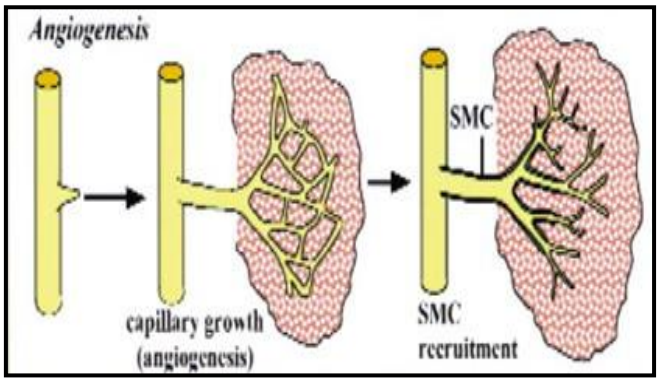

Figure 1: The mechanism of angiogenesis

The new vessels are growing from the original existing vessels.(Carmeliet 2000)

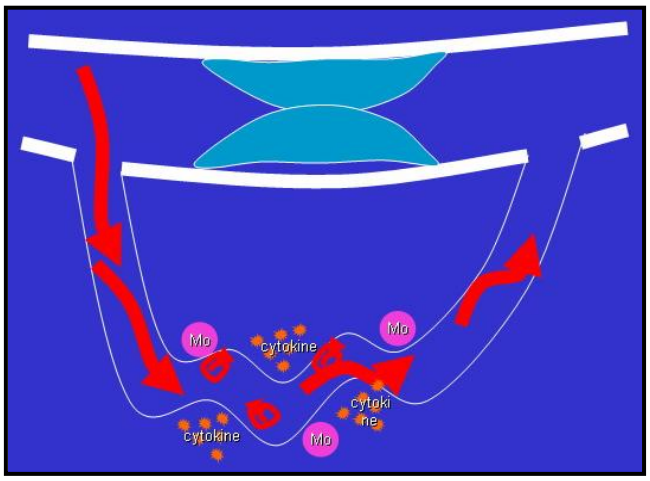

Figure 2: The mechanism of arteriogenesis

After the occlusion of the original major stem vessels, the blood will pass through the original arterioles, with the shear force of the blood stream and the accumulation of the monocytes and the cytokines, the arterioles become collateral arteries(Ito 2003).

1.5 Rat hindlimb ischemia model

The hindlimb ischemia model is an ideal model for the circulation research in the legs. Basically, it is performed by ligation of the femoral artery with surgical procedures. After the ligation, collateral vessels will start to grow and the perfusion below the ligation part will be reduced.

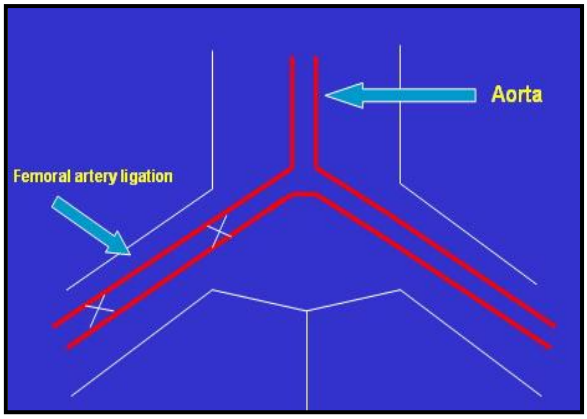

Figure 3: Rat hindlimb ischemia model
The femoral artery was isolated and carefully ligated 1cm apart with surgical procedures

1.6 Micro computer tomography system

Micro computer tomography (Micro-CT) is a new computer tomography technology for the preclinical imaging study. The construction and the principle of the micro-CT is basically the same as the normal computer tomography we use in clinic. As described in figure 4, the system is consisting of three major parts: micro X-ray source; object holding and rotating system; image capture and reconstruction system. We can first get a 360° radioscopic X-ray image database. Then, the database is used for reconstruction of the cross-sectional images by the computer reconstruction system.

Compared to the normal clinical used CT, the spatial resolution of the micro-CT is fairly high, which is usually from 5 μm to 50 μm. The term micro is used to describe the pixel size of the projection, which means, with the micro-CT, one pixel from the pixel image equals to 5 μm to 50 μm. Also, as described in figure 5, the size of the micro-CT machine is much smaller than the normal medical CT. Micro-CT is usually used for preclinical studies for small objects like rat and mice.

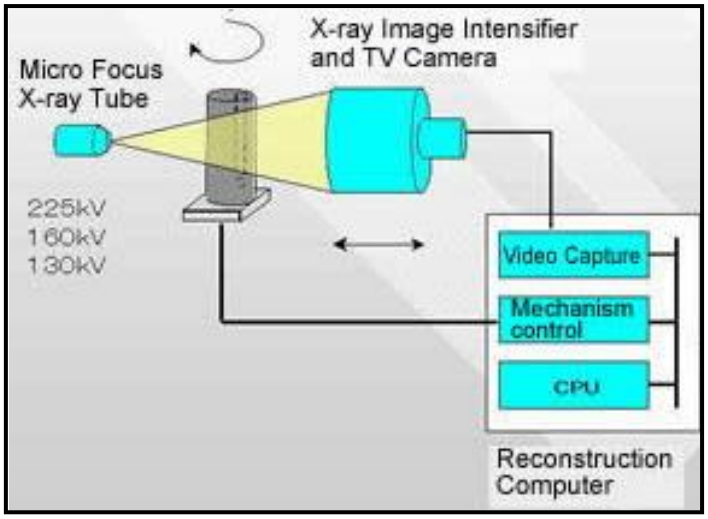

Figure 4: Basic principle of the micro CT
The system is consisting of three major parts: micro X-ray source; object holding and rotating system; image capture and reconstruction system.
(Modified from www.toshiba-itc.com)

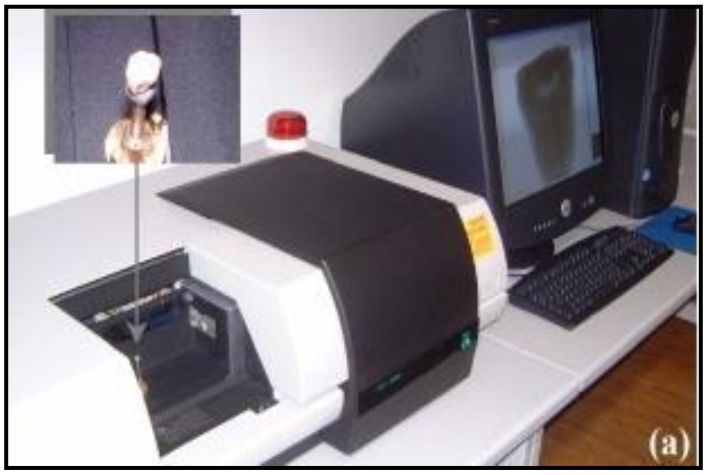

Figure 5: Skyscan1072 micro-CT system (SkyScan, Arstselaar, Belgium)

1.7 Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU)

It is important to measure the cell proliferation in cell biology research. Bromodeoxyuridine (5-bromo-2-deoxyuridine, BrdU) is an analogue of thymidine. It can be incorporated into the DNA of replicating cells in the place of thymidine during the systemization (in the S phase of the cell cycle). By using this feature, BrdU is widely used to detect the proliferating cells in living tissues. With the monoclonal antibodies against BrdU and a secondary fluorescence-conjuncted

antibody, we can quickly detect the proliferating cells in the tissue. In our study, we use BrdU to detect the proliferating cells inside the vessel wall.

1.8 Fluorescent microspheres

Microspheres are spherical particles with diameters in the micron range. There is a variety of different forms of microspheres: glass microspheres, ceramic microspheres, polystyrene microspheres and so on. In our research, we used polystyrene microspheres containing a red fluorescence dye for blood flow detection. They are 15 μm in diameter, with 0.2% (w/w) suspension in 10 ml (Molecular Probes, Invitrogen) of saline, with 0.02% Tween-80® and 0.02% thimerosal added. The red fluorescent dye has the maximal excitation wavelength of 566nm and the maximal emission wavelength of 598 nm, which can be seen by a fluorescent microscope. When a certain kind of microspheres is used, regional blood flow can be expressed by the number of microspheres trapped in the region of interest(Gonzalez and Bassingthwaighte 1990). Although radioactive microspheres are considered to be the classic method for blood flow detection, the safer fluorescent microspheres method is considered to be a better way in recent researches (Jacobi et al. 2004).

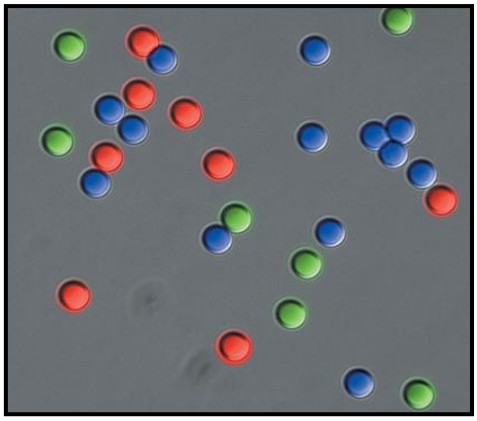

Figure.6: Fluorescent microspheres
A photomicrograph of a multicolor mixture of Molecular Probes' FluoSpheres® fluorescent microspheres (modified from www.invitrogen.com)

2. Material and Methods

2.1 Cell clonogenic assay

The spontaneously immortalized rat endothelial cell line was used. Cells were grown under standard cell culture conditions in a 5% CO_2 incubator at 37°C in DMEM supplemented with 10% fetal calf serum, glutamine, and antibiotics. Confluent monolayers were split routinely 1:2 after washing with PBS and treating with trypsin-EDTA (1×).

Cells were cultured until 80% confluence. Cells were then trypsinized and 1 million cells were transfected with GFP plasmid by Amaxa nucleofection technology with Amaxa transfection kit L. One day after transfection, cells were sorted by fluorescent-activated cell sorting to place one single cell per well in a 96 well tissue culture plate. Each well was filled with 100μm DMEM+10%FCS. Cell growth was examined and counted everyday by microscope. The wells with more than 100 cells after 7 days were selected and subcultured in 24 well plates; only the cells from the best growing well were selected for the further research.

2.2 Culture of highly proliferative cell clone

After cultured in the 24 well plates, the cells were then transferred to a 6 well plate. After confluence, cells were seeded into a new T150 flask. Cells were observed every day by microscope and the morphology changes were recorded.

2.3 Cell growth measurement

Cell growth was detected by CASY cell counting system. 1000 cells were seeded in each well within a 24 well plate. 3 wells were counted and averaged each day for 7 days.

2.4 Rat hindlimb ischemia model

This study was performed according to Section 8 of the German Law for the Protection of Animals, and with the permission of the State of Schleswig-Holstein. Experiments were performed on male Sprague-Dawley rats (weight 150-200 g, Charles River Laboratories, Germany). Anesthesia was induced by intraperitoneal injections of Ketamine (100 mg/kg body wt, Atarost) and 2% Xylazine (5mg/kg body wt, Bayer). Femoral artery occlusion was performed as previously described (Herzog et al. 2002). Briefly, the right femoral artery was isolated carefully without damaging the vein and nerve and ligated with two sutures 1 cm apart. The wounds were then closed, and the animal was allowed to recover.

2.5 Visualization micro-CT preparation

For micro-CT research, after femoral artery occlusion, rats were divided into two groups: experimental group (n=7) and control group (n=6). 25 million cells in 200μl PBS with favorable characteristics (stable growth, expression pattern of CD133+, Flt1+,Flk1+) were injected into the collateral vessel growing zone(upper part of the hindlimb) in the experimental group, and the control group was injected

with the same volume of PBS. The micro-CT angiographies were done as described before with some improvements(Herzog et al. 2002).Gelatin (12 g, type A, from porcine skin, Sigma) and barium sulfate (40g ,Merck) were dissolved in 100 ml of heated distilled water under continuous steering. Rats were anesthetized again seven days after the femoral artery ligation and anticoagulated with heparin (100IU/L), and the aorta was cannulated. Subsequently, the vasculature was flushed with 0.9% normal saline, then with 10% neutral buffered formalin for fixation (37°C at 250 mmHg). After clearance of the blood from the non-ligated leg, the rats were put into the warm water (37°C) and the contrast medium was then injected with the pressure of 250 mmHg until filling of the distal femoral stump was observed. The animals were then immediately placed on crushed ice, and the contrast medium was allowed to harden under continuous pressure. Then the ligated leg was cut off and fixed with formalin for 12 hours before the micro-CT detection.

2.6 Collateral artery visualization

For the visualization and quantification of the vessels, we used a high resolution desktop X-ray micro-CT system, SkyScan1072. (SkyScan, Belgium). The system contains an 80 kV microfocus tube with a focal spot size of 8μm. Its imaging system consists of an X-ray source and a 2-dimensional X-ray detector creating projection images (cone beam reconstruction mode). The field of view is vertically adjustable by translation of the sample holder. The magnification is

determined by the adjustable position of the sample holder between the detector and the X-ray source. In our research the voxel size was set at 18 μm. Aluminum-Filter and an exposure time of 4 seconds were used for best resolution. The sample holder rotation step was set at a minimum of 0.45°, which corresponds with 400 views or projections. From these projection images the virtual 3D-Model of the object is recreated. DataViewer (Version1.3.2 SkyScan, Belgium) was used for the 3D-Model visualization of the collateral vessels from all the projections. And the collateral arteries were visualized by the animation view of the projections. The reconstruction of the raw projections data was obtained by the NRECON RECONSTRUCTION (v 1.5.1 SkyScan). The whole Z axis is reconstructed with the image dynamic range from 0.0015 to 0.03 to give the best visualization of the vessel structure on reconstruction slices. All the reconstruction sections were stored and used for the arteriogenesis and angiogenesis quantification. Quantification details are shown in the results part 3.6.

2.7 Animal preparation for proliferation and blood flow research

For collateral artery proliferation and lower hindlimb blood flow research, animals (n=12 in total, n=6 in both experimental group and control group) were supplied with osmotic minipumps filled with BrdU after anesthesia. Osmotic pump preparation was conducted according to the instructions of Alzet. Osmotic minipumps (model 2Ml1, Alzet, 10 ml/h, 7 days) were filled with 62 mg BrdU dissolved in 3 ml of 0.5 M NaHCO3 buffer (pH 9.8) under sterile conditions. They

were implanted subcutaneously in the back region of the rats. Femoral artery ligations were then performed, after ligation, cells are injected as described above in the experimental group, and the control group was treated with PBS. On the sacrifice day, surgical preparation was done in the early morning: a catheter (PE-50 tubing) was placed in the arch of the aorta via the left carotid artery for infusion of the fluorescent microspheres (red, 15μm, invitrogen). The catheter was then filled with heparinized saline (100 IU/ml) and led under the skin to the back of neck. The animals were then allowed to rest for at least 4 hours before running on the treadmill. The red fluorescent microspheres (0.45ml containing 450,000 microspheres) was then prepared by ultrasonicated and vortexed for at least 5 minutes. After running (15 m/min at 15% grade) on the treadmill for 1 minute, a well-mixed microspheres solution was infused through the carotid catheter, followed by a saline flush, for 20s(Yang et al. 2001). The rats were sacrificed after the infusion. For the proliferation research, the midzone part of the collateral vessels was removed; for the blood flow study, gastrocnemius, soleus and middle part of both kidneys were collected. Samples were then embedded in Tissue Tek (OCT compound, Sakura Finetek) and shock frozen in liquid nitrogen for further research.

2.8 Collateral proliferation assay

The midzone part of the collateral vessel growth zone, including the surrounding tissue, was embedded in Tissue Tek (OCT compound, Sakura

Finetek). Cryostat sections (10 μm thick) were fixed in glycine and ethanol (3:7, pH 2.0) at -20°C for 20 min. After drying the sections for 30 min, sections were washed in phosphate-buffered saline (PBS). Then 1% BSA (bovine serum albumin, Fluka) was applied 30min for blocking. For BrdU staining, we used the BrdU working solution as supplied by the 5-Bromo-2'-Deoxyuridine Labeling and Detection Kit 2 (Roche Diagnostics, Germany) according to the protocol supplied by the manufacturer. As a secondary antibody, we used a FITC-conjugated goat anti-mouse IgG (Catalog No: R1611F, Acris Antibodies GmbH, Germany) diluted 1:500 in 1% BSA. Nuclear staining was obtained using a 0.001% Hoechst3334 nuclei dye (cambrex). Before analysis under the fluorescent microscope, slides were embedded in Mowiol (Calbiochem) und 1, 2-phenylenediamine (PPD; Merck). For counting purposes, pictures were taken from five sections of three different midzone segments of the main collateral vessel. The proliferative index was calculated as the number of BrdU-positive nuclei (green-fluorescence) to the total number of nuclei (blue fluorescence). The proliferative index was determined in 6 animals after 7 days of unilateral femoral artery occlusion and continuous BrdU infusion.

2.9 Microspheres blood flow detection

All the microspheres were counted with a fluorescent microscope (Nikon Eclipse TS100, Japan) with the frozen section thickness of 60μm. Kidney samples with more than 15% differences of microspheres from both kidney samples are

excluded from the blood flow detection. The numbers of microspheres from both ligated and no-ligated legs were then calculated, blood flow was expressed as occluded and nonoccluded hindlimb perfusion ratios (microspheres) of the soleus and gastrocnemius.

2.10 Statistic assay

All analyses were repeated at least three times. Data are shown as means±SD. Statistical comparisons between groups were performed with Student's t-test. Differences among means were considered significant when $P<0.05$

3. Results

3.1 Single cell clonogenic assay

Cells were transfected with green fluorescence protein plasmid and then went through FACS (Fig 7 a). After seeding the single cells in 96 well plates for 7 days, cell growth was recorded everyday by microscope. The well with the fastest growing clones was chosen as the well which contains vascular resident endothelial progenitor cells (VREPCs). As described in figure 7, from the fastest growing single cell, more than 100 cells were observed in one single well (Fig 7 b).

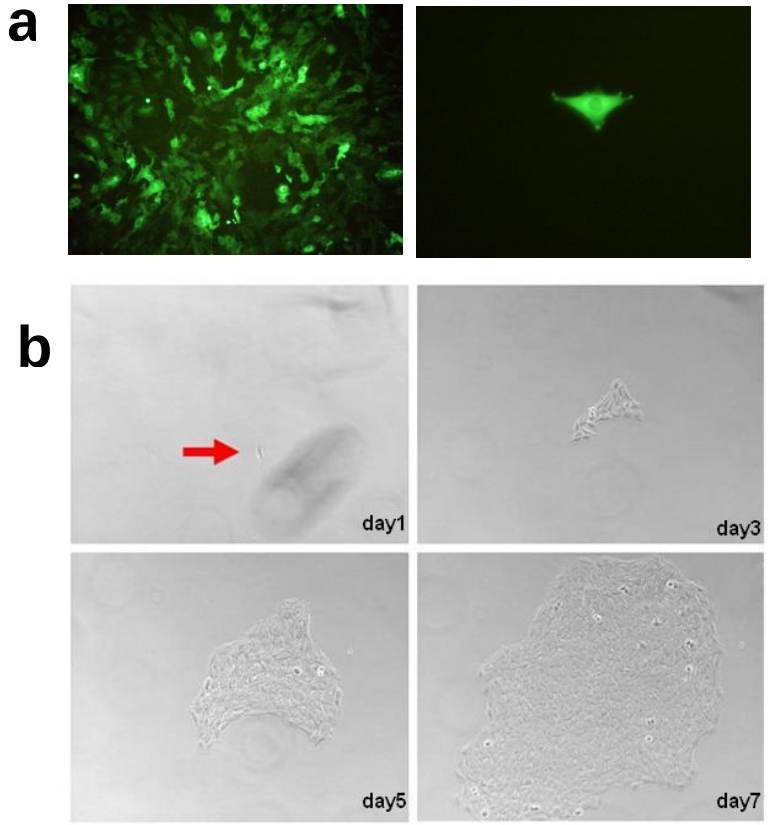

Figure 7: Single cell clonogenic assay
(a)After transfection, GFP positive cells were sorted by FACS and single cell clone was placed in one well. (b)Single cell clonogenic assay: single cells were seeded in 96 wells plate, after 7days, cell colony with more than 100 cells can be observed.

3.2 Culture and cell growth of VREPCs

After seeding in a T150 flask, cells attached to the bottom of the flask after one day, on day 3, cells began to form colony like structures. (Fig 8 a right). As described in the cell growth curve, we obtained around 700 times more cells in one week.(Fig 8 b)

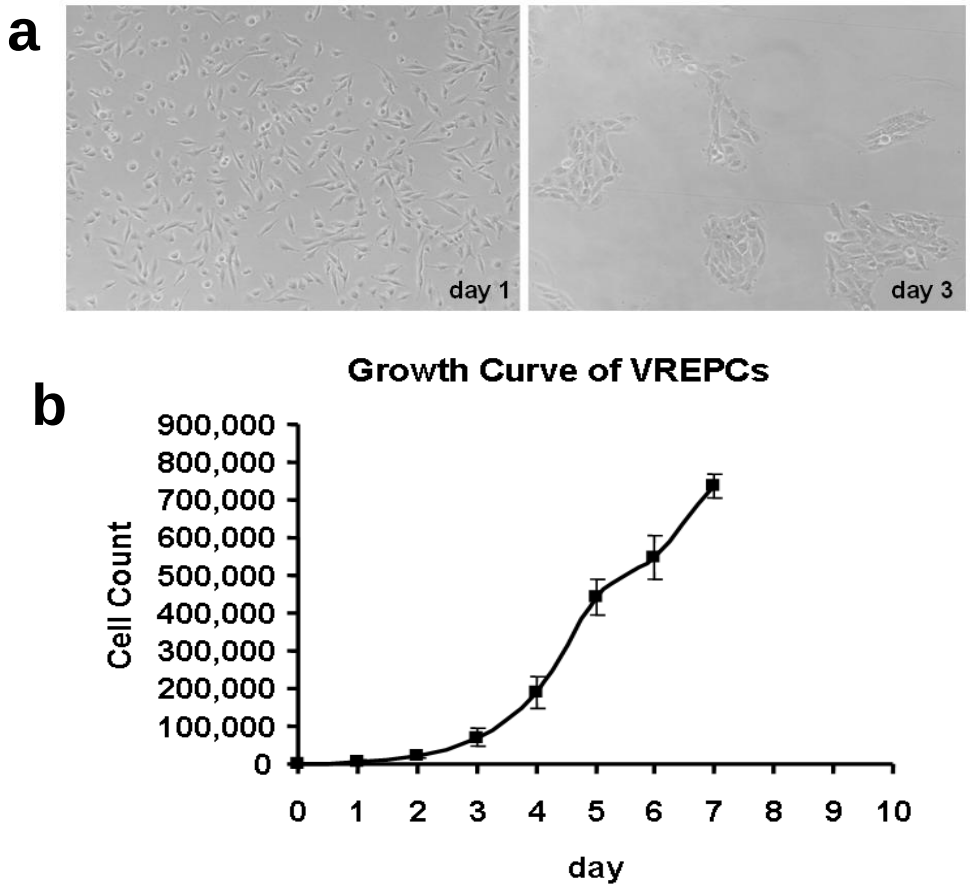

Figure 8: Cell morphology and cell growth curve
(a) Highly proliferative cells form colony like structures 3 days after culture. (b) VREPCs Cell growth curve

3.3 VREPCs enhanced collateral growth

To further investigate the in vivo effect of VREPCs, we applied the VREPCs intramuscularly to ischemic rat hindlimbs. After the CT angiographies, the ischemic hindlimbs were analyzed by micro CT. The collateral growth was checked by a high

resolution micro-CT system and the ischemia zone was reconstructed with 1024 cross section images. Visible collateral artery number was counted in both the original X-ray projections and the three dimensional reconstruction animation (Fig 9. a) as described before with modifications (Herzog et al. 2002; Khmelewski et al. 2004). Only the collaterals with a clearly stem zone, middle zone and reentry zone were calculated as collateral arteries. Following VREPCs administration, there are more visible collateral arteries (P<0.01).

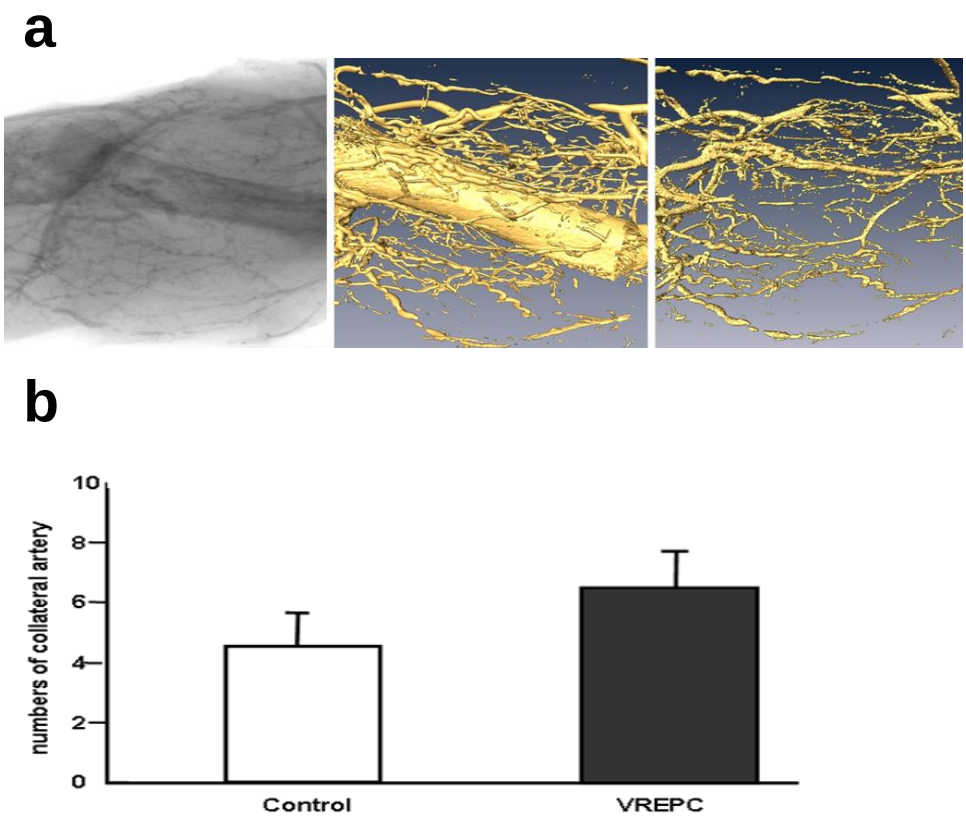

Figure 9: Detection of in vivo neovascularization by micro-CT system for VREPCs
(a) The process of the CT construction: left image shows the original X-ray projection of the ischemic hindlimb, middle image shows the three dimensional reconstruction of the hindlimb, right image shows only the vessel structures. (b) Collateral counting result shows that after the injection of the VREPCs, collateral growth was improved (P<0.01)

3.4 Both angiogenesis and arteriogenesis were enhanced after VREPC injection

To further evaluate whether VREPCs have the ability to stimulate the postnatal neovascularization, we performed arteriogenesis and angiogenesis quantification analyses after VREPCs administration to rat hindlimbs. Here, the terms arteriogenesis and angiogenesis were used to discriminate different sizes and formation pattern of the vessels. Arteriogenesis quantification mainly focused on the quantification of the typical medium size collateral vessels which were mainly formed by shear force. The angiogenesis quantification, focused on the thin-walled small size vascular network. The quantifications were done by the measurement of the voxels numbers in the ischemia zone from the 600 cross section reconstructed images below the proximal end of the ligation. Two different thresholds 20 and 40 from the Amira4.0 software were used for the quantification, threshold 20 was used to quantify both small size and medium size vessels (Fig 10 a 3), and threshold 40 was used to quantify only the medium size vessels (Fig 10 a 4); the voxel number in the selected region was counted. Total voxel number of arteriogenesis was counted with threshold 40 and total voxel number of angiogenesis was calculated by subtracting the value of threshold 40 from the value of threshold 20. After the VREPCs injection, both arteriogenesis and angiogenesis were improved (Fig 10 b angiogenesis<0.05, arteriogenesis<0.01), which indicate an improvement in the medium size vessel and related small thin vascular networks.

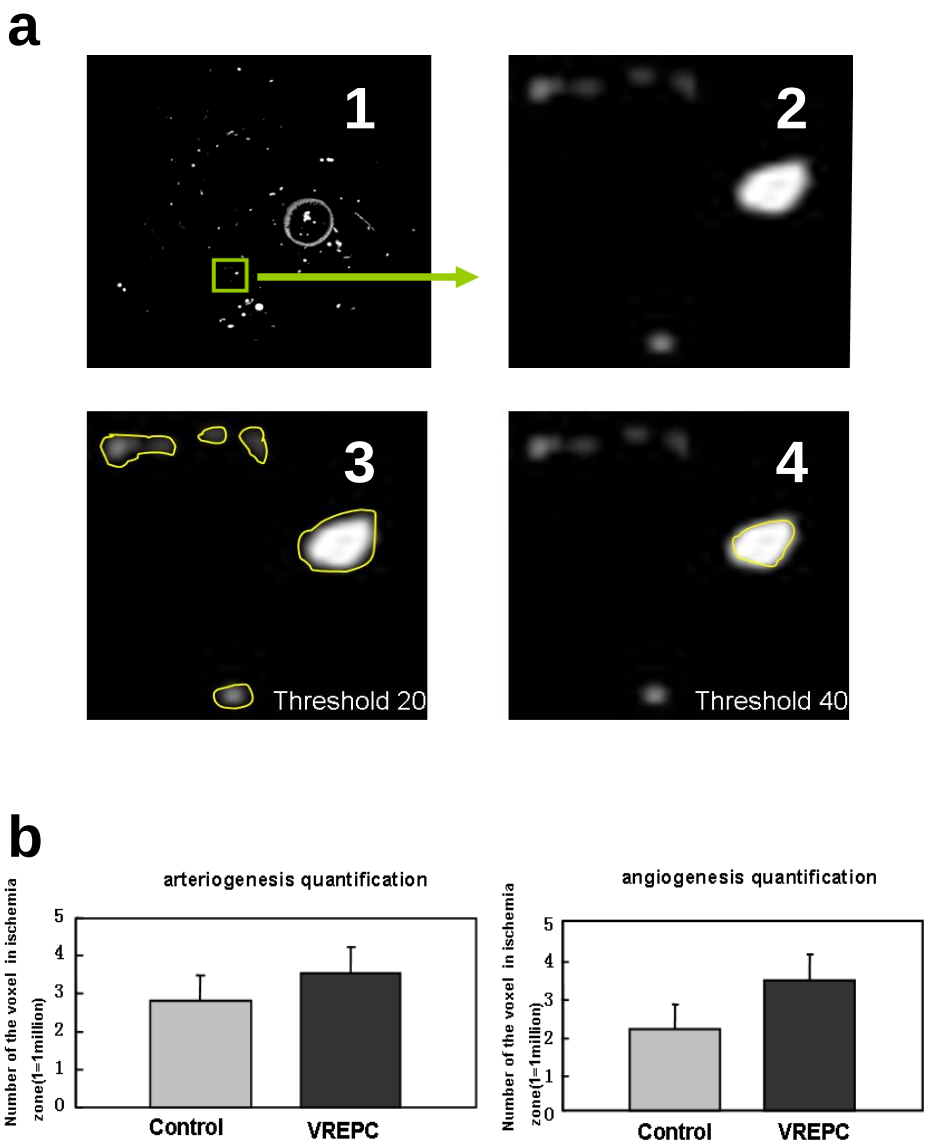

Figure 10: Angiogenesis and arteriogenesis assay

(a) The ischemia zone was defined as 600 slices below the proximate ligation point. Quantification was done with two different thresholds: 20 and 40 from the Amaxa software. With threshold 40, only the voxels within the high diameter were counted, with threshold 20, both the voxels within the high and low diameters were counted. (b)Both angiogenesis and arteriogenesis were enhanced after VREPCs injection (angiogenesis $P<0.05$, arteriogenesis $P<0.01$).

3.5 Collateral proliferation was improved by VREPCs

We proved that proliferation kinetics was related to collateral growth. One week after femoral artery ligation, the collateral proliferation would rise to maximum(Herzog et al. 2002). Thus, to further examine the function of the VREPCs, we performed proliferation assay after VREPCs administration. BrdU positive nuclei were with green fluorescence (Fig 11a upper row). The total nuclei were stained with Hoechst nucleus dye (Fig 11a lower row). Proliferation indexes which represent the ratio of the proliferation cells compared to the total number of endothelial and smooth muscle cells within the collateral arteries. Positively BrdU stained nuclei and the total amount of the nuclei were counted. The accumulative proliferation indexes were then calculated. After VREPCs injection, the collateral proliferation was enhanced (Fig 11 b $P<0.01$).

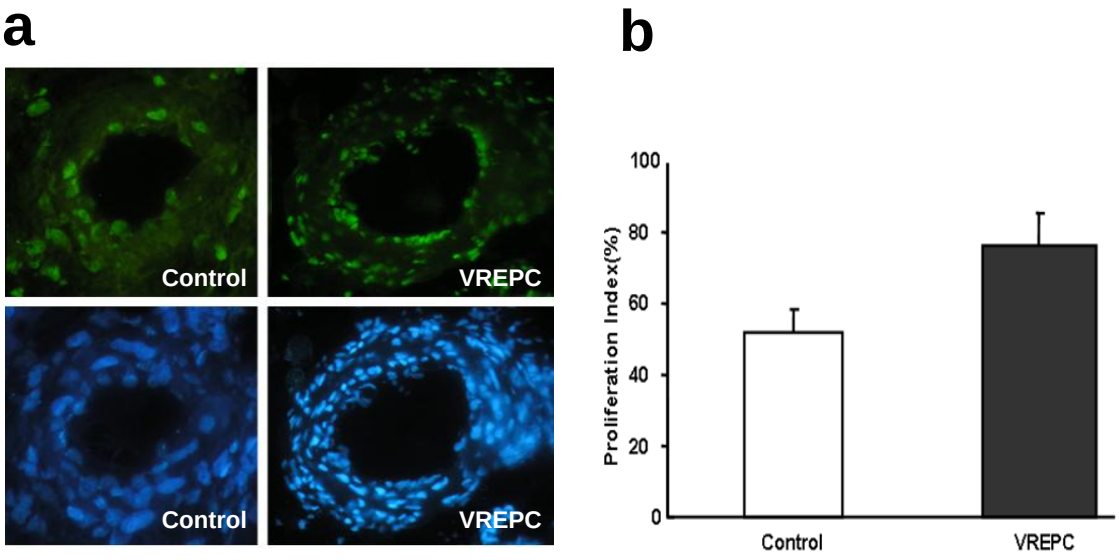

Figure 11: Effects on proliferation of VREPCs

(a) BrdU positive nuclei were with green fluorescence (upper row). The total nuclei were stained with Hoechst nucleus dye (lower row). (b)The injection of the VREPCs enhanced the collateral proliferation ($P<0.01$).

3.6 Lower hindlimb blood flow was increased by VREPCs

To examine the blood flow in the lower ischemic hindlimbs, fluorescent microspheres were infused via carotid artery one week after VREPCs administration. Because the diameter of the microspheres is similar to the small arteries, they are trapped inside the lumen of the small arteries (Fig12a left: normal filter; middle: Cy3 filter; right: GFP filter). The numbers of microspheres were related to the blood flow in these small arteries. Since we mainly focused on the improvement of blood flow after cell administration, we only evaluated the relative perfusion ratio. Thus, microspheres from both soleus and gastrocnemius (ligated and non ligated hindlimb) were counted by fluorescent microscope to get the perfusion ratio from each experimental rat and control rat. Blood flow from both soleus and gastrocnemius were significant improved after the VREPCs administration (Fig 12b soleus: $P<0.001$ gastrocnemius $P<0.001$).

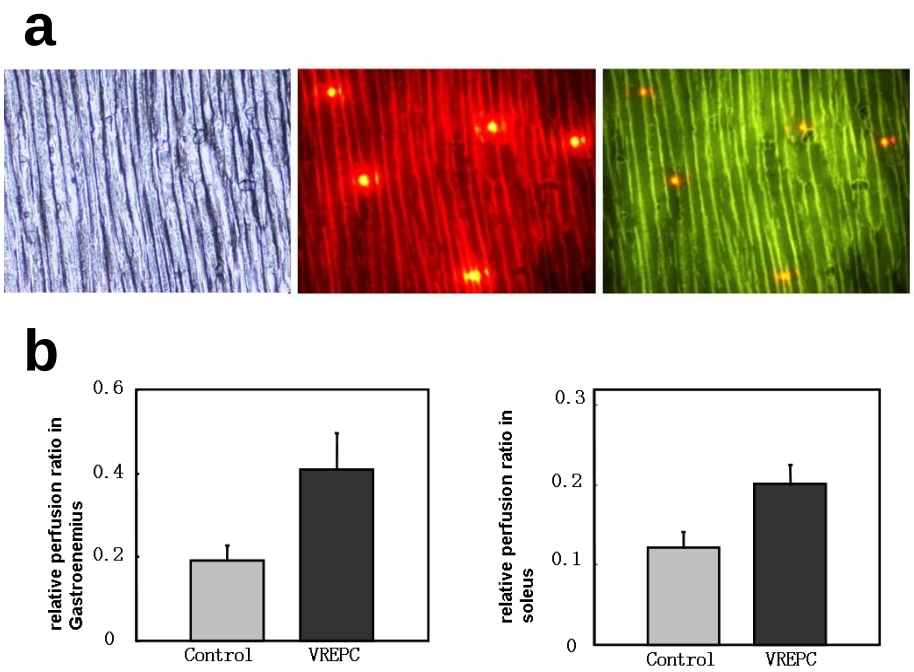

Figure 12: Lower hindlimb blood flow was increased by VREPCs

(a) Red fluorescent microspheres were trapped in gastrocnemius and soleus: the same unstained 60 μm cryostat section region showed red microspheres appearing as orange in Cy3 (middle) and GFP (right) microscopy filter. With normal light, no microspheres were detected (left). (b)Blood perfusion ratios: Results are shown as left: gastrocnemius perfusion ratio between control group and VREPC group. Right: soleus perfusion ratio between control group and VREPC group. Both blood flow in soleus and gastrocnemius were improved (soleus: $P<0.001$ gastrocnemius $P<0.001$).

4. Discussion

In the present study, we successfully isolated a vascular resident endothelial progenitor cell population from rat heart microvasculature and proved its important in vivo functional role in collateral growth. We presented several lines of evidences that these cells are vascular resident endothelial progenitor cells which own a high differentiating ability in vitro and contribute to the postnatal neovascularization after in vivo administration. First, although the cells have single cell origin, they can give rise to highly proliferative different functional progeny. Second, they showed tube formation ability in vitro (data not show). Third, when applied in vivo, injection of vascular resident endothelial progenitor cells increased the collateral growth by visible collateral artery counting, moreover, the VREPCs improved both the arteriogenesis and angiogenesis in the ischemia zone and blood flow in the lower hindlimb. Thus, VREPCs can be a new source for the postnatal neovascularization research.

There are usually two ways to define EPCs, one is mainly based on the expression of the cell surface marker CD133 and Flk1 (Asahara et al. 1997; Urbich and Dimmeler 2004). These cells have high differentiation ability both in vitro and in vivo, but have low proliferation rates. The second way is based mainly on the theory that stem/progenitor cells can give rise to highly proliferative functional progeny (Ingram et al. 2004; Yao et al. 2007; Hirschi et al. 2008). Our VREPCs are the first EPCs that qualified both standards, from the single cell cloning assay; we

proved they own high proliferation ability (Fig7). The FACS analyses of VREPCs showed highly differentiation ability of the cells (data no showed). Thus, the cells are highly proliferative endothelial progenitors.

From the FACS results, our cells are a mixture of different kinds of progenitor/stem cells. We think this mixture of stem/progenitor cell types is good for the cell performance in vivo. A lot of studies have proven the synergetic effects of different kinds of stem/progenitor cells. Coadministration of early EPCs and late outgrowth EPCs was proven to be more effective than administrating them alone (Yoon et al. 2005). A recent study showed that the coadministration of endothelial and smooth muscle progenitor cells have synergetic effects (Foubert et al. 2008). In these studies, the progenitor cells are usually a mixture of cells in different stages of cell differentiation.

Our findings are also supported by (Hirschi et al. 2008). In his review, the authors evaluated all basic methods for endothelial progenitor cell identification and described the in vivo functions of the EPCs. They concluded that EPCs should have two important characters: 1) they are able to produce endothelial progeny; 2) they can form tube like structures in vitro and contribute to the de novo formation of vascular structures in vivo. Considering this definition, our cells are strictly EPCs. Interestingly, our cells showed some similarities to endothelial colony forming EPCs (late outgrowth EPCs) which were indicated in the review to be the only “real” EPCs, both cell types are colony forming, form cobblestone patterns and show high proliferation ability.

For the in vivo collateral growth assay, we used a computer assistant micro-CT system. Using micro CT for the collateral growth research is a newly developed method; it has been proven to be effective and objective (Duvall et al. 2004; Timmermans et al. 2007). Different binarization threshold settings can be used for detection of different size of vessels. Since angiogenesis and arteriogenesis are related to two different kinds of vessels during the collateral growth (van Royen et al. 2001; Duvall et al. 2004) , we quantified the different size of vessels during the collateral growth for the arteriogenesis and angiogenesis quantification. Numerous studies analyzed postnatal neovascularization in a hindlimb ischemia model; few of them also focused on the different roles for angiogenesis and arteriogenesis. One of the major reasons is that with the x-ray angiography system, it is nearly impossible to analyze all the different vessels in the ischemia zone. Here, with micro-CT system and the computer 3D reconstruction, arteriogenesis and angiogenesis can be analyzed separately. In our previous study, we proposed the presumption that during the collateral growth, a vascular network has to be build de novo to meet the need for the remodeling of collateral arteries. In our present study, by using of micro CT technology we proved that angiogenesis and arteriogenesis should coordinate during the collateral growth process.

The collateral proliferation is another parameter we checked in vivo. After intramuscular injection, we detected more proliferation in the collateral artery growing zone; this indicates that EPCs can improve the collateral growth. But, how these EPCs worked in vivo still remained unknown. Our next step is to investigate

whether these cells resident in the neovascular site and influence the neovascularization by paracrine effects or directly incorporate into the collateral vessel. If they can incorporate into the newly formed vessels, then our result is in accordance with the latest discovery by (Sieveking et al. 2008) that only the late-out growth EPCs can incorporate into the vessel intima and function as endothelial cells.

Another intriguing phenomenon is that even after the EPC injection, there are still some no proliferation vessels which locate near the collateral vessels. This indicates that the paracrine/incorporation effect of the EPCs is specific for the collateral vessels. As a result, this proves again that the initial signal for the collateral growth is inside the vessel other than from the outside. We believe the whole process should start with the change of the hemodynamic forces, then the collateral vessels start to send signals outside the vessels, all the collateral growth helpers including cells, cytokines will start to secrete or migrate to the collateral growth zone. If so, there must be certain kinds of the unknown proteins involved in the early stage of the collateral growth; this is also worth for further identification.

In summary, in our present study, we identified for the first time a group of vascular resident EPCs from a group of self immortalized rat heart microvasculature cells. We believe the existence of the EPCs inside the original cell population is the reason of the self immortalization. Moreover, after the administration of the mixture cell population, the collateral growths were significantly enhanced. However, although we proved progenitor characters of the

cells in vitro and in vivo, further investigations of the cells are still needed. Such as how the cells actually work in vivo and why they own high proliferation ability? In spite of that, the present study provides us a new source of EPCs for the therapeutic treatment in circulation research.

5. Abstract

Endothelial progenitor cells (EPCs) are used for neovascularization research for over a decade since their first discovery in 1997. More and more evidences have showed that only the EPCs which own high proliferation ability can work in vivo, such as late outgrowth EPCs and endothelial colony forming cells. However, until now, the exact definition and location of EPCs are still under debate. Thus, in our present study, we successfully identified a new hierarchy of highly proliferative vessel wall resident EPCs by single cell clonogenic assay. These cells are mainly consisted of CD133+, Flt1+ and Flk1+ cells. They can form colonies in culture and form tube like structures in vitro. In the in vivo test, we checked the arteriogenesis and angiogenesis in a rat hindlimb ischemia model. Both angiogenesis and arteriogenesis were quantified by a high resolution micro-CT system, after one week, both of them were increased in the cell injection group (arteriogenesis quantification $P<0.01$; angiogenesis quantification: $P<0.05$; collateral artery counting: $P<0.01$).The blood flow in the lower hindlimb was measured by fluorescent microspheres, the ischemia perfusion versus non ischemia perfusion ratios were improved in both soleus and gastrocnemius after application of the cells($P<0.001$). The proliferation inside the collateral arteries was also increased after one week ($P<0.01$). In conclusion, vessel wall resident endothelial progenitor cells can be a prominent therapy choice in circulation research.

6. Zusammenfassung

Mehr und mehr Beweise wurden geliefert, dass nur EPCs, die eine hohe Fähigkeit zur Proliferation haben, wie late outgrowth EPCs und koloniebildende Zellen, in vivo arbeiten. Bis heute werden die exakte Definition und Lokalisation der EPCs noch stark diskutiert. In vorliegender Studie haben wir erfolgreich eine neue Hierarchie der stark proliferativen EPCs, lokalisiert in Gefäßwänden, durch Single Cell Clonogenic Assay identifiziert. Diese Zellen sind hauptsächlich CD133+, Flt1+ und Flk1+ Zellen. Sie können Kolonien in Kulturen und röhrenförmige Strukturen in vitro formen. In dem in vivo Test prüften wir die Arteriogenese und Angiogenese an einem ischemia model der hinteren Extremität von Ratten. Angiogenese und Arteriogenese wurden anhand eines hoch auflösenden Micro-CT-Systems quantifiziert; nach einer Woche waren beide in der cell injection Gruppe gestiegen (Arteriogenese Quantifizierung $P<0.01$; Angiogenese Quantifizierung: $P<0.05$; Zählung kollateraler Arterien: $P<0.01$). Der Blutfluss in der unteren hinteren Extremität wurde durch fluoreszente Mikrokugeln gemessen, das Verhältnis ischämische versus nicht-ischämische Perfusion wurde sowohl im M. soleus als auch im M. gastrocnemius nach Verwendung der Zellen verbessert ($P<0.001$). Die Proliferation innerhalb der kollateralen Arterien wurde ebenfalls nach einer Woche verbessert ($P<0.01$). Fazit: Die in der Gefäßwand lokalisierten endothelialen Vorläuferzellen können eine bedeutende Therapiewahl im Bereich der Forschung auf dem Gebiet der Durchblutung sein.

7. References

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8. Appendix

8.1 Curriculum vitae

I. Personal data

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II. Education

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III. Congress presentations

1.**Z. Zhang**, A Slobodianski, A Kathöfer,J.Frenz G Middeler, W.D Ito,HG Machens Analyses of collateral vessel growth in a rat cell therapy model of ischemia hindlimb: a research about computer tomography based angiogenesis and arteriogenesis assessment. 12th European Conference of Scientists and Plastic Surgeons (ECSAPS) September 2008, Bern, Switzerland. (Oral presentation)

2, A. Slobodianski,**Z. Zhang** ,A. Kathöfer, J. Frenz, G. Middeler, H.G. Machens Development of “therapeutic angiogenesis” - a technique to induce angiogenic reaction in ischemic tissue12th European Conference of Scientists and Plastic Surgeons (ECSAPS) September 2008, Bern, Switzerland. (Oral presentation)

3, N. Lund, U. Gehling, **Z.Y Zhang**, H. Schunkert,Machens HG,W.D.Ito Cloning and functional characterization of vascular progenitor cells from rat heart tissue 74 annual conference of the German Cardiac Society (GCS) March 2008 Mannheim Germany (poster presentation)

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When I was young, I dreamed of to be a doctor, to help people, to get people out of the sickness. After five years of medical education and clinic training, I have learned a lot in the mechanisms of the diseases and how to treat the diseases. But the more I have learned, the more “whys” appeared in my mind. Fortunately, I got the chance to go abroad after my graduate school and continue my medical life as a researcher, on this point, I feel very grateful for the support from the China Scholarship Council and my home university, Tongji Medical College of Huazhong University of Science and Technology.

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