Differentiation potential and functional properties of a CD34$^-$CD133$^+$ subpopulation of endothelial progenitor cells

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Abstract. Endothelial progenitor cells (EPCs) promote angiogenesis and play an important role in myocardial and vascular repair after ischemia and infarction. EPCs consist of different subpopulations including CD34$^-$CD133$^+$ EPCs, which are precursors of more mature CD34$^+$CD133$^+$ EPCs and functionally more active in terms of homing and endothelial regeneration. In the present study we analyzed the functional and differentiation abilities of CD34$^-$CD133$^+$ EPCs. Isolation of EPC populations (CD34$^+$CD133$^+$, CD34$^-$CD133$^+$) were performed by specific multi-step magnetic depletion. After specific stimulation a significant higher adhesive and migrative capacity of CD34$^-$CD133$^+$ cells could be detected compared to CD34$^+$CD133$^+$ cells ($P<0.001$, respectively). Next to this finding, not only significantly higher rates of proliferation ($P<0.005$) were detected among CD34$^-$CD133$^+$ cells, but also a higher potential of cell-differentiation capacity into other cell types. Next to a significant increase of CD34$^-$CD133$^+$ EPCs differentiating into a fibroblast cell-type ($P<0.001$), an enhancement into a hepatocytic cell-type ($P=0.033$) and a neural cell-type ($P=0.016$) could be measured in contrast to CD34$^+$CD133$^+$ cells. On the other hand, there was no significant difference in differentiation into a cardiomyocyte cell-type between these EPC subpopulations ($P=0.053$). These results demonstrate that EPC subpopulations vary in their functional abilities and, to different degrees, have the capacity to transdifferentiate into unrelated cell-types such as fibroblasts, hepatocytes, and neurocytes. This indicates that CD34$^-$CD133$^+$ cells are more pluripotent compared to the CD34$^+$CD133$^+$ EPC subset, which may have important consequences for the therapy of vascular diseases.

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Introduction

Endothelial progenitor cells (EPCs) play a pivotal role in neoangiogenesis and mediate recovery and repair of damaged endothelium (1-4). A meta-analysis of cell-based therapies in patients with refractory angina found improvements in cardiovascular outcome (5). Different surface markers can be used to subcategorize the heterogeneous EPC family, two of the most commonly employed being CD34 and CD133 (6-9). Recently, intramyocardial transplantation of CD133$^+$ EPCs was shown to improve heart function after severe myocardial infarction (10,11). We identified a novel CD34$^-$CD133$^+$ EPC subpopulation, which gives rise to more mature CD34$^+$CD133$^+$ EPCs, and more potently mediates homing and vascular reparation than the latter EPC subset (6,12-14). Subsequently, numbers of CD34$^-$CD133$^+$ EPCs were found to be decreased in patients with vascular disease and diabetes in contrast to numbers of CD34$^+$ EPCs (15), which might indicate a clinical relevance of the former EPC subset.

Hematopoietic precursors have been found to have the potential of transdifferentiation into non-hematopoietic cells, i. e. hepatocytes (16), neurons (17), and cardiomyocytes (18). Interestingly, following heart transplantation a proportion of cardiomyocytes from donor organs originated from non-cardiac sources (19), which may include the bone marrow (20). It has previously been shown that EPCs can be transdifferentiated *in vitro* into functionally active cardiomyocytes when co-cultivated with rat cardiomyocytes (21), a process depending on E-cadherin (22).

In our present study we sought to analyze CD34$^-$CD133$^+$ and CD34$^+$CD133$^+$ EPC subpopulations for their differentiation potential into non-endothelial cell-types, which might have important implications for regenerative therapeutic purposes.

Materials and methods

Purification of EPC subpopulations from human peripheral blood. The local ethics committee (Ethik-Kommission der Ärztekammer des Saarlandes) approved all investigations (compliance no. 122/09), which were carried out as specified by institutional guidelines. All volunteers provided written informed consent. Peripheral blood mononuclear cells (PBMNCs) were obtained from peripheral blood sampled from healthy volunteers (n=7, age 32±1.7 years, 5 male, 2 female)

followed by separation into $CD34^-CD133^+$ and $CD34^+CD133^+$ EPCs as previously described (6,9,23,24). A detailed description of the two EPC separation methods used (MACS® Cell separation and EasySep® Cell separation) can be found in the supplementary material.

MACS® cell separation. This separating system is based on microbeads, which are superparamagnetic particles. Magnetically labelled cells are separated over a column placed in a specific separator. Cells are then retained on the column, while unlabeled cells pass through. These cells are then collected as the unlabeled fraction and the retained cells are eluted from the MACS Column after removal from the magnet. For magnetically labelling cells were initially suspended in 300 μl MACS buffer (PBS, pH 7.2 with 0.5% BSA and 2 mM EDTA) per 108 cells. Then 100 μl FcR block reagent was added in order to avoid non-specific and Fc-receptor mediated binding of the antibody, as well as 100 μl CD34 MicroBeads. The cell suspension was then mixed well and incubated for 30 min at 4˚C. After ten minutes washing in 15 ml MACS buffer, the cell pellet was suspended in 500 μl MACS buffer per 10^8 cells. Two columns were then prepared by being flushed with buffer. Then, cell suspension was added to one of the columns, which had been previously placed in the MACS separator. The $CD34^+$ cells were retained by the magnet in the column. The column was then rinsed with buffer 3 times and then placed on the second prepared column. To increase purity three rinse steps were repeated once more, and finally $CD34^+$ cells get transferred in a new tube. In order to these cells once again magnetically, it was necessary to remove the microbeads from the cells. For this, 20 μl MACS multisort release reagent were added to the cell suspension and incubated for 10 min. Using a pre-rinsed MS column the remaining magnetically labelled cells were removed. After another washing, the cell pellet was taken up in 50 μl MACS buffer per 10^7 cells. Afterwards $CD34^+$ and $CD34^-$ cells were labelled with CD133-microBeads as described above.

EasySep® cell separation. This separating system is based on Tetrameric Antibody Complexes (TAC) recognizing specific cell surface antigens and dextran-coated magnetic particles for selection or depletion of cells. Labelled cells are linked to magnetic particles and are separated using a special magnet. For positive cell isolation, cells of interest are labelled and remain in the tube in the magnet, while unlabeled cells are poured off. For negative cell isolation, unwanted cells are labelled for depletion while cells of interest are poured off into a new tube, and are untouched. The subpopulations were isolated using the EasySep® system (StemCell Technologies) in two steps. Mononuclear cells were at first separated into CD34-positive and CD34-negative cells. For this, $2x10^8$ cells were resuspended in 1 ml of a special buffer (PBS with 2% FBS and 1 mM EDTA) and incubated at room temperature for 15 min with 100 μl EasySep® CD34-positive selection cocktail. Then, 50 μl magnetic nanoparticles were added and incubated for 10 min. Then, buffer was added and the tube was placed in the EasySep® magnet. After 15 min, the supernatant was poured off and the tube was filled again with buffer. This procedure was repeated a total of four times. The remaining cells in the tube (CD34+) population were suspended in 500 μl of PBS. The supernatant (CD34-) was further separated into CD133-positive and-negative cell populations. They were incubated for 15 min with 100 μl human FcR-blocking Reagent and 100 μl PE-conjugated antibody at room temperature, then for 15 min with PE EasySep® Selection cocktail and for another 10 min with 50 μl EasySep® Magnetic Nanoparticles. The procedure of the first separation was repeated and the supernatant consisted of $CD34^-CD133^-$ cells and in the tube remained $CD34^-CD133^+$ cells.

EPC culture. Growth capacity was tested using the commercially available cell media endothelial basal medium (EBM), DMEM, RPMI (Roswell Park Memorial Institute), and StemSpan® (H3000, StemCell Technologies).

Migration and adhesion assays. We measured the migratory and adhesive capacities of EPC-subsets using modified Boyden chambers, fibronectin-(Sigma) coated plates, and a parallel-plate, laminar-flow chamber (Immunetics, Cambridge, MA, USA) as previously described (6,25).

Differentiation assays. Differentiation capacity of EPC-subpopulations ($CD34^+CD133^+$, $CD34^-CD133^+$, $CD34^-CD133^-$) into fibroblasts, hepatocytes, cardiomyocytes, endothelial cells, and neuroblasts was analyzed by stimulation with the indicated growth factors followed by specific staining. The indicated cell-types ($1x10^5$) were cultured in 100 μl DMEM in 96-well plates, and then stimulated with fibroblast growth factor (FGF, 1 ng/ml) for fibroblasts, hepatocyte growth factor (HGF, 30 ng/ml) for hepatocytes, Cardiogenol C (100 nM) for cardiomyocytes, beta-nerve growth factor (β-NGF, 2 ng/ml) for neuroblasts, and vascular endothelial growth factor (VEGF, 4 ng/ml) for endothelial cells. Differentiation was assessed by cell morphology and immunohistochemistry. For the latter, the following monoclonal antibodies were used: Anti-FGF-2/basic-FGF for fibroblasts (clone bFM-2, Millipore, Billerica, USA), anti-α-Fetoprotein (AFP) for hepatocytes (clone AFP-11, R&D Systems, Minneapolis, USA), anti-α-sarcomeric actin (clone 5C5, Sigma Aldrich, Steinheim) for cardiomyocytes, anti-CD31 PE (clone WM59, BD, Heidelberg) for endothelial cells, and anti-neuron specific enolase (NSE, clone EPR3377, Abcam, Cambridge, UK) for neuronal cells. Specificity of the antibodies was previously tested in positive control experiments. For this purpose, murine fibroblasts (LMTK, LGC Standards GmbH), human neuroblastoma cells (SH-SY5Y (ATCC® CRL-2266™), LGC Standards GmbH), rat cardiomyoblasts (H9c2, LGC Standards GmbH), human endothelial cells (HUVECs), and human liver-carcinoma cells (HuH7) were used. Authentification of SH-SY5Y (ATCC® CRL-2266™) was performed using short tandem repeat (STR)-profiling with a commercially available PCR amplification kit (Applied Biosystems). Culture media were employed according to manufacturer's instruction (LMTK and H9c2: DMEM, 10% FCS, 1% PenStrep; SHSY5Y: DMEM, 10% FCS, 1% PenStrep, 1% MEM; HUVEC: ECGM; HuH7: RPMI, 10% FCS, 1% PenStrep, 5 μl/ml Ciprobay, 1% L-glutamine).

Statistical analysis. The SigmaStat program was used or all statistical analyses in the present study. Mean ± standard error of the mean (SEM) of the data was calculated for statistical analysis. Following this, the Kolmogorov-Smirnov test was

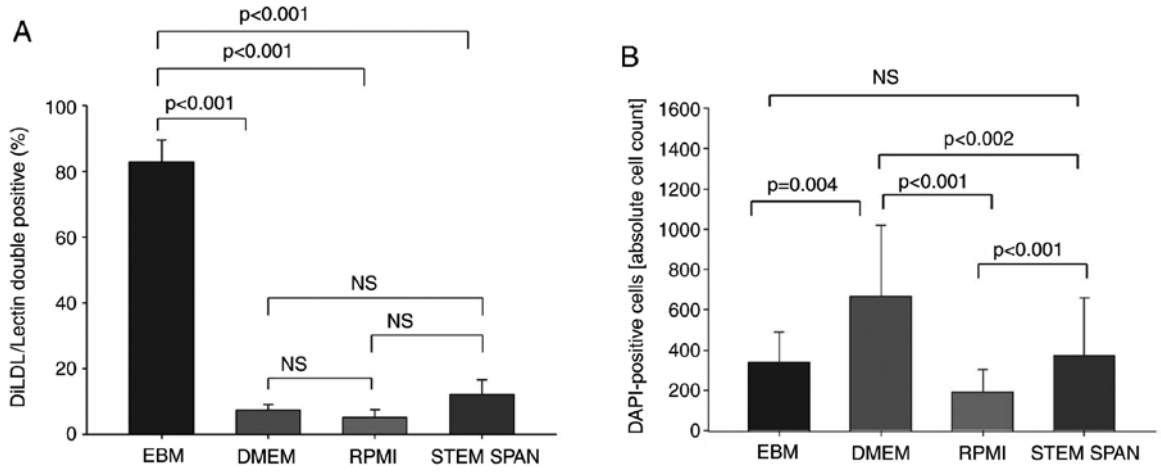

Figure 1. Analysis of PBMNC growth capacity in different media to determine optimized culture conditions for subsequent expansion. (A) PBMNCs remained undifferentiated with respect to classical endothelial staining with DiLDL/Lectin when cultured in DMEM or RPMI in contrast to EBM (P<0.001) or StemSpan® (ns). (B) Growth capacity of PBMNCs in different media showed that PBMNCs expanded significantly more when cultured in DMEM as compared with cultures with EBM, RPMI or StemSpan® (left panel). Moreover, DMEM-cultured PBMNCs displayed significantly higher proliferation rates (right panel). Importantly, PBMNCs remained undifferentiated with respect to classical endothelial markers when cultured in DMEM or RPMI in contrast to EBM or StemSpan®. PBMNCs, peripheral blood mononuclear cells; EBM, endothelial basal medium; ns, not significant.

used to analyze for normal distribution and compared with the one-way ANOVA test, which was also used for comparisons of categorical variables, and the Bonferroni post hoc test was used. The null hypothesis was rejected at P<0.05 as it was considered to indicate a statistically significant difference.

Results

Growth capacity of PBMNCs in different media. First, growth capacity of PBMNCs was analyzed in different media in order to determine optimized culture conditions for subsequent expansion. After four days in culture with DMEM or RPMI, PBMNCs did not differentiate into any endothelial-specific cell-types such as EPCs as defined by double-positivity in the DiLDL/Lectin staining (Fig. 1A). However, PBMNCs expanded significantly into EPCs when using endothelial-specific media such as EBM or, to a lower extent, in stem cell-specific media such as StemSpan®. In contrast to EBM, RPMI, and StemSpan®, cells expanded significantly superior (DAPI-positivity, Fig. 1B) and had a higher proliferation rate as assessed by Ki67-staining when cultured in DMEM (Fig. S1A and B). Therefore, DMEM supplemented with specific growth factors was used for subsequent differentiation experiments.

Identification and characterization of EPCs. Formation of vascular tubes was measured by matrigel assay (BD Biosciences) as instructed by the manufacturer. After four days in culture, the growth morphology of EPC colonies was characterized by the formation of spindles and clusters. Closed network units were quantified by counting in four adjacent wells. $CD34^-CD133^+$ EPCs formed significantly more CFUs per well than $CD34^+CD133^+$ cells (Fig. S2A). EPCs were then defined by binding of FITC-labeled *Ulex europaeus*-lectin and the uptake of DiI-Ac-LDL (Fig. S2B). Double-positivity of EPCs (yellow-merged images) was analyzed by immunofluorescence staining and characterized most of the EPCs. These results confirm our data from previous studies (6,9).

Cell separation of EPC subpopulations. Next we compared MACS® Cell separation vs. EasySep® Cell separation, by analyzing the purity of obtained cells in terms of the distinct EPC-subpopulations ($CD34^+CD133^+$ and $CD34^-CD133^+$). Both methods show a nearly equal purity for cell separation of $CD34^+$ cells (Fig. 2). In contrast to this, purity of $CD133^+$ cells was significantly higher using EasySep® (P<0.001; Fig. 2, right panel). Among these cells, $CD34^-CD133^+$ EPCs were reliably gained to a higher extent using the EasySep® Cell separation method when compared with the MACS® Cell separation method (P<0.001; Fig. 2).

Further characterization of EPC subpopulations. FACS analysis of the endothelial markers CD31 (PECAM) and VEGFR-2 revealed them to be present on both $CD34^-CD133^+$ and $CD34^+CD133^+$ cells, but to a significantly higher extent (P<0.001) on $CD34^+CD133^+$ cells (Fig. S3). The monocytic surface marker CD14 was significantly higher expressed on $CD34^+CD133^+$ cells (P=0.002; Fig. S4), whereas the lymphocytic markers In contrast, CD3 expression was significantly higher on $CD34^-CD133^+$ cells (P<0.001; Fig. S4).

Proliferation capacity of EPC subpopulations. Staining of EPC subpopulations with the proliferation marker Ki67 was significantly higher in cell cultures originating from $CD34^-CD133^+$ EPCs compared to cell cultures originating from $CD34^+CD133^+$ (P<0.005; Fig. S5).

Apoptosis and Necrosis in EPC subpopulations. The apoptosis marker Annexin-V was detectable to nearly the same extent in both subpopulations (P=0.469; Fig. S6A). In contrast, the necrosis marker Propidium-Iodide was detectable in a significantly higher amount (P=0.005) on $CD34^+CD133^+$ cells compared to $CD34^-CD133^+$ cells (Fig. S6B and C). Representative FACS plots of $CD34^-CD133^+$ and $CD34^+CD133^+$ cells as well as of apoptosis (lower right section) and necrosis (upper right section) in $CD34^+CD133^+$ cells are presented in Fig. S6C and D, respectively.

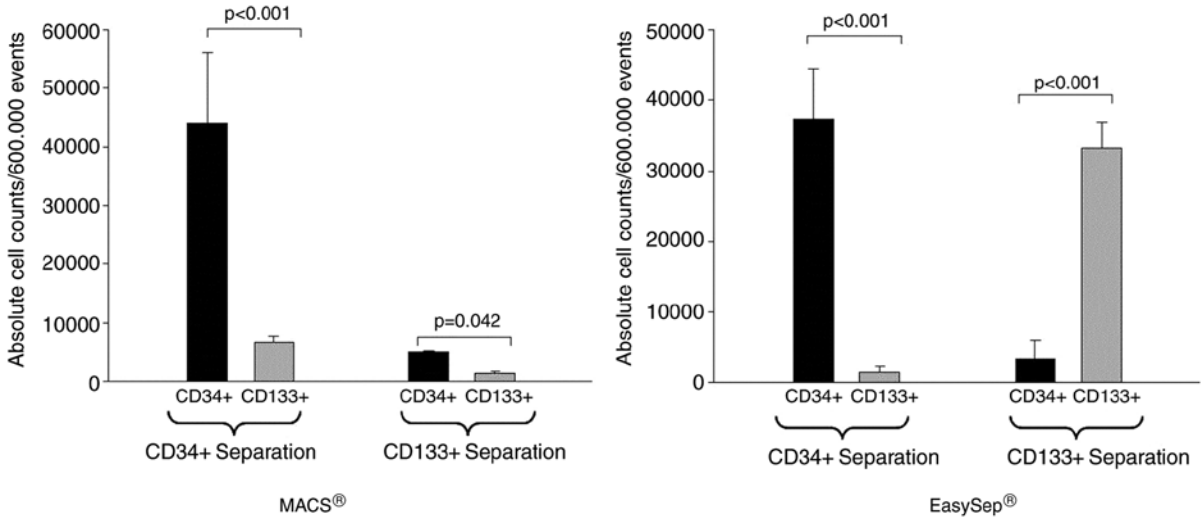

Figure 2. Comparison of the two different microbead-based cell separation procedures (MACS® cell separation compared with EasySep®) investigating purity of cell separation by fluorescence activated cell sorting analysis. Both separation methods showed a nearly equal purity for cell separation of $CD34^+$ cells. In contrast, the purity of $CD133^+$ cells was significantly higher using EasySep® ($P<0.001$).

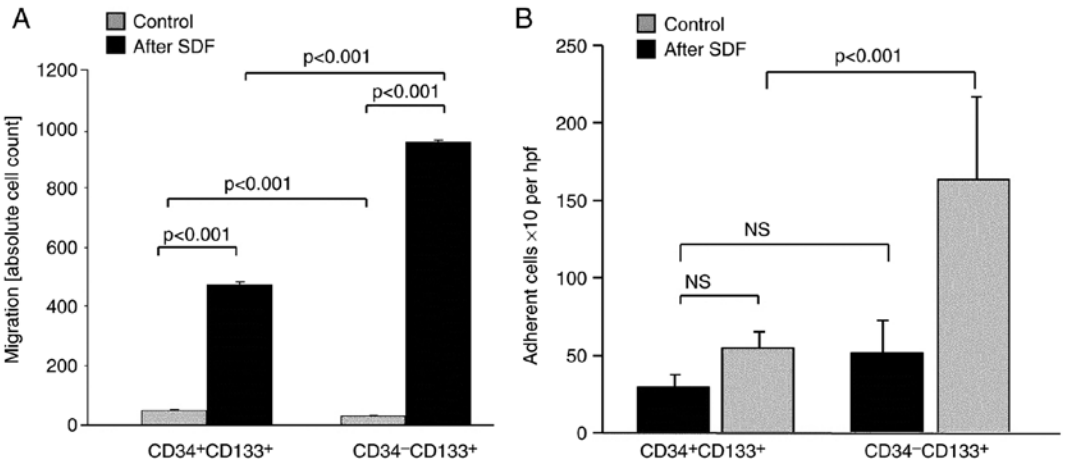

Figure 3. Migration and dynamic adhesion of the indicated endothelial progenitor cell subsets in response to SDF-1 (100 nmol/l) were analyzed using modified Boyden chambers and a parallel-plate, laminar-flow chamber, respectively. (A) Migration was microscopically quantified (magnification, x10) after staining with DiLDL. A significant increase was detected after stimulation with SDF-1 in both $CD34^+CD133^+$ cells and $CD34^-CD133^+$ cells (both black panels) compared with unstimulated cells ($P<0.001$). Importantly, SDF-1-triggered migration was significantly higher in $CD34^-CD133^+$ cells compared with $CD34^+CD133^+$ cells ($P<0.001$). (B) Under physiological flow conditions (shear stress 2 dynes/cm^2), addition of SDF-1 significantly increased dynamic adhesion of $CD34^-CD133^+$ cells ($P<0.01$), while it had no significant effects on $CD34^+CD133^+$ cells. SDF, stromal cell-derived factor; ns, not significant.

Migration capacity of EPC subsets. Assays of SDF-1-triggered migration of EPC subsets revealed than $CD34^-CD133^+$ EPCs display a higher migratory capacity than $CD34^+CD133^+$ EPCs ($P<0.001$, Fig. 3A; Fig. S7, top panel).

Adhesion capacity of EPC subsets. Then, adhesion assays of EPC subsets were performed under static and dynamic conditions. Under static conditions, $CD34^-CD133^+$ EPCs cells display higher levels of SDF-1-triggered static adhesion as compared with $CD34^+CD133^+$ EPCs ($P<0.027$; Fig. S7, bottom panel). These findings could be verified under dynamic adhesion conditions using a flow chamber (Fig. 3B).

Ex vivo differentiation capacity of EPC subpopulations. Compared to $CD34^+CD133^+$ cells, the $CD34^-CD133^+$ progenitor subset showed a significantly higher differentiation capacity into unrelated cell-types such as fibroblasts, hepatocytes, cardiomyocytes, and neurocytes. Prior to these experiments, positive controls for the specific staining of each cell type were performed (Fig. S8).

Fibroblast cell-type. Differentiation of $CD34^-CD133^+$ EPCs (30%) into a fibroblast cell-type could be detected after two days in culture in contrast to $CD34^+CD133^+$ cells (2%) ($P<0.001$, Fig. 4A; Fig. S9). After four days, 71% of $CD34^-CD133^+$ cells were positive for the fibroblast-marker FGF in comparison to only 20% of the $CD34^+CD133^+$ cells (Fig. S9). After eight days in culture, $CD34^-CD133^+$ cells were significantly more positive than $CD34^+CD133^+$ cells, but after twelve days both subpopulations were nearly completely differentiated into fibroblast-like cells (95% vs. 97%; Fig. 4A).

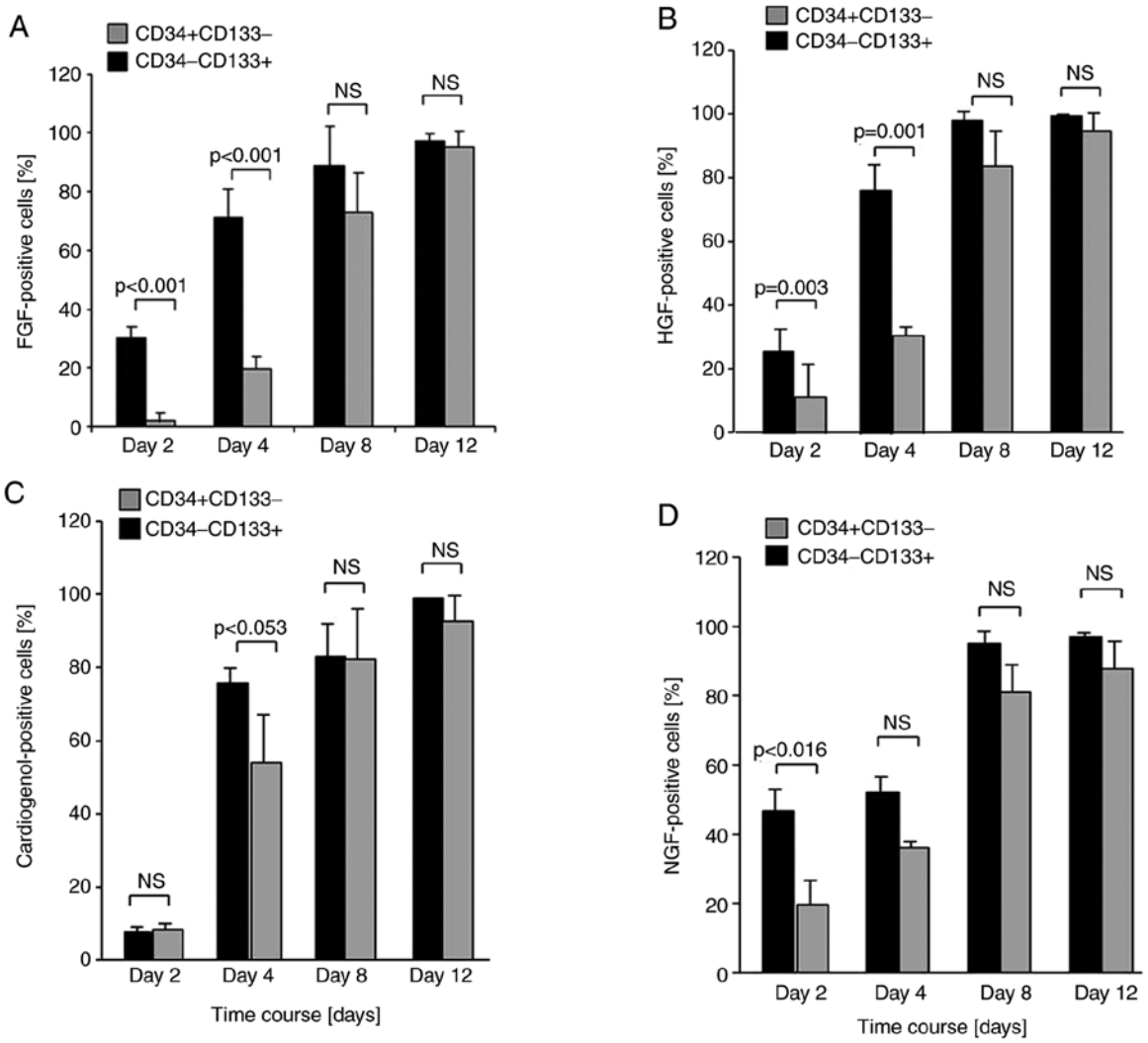

Figure 4. *Ex vivo* differentiation capacity of $CD34^{-}CD133^{+}$ (black panel) and $CD34^{+}CD133^{+}$ (grey panel) EPCs into different cell-types. (A) Fibroblast cell-type: After 2 days of culture, 30% of $CD34^{-}CD133^{+}$ cells stained positive for the fibroblast-marker FGF compared to $CD34^{+}CD133^{+}$ cells with 2%. After 4 days, 71% of $CD34^{-}CD133^{+}$ cells were FGF-positive in comparison to only 20% of the $CD34^{+}CD133^{+}$ cells. After 8 days in culture, $CD34^{-}CD133^{+}$ cells were significantly more FGF-positive than $CD34^{+}CD133^{+}$ cells, but after 12 days both subpopulations were nearly completely differentiated into fibroblast-like cells (95% compared with 97%). (B) Hepatocyte cell-type: After two days of culture, 26% of $CD34^{-}CD133^{+}$ differentiated compared to $CD34^{+}CD133^{+}$ with 10%. After 4 days, the disparity between both cell types increased (77% compared with 26%; P=0.001). In the following differentiation period into HGF-positive cells up to 12 days, no significant difference could be detected between the two EPC subsets (P=0.204 and P=0.442). (C) Cardiomyocyte cell-type: Differentiation took place after 4 days and showed a trend towards a difference between $CD34^{-}CD133^{+}$ and $CD34^{+}CD133^{+}$ cells, but to no significance (P=0.053). In the subsequent 8 days differentiation into cardiomyocyte-like cells was equal in both subpopulations ending up in 93 and 99% (ns; P=0.193), respectively. (D) Neural cell-type: After 2 days in culture, $CD34^{-}CD133^{+}$ cells were significantly more positive for NGF-staining in contrast to $CD34^{+}CD133^{+}$ cells (P=0.016). The difference between the EPC subsets was not traceable anymore in the following culture period up to day 12, when up to 95-97% of the cells were neurocyte-like in both subpopulations. EPC, endothelial progenitor cell; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; NGF, nerve growth factor; ns, not significant.

Hepatocyte cell-type. Differentiation capacity into hepatic-like cells was significantly increased in $CD34^{-}CD133^{+}$ EPCs in contrast to $CD34^{+}CD133^{+}$ cells after two days being in culture (P=0.003, Fig. 4B; Fig. S9). After four days, the disparity between both cell types increased (77% vs. 26%, P=0.001, Fig. 4B; Fig. S9). In the following differentiation period into HGF-positive cells, no significant difference could be determined (P=0.204 and P=0.442).

Cardiomyocyte cell-type. Differentiation into a cardiomyocyte cell-type could be detected after four days and showed a non-significant difference between $CD34^{-}CD133^{+}$ and $CD34^{+}CD133^{+}$ cells (P=0.053, Fig. 4C; Fig. S9). In the subsequent eight days, differentiation into cardiomyocyte-like cells was equal in both subpopulations ending up in 93 and 99% (P=0.193; Fig. 4C).

Neural cell-type. After two days in culture with β-NGF, $CD34^{-}CD133^{+}$ cells were significantly more positive for NGF-staining in contrast to $CD34^{+}CD133^{+}$ cells (P=0.016, Fig. 4D; Fig. S9). The difference was not traceable anymore in the following four to twelve days, when finally 95-97% of the cells were neurocyte-like in both subpopulations (Fig. 4D).

Endothelial cell-type. Growth into endothelial cells was significantly higher for $CD34^{-}CD133^{+}$ cells after four days of culture (P=0.080; Fig. S10), and highly significant after eight days (P<0.001; Fig. S10). After twelve days, both subpopulations,

$CD34^-CD133^+$ and $CD34^+CD133^+$ cells, were positive for VEGF in 97-99%. Additionally, endothelial staining using DiLDL/Lectin revealed a higher amount of double-positivity in $CD34^-CD133^+$ EPCs compared to $CD34^+CD133^+$ cells (Fig. S2).

Discussion

To our knowledge, our data is the first to indicate that $CD34^-CD133^+$ and $CD34^+CD133^+$ EPCs can transdifferentiate into other cell-types such as hepatocytes, fibroblasts, and neuronal cells under specific selection pressure. Moreover, our data present novel evidence that the $CD34^-CD133^+$ EPC subpopulations is characterized by a significantly faster differentiation capacity than $CD34^+CD133^+$ EPCs. This might be important to rapidly tailor differentiation of $CD34^-CD133^+$ EPC according to the local need of the injured tissue. In a recent study (26), co-culturing EPCs with VEGF-secreting mesenchymal stem cells (MSCs) enhanced endothelial marker expression (CD31, von Willebrand factor), which points to a VEGF-mediated role for MSCs in EPC differentiation. Clearly, more studies are needed to further discriminate the complex network of regulators of EPC differentiation including paracrine factors as well as stem and progenitor cells.

In mice with hind limb ischemia, co-administration of endothelial colony-forming cells (ECFCs) with MSCs significantly increased vessel density and foot perfusion by a CD105 (endoglin)-dependant mechanism (27). Interestingly, endoglin was found to play a critical role in integrin-mediated adhesion of mural cells to endothelial cells in mice (28). We found that $CD34^-CD133^+$ EPCs display significantly higher adhesion capacity to TNFα-activated HUVECs and to fibronectin-coated surfaces in response to SDF-1 than $CD34^+CD133^+$ EPCs. The cell-cell adhesion activity of $CD34^-CD133^+$ EPCs and $CD34^+CD133^+$ EPCs towards pericytes/vascular smooth muscle cells as well as the role of endoglin in that respect will have to be analyzed in future studies.

The EPC family consists of diverse members who are characterized by different functional potential and have been implicated in several pathologies. In that respect, $CD133^+$ from the bone marrow (BM) were shown to contain more $VEGFR^+$ cells, a higher distribution of primitive progenitors, and a higher proliferation activity than the $CD34^+$ BM population or the corresponding mobilized peripheral blood cells (29). Our findings shed more light onto these data by showing that $CD133^+$ cells consist of $CD34^+$ and $CD34^-$ subopulations, which are characterized by specific adhesive, migrative, and transdifferative potentials. In patients with congestive heart failure, numbers of $CD34^+$, $CD133^+$, and $CD34^+CD133^+$ cells in peripheral blood are regulated differently (30). In endarterectomized tissue from patients with chronic thromboembolic pulmonary hypertension, a putative EPC subpopulation of $CD34^+CD133^+$ fetal liver kinase-1^+ (flk-1) cells could be identified (31). In patients 6 months after coronary stent implantation, subpopulations of $CD34^+CD133^+$, $CD34^+$ human $VEGFR^+$, and $CD34^+CD133^+$ human $VEGFR^+$ EPCs inversely correlated with plaque volume and plaque area (32). In patients with acute ischemic stroke, elevated inflammatory parameter levels negatively correlated with circulating $CD133^+VEGR2^+$ EPCs (33). These studies as well as our data underscore the ongoing debate on the exact definition of EPCs, which is even more complicated by the diversity of markers used and the regulation of EPCs in health and disease.

Moreover, genetic factors may be of importance in EPC regulation as a recent study reported increases of $CD34^+$ and $CD34^+VEGFR2^+$ EPCs and diverse subsets in individuals with at least one specific polymorphism allele of KLOTHO KL-VS in comparison with individuals with wild-type alleles (34). Besides this, obese teenagers were shown to have higher levels of $CD34^-$ EPCs than $CD34^+$ EPCs which correlated with elevations of systolic blood pressure, hsCRP, HbA1c, and lower HDL levels (35). In conclusion, in overweight adolescents $CD34^-$ EPCs may serve as markers for vascular injury and may point to increased cardiovascular risk.

In summary, immature $CD34^-CD133^+$ EPCs are characterized by a particularly high capacity of differentiating into various kinds of non-endothelial cell types under specific selection pressure and according to the actual need. The significant higher capacity of proliferation of $CD34^-CD133^+$ EPCs may make it possible to gain higher numbers of EPCs, thus possibly increasing the potential for therapy with EPCs.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

CB performed the experiments. KB analysed and interpreted the data. EF supervised the study. KB and EF designed the current study and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The local ethics committee (Ethik-Kommission der Ärztekammer des Saarlandes) approved all investigations (compliance no. 122/09), which were carried out as specified by institutional guidelines. All volunteers provided written informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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