Human CD133+ Progenitor Cells Promote the Healing of Diabetic Ischemic Ulcers by Paracrine Stimulation of Angiogenesis and Activation of Wnt Signaling

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Abstract—We evaluated the healing potential of human fetal aorta–derived CD133+ progenitor cells and their conditioned medium (CD133+ CCM) in a new model of ischemic diabetic ulcer. Streptozotocin-induced diabetic mice underwent bilateral limb ischemia and wounding. One wound was covered with collagen containing 2×10^4 CD133+ or CD133− cells or vehicle. The contralateral wound, covered with only collagen, served as control. Fetal CD133+ cells expressed high levels of wingless (Wnt) genes, which were downregulated following differentiation into CD133− cells along with upregulation of Wnt antagonists secreted frizzled-related protein (sFRP)-1, -3, and -4. CD133+ cells accelerated wound closure as compared with CD133− or vehicle and promoted angiogenesis through stimulation of endothelial cell proliferation, migration, and survival by paracrine effects. CD133+ cells secreted high levels of vascular endothelial growth factor (VEGF)-A and interleukin (IL)-8. Consistently, CD133+ CCM accelerated wound closure and reparative angiogenesis, with this action abrogated by coadministering the Wnt antagonist sFRP-1 or neutralizing antibodies against VEGF-A or IL-8. In vitro, these effects were recapitulated following exposure of high-glucose-primed human umbilical vein endothelial cells to CD133+ CCM, resulting in stimulation of migration, angiogenesis-like network formation and induction of Wnt expression. The promigratory and proangiogenic effect of CD133+ CCM was blunted by sFRP-1, as well as antibodies against VEGF-A or IL-8. CD133+ cells stimulate wound healing by paracrine mechanisms that activate Wnt signaling pathway in recipients. These preclinical findings open new perspectives for the cure of diabetic ulcers. (Circ Res. 2009;104:1095-1102.)

Key Words: ischemia ■ wound healing ■ diabetes ■ stem cells ■ angiogenesis

Chronic wounds represent a relevant clinical and socioeconomic burden, with diabetic foot ulcers alone causing costs of 300 million pounds per annum to the United Kingdom National Health System.1 Diabetic patients with foot ulcers associated with peripheral vascular disease manifest the worst outcome, with higher amputation and mortality rates than patients carrying nonischemic ulcers.2,3 Although the efficacy of a topical gel formulation of recombinant human platelet-derived growth factor-BB was recently demonstrated in patients with nonischemic neuropathic ulcers,4 most ischemic ulcers are refractory to conventional treatment and growth factor (GF) therapy.5 Therefore, new strategies for the cure of life-threatening ischemic ulcers are urgently awaited.

Preliminary evidence supports the potential of adult or fetal stem/progenitor cells for the healing of skin ulcers.6–8 However, because of the lack of an appropriate preclinical model, no information is available regarding the effectiveness of cell therapy on ischemic diabetic foot ulcers. The healing activity of stem cells is credited to their ability to transdifferentiate into the vascular and nonvascular components of injured tissue, as well as to secretion of GFs, which may activate endogenous modulators of angiogenesis in the recipient.9–11 Notably, fetal stem cells show significant advantages over their adult counterparts in terms of proliferative capacity, engraftment kinetics, and differentiation plasticity. Fetal stem cells abundantly express CD133, an antigenic marker associated with high clonogenic potential and asymmetrical divi-

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sion; both of these are typical “stemness” features. In a non-diabetic murine ischemic hindlimb model, we recently reported that transplantation of a low number of CD133+ human fetal aorta–derived vascular progenitor cells promotes reparative neovascularization and skeletal myocyte regeneration, thereby supporting limb salvage. We also showed that fetal CD133+ cells release large amounts of vascular endo-
thelial growth factor (VEGF)-A. VEGF-A is a potent stimulator of the phosphatidylinositol 3-kinase–protein ki-
nase B (Akt) pathway, which exerts proangiogenic and prosurvival effects through, among others, phosphorylation/activation of endothelial nitric oxide synthase and phosphor-
ylation/inactivation of glycogen-synthase kinase 3β and fork-head box O (FOXO) transcription factors. Expanding our
present knowledge of stem cell action, we analyzed the role of Wingless (Wnt) gene products, which have previously been implicated in stem cell self-renewal. Wnt proteins of the canonical pathway bind to Frizzled (Fz) receptors, which then form a complex with the coreceptor LRP (LDL receptor–related protein). Through several cytoplasmic relay com-
ponents, the signal is transduced to β-catenin, which enters the nucleus to modulate the expression of target genes. The
noncanonical pathway is independent of LRP/β-catenin and encompasses the Wnt/Ca2+- and Wnt/planar cell polarity
pathways. Wnts play a key role in embryonic vasculogenesis, by modulating the expansion of primitive VEGF recep-
tor 2–positive vascular progenitor cells, as well as in postnatal angiogenesis. In addition, Wnt/β-catenin signaling is implicated in physiological and pathological wound cicatrisation.

Here, we used a newly developed mouse model of diabetic ischemic foot ulcer to study the therapeutic activity of fetal
CD133+ cells and their conditioned medium. We furthermore elucidated mechanistic aspects of CD133+ cell action in the
setting of diabetic wound healing.

Materials and Methods

Human Fetal Cells

Aortas from 11- to 12-week-old human fetuses (n=15) were ob-
tained according to the ethical guidelines of the Network for
European CNS Transplantation and Restoration (NECTAR) as
described before. The experimental protocol was approved by the
ethics committees of the National Neurological Institute “Carlo
Besta” (Milan, Italy). CD133+ cells were generated from CD133+ cells by serum-induced differentiation, as previously reported. Cell-conditioned medium (CCM) was obtained from cultures of
2×105 cells/mL after 48 hours of incubation.

Animal Procedures

All procedures complied with the standards stated in the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory
Animal Resources, National Academy of Sciences, Bethesda, Md.
1996) and were covered by ethical approvals from the Italian
Ministry of Health and the United Kingdom Home Office. In 6- to
7-week-old male CD1 mice (Charles River Laboratories, Milan,
Italy, and Morgate, UK) diabetes was induced by streptozotocin (Sigma), as described. Persistence of glycosuria of ≥10 g/L was checked over the duration of the experiments.

Four weeks after diabetes induction, bilateral hindlimb ischemia was induced by ligation of the proximal end of femoral arteries. At the same occasion, full-thickness wounds were created in the thigh dorsal skin of both legs using a sterile 5-mm-wide biopsy punch. The wounds were covered with type I collagen (Sigma) alone or collagen containing 2×104 CD133+ or CD133- cells. In separate experiments, wounds were covered with Extracel–HP hydrogel (Tebu-Bio, Le Perray en Yvelines, France), containing undiluted CD133+ or CD133- CCM with or without the Wnt inhibitor secreted frizzled-related protein (sFRP)-1, or CD133+ CCM was applied together with neutralizing antibodies against VEGF, interleukin (IL)-6, or IL-8. Contralateral wounds were covered with hydrogel containing nonconditioned culture medium (NCCM). After surgery, animals were maintained in individual cages with food and water ad libitum and in a temperature and humidity-controlled environment. Clinical outcome was established by determining the rate of wound closure.

An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Results

Characterization of CD133+ and CD133- Cells

Progenitor cells derived from human fetal aorta, expressed CD133, CD117 (c-Kit), and CXCR4 but only low levels of mature endothelial markers, such as CD31 and CD146, as previously reported. Exposure to serum-induced differenti-
ation associated with the loss of CD133 and CD117 and the
acquisition of endothelial antigens (Online Figure I, A and B, in the online data supplement, available at http://circres. ahajournals.org). Gene expression analysis verified down-
regulation of several stem cell–associated genes during
serum-induced maturation (Online Table I).

Furthermore, differentiation of CD133+ into CD133- cells was associated with downregulation of Wnt4, Wnt5A, Wnt7A, Wnt7B, and Wnt10A and upregulation of sFRPs (Online
Figure I, C). Inversely, culture of CD133- cells in the presence of sFRP-1 caused a 25% reduction in CD133 expression (Online Figure I, D), without altering the abundance of CD31 (data not shown).

CD133+ Cells Accelerate Wound Closure in Diabetess

To assess the added effect of ischemia on diabetic wound
healing, limb wounds were produced bilaterally together with unilaterial femoral artery occlusion in streptozotocin-induced diabetic mice. Laser Doppler flowmetry confirmed an initial reduction of limb blood flow on the side of artery occlusion by 82%. Blood flow was still reduced to 52% as compared to the contralateral side on day 7. Wound closure was signifi-
cantly delayed in ischemic as compared to nonischemic wounds (Online Figure II).

Next, to study the impact of cell or medium administration on wound healing, we produced wounds and limb ischemia bilaterally. The right side was covered with plain collagen gel alone and served as internal control, whereas gel on the left side wound contained either 2×104 CD133+ or CD133- cells or the vehicle. Transplantation of CD133+ cells accelerated the rate of wound closure in streptozotocin-induced diabetic mice, whereas no effect was observed in groups given CD133- cells or collagen as compared with the contralateral side (Figure 1A and Online Figure III). Two-way ANOVA detected a treatment effect among groups (P<0.05), with no interaction between treatment and time. In addition, Bonferroni post test analysis revealed an improved clinical outcome in the CD133+ treatment group as compared with collagen or
CD133- cells. Neither cell therapy nor collagen accelerated hindlimb hemodynamic recovery (data not shown).

Capillarization was increased at days 3 and 7 in wounds transplanted with CD133+ cells as compared with CD133- cell- or collagen-treated ulcers (Online Figure IV and Figure 1B) but returned to levels comparable to CD133+ cells or collagen gel alone by day 14 (C) (n=12 mice per group). *P<0.05 vs collagen, +P<0.05 vs CD133+ cells. Bar=20 μm.

CD133+ Cells Mediate Wound Healing by Paracrine Mechanisms

Human cells derived from transplanted CD133+ or CD133- cells were rarely recognized in the wound granulation tissue harvested 3 days after transplantation (5.4±1.7 and 4.3±2.4 cells/mm², respectively, P=NS). Fluorescence-activated cell-sorting (FACS) analysis, as well as quantitative PCR of excised wounds, confirmed that only low numbers of cells remained in the wounds after 3 days, with no difference between cell groups (CD133+ donor cells/wound: 34±9 [FACS], 43±7 [quantitative PCR]; CD133- donor cells per wound: 36±11 [FACS], 18±6 [quantitative PCR]; P=NS).

We have previously shown that CD133+ cells produce large amounts of VEGF-A.13 By cytokine bead array, we verified this and identified additional proangiogenic factors, secreted at higher levels by CD133+ cells compared to CD133- cells. Highest levels were detected for IL-6 and IL-8 among interleukins, VEGF-A and granulocyte-colony stimulating factor (G-CSF) among GF, and monocyte chemoattractant protein-1 (CCL2) among chemokines (Figure 3). In accordance with these findings, administration of CD133+ CCM instead of cells supported wound closure, whereas CD133- CCM was ineffective (Figure 4A). The healing action of CD133+ CCM was associated with increased wound vascularization at 7 days as compared with wounds given CD133- CCM or NCCM (Figure 4B and 4D). However, no difference in capillary density was detected among groups at 14 days (Figure 4C).

CD133+ CCM Promotes Endothelial Cell Migration and Survival In Vitro

To gain insight into the mechanisms underlying CD133+ CCM-induced capillarization, we first performed a Matrigel-
based in vitro angiogenesis assay. Corresponding to in vivo capillary density data, CD133+ CCM potentiated human umbilical vein endothelial cell (HUVEC) network formation as compared to NCCM or CD133− CCM (Figure 5A).

Network formation, even in a simplified in vitro assay, relies on the interplay of several distinct cellular processes, which we addressed separately. First, we focused on directed migration of ECs, which is crucial for organized capillary growth. HUVECs migrated toward CD133+ CCM in gap closure (“scratch”), as well as in “transwell” chemotaxis assays (Figure 5B and 5C). Next, we analyzed apoptosis, which is typically activated in ECs from diabetic patients or after exposure to hyperglycemic (HG) culture conditions. Consistently, we found higher activities of caspases 3 and 7 in HG-cultured ECs, which was prevented by the addition of CD133+ CCM, but not CD133− CCM, to the HG medium (Figure 5D).

One critical nexus in the network controlling migration, proliferation, and apoptosis is governed by the protein kinase Akt. In the presence of CD133+ CCM, we detected higher phosphorylation states of Akt at Ser473, which has been described to be crucial for its activity (Online Figure VI, A).14 Accordingly, transfection of HUVECs with dominant-negative Akt reduced CD133+ CCM-mediated survival in HG conditions by 50% (P<0.05 versus green fluorescent protein–transfected cells, data not shown). Akt itself exerts its effects in part via the phosphorylation of endothelial nitric oxide synthase (associated with activation and increased generation of the endothelial survival factor nitric oxide [NO]) and via the forkhead transcription factor FOXO1 (associated with inactivation and blockade of its proapoptotic function). In the presence of CD133+ CCM, endothelial nitric oxide synthase and FOXO1 showed higher phosphorylation levels as compared to NCCM, a result that agrees with the above described overall prosurvival action of CD133+ CCM (Online Figure VI, B and C). We could not detect significant difference among treatment groups with regard to glycogen-synthase kinase 3β phosphorylation (Online Figure VI, D).

Paracrine Mechanisms Implicated in CD133+ CCM-Regenerative Action

As described above, we detected high levels of VEGF-A, IL-6, and IL-8 in the CCM of CD133+ cells and therefore suspected their involvement in the therapeutic and angiogenic action of CD133+ CCM. In vivo, neutralizing antibodies against VEGF-A or IL-8 inhibited the healing effect of CD133+ CCM, thus confirming the critical role of both factors in wound closure and capillarization, whereas capturing IL-6 did not affect CD133+ CCM-induced wound closure or capillarization (Online Figure VII, A and B). In in vitro scratch assays, neutralizing antibodies against VEGF-A, IL-6, or IL-8, however, failed to suspend the protective action of CD133+ CCM on HG-induced apoptosis (data not shown).

CD133+ Cells Activate Wnt Signaling In Vivo and In Vitro

Recent evidence suggests a link between the VEGF-A and Wnt pathways, with Wnt potentiating the susceptibility of
ECs to VEGF-A signals. The findings that CD133^+ cells express Wnt genes and release VEGF-A prompted us to investigate whether CD133^+ cells may paracrinally activate the Wnt signaling pathway in the recipient’s wounds.

To this aim, we screened for Wnt genes regulated by the treatment with CD133^+ cells in diabetic wounds. RNA was extracted from tissue collected at day 3 postwounding and subjected to quantitative RT-PCR. Wnt4, Wnt5A, Wnt5B, Wnt7A, and Wnt7B genes were present in all wound samples (data not shown). Interestingly, the expression of only Wnt7A was increased in CD133^+ cell–treated wounds as compared with either CD133^-cell– or collagen-treated wounds (Online Figure VIII).

Next, we evaluated the involvement of Wnt in promotion of angiogenesis by human progenitor cells. HUVECs cultured under HG conditions expressed Wnt2B, Wnt3, Wnt4, Wnt5A, Wnt9A, and, to a lesser extent, Wnt8B and Wnt16 (data not shown). Following exposure to CD133^+ CCM, we observed an upregulation of Wnt3, Wnt5A, and Wnt9A as compared with HUVECs exposed to CD133^- CCM or control NCCM (Online Figure IX). Importantly, addition of anti–VEGF-A antibodies to CD133^+ CCM prevented the induction of Wnt3, Wnt5A, and Wnt9A by CD133^+ CCM (data not shown), thus implying a role of VEGF-A in the modulation of Wnt expression.

To verify the importance of Wnt signaling in vivo, we next applied the Wnt antagonist sFRP-1 together with CD133^+ CCM onto diabetic wounds. Importantly, sFRP-1 abolished the facilitation of wound closure and reparative angiogenesis by CD133^+ CCM (Figure 6). Consistent with in vivo data, the supportive action of CD133^+ CCM on in vitro network formation by HUVECs was negated in the presence of sFRP-1 (Online Figure X). However, if sFRP-1 was added to NCCM, HUVEC network formation was facilitated, in agreement with results published before. Similarly, CD133^+ CCM-induced HUVEC migration was blunted by sFRP-1 (Online Figure XI). No effect of sFRP-1 on HUVEC survival in the presence of CD133^+ CCM under HG conditions was detected (data not shown). To elucidate further distinct Wnt signaling mechanisms mediated by CD133^+ CCM, we studied network formation, as well as gap closure and survival in HUVECs, in the presence or absence of Dkk-1. Dkk-1 inhibits canonical Wnt signaling by binding to LRP, which is thereupon removed from the membrane via kremen proteins. Dkk-1 tended to reduce HUVEC network formation facilitated by CD133^+ CCM (Online Figure X), whereas HUVEC gap closure mediated by CD133^+ CCM was not affected by addition of Dkk-1 (Online Figure XI).

In CD133^+ CCM-stimulated ECs, we performed a luciferase-based reporter assay to detect β-catenin–induced gene expression via binding to Tcf/Lef elements. Surprisingly, we could not find transcriptional activation in response to either CD133^+ or CD133^- CCM (Online Figure XI), thus suggesting the involvement of β-catenin-independent mechanisms in CD133^+ CCM-induced effects.

**Discussion**

Great enthusiasm has been generated by recent preclinical stem cell therapy trials on wounds created by punching the interscapular region of diabetic mice. In diabetic patients, lesions of this kind are caused by accidental nonadverted traumas and easily heal with rest and conventional treatment. Therefore, extrapolation of those promising results to the precarious situation of ischemic foot ulcers is premature. To recreate a situation analogous to the ischemic diabetic foot, we developed a new model, which consists of...
excisional full-thickness wounds in ischemic limbs of streptozotocin-induced diabetic mice. We verified that the association of diabetes and ischemia leads to a severe impairment in wound cicatrisation. In this model, transplantation of angiocompetent cells, derived from differentiation of peripheral blood mononuclear cells of healthy donors, were unable to facilitate wound closure (P. Madeddu, L. Barcelos, unpublished observation, 2008). We evaluated whether fetal progenitor cells, which previously proved to be therapeutically effective in a limb ischemia model,13 succeed where adult cells failed. Besides CD133, those fetal progenitors expressed stemness markers not shared by their CD133− progeny, such as TERT, Syk, and uPAR, which have previously been described to be involved in stem cell maintenance, angiogenesis, and vasculogenesis.31–35

Although fetal CD133+ cells express Wnt, under differentiation, they strongly downregulate Wnt4 and Wnt7A and upregulate of sFRP-1, sFRP-3, and sFRP-4. Interestingly, sFRP-1 was able to reduce CD133 expression in fetal progenitor cells without inducing the acquisition of mature endothelial markers. Given the complexity of signaling events governing differentiation, we would not expect the identical mimicking of CD133− cell phenotype by only the presence of this one Wnt antagonist. The reduction in CD133 expression, however, verifies maintenance of CD133+ identity in our model to strongly rely on Wnt signaling, as has been shown for other stem cells before,36 and suggests that Wnt antagonism may trigger initial phases of differentiation by overriding the negative control exerted by Wnt.37 Maintenance of stemness was relevant to the outcome of our preclinical trial: topical application of CD133+ cells onto ischemic diabetic limb ulcers accelerated healing, whereas CD133− cells were ineffective.

Impaired wound healing in diabetic patients results from multifactorial deficits, including inefficient reparative angiogenesis,26,38 as well as aberrant control of cell survival39; thus it may be clinically relevant that transplantation of fetal CD133+ cells restored reparative angiogenesis in murine diabetic ulcers through stimulation of EC survival, proliferation, and migration.

The therapeutic efficiency of cell therapy depends on the adequate recruitment of applied cells to the target tissue and their ability to produce substances capable of supporting the healing process.10 Using in vivo imaging methods, the extent of homing was shown to be rather low in most experimental and clinical studies (reviewed elsewhere40). Consistently, by 3 different assays, we showed a low rate of incorporation of CD133+ cells in diabetic wounds, which might result from the unfavorable environment at site of application. Our data do not exclude that vascular progenitor cells might directly support reparative angiogenesis under different circumstances, as supported by our previous observation that intramuscularly injected human fetal CD133+ cells were able to incorporate in limb muscle neovascularature.11 In view of the short persistence after application onto diabetic wounds, it is plausible that CD133+ cells exert favorable effects on healing through a burst release of remedial factors and/or stimulation of the sustained paracrine reaction of the tissue of the recipient, as reported previously for other progenitor cells.9

We specified a complex combination of cytokines and GFs produced by CD133+ cells. Similarities could be found with substances released by endothelial progenitor cells41; however, much higher concentrations were detected in the fetal CD133+ CCM, thus accounting for the greater healing potential of these cells. We found that CD133+ CCM elicited promigratory and prosurvival effects on human ECs. Neutralizing antibodies against VEGF-A, IL-6, and IL-8 reduced HUVEC migration but failed to suspend the protective action of CCM on high glucose–induced apoptosis (data not shown). The Akt-FOXO pathway seems to be implicated in the latter phenomenon because transfection of HUVECs with dominant-negative Akt reduced CD133+ CCM-mediated survival in HG conditions. Other factors that were found increased in CD133+ CCM, such as angiogenin, reportedly activate Akt-dependent survival pathway in ECs.42 Thus, the combination rather than a single released agent seems to be responsible for the overall effects of the CD133+ CCM on HUVECs. In vivo, cicatrisation promoted by CD133+ CCM seems to be mainly ascribed to VEGF-A and IL-8, as denoted by the use of neutralizing antibodies.

Wnts are potent regulators of stem cell fate and skin maintenance and regeneration,22 as well as angiogenesis.20,21 This study provides pioneering evidence linking stem cell paracrine action to activation of Wnt signaling in the host. CD133+ cells induced endogenous expression of distinct Wnt genes in vivo and in vitro. Therefore, it appears that besides expressing both canonical and noncanonical components of the Wnt family, CD133+ cells also selectively upregulate some of the Wnt genes that are constitutively expressed by ECs. The importance of these expressional changes is supported by our finding that stimulation of wound cicatrisation and neovascularization by CD133+ CCM is inhibited by the Wnt antagonist sFRP-1. These results were mirrored by in vitro functional assays, in which the same antagonist abrogated the stimulatory effect of CD133+ CCM on HUVEC migration and network formation.

At least 2 classes of Wnt antagonists have been reported.29 The first class includes sFRP-1, which acts as scavenger of Wnt. The second class encompasses members of the Dkk family. Dkk-1 binds Kremen and the coreceptor LPR5/6, which is essential for canonical signaling, but does not prevent Wnt from associating with Fz. Dkk-1 was not efficient as sFRP-1 in inhibiting EC migration and network formation stimulated by CD133+ CCM. By using a reporter assay, we could not demonstrate any change in β-catenin–induced transcription activity in HUVECs stimulated by CD133+ CCM. Altogether, these results indicate that the mechanisms implicated in CD133+ CCM-induced stimulation of capillary-like network formation involve the interaction of Wnt with Fz on ECs and support the emerging concept that noncanonical Wnt pathways play a role in angiogenesis.43

Our results indicate the participation of VEGF-A in the stimulatory effect of CD133+ CCM on Wnt expression in human ECs. Reciprocally, the Wnt signaling pathway was found to strongly upregulate VEGF-A and IL-8, thereby supporting angiogenesis.44,45 However, recent microarray analyses revealed the complexity of Wnt targetome, the primary level including proteolytic enzymes (eg, matrix
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metalloproteinase-7), transcription regulators (eg, c-Myc), and pathway regulators (eg, VEGF-A), the secondary level being either effectors (eg, the c-Myc target gene p21) or target pathways (eg, VEGF receptor tyrosine kinase pathway) and the tertiary level encompassing targets of the target pathways (eg, the VEGF target gene DSCR1) (reviewed elsewhere*). Exploring these mechanisms was beyond the focus of the present study.

In conclusion, this is the first study to demonstrate the efficacy and associated healing mechanisms of local therapy with CD133+ progenitor cells in a preclinical model of diabetic ischemic foot ulcer. The fetus-derived cells would be difficult to obtain for therapeutic applications. However, the finding that CD133+ CCM is also effective in stimulating wound cicatrization, together with the discovery that the healing effect is associated with activation of the Wnt signaling pathway in the host, may have important implications for the cure of the ischemic complications of diabetes. Fetal CD133+ cells might be used in the future as a "factory" of therapeutic substances. Alternatively, synthetic replica of the CCM could be produced to obviate ethical concerns surrounding the direct use of fetal stem cells.

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Disclosures

None.

References


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**Human fetal cells**

Aortas from 11- to 12-week-old human fetuses (n=15) were obtained according to the ethical guidelines of the Network for European CNS Transplantation and Restoration (NECTAR) as described before. The experimental protocol was approved by the ethics committees of the National Neurological Institute "Carlo Besta" (Milan, Italy). CD133\(^-\) cells were generated from CD133\(^+\) cells by addition of 2% FBS (Gibco, N. Y., USA) to the culture medium, as previously reported.\(^1,2\) Paired samples of CD133\(^-\) and CD133\(^+\) were derived from the same fetal aorta preparation. Cell-conditioned medium (CCM) was obtained from cultures of 200,000 cells/mL after 48 hours of incubation and kept frozen in small aliquots at -80°C until use.

**Animal procedures**

All procedures complied with the standards stated in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, National Academy of Sciences, Bethesda, Md, 1996) and were covered by ethical approvals from the Italian Ministry of Health and the UK Home Office. Six to seven week old male CD1 mice (Charles River Laboratories, Milan, Italy and Morgate, UK) were made diabetic by streptozotocin (STZ, Sigma), as described.\(^3\) Persistence of glycosuria ≥10 g/L was checked over the duration of the experiments.

Four weeks after diabetes induction, mice were depilated in their hindlimb area and underwent bilateral hindlimb ischemia by ligature and electro-coagulation of the...
proximal end of femoral arteries under anesthesia (Avertin, 880 mmol/kg, i.p., Sigma). At the same occasion, full thickness wounds were created in the thigh dorsal skin of both legs using a sterile 5-mm-wide biopsy punch. The wounds were covered with type I collagen (20 µl, Sigma) alone or collagen containing 2x10^4 CD133^+ or CD133^- cells. In separate experiments, wounds were covered with Exracel-HP hydrogel (Tebu-Bio, Le Perray en Yvelines, France), which allows for controlled growth factor delivery, containing undiluted CD133^- or CD133^+ CCM (10µL) with or without the Wnt inhibitor sFRP1 (5 µL, 1 µmol/L). In separate experiments, CD133^+ CCM was applied onto ulcers together with neutralizing antibodies against VEGF (5 µL, 1 µg/mL, R&D), interleukin 6 (IL-6) (5µL, 100ng/mL, R&D), or interleukin 8 (IL-8) (5µL, 10µg/mL, R&D). Contralateral wounds were covered with hydrogel containing non-conditioned culture medium (NCCM). After surgery, animals were maintained in individual cages with food and water ad libitum and in a temperature and humidity-controlled environment. Clinical outcome was established by determining the rate of wound closure. Laser Doppler perfusion image analysis (Perimed, Stockholm, Sweden) was performed to confirm and monitor limb ischemia.

Flow cytometry

Antigenic characterization of fetal aorta-derived cells was carried out using a FACS Calibur flow cytometer and the CellQuest software (BD Pharmingen). Cells were incubated with phycoerytrin (PE)-conjugated anti-CD133 (Miltenyi), Allophycocyanin (APC)-conjugated anti-CD117 (BD Pharmingen), APC-conjugated anti-CXCR4, or PE-conjugated anti-CD146 or anti-CD31 monoclonal antibodies (R&D systems).
Fluorescence-conjugated, isotype-matched IgG with the same dye were used (BD Pharmingen) was used as control.

**Serum and sFRP-1 induced differentiation**

Fetal aorta derived CD133\(^+\) were plated in triplicate in a 12 well plate (30,000 cells per well) with NeuroCult (Stem Cell Technologies, proliferation kit enriched with EGF 20 ng/ml and FGF 10 ng/ml). After 24 hours, either sFRP-1 (6 µg/mL) or serum (2% as a positive control for differentiation) or nothing was added to the medium. After 4 days of culture, pictures were taken in bright field microscope, cells were then detached using TryPLE (Invitrogen) and stained for analysis of CD133 and CD31 expression using a FACS Calibur flow cytometer.

**Quantitative RT-PCR analysis for differential gene expression between CD133\(^+\) and CD133\(^-\) cells**

Total RNA from undifferentiated and serum-differentiated fetal cells was isolated with TRIzol\textsuperscript{\textregistered} reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. RNA from each sample was reverse transcribed with random hexamer primers and Superscript III reverse transcriptase (Invitrogen) following the manufacturer’s instructions.

The Human Angiogenesis RT\textsuperscript{2} Profiler\textsuperscript{TM} PCR Array was performed in triplicate for each sample using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Data analysis is based on the ΔΔCt method with normalization of the raw data to housekeeping genes included in the array.
**Multiplex cytokine analysis**

The following cytokines were assayed by the use of the multiplex cytometric bead array CBA flex, BD Biosciences, Heidelberg, Germany: angiogenin, interleukin-10 (IL-10), macrophage inhibitory protein-1α (MIP-1 α/CCL3), interleukin-4 (IL-4), interleukin-7 (IL-7), interleukin-1β (IL-1β), monocyte chemoattractant protein-1 (MCP-1/CCL2), regulated upon activation normal T cell-expressed and -secreted cytokine (RANTES/CCL5), interleukin-3 (IL-3), interferon gamma-induced protein 10 (IP-10/CXCL10), interleukin-2 (IL-2), granulocyte colony-stimulating factor (G-CSF), vascular endothelial growth factor-A (VEGF-A), interleukin-5 (IL-5), granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-8 (IL-8/CXCL8), interleukin-12p70 (IL-12p70), monokine induced by interferon-gamma (MIG/CXCL9), interleukin-6 (IL-6), macrophage inhibitory protein-1α (MIP-1 β/CCL4), and interleukin-9 (IL-9). The test was performed and analyzed according to the manufacturer's instructions. In brief, 50 µL of premixed capture beads were mixed with 50 µL PE-detection reagent. After addition of 50 µL of the provided standards or sample (diluted 1:4) the mixture was incubated in the dark for 3 h at room temperature. After incubation the mixture was washed, centrifuged (at 200x g for 5 min) and the pellet was resuspended in 300 µL of wash buffer. The BD FACSCalibur flow cytometer was calibrated with setup beads and 8,500 events were acquired for each sample. Individual analyte concentrations were indicated by their fluorescence intensities (FL-2) and were computed by using the respective standard reference curve and BD CBA software 1.1.
Histological assessment

At animal sacrifice by an overdose of anesthetic, wounds and surrounding skin were removed and perpendicularly cut into two halves. One half was immediately frozen for molecular biology studies while the other was fixed in 4% buffered formalin solution and further processed for histology or immunohistochemical analyses.

Capillary profiles were recognized by immunohistochemical staining using rabbit von Willebrand Factor (vWF, 1:100, over night; Dako) followed by FITC- conjugated donkey anti-rabbit IgG (1:20; Jackson Immunoresearch). Capillary density was determined in a blind manner as described.\(^6\)

Proliferating capillary endothelial cells were detected by co-staining for rabbit MCM-2 (1:50; Santa Cruz), followed by TRITC- conjugated goat anti-rabbit IgG (1:50; Sigma) and vWF, as described above. Detection of DNA strand breaks as a measure of apoptosis was performed by using the TUNEL method and Biotin-16-dUTP followed by streptavidin-TRITC secondary antibody together with staining for vWF, as described above.

Detection of donor CD133\(^+\) and CD133\(^-\) cells in wounds

In paraffin-sections of wounds, human cell engraftment was identified using mouse mAb against human nuclear antigen (hNA; 1:50; Chemicon) followed by FITC-conjugated goat anti–mouse IgG (1:20, Sigma) staining.

Cultured human CD133\(^+\) and CD133\(^-\) cells were stained with the red fluorescent membrane tracer DiI and washed thoroughly immediately before transplantation. Two days after transplantation, wounds were excised, cut into small pieces, washed in HBSS.
digested for 30 min under agitation at 30°C in DMEM containing 0.2% collagenase II, washed in PBS/5mM EDTA and digested for another 10 min in PBS/2mMEDTA/0.25% trypsin. The reaction was stopped by adding in excess PBS/10% FBS and cells spun down at 300g for 10 min. Cells were then resuspended in PBS/1% BSA and passed through a 100µm filter. After another spin at 300g for 10 min, single cells were fixed in 100µL PBS/0.5% paraformaldehyde. Before FACS data acquisition, 100µL of counting beads were added to each sample according to the manufacturer’s instructions (Caltag). Approximately 180µL of each sample were acquired in a FACS Canto II (BD). For data analysis, counting beads were roughly identified by their small size in the FSC/SSC and the 2 separate bead types (A- and B-beads) further defined by their different FL2-fluorescence and side scatter (Online Figure XIII-A & B). Cells were first defined by their FSC/SSC characteristics. DiI+ cells were then identified by their high FL2 fluorescence (Online Figure XIII-B). Using the known number of beads per total sample (and therefore per wound), the number of DiI+ cells was calculated for each wound (Online Figure XIII-C). Right side wounds which had not received any cells served as negative controls.

Results of cell incorporation were confirmed by quantitative PCR for human genomic DNA within the mouse wounds. Briefly, genomic DNA and total RNA were isolated sequentially from excised murine wounds using the AllPrep DNA/RNA mini kit (Qiagen) according to the manufacturer’s instructions. The amount of human gDNA within each murine sample was detected by qPCR using specific primers against the most conserved region of the human Alu sequence (Forward: 5’-TGA GGC AGG CGA ATC GCT TGA A-3’, Reverse: 5’-GAC GGA GTT TCG CTC TTG TTG-3’). 45 cycles of 95°C (15s), 58°C (30s), 6
and 72°C (15s) were run in a LightCycler 480 (Roche) with fluorescence acquisition after each cycle. Quantification of human DNA in mouse tissue was based on a standard curve using serial dilution of human CD133⁻ cell gDNA equivalents, spiked with mouse gDNA, calculated according to assumption that one human cell contains 6.6 pg of gDNA. Using serial dilution, the method was tested to evaluate sensitivity up to one cell.

**Cell culture**

Human umbilical vein endothelial cells (HUVEC) were grown in EGM-2, containing 2% FBS (all Cambrex) and used between passages 3 and 6.

**Functional assays on cultured HUVEC**

**Apoptosis assay.** Subconfluent HUVEC (Cambrex) were harvested with trypsin/EDTA, seeded into 6-well collagen-coated plates at 4x10⁵ cells / well and incubated overnight to allow adhesion. Adherent cells were then incubated under high glucose concentration (30mM) in EBM-2 (Cambrex) for 72 h. HUVEC grown under normal glucose concentration (5.55 mM) were used as basal control. The effect of NCCM, CD133⁺ or CD133⁻ CCM (diluted 1:3) on high glucose-induced endothelial cell apoptosis was examined by the Caspase-Glo® 3/7 assay (Promega).

**Migration assays.** HUVEC migration towards CCM of CD133⁺ or CD133⁻ or NCCM was assayed with a modified 96-well Boyden chamber (Neuro Probe) using an 8-µm pore-size polyvinylpyrrolidone-free polycarbonate membrane (Neuro Probe). The membrane of a Boyden chamber was precoated with type I collagen (10 µg/mL in PBS, Sigma) at room temperature for 1 h or with fibronectin (100µg/mL in PBS, Sigma) at 4°C overnight, respectively, and then washed with PBS. Subconfluent HUVEC were starved
in EBM-2 (Cambrex), respectively, under high glucose (30mM) for 24 h. HUVEC were
detached with 0.02% PBS/EDTA, resuspended in EBM-2 (Cambrex), respectively, and
then placed in the upper chamber at 5,000 cells/well. The stimuli (NCCM, CD133+ or
CD133− CCM (diluted 1:5 in EBM-2) were added to the lower chamber. During all the
starvation and experimental period, HUVEC were incubated in EBM-2 containing 0.1%
FBS. The chambers were maintained at 37°C and 5% CO₂ for 4 h. The upper surface
adherent cells were removed by scrapping and the lower adherent cells were considered
to be migrated. The membranes were fixed with 100% methanol at room temperature for
5 minutes and then mounted in glass slides with Vectashield® mounting medium with
DAPI (Vector Laboratories). The numbers of stained nuclei were counted in five high-
power fields per each well under a fluorescence microscope.

In addition, the ability of CCM to stimulate HUVEC migration was evaluated in
the scratch assay. HUVEC grown to a confluent monolayer in 100µg/mL fibronectin / 0.5%
gelatin-coated 8-well slide chambers were starved in EBM-2 containing 0.1% FBS
(Cambrex) under high glucose concentration (30mM) for 24 h. A central scratch was
created by scraping cells away with a p1000 pipette tip. After removal of debris by
washing the wells with DPBS, cells were incubated with EBM-2 containing 2mM of
hydroxyurea (Sigma) to induce growth arrest in the presence of 1:5 diluted NCCM,
CD133+ or CD133− CCM. After 24h of incubation, cells were washed, fixed with
methanol and subjected to hematoxylin/eosin staining. Scratches were photographed at
4x magnification at 25%, 50% and 75% of the scratch length and distance between
migrating fronts was measured using the Image Pro Plus software (Media Cybernetics).
Scratches fixed and stained immediately after scratching served to calculate initial gap
width (0% closure). Briefly, length was calibrated using a 1mm size standard photographed in the same camera-microscope-setting. Migrating fronts were traced and average distances were calculated by the software (Online Figure XIV). Each condition was run in quadruplicate and the assay was repeated three times.

**In vitro angiogenesis assay.** Subconfluent HUVEC were incubated overnight with EGM-2 (Cambrex) plus 2% FBS containing NCCM, CD133⁺ or CD133⁻ CCM diluted 1:5 and then detached with trypsin/EDTA and resuspended in EBM-2 (Cambrex) plus 0.1% FBS containing NCCM or CCM diluted 1:5. The formation of network structures was assessed using the growth factor reduced Matrigel™ (BD Biosciences) thick gel method according to the manufacturer’s instructions. HUVEC were seeded at 3x10⁴ cells / well in 8-well slide chambers containing 1:5 diluted NCCM, CD133⁺ or CD133⁻ CCM in 100µL of Matrigel. The chambers were incubated at 37°C and 5% CO₂ overnight. The wells were then photographed under a phase-contrast inverted microscope at 4x and 10x magnification. For each condition, network extension was measured using the Image Pro-Plus software (Media Cybernetics) as recently described. Each condition was run in quadruplicate and the assay was repeated two times.

**Inhibition of signaling pathways.** The involvement of Wnt in the effects exerted by CD133⁺ CCM in HUVEC functional assays was evaluated by the use of the Wnt inhibitors sFRP-1 (recombinant bovine, 10 nmol/L; from Cecile Duplaa) or Dkk-1 (recombinant human, 250ng/mL; R&D Systems). NCCM, CD133⁺ or CD133⁻ CCM (diluted 1:5 in EBM-2) was mixed with sFRP-1 or vehicle 30min prior to the addition of the mixture to stimulate HUVEC in tube formation, migration and apoptosis assays.
separate experiments, HUVEC were pre-incubated with Dkk1 for 1h before adding NCCM, CD133⁺ or CD133⁻ CCM (diluted 1:5) as a stimulus.

In separate experiments of apoptosis and migration, NCCM, CD133⁺ or CD133⁻ CCM (diluted 1:5 in EBM-2) was mixed with anti-VEGF-A (500ng/mL), anti-IL-6 (20µg/mL) or anti-IL-8 (15µg/mL) neutralizing antibodies (R&D System) 30min prior to stimulation of HUVEC. Antibody final concentrations were 8- to 25-fold greater than the concentrations for 100% inhibition of the highest values of those cytokines in CCM.

In order to determine the involvement of Akt in the pro-survival action of CD133⁺ CCM, HUVEC were infected with Ad.DN-Akt before stimulation. In brief, HUVEC were seeded into white-walled 96-well culture plates at 3.5x10³ cells / well and, after overnight incubation to allow adhesion, cells were infected with either Ad.Lac-Z or Ad.DN-Akt at MOI of 250. After 6 hours, virus was removed and stimuli (NCCM, CD133⁺ or CD133⁻ CCM) were added in the presence of high glucose (30mM) in EBM-2 containing 0.1% FBS (Cambrex) for 72 h. HUVEC grown under normal glucose concentration (5.55 mM) were used as basal control.

**Western Blot analysis**

Subconfluent HUVEC were harvested with trypsin/EDTA, seeded into 6-well culture plates at 5x10⁵ cells/mL and incubated overnight to allow adhesion. Adherent cells were then starved in EBM-2 (Cambrex) under high glucose concentration (30mM) for 24 h and then stimulated with NCCM, CD133⁺ or CD133⁻ CCM diluted 1:5 in EBM-2 as indicated. Cells were washed with PBS and immediately resuspended in lysis buffer (50 mM Hepes pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 25 mM NaF, 5mM...
NaPPi, 1% Triton X-100, 1% NP-40, 0.25% sodium deoxycholate, protease and phosphatase inhibitors) on ice. Protein whole extracts (50 µg) were separated by SDS-PAGE and then transferred to PVDF membrane (Bio-Rad Laboratories). Membranes were probed with primary antibodies for phospho-Akt, total Akt, phospho-GSK3β, total GSK3β, phospho-eNOS, phospho-FOXO1, tubulin (all from Cell Signaling Technology), total eNOS (Santa Cruz Biotechnology) and total FOXO1 (Upstate), followed by secondary antibody horseradish peroxidase conjugated-anti-rabbit IgG (Amersham Bioscience; 1:5000).

**Quantitative RT-PCR for Wnt gene expression.**

Total RNAs was isolated using Tri Reagent (Euromedex) and DNase (Promega) treatment according to manufacturer’s instructions. 500 ng of total RNA were reverse-transcribed using M-MLV Reverse Transcriptase (Promega) as previously described.9 PCR was done using IQ SYBR Green supermix (Bio-Rad). An MJ Research Opticon and the following parameters were used for real time PCR: 95°C for 5 min followed by 35 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 15 s. Negative controls without RT were prepared in parallel for each RNA sample. All experiments were done in triplicate and target mRNA levels were normalized to expression of β actin in each sample and expressed as fold of indicated control samples (ΔΔCt method). Primers used were:

*Human primers:*

h Wnt2B

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<th>Primer 2</th>
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h Wnt3

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R  5’- CGTAGATGCGAATACACTCC -3’
h Wnt4  F  5’- AGGATGCTCTGACAAACATCG -3’
R  5’- TTACCTCACAGGAGCCTGAC -3’
h Wnt5A  F  5’- AGCATCAGTCCACAAACAC -3’
R  5’- TCACCATTCCACAGGAGAG -3’
h Wnt 7A  F  5’- GCAGTGCAACTGTAAGTTCC -3’
R  5’- CCTCAGCAGAAAGACAAGGC -3’
h Wnt 7B  F  5’- CCTGGATCATGCACAGAAAC -3’
R  5’- CCTCCCCAATCACAATGATG -3’
h Wnt8B  F  5’- TCTCTTCTGCACAGCCTCCTC -3’
R  5’- TGGTGTTCAAGTTAGCACTCC -3’
h Wnt9A  F  5’- ACAGCAGCAAGTTCGTCGAG -3’
R  5’- TTGCCACCTCATGGAAAGG -3’
h Wnt10A  F  5’- CACACCCCTAAAACAAGCCTC -3’
R  5’- GAATGATGAGGGGAATGGTG -3’
h Wnt16  F  5’- TGGAGAGGTGTGAGTGTAAG -3’
R  5’- ATTCACACTGCAAGTAGCAAC -3’
h sFRP1  F  5’- TGCCCCTGCTCAACAAGAAC -3’
R  5’- AAGCCGAAAGACTGCATGAC -3’
h sFRP3  F  5’- AGCAGTGAAACGCTGTAATG -3’
R  5’- AATCTCTTCACCTCCACTAC -3’
h sFRP4  F  5’- GTAATCCCCCAAACAAAG -3’
**Supplemental Material**

Barcelos LS *et al.*

R 5'-TGTAAGGAAGTCGGAAGTCTC -3’

h cyclinD1 F 5’- CACACACACACACACACAC -3’

R 5’- GCCAAACAGGCTGAATCAATG -3’

**Murine primers:**

m Wnt4 F 5’- TTCACAACAAACGAGGCTGGCAG -3’

R 5’- CACCGTCAAACCTTCTCCTTTAGCG -3’

m wnt5A F 5’- GGCATCAAGGAATGCCAGTA -3’

R 5’- GTACGTGAAGGCCGTCTCTC -3’

m Wnt7A F 5’- GCTCCTATACCTTTTGCCCTTTACAG -3’

R 5’- GCCTCTTATCCAGTGGTTTCACG -3’

m Wnt7B F 5’- AAGATTTACTGGAGACCCCACGGC -3’

R 5’- CAGAAGAAGGACAAAAACCCAAGG -3’

β actin F 5’- GTTCCGATGCCCGAGGCTC -3’

R 5’- GCATTTGCGGTGCACGATGGA -3’

**Luciferase reporter assay**

For TOPFLASH reporter assays, MS1 cells were transfected in 24-well plates at 2x10^5 cells/cm^2 density with Super8XTOPFLASH luciferase reporter plasmid (375 ng/well) containing LEF/TCF consensus binding sites (Randal T. Moon laboratory). pRL-CMV (Promega) was cotransfected (90 ng/well) to normalize samples for transfection efficiency (Dual luciferase kit, Promega). Transfections were performed with Lipofectamine 2000 (Invitrogen) reagents according to the manufacturers’ recommendations. After 24 hours, the medium was replaced with non conditioned
medium or with conditioned media obtained either from CD133+ or CD133− cells diluted 1:5 with medium containing 1% FCS and 0.5% BSA. Cells were harvested 24 h later, and luciferase activity was determined with a Turner Designs luminometer. The data are presented as the means ± SD of triplicate well measurements for one representative experiment.

**Statistical analysis**

Results are presented as the mean ± SEM. Wound closure data were analyzed using two-way ANOVA and the interaction between treatment and time was considered, followed by Bonferroni posttest analysis. For other data sets, comparisons were carried out using one-way analysis of variance (ANOVA) and differences between groups assessed using the Newman-Keuls post-test. A p-value less than 0.05 was considered significant.
**Supplementary Results**

**Supplementary Table I:** Changes in gene expression after differentiation of CD133⁺ cells into CD133⁻ cells, induced by serum addition to the medium.

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<th>Accession No</th>
<th>Gene Name</th>
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Online Figure Legends

**Online Figure I:** Characterization of studied cell populations (CD133+ and CD133− cells) verified loss of stem/progenitor associated markers (CD133, CD117, CXCR4) during serum-induced differentiation of CD133+ cells into CD133− cells (A & B). Furthermore RT-PCR revealed that Wnt genes, highly expressed in CD133+ cells, were downregulated in CD133− cells, while expression of Wnt antagonists sFRPs was induced (C). Addition of sFRP-1 to CD133+ culture medium resulted in the reduction of CD133 expression (D). Data are expressed as mean±SEM, n=3 per group; * P<0.05 vs. CD133+ cells. Grey areas in B and D mark the negative range determined by isotype control staining.

**Online Figure II:** Ischemia impedes wound closure in diabetic mice. Data are expressed as mean±SEM, n=10 per group; ***P<0.005 vs. non-ischemic.

**Online Figure III:** Photographic documentation of wounds covered by collagen gel alone, or collagen gel containing CD133+ or CD133− cells at days 0, 7 and 14.

**Online Figure IV:** CD133+ cells improve neovascularization in diabetic ischemic ulcers collected at day 3 post-transplantation. Representative immunohistochemical images showing higher abundance of von Willebrand factor (vWF) positive endothelial cells in wounds treated with CD133+ cells as compared with CD133- cells or collagen. The bar length corresponds to 20 µm.

**Online Figure V:** Representative micro-photographs showing the effect of CD133+ (A) and CD133− cells (B) on endothelial cell proliferation, as assessed by co-staining for MCM-2 (red fluorescence) and vWF (green fluorescence), in wounds harvested at 7 days from cell transplantation. Human cells identified by staining for human nuclear antigen
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(purple fluorescence) was rarely documented at 7 (C) but not at 14 days (D) post-transplantation of CD133^+ cells. Vessels were surrounded by erythrocytes (yellow fluorescence). Bar corresponds to 10µm.

**Online Figure VI:** Western blot analysis demonstrates higher phosphorylation levels of Akt (A), eNOS (B) and FOXO1 (C) in HUVEC after stimulation with CD133^+ CCM vs. control with NCCM. CD133^- CCM stimulated Akt phosphorylation to a lesser extent than CD133^+ CCM (A) and did not induce FOXO1 phosphorylation (C). GSK3β phosphorylation was not altered by CD133^+ or CD133^+ CCM (D). * P<0.05, ** P<0.01, *** P<0.005 vs. NCCM, ### P<0.005 vs. CD133^- CCM. Data are expressed as mean±SEM of triplicates of one representative experiment which was repeated three times.

**Online Figure VII:** Wound closure, accelerated by CD133^+ CCM, was hampered when VEGF or IL-8 were neutralized, but not after capturing of IL-6 (A). Furthermore, capillary density of wounds was not different between vehicle and CD133^+ CCM-treatment when VEGF or IL-8 had been neutralized, while CD133^+ CCM was still enhancing capillarization after elimination of IL-6 (B). HUVEC migration, as assessed by scratch assay, was stimulated by CD133^+ and, to a lesser extent, by CD133^- CCM. Neutralization of VEGF-A, IL-6, or IL-8 blunted the pro-migratory effect of CD133^+ CCM (C). Data are expressed as mean±SEM of triplicates of one representative experiment, which was repeated three times (C) or of n=7 animals per group (A&B). *** P<0.01, *** P<0.001 vs. NCCM, $P<0.05$ vs. plain CD133^+ CCM.
Online Figure VIII: Quantitative RT-PCR revealed increased Wnt7A mRNA levels in wounds treated with CD133⁺ CCM. Data are expressed as mean±SEM, n=5 per group.* P<0.05 vs. collagen, $ P<0.05$ vs. CD133⁺ cells.

Online Figure IX: Culture of HUVEC in the presence of CD133⁺ CCM increased expression of Wnt3, Wnt5A and Wnt9A, while CD133⁻ CCM did not influence Wnt expression as compared to NCCM. Non-quantitative (A) and quantitative PCR results (B) and are shown. Data are expressed as mean±SEM of triplicates of one representative experiment which was repeated two times. **P<0.01 vs. NCCM-treated HUVEC, $ $P<0.01 vs. CD133⁻ CCM-treated HUVEC.

Online Figure X: CD133⁺ CCM, and to a lesser extent also CD133⁻ CCM, facilitate HUVEC capacity to form network structures. While CD133⁻ CCM effect was independent of Wnt signalling, addition of Wnt antagonist sFRP1 to CD133⁺ CCM reduced its capacity to stimulate HUVEC network formation. Representative bright-field photographs are displayed in the lower panel. Data are expressed as mean±SEM of quadruplicates of one representative experiment which was repeated twice. *P<0.05, ***P<0.001 vs. NCCM without antagonists; $ $P<0.05, $ $$P<0.01$ vs. CD133⁺ CCM without antagonists.

Online Figure XI: CD133⁺ CCM, and to a lesser extent also CD133⁻ CCM, facilitate HUVEC gap closure, the effect of CD133⁺ CCM being reduced by sFRP-1. Representative bright-field photographs of gaps after hematoxylin/eosin staining are displayed in the lower panel. Data are expressed as mean±SEM of quadruplicates of one representative experiment, which was repeated twice. *P<0.05, ***P<0.001 vs. NCCM without antagonists; $ $P<0.05 and $ $$P<0.01$ vs. CD133⁺ CCM without antagonists.
**Online Figure XII:** CD133$^+$ and CD133$^-$ CCM did not activate β-catenin as assessed by luciferase reporter assay using a plasmid containing LEF-1/TCF binding sites. Lithium was used as positive control. ***P<0.001 versus NCCM.

**Online Figure XIII:** The number of DiI-labelled donor cells remaining in the wounds was assessed in digests by flow cytometry. For data analysis, first, type A and B counting beads were identified by their size in FSC/SSC (A) and FL2-fluorescence intensity/SSC (B). Cells were defined by their FSC/SSC characteristics and DiI$^+$ cells were subsequently identified by their high FL2 fluorescence (B). Using the known number of beads per total sample (and therefore per wound), the number of DiI$^+$ cells was calculated for each wound (C). Right side wounds which had not received any cells served as negative controls.

**Online Figure XIV:** Gap closure was calculated on images taken from hematoxylin/eosin-stained gaps 24h after scratching. Using Image Pro Plus (Media Cybernetics Inc.), migrating fronts were traced and average distances were calculated by the software.
References


Online Figure I

A

![Bar graph showing antigen expression in CD133+ and CD133- cells.](image)

B

![Flow cytometry analysis of CD133 and CD117 expression.](image)

C

![Western blots showing expression of Wnt4, Wnt5A, Wnt7A, Wnt7B, Wnt10A, sFRP1, sFRP3, sFRP4, and β-actin.](image)

D

![Flow cytometry analysis of CD133 expression with and without sFRP treatment.](image)
Online Figure II

![Graph showing wound area (percent of basal) over time (days)].

- **Ischemic**
- **Non-ischemic**

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<th>Time (days)</th>
<th>Wound Area (percent of basal)</th>
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<td>3</td>
<td>50</td>
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<td>7</td>
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Significance levels: ***
Online Figure III

day 0   day 7   day 14

collagen

CD133+ cells

CD133- cells
Online Figure IV

Collagen  CD133+ cells  CD133- cells
Online Figure V
**Online Figure VI**

**A**

- **kDa**
  - 60
  - 140

- **pAkt**
  - Akt

**B**

- **kDa**
  - 60
  - 140

- **peNOS**
  - eNOS

**C**

- **kDa**
  - 80
  - 140

- **pFOXO1**
  - FOXO1

**D**

- **kDa**
  - 46
  - 140

- **pGSK3β**
  - GSK3β

**Legend:**
- **NOCM**
- **CD133**
- **CD133**

**Statistical Analysis:**

- **PhosphoAkt / total Akt (arbitrary value)**
- **PhosphoNOS / total NOS (arbitrary value)**
- **PhosphoFOXO1 / total FOXO1 (arbitrary value)**
- **PhosphoGSK3β / total GSK3β (arbitrary value)**

- **Significance Levels:**
  - * (p < 0.05)
  - ** (p < 0.01)
  - *** (p < 0.001)
  - ### (p < 0.0001)
Online Figure VII

A

[Graph showing wound area over time for different treatments: NCCM, CD133^+CCM, CD133^+CCM + anti-VEGF-A, CD133^+CCM + anti-IL6, CD133^+CCM + anti-IL8. Each graph compares the wound area at times 0, 3, and 7 days.]

B

[Bar graph showing wound capillary density for NCCM and CD133^+CCM treated with different antibodies: anti-VEGF-A, anti-IL-6, and anti-IL8. Each bar is labeled with statistical significance symbols: *** for P < 0.001, ** for P < 0.01, * for P < 0.05, and $ for P < 0.001.]
Online Figure VII

C

Gap closure [%]

NCCM  |  CD133⁺ CCM  |  CD133⁻ CCM

+ anti-VEGF-A  |  ***  |  $  |  $

+ anti-IL-6  |  $  |  $  |  **$

+ anti-IL8  |  |  |  

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Online Figure VIII

![Graph showing Wnt7A mRNA expression in different conditions.

- Collagen
- CD133+ cells
- CD133- cells

The graph indicates a significant increase in Wnt7A mRNA expression in CD133+ cells compared to collagen and CD133- cells. Statistical significance is denoted by asterisks (*) for CD133+ cells and a dollar sign ($) for CD133- cells.]
**Online Figure IX**

**A**

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**B**

Gene product [normalized to β-actin, n-fold of NCCM]

- **Wnt3**
  - NCCM-treated HUVEC
  - CD133+ CCM-treated HUVEC
  - CD133- CCM-treated HUVEC

- **Wnt5A**
  - NCCM-treated HUVEC
  - CD133+ CCM-treated HUVEC
  - CD133- CCM-treated HUVEC

- **Wnt9A**
  - NCCM-treated HUVEC
  - CD133+ CCM-treated HUVEC
  - CD133- CCM-treated HUVEC

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Online Figure X

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Online Figure XI

Gap Closure [%]

NCCM | CD133^+ CCM | CD133^- CCM

+ Dkk-1 | + sFRP-1

Normalized to 100% for each condition.
Online Figure XII

Luciferase activity [n-fold vs. NCCM]

NCCM  NCCM  CD133⁺  CD133⁻  + LiCl  CCM  CCM

***
Online Figure XIII

Panel A: Specimen 001-DSL
- Left wound (cells)

Panel B: Specimen 001-DSL
- Right wound (control)

Panel C: Specimen 001-DSL
- Tube: DIL

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Online Figure XIV