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PHD-2 Suppression in Mesenchymal Stromal Cells Enhances Wound Healing

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Abstract

Background—Cell therapy with mesenchymal stromal cells is a promising strategy for tissue repair. Restoration of blood flow to ischemic tissues is a key step in wound repair, and MSCs have been shown to be proangiogenic. Angiogenesis is critically regulated by the hypoxia-inducible factor (HIF) superfamily, consisting of transcription factors targeted for degradation by prolyl hydroxylase domain-2 (PHD-2). The aim of this study is to enhance the proangiogenic capability of MSCs and to utilize these modified cells to promote wound healing.

Methods—MSCs harvested from mouse bone marrow were transduced with shRNA against PHD-2; control cells were transduced with scrambled shRNA (shScramble) construct. Gene expression quantification, Human umbilical vein endothelial cell (HUVEC) tube formation assays, and wound healing assays were used to assess the effect of PHD knockdown MSCs on wound healing dynamics.

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Results—PHD-2 knockdown MSCs overexpressed HIF-1 α and multiple angiogenic factors compared to control (* p <0.05). HUVECs treated with conditioned medium from PHD-2 knockdown MSCs exhibited increased formation of capillary-like structures and enhanced migration compared with HUVECs treated with conditioned medium from shScramble transduced MSCs (* p <0.05). Wounds treated with PHD-2 knockdown MSCs healed at a significantly accelerated rate compared to wounds treated with shScramble MSCs (* p <0.05). Histological studies revealed increased blood vessel density and increased cellularity in the wounds treated with PHD-2 knockdown MSCs (* p <0.05).

Conclusions—Silencing PHD-2 in MSCs augments their proangiogenic potential in wound healing therapy. This effect appears to be mediated by overexpression of HIF family transcription factors and upregulation of multiple downstream angiogenic factors.

Keywords

hypoxia-inducible factor (HIF); small hairpin RNA (shRNA); angiogenesis

Introduction

Bone marrow-derived mesenchymal stromal cells (MSCs) are attractive candidates for cell-mediated ischemic tissue repair. Because of their multipotency, MSCs have been tested in numerous disease models including acute myocardial infarction, peripheral vascular disease, and wound healing.¹⁻³ MSCs localize to areas of ischemia and tissue injury,⁴ a property that allows for tissue-specific engraftment. Furthermore, these cells are also believed to be immune privileged,⁵ making them attractive candidates for the development of both autologous and allogeneic cell-based therapies. Many studies have documented that MSCs contribute to the restoration of blood flow during ischemic events.^{6,7} However, several clinical trials testing administration of MSCs in ischemic diseases yielded equivocal results,⁸ suggesting that the endogenous growth factors produced by MSCs alone are insufficient to produce significant improvements in blood flow. In addition, the treatment of ischemic diseases with recombinant growth factors is limited by low biostability and risk of toxicity at high doses,⁹ often resulting in incompetent or “leaky” vasculature.¹⁰ Therefore, genetic modification of MSCs may allow further improvement of their therapeutic potential.

Restoring blood flow to the site of injured tissue is a prerequisite for mounting a successful healing response. At the molecular level, the angiogenic switch is regulated by hypoxia-inducible factor (HIF), a family of transcriptional activators that function as critical regulators of cellular adaptive responses to hypoxia. HIF-1 α is critical for expression of multiple angiogenic growth factors, cell motility, and recruitment of endothelial progenitor cells.¹¹⁻¹³ The importance of this pathway in wound healing has been demonstrated previously.^{14,15} HIF-1 α is normally targeted for degradation by prolyl hydroxylase domain-2 (PHD-2) protein, its main negative regulator.¹⁶ Several studies have shown that inhibition of PHD-2 recapitulates various cellular and physiologic responses to hypoxia including HIF-1 α stabilization, induction of hypoxia-inducible genes, stimulation of angiogenesis, and protection against metabolic stress.^{17,18} Other members of the PHD family were shown to play similar roles in regulating HIF-1 α , including PHD-1 and PHD-3.¹⁹⁻²²

Previous studies have shown that specific knockout of PHD-2 in endothelial cells affects cell proliferation, and that heterozygosity of PHD-2 in normal mouse endothelial cells influences vessel maturation and normalization in hypoxia.^{23,24} Furthermore, PHD-2 has been studied in wound healing and can increase wound healing capabilities,²⁵⁻²⁷ though there was augmentation of cells known to independently increase wound healing, such as MSCs. Recently, we showed that inhibition of PHD-2 alone via short hairpin RNA accelerated wound healing in diabetic mice and improved perfusion of ischemic hind limbs.²⁸

We hypothesized that the application of MSCs containing knocked down PHD-2 could have a synergistic positive effect on wound healing. In this study, we demonstrate that overexpression of HIF-1 α by silencing PHD-2 in MSCs markedly augments the cells' proangiogenic potential, and that the transplantation of such modified MSCs significantly enhances wound healing.

Materials and Methods

Isolation and Culture of MSCs

Bone marrow-derived cells were collected by flushing the femurs and tibias from 5-week-old C57BL/6 male mice (The Jackson Laboratory, Bar Harbor, Maine). Cells were cultured in DMEM medium (high glucose) supplemented with 10% fetal bovine serum (Gibco, Life Technologies, Grand Island, New York) and 1% penicillin/streptomycin (Gibco). After 48 hours, non-adherent cells were removed and fresh medium was added to the cells. Medium was changed every 2 or 3 days. Adherent spindle-shaped cells were further propagated.

Differentiation of MSCs

MSCs were cultured in osteogenic differentiation media composed of DMEM supplemented with 10% fetal bovine serum (FBS), 10 μ M glycerol β -phosphate, 0.25 μ M ascorbic acid (Sigma-Aldrich, St. Louis, Missouri), and 1% penicillin/streptomycin. After three weeks of differentiation, cells were stained with Alizarin red. For adipogenic differentiation, cells were maintained in adipogenic media (DMEM supplemented with 10% FBS, 10⁻⁷ M dexamethasone (Sigma-Aldrich), 10 mM β -glycerol phosphate, 50 μ M ascorbate-2-phosphate). After 10 days of differentiation in adipogenic media, cells were visualized by light microscopy to confirm lipid vesicle formation within cells.

Flow Cytometric Analysis of MSC Immunophenotype

Immunostaining was performed by incubation of MSCs with the following fluorescently conjugated monoclonal antibodies: CD45 (Pacific Blue), CD11b (phycoerythrin (PE)-Cy5), stem cell antigen-1 (Sca-1, PE-Cy7), CD29 (AF700), CD44 (allophycocyanin), and CD105 (PE). All antibodies were purchased from BD Biosciences (Bedford, Massachusetts). Passage 3 cells were trypsinized, washed, and then run on a Becton Dickinson-LSR Flow Cytometer (Becton Dickinson, Franklin Lakes, New Jersey). Data were analyzed using FlowJo software.

Lentiviral Vector Production and Cell Transduction

For shRNA knockdown of PHD-2 in MSCs, pGIPZ lentiviral plasmids expressing microRNA-adapted shRNA specific for PHD-2/green fluorescent protein (GFP) bicistronic constructs and a nonsilencing control were used (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). Lentiviruses expressing these shRNA constructs were generated in human embryonic kidney cells (HEK 293T) by transfecting these vectors together with the VSV-G envelope-encoding plasmid pMD.G, and packaging plasmid CMV R8.74 using calcium phosphate method. Supernatants were harvested 48 and 72 h post-transfection, pooled, passed through a 0.45 μ m filter, ultracentrifuged for 2 h 20 min at 19,200 rpm in an SW28 rotor, and used to infect passage 2 MSCs in the presence of 8 μ g/mL polybrene for 24 h. MSCs stably expressing the shRNAs were selected by incubation with puromycin (10 μ g/mL) for 7 days. Selection efficiency was controlled by monitoring green fluorescence expression associated with co-expression of GFP encoded by plasmids.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA from treated cells was extracted using the RNeasy Plus Mini Kit (Qiagen, Venlo, Limburg, Netherlands) and reverse transcribed by the TaqMan Reverse Transcription Reagents (Applied Biosystems, Life Technologies, Grand Island, New York). Real-time quantitative PCR was run on ABI Prism 7900HT Sequence Detection System (Applied Biosystems) using *Power* SYBR Green Master Mix (Applied Biosystems) as the detection method. PCR was performed by denaturing at 95°C for 15 min, followed by 40 cycles of denaturation at 95°C for 30 s and annealing at 60°C for 1 min. Data were normalized to β -actin. Reactions were run in triplicate. Primer sequences are listed in Supplemental Digital Content 1, See table, which shows the Gene Primer Sequences Used for Quantitative, Real-Time Polymerase Chain Reaction,.

Western Blotting

Western blotting was performed as previously described.²⁵

Vascular Endothelial Growth Factor (VEGF) Enzyme-Linked Immunosorbent Assay (ELISA)

Quantikine mouse VEGF ELISA kits (R&D Systems, Minneapolis, Minnesota) were used according to the manufacturer's protocol. Briefly, VEGF standards (1 to 1000 pg/mL) and samples were placed by pipette into wells coated with antibody specific for mouse VEGF. After a wash, an enzyme-linked polyclonal antibody specific for mouse VEGF was added to the wells. After a second wash, a substrate solution was added. The absorbance of standards and samples was measured spectrophotometrically at 450 nm with a wavelength correction set to 570 nm using a microplate reader. VEGF concentrations were calculated (in pg/mL) with the standard curve via four parameter logistic (4-PL) curve-fit model using GraphPad Prism (GraphPad Software, La Jolla, California), and then adjusted for protein concentrations. Total protein concentrations in each sample were determined by the Bradford assay (Bio-Rad Laboratories).

Angiogenesis Antibody Array

Proteome Profiler Mouse Angiogenesis Antibody Array (R&D Systems) was used according to the manufacturer's protocol. In brief, the nitrocellulose membranes were first incubated in blocking buffer for 1 h. A cocktail of biotin-labeled antibodies against different individual angiogenesis-related proteins was incubated with approximately 1 mL of conditioned media prepared from MSCs after normalization with equal amounts of total protein. The sample/antibody mixture was then incubated with the membrane overnight at 4°C. After multiple washes to remove unbound materials, the membranes were incubated with horseradish peroxidase (HRP)-conjugated streptavidin for 1 h at room temperature. After washing the membranes, the signals were detected by electrochemiluminescence (ECL) system (GE Healthcare). The array image was then semiquantitated by reverse image scanning densitometry (Adobe Photoshop CS4, Adobe Systems, San Jose, California).

In Vitro Angiogenesis Assay

Fifty thousand cells of transduced MSCs were plated in 1 mL of Endothelial Basal Media with 2% FCS. Conditioned media was collected two days later. Human umbilical vein endothelial cells (HUVECs) were purchased from Lonza (C2517A; Basel, Switzerland). Cells were maintained in culture in the supplier's recommended complete medium (EGM-2) at 37°C, 5% CO₂. Five to ten thousand HUVECs were plated in 100 mL of conditioned media on Matrigel (Millipore, Billerica, Massachusetts). Cells were incubated for 4 to 6 h at 37°C and were visualized by light microscopy. The amount of angiogenesis was quantified by counting the number of cells in branch point capillaries in five random fields per replicate.

Five thousand transduced MSCs were also co-plated with five thousand PKH26-labeled murine bEND.3 endothelial cells (American Type Culture Collection, Manassas, Virginia) on Matrigel. Ten thousand PKH26-labeled mouse bEND.3 endothelial cells were also plated alone under the same conditions. Experiments were performed in triplicates. Fluorescence microscopy was performed with a Leica DMI4000 B microscope, and the amount of tubulogenesis was quantified as described above.

Excisional Wound Model

All experiments were performed in accordance with the Stanford University Veterinary Department and approved by the Stanford University Institutional Animal Care and Use Committee (APLAC #21308).

C57BL/6 mice (female, 12-15 weeks old, The Jackson Laboratory) were housed five per cage in a 12-h light/dark cycle and provided *ad libitum* with standard food and water. Full-thickness excisional wounds extending through the panniculus carnosus were made on the dorsa of mice as previously described.^{25,29} For studies using genetically modified MSCs, each wound received 2.5×10^5 cells: 1.25×10^5 cells in 60 μ L of PBS injected subdermally around the wound at four injection sites and 1.25×10^5 cells in 20 μ L of growth factor reduced Matrigel (BD Biosciences) applied topically onto the wound bed. The animals were randomly divided into four treatment groups: MSCs transduced with PHD-2 silencing shRNA, MSCs transduced with scrambled shRNA, untransduced MSCs, and matrigel/PBS

vehicle only (n=6 to 10 wounds per group). Wound healing was analyzed as previously described.²⁵ The wet wound area was measured as a ratio to the inner diameter of the silicone ring, as a percentage of original wound by two independent blinded observers using large, full-scale images.

Histology, Immunohistochemistry Staining, and Evaluation

Animals were euthanized in accordance with institutional protocols and the wound tissue was excised. Tissue processing and immunofluorescence was performed as previously described.²⁵

Statistical Analysis

All data are expressed as mean $\pm$ SEM. Student's t test or Wilcoxon rank sum tests were used to determine significance. A p value < 0.05 was considered statistically significant.

Results

Immunophenotypic Characterization of MSCs

Bone marrow-derived MSCs are generally selected for by their characteristic spindle-shaped appearance and adherence to plastic, but these cells are also known to be multipotent and express characteristic cell surface receptors.³⁰ To verify the identity of MSCs, their differentiation ability was confirmed by culturing them in osteogenic or adipogenic differentiation media (See Figure, Supplemental Digital Content 2, which shows MSCs differentiate into bone and adipocytes. **(A)** Cultured in osteogenic media, MSCs differentiate into osteoblasts, with mineralization of extracellular matrix as indicated in red by alizarin red staining. **(B)** Cultured in adipogenic media, MSCs differentiate into adipocytes, which are indicated by lipid vesicles within the cells). Early passage bone marrow-derived MSCs (passage 3) were further defined through fluorescence-activated cell sorting (FACS) analysis. Results confirmed that cells lacked lineage cell markers CD45 and CD11b and strongly expressed typical surface markers including Sca-1, CD29, CD44, and CD105 (See Figure, Supplemental Digital Content 3, which shows the Flow cytometry characterizes cultured bone marrow-derived MSCs. MSCs were evaluated for the presence of markers CD11b, CD29, CD44, CD45, CD105, and Sca-1. Red curves represent cells incubated with antibody against the noted marker, whereas blue curves show unstained cells as control).

Characterization of PHD-2 Knockdown MSCs

The experimental design is outlined in (See Figure, Supplemental Digital Content 4, which shows PHD-2 silenced MSCs accelerate wound healing. For each round of experiments, bone marrow-derived MSCs were harvested from 40 C57BL/6 mice at 5 weeks of age. These cells were selected for their adherence to plastic and were expanded in culture over a period of 3 to 4 weeks. FACS analysis for cell surface markers confirmed the MSC immunophenotype. Cells were then stably transduced with plasmid via lentiviral transduction and used to treat excisional wounds. Wound healing kinetics were followed until full re-epithelialization). After isolation of bone marrow-derived MSCs from the tibias and fibulas of 5 to 6 week old male mice, cells were expanded in culture. Lentiviral-mediated transduction and expression of three different shRNA against PHD-2/GFP

bicistronic constructs in these MSCs was confirmed by real-time PCR. Quantitative real-time PCR data showed that stable transduction of constructs A and B in MSCs resulted in a 35-50% decrease in PHD-2 mRNA expression (Figure 1A). Western blot of the cell lysates demonstrated that transduction with construct A resulted in stabilization of HIF-1 α protein in normoxic conditions (Figure 1B). Thus, construct A was chosen to be used in subsequent experiments.

After it was determined that MSCs transduced with shRNA against PHD-2 (shPHD-2) overexpress HIF-1 α protein, the impact of PHD-2 silencing on VEGF secretion by the transduced cells was investigated using ELISA. It was confirmed that PHD-2 knockdown MSCs (shPHD-2 MSCs) led to a significant accumulation of VEGF in the supernatant compared to MSCs transduced with scramble constructs (shScramble MSCs) (Figure 1C). In addition, qRT-PCR demonstrated that VEGF-A and angiopoietin-1 (Ang-1), genes critical for angiogenesis, were significantly upregulated in shPHD-2 MSCs compared to shScramble MSCs (* p <0.05) (Figure 1D). The finding of elevated angiogenic gene expression was confirmed at the protein level using the angiogenesis antibody array. Multiple common angiogenesis-related proteins were overexpressed in the supernatant of the shPHD-2 MSCs compared to that of shScramble MSCs (Figure 1E).

Angiogenesis In Vitro

Given that PHD-2 gene silencing significantly increased the angiogenesis-related protein expression by MSCs, the effect of the modified stem cells on primary endothelial cells was determined using a short-term *in vitro* HUVEC assay. This assay monitors the extent of tube assembly in HUVECs plated on Matrigel in response to appropriate cues. As shown in Figure 2A, conditioned media from PHD-2 knockdown MSCs, in comparison to conditioned media from shScramble MSCs, significantly increased the number of branch points per field of view (* p <0.05), indicating elevated tubulogenesis.

To further assess whether PHD-2 knockdown MSCs are able to augment angiogenic activity, MSCs were co-cultured with bEND.3 mouse endothelial cells. Unlike primary endothelial cells, bEND.3 mouse endothelial cells lack the ability to organize on Matrigel and form only clumps of cells.³¹ However, when shPHD-2 MSCs were co-cultured with mouse endothelial bEND.3 cells, complex mesh-like structures formed in which the endothelial cells exhibited enhanced ability to migrate and organize into cord structures on Matrigel, in marked contrast to shScramble MSCs in co-culture with bEND.3 cells remaining as round cells (Figure 2B, panels a through c with quantification in panel d). Under fluorescence microscopy, transduced shPHD-2 MSCs that express the bicistronic GFP transgene were found organizing in concert with PKH26-labeled bEND.3 cells within the tubular structures, suggesting that there is a cooperative interaction between the two cell types that may lead to enhanced angiogenesis (Figure 2B, panel e).

PHD-2 Knockdown MSCs Enhance Wound Healing

A well-described, reproducible wound healing model that minimizes contraction of the murine wound was used to more closely mimic wound healing as it occurs in humans.²⁹ The *in vivo* experiments tested a cell-based gene therapy approach to plasmid delivery. Mice

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were divided into four groups: animals receiving shPHD-2 MSCs, shScramble MSCs, untransduced MSCs, or vehicle medium with PBS and Matrigel. There were no deaths or other adverse side effects from cell therapy. Wounds treated with PHD-2 knockdown MSCs exhibited significantly accelerated wound closure compared to wounds treated with shScramble MSCs, untransduced MSCs, and vehicle medium (* $p<0.05$) (Figure 3) (See Figure, Supplemental Digital Content 5, which shows PHD-2 knockdown MSCs enhanced wound healing. Full-thickness excisional wounds on wild type mice were treated with shPHD-2 MSCs, shScramble MSCs, untransduced MSCs, or PBS/Matrigel. Representative photographs of wounds in different treatment groups. Treatment with shPHD-2 MSC heals wounds significantly faster compared to control wounds. Note the shPHD-2 MSC-treated wounds (top row) were healed by Day 8).

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Histology confirmed increased angiogenesis in the wound bed. Vascular structures in the wound were visualized by fluorescent immunostaining specific for the endothelial marker CD31. The CD31 positive endothelial cells (red) lining the blood vessels were found in the highest density in wounds treated with PHD-2 knockdown MSCs compared with shScramble MSC- or sham PBS/matrigel-treated wounds (* $p<0.05$) (Figure 4A) (See Figure, Supplemental Digital Content 6, which shows Immunofluorescence for endothelial cells. Wound sections were stained with an anti-CD31 antibody and detected with Fluor 594 (red). Wounds from shPHD-2 MSC-treated mice had significantly higher vessel density compared with that from PBS/Matrigel-treated mice (* $p<0.05$) (Fig. 4A). Although a significantly enhanced wound healing rate in shPHD-2 MSCs compared to control treated wounds was demonstrated, the question remained as to whether the injected MSCs remained viable and active in the wound. To assess the engraftment of transduced MSCs into the wounded skin, we performed immunostaining for GFP on wounds treated with shPHD-2 MSCs. Day 14 wounds were chosen to study cell survival due to the fact that all groups were healed by two weeks post-wounding. In day 14 wounds, GFP positive PHD-2 knockdown MSCs (green) were mostly found scattered in the newly formed dermis (Figure 4B). The GFP positive MSCs frequently appeared in proximity to blood vessels but rarely contributed (Figure 4B). Hence, the enhancement of angiogenesis mediated PHD-2 knockdown MSCs appears to be mainly due to paracrine effects. Additional immunofluorescence studies of wounds treated with shPHD-2 MSCs confirm that MSCs in the wound bed at the time of wound closure are metabolically active, expressing PCNA (Figure 4C), and express α -SMA, which labels pericytes and myofibroblasts (Figure 4D).

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In addition to the increased vasculature, analysis of wounds taken at day 14 demonstrate that PHD-2 knockdown MSC-treated wounds have significantly enhanced cellularity with a densely populated cell layer in the wound bed compared to PBS/Matrigel-treated controls, indicating an enhanced proliferative response in the wound repair process elicited by the transplanted cells (Figure 5). During the proliferative and remodeling stages of wound repair, increased dermal cellularity is most likely attributed to a mixed population of fibroblasts and myofibroblasts.

Discussion

MSCs derived from bone marrow have been proposed as a promising cell-based therapeutic strategy for injury in various tissues including heart, ischemic limb, and skin. Our current study demonstrates that genetic modification of MSCs to suppress PHD-2 increases HIF-1 α and angiogenic growth factor release, which promotes an endothelial cell angiogenic response. Application of PHD-2 knockdown MSCs in cutaneous wounds leads to significant acceleration in the rate of closure, with enhanced cell infiltration and angiogenesis. Given that the wild-type mice heal at near maximal rate, the acceleration in the rate of closure in the wild-type mice by the application of PHD-2 silenced MSCs is a significant and promising finding.

Although our study shows that unaltered MSCs had little influence on healing, PHD-2 knockdown MSCs produce markedly enhanced angiogenesis and wound repair. Remarkably, relatively few PHD-2 knockdown MSCs (2.5×10^5 cells) applied to wounds generate the robust angiogenic response in the wound bed and significantly accelerate closure. Angiogenesis is a critical component of the wound healing process. Endothelial cells adjacent to wounds migrate, proliferate, and sprout within the granulation tissue.³² MSCs have been shown to be proangiogenic and produce a number of cytokines involved in vessel development.³³ Approaches toward enhancing the angiogenic properties of MSCs could significantly contribute to the repair of post-ischemic tissue injury. In this regard, recent approaches have included genetic modification of MSCs to express proangiogenic factors, including VEGF and Ang-1.³⁴ Our group has previously shown that depletion of PHD-2 in skin wound fibroblasts using a Cre-lox system results in accelerated wound closure and reduced ischemic injury.²⁷ We recently demonstrated similar results with silencing of PHD-2 by delivery of naked short hairpin RNA.²⁸ Finally, we have also found that increasing HIF-1 α or VEGF enhances wound healing.^{14,35} In the present study, rather than targeting a single growth factor, we have focused on PHD-2, the main regulator upstream to the HIF pathway. Silencing PHD-2 in MSCs by RNA interference induced the cells to stabilize HIF-1 α and to overexpress its multiple downstream angiogenic targets, including VEGF-A and Ang-1. This strategy may be more effective in delivering potent angiogenic factors that stimulate new vessel growth.

The cell-based therapeutic strategy presented in this study is promising not only for its potent angiogenic effects, but also due to the practicality of the approach. Using MSCs for cell therapy has great translational potential given the ability to easily harvest cells from a patient through bone marrow aspirate, genetically manipulate the cells, and re-implant the cells back into the same patient. Furthermore, the use of stem cells derived from adult tissue avoids political and ethical controversies associated with embryonic stem cell therapy.

Unfortunately, human MSC harvest involves a painful procedure that can result in donor site morbidity. Stromal/stem cells can be harvested from other, more convenient sources to be used for cell-based therapy. Adipose-derived stromal cells, for example, are isolated from the stromal vascular fraction of adipose tissue and represent another source of multipotent cells.³⁶ ASCs are easy to isolate in large numbers through a less invasive approach, lipoaspiration, which can be safely and repeatedly performed in the same donor. Liposuction

has maintained its position as one of the top five cosmetic procedures performed over the past decade,³⁷ and the aspirate is often discarded as medical waste. These cells share many of the same advantages that MSCs possess; they are thought to be immune privileged, release angiogenic growth factors, and home to areas of tissue injury and ischemia.³⁸ Recent studies show that ASCs possess 90% immunophenotypic homology to bone marrow MSCs;³⁶ therefore, the therapeutic strategy presented in this study may be applied to this more abundant and easily acquired cell source.

Furthermore, other methods of gene transfer, including non-viral approaches, such as minicircles, nanoparticulate vectors, and small molecules, may be utilized to decrease the risk of insertional mutagenesis and tumorigenesis, thereby allowing this approach to gain momentum as a promising clinical wound care strategy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Ang-1	angiopoietin-1
ASC	adipose-derived stromal cell
ECL	electrochemiluminescence
ELISA	enzyme-linked immunosorbent assay
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GFP	green fluorescent protein
HIF	hypoxia-inducible factor
HRE	hypoxia response element
HRP	horseradish peroxidase
HUVEC	human umbilical vein endothelial cell

MSC	mesenchymal stromal cell
OCT	optimal cutting temperature
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PE	phycoerythrin
PHD-2	prolyl hydroxylase domain-containing protein-2
qRT-PCR	quantitative reverse transcriptase-polymerase chain reaction
SDS	sodium dodecyl sulfate
shRNA	small hairpin RNA
siRNA	small interfering RNA
VEGF	vascular endothelial growth factor

Fig. 1.

Effect of PHD-2 knockdown on bone marrow-derived MSCs. **(A)** Quantitative real-time PCR of PHD-2 transcript expression in MSCs transduced with different lentiviral constructs (A through C). The results were normalized to the β -actin mRNA level, and shown relative to values from cells transduced with scrambled construct D. Constructs A and B showed statistically significant PHD-2 knockdown compared to shScramble ($*p < 0.05$). **(B)** Western blot of HIF-1 α in MSCs transduced with construct A, shRNA to PHD-2 compared to MSCs transduced with construct D, scramble shRNA under normoxic conditions. **(C)** VEGF expression as detected by ELISA in MSCs stably transduced with shPHD-2 or shScramble shows increased VEGF protein concentration in conditioned media from cells stably transduced with shPHD-2 ($*p < 0.05$). **(D)** qRT-PCR analysis of common angiogenic genes in MSCs stably transduced with shPHD-2 construct versus those transduced with shScramble. Results are shown as percent of levels compared to levels of shScramble MSCs ($*p < 0.05$). **(E)** Angiogenesis antibody array using conditioned medium from MSCs stably transduced with shPHD-2 or shScramble. Membrane images of antibody array treated with conditioned media of stably transduced cells (left). Specific proteins captured on the array are labeled 1 to 13. Semiquantitative profiles of proteins 1 to 13 on the angiogenesis antibody array using reverse image scanning densitometry (right). There was a significant increase in the

expression of multiple angiogenic proteins by shPHD-2 MSCs in comparison to shScramble MSCs (*p<0.05).

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Fig. 2.

PHD-2 knockdown MSCs enhance *in vitro* angiogenesis. **(A)** Representative photographs of human umbilical vein endothelial cells seeded onto Matrigel in conditioned medium from shPHD-2 MSCs or shScramble MSCs (left). Quantitative assessment of branch points (right). Conditioned medium from shPHD-2 significantly increased tube formation compared to controls (* $p < 0.05$). **(B)** Representative images of shPHD-2 MSCs versus shScramble MSCs in co-culture with bEND.3 endothelial cells on Matrigel (**a**, **b**, and **c**). shPHD-2 MSCs seeded with bEND.3 cells form complex mesh-like tubule structures in marked contrast to control cells that remain largely as poorly organized round cells (quantification shown, **d**). shPHD-2 MSCs express GFP transgene (green), which can be seen aligning with PKH26 dye-labeled endothelial cells (red) within the tubular structures (**e**).

Fig. 3.

PHD-2 knockdown MSCs enhanced wound healing. Full-thickness excisional wounds on wild type mice were treated with shPHD-2 MSCs, shScramble MSCs, untransduced MSCs, or PBS/Matrigel (Fig. S4). Wound closure rate over time is depicted in the graph. There is a significant acceleration of wound healing in the PHD-2 knockdown MSC treatment group (n=6) (*p<0.05).

Fig. 4.

shPHD-2-transduced MSCs increase blood vessel formation in the wound bed, contribute to newly formed blood vessels, and remain viable and metabolically active in the wound bed after complete closure. **(A)** Graph depicting immunofluorescence for endothelial cells. Wound sections were stained with an anti-CD31 antibody and detected with Fluor 594 (red) (Fig. S5). Software-assisted quantification of vessel density in the entire area of residual wounds at day 14, as percent fluorescence. Wounds from shPHD-2 MSC-treated mice had significantly higher vessel density compared with that from PBS/Matrigel-treated mice (* $p < 0.05$). **(B)** Engraftment of bone marrow-derived MSCs. Immunofluorescence of wounds treated with shPHD-2 MSCs shows that GFP expressing MSCs (green) were found in the dermis of residual wounds. MSCs are scattered in the dermis with little evidence for co-localization of CD31 positive blood vessels (red). Some MSCs appear intimately aligned with blood vessels (**arrow**). **(C)** Viability of engrafted MSCs. Immunofluorescence of wounds treated with shPHD-2 MSCs shows that GFP expressing MSCs (green) co-localizes with PCNA markers (red) indicating an active metabolic state of engrafted MSCs. **(D)** Engrafted GFP expressing MSCs (green) express the pericyte/myofibroblast marker α -SMA (red) as shown by co-localization study of wounds treated with shPHD-2 MSCs.

Fig. 5. H&E stains of wound tissue after full closure at 14 days post-wounding. PHD-2 knockdown MSC-treated wounds have higher dermal cellularity in the wound bed compared to PBS/Matrigel-treated control wounds. Black box indicates the area of healed wound depicted under high power view. Quantification of dermal cellularity is shown (right) (* $p < 0.05$).