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Overexpression of Hif-1α in Mesenchymal Stem Cells Affects Cell-Autonomous Angiogenic and Osteogenic Parameters

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ABSTRACT
Reconstruction of large bone defects still represents a major medical challenge. In recent years tissue engineering has developed techniques based on adult mesenchymal stem cells (MSCs) that could represent an attractive therapeutical option to treat large bone defects in the future. It has been demonstrated in various animal models that ex vivo expanded MSCs are capable of promoting the regeneration of skeletal defects after implantation. However, for the efficient regeneration of bone in tissue engineering applications, a rapid vascularization of implanted grafts is essential to ensure the survival of cells in the early post-implantational phase. A promising strategy to enhance vascularization of MSC-containing implants could consist of overexpression of the angiogenic master transcription factor Hypoxia-inducible factor 1 (Hif-1) in the MSCs in order to induce angiogenesis and support osteogenesis. In the present study, we overexpressed Hif-1α in MSCs by using recombinant adenoviruses and investigated cell-autonomous effects. Overexpression of Hif-1α enhanced proliferation, migration, cell survival and expression of pro-angiogenic genes. Other parameters such as expression of the osteogenic markers BMP-2 and RunX2 were decreased. Hif-1α overexpression had no effect on invasion, senescence and osteogenic differentiation of MSCs. Our experiments revealed multifarious effects of Hif-1α overexpression on cell-autonomous parameters. Therefore, Hif-1α overexpression may represent a therapeutic option to improve cellular functions of MSCs to treat critical sized bone defects. J. Cell. Biochem. 117: 760–768, 2016. © 2015 Wiley Periodicals, Inc.

KEY WORDS: Hif-1α; ADENOVIRUS; ANGIOGENESIS; OSTEOGENESIS; MIGRATION

Bone marrow derived mesenchymal stem cells (MSCs) have gained much interest in regenerative medicine [Caplan and Hariri, 2015]. These cells are capable of self-renewal and can differentiate into several types of mesenchymal tissues including cartilage, adipose tissue, and bone [Pittenger et al., 1999; Sotiropoulou et al., 2006]. Moreover, these cells are very attractive for tissue engineering applications because of their high ex vivo proliferation capacity and the low donor-site morbidity of the harvesting procedure [Wakitani et al., 1994]. It has been demonstrated in various animal models that ex vivo expanded MSCs are capable of promoting the regeneration of skeletal defects after implantation [Bruder et al., 1998; Gazit et al., 1999; Kon et al., 2000]. However, for the efficient regeneration of bone in tissue engineering applications, a rapid neovascularization of implanted grafts is essential to ensure the survival of cells in the early post-implantational phase [Shieh and Vacanti, 2005]. The long-term survival of engineered grafts is strongly dependent on oxygen delivery and nutrient exchange, functions, which are provided by the neovasculature. A promising strategy to enhance vascularization of implants containing MSCs could consist of overexpression of the proangiogenic master transcription factor Hypoxia-inducible factor 1 (Hif-1) in MSCs in order to induce an angiogenic response in the surrounding host tissue.

Hif-1 is composed of two subunits, the oxygen-dependent subunit Hif-1α and the constitutively expressed Hif-1β [Wang and Semenza, 1995; Wang et al., 1995; Jiang et al., 1996] which heterodimerize and bind to the so called hypoxia response element in promoters of several genes that influence angiogenesis, erythropoiesis and cell metabolism in response to tissue hypoxia [Huang et al., 2010]. Under normoxic conditions, prolyl hydoxylase is activated and hydroxylates proline residues 402 and 564 of Hif-1α thereby linking Hif-1α to the von Hippel-Lindau protein (pVHL), an E3 ubiquitin ligase. This leads to degradation of HIf-1α in the proteosome [Kallio et al., 1999; Maxwell et al., 1999; Ivan et al., 2001]. Under hypoxic conditions, Hif-1α hydroxylation is inhibited and Hif-1α accumulates in the nucleus where it forms a heterodimer with the Hif-1β subunit and activates several Hif-responsive pro-angiogenic genes such as VEGF [Kallio et al., 1998]. Hif-1α plays a very important role in several

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pathological conditions, such as tumor growth [Liao and Johnson, 2007]. If a solid tumor is not connected to the host vasculature, then there exists a balance between proliferating cancer cells at the margin of the tumor and apoptotic cancer cells in the center. Due to extreme hypoxic conditions in the center, Hif-1α is activated in central cancer cells, which leads to overexpression of paracrine-acting pro-angiogenic genes that induce ingrowth of adjacent blood vessels. Only after the solid tumor is vascularized, tumor growth and metastasis progression can occur.

Hif-1 also plays an important role in coupling osteogenesis and angiogenesis during skeletal development and bone regeneration. In this context, it was shown that deletion of pVHL in osteoblasts, resulting in increased Hif-1 activation, significantly improved bone regeneration and bone vascularization [Wang et al., 2007].

Therefore, a gene therapeutic approach to overexpress Hif-1α in MSCs could represent an attractive therapeutic option to improve vascularization and possibly also osteogenesis of MSC-seeded bone implants.

In the present study, we intended to overexpress Hif-1α in MSCs by using recombinant adenoviruses and to investigate putative cell-autonomous effects such as proliferation, migration, invasion, apoptosis, senescence, extracellular matrix production and expression of genes playing a role in angiogenesis and osteogenesis.

MATERIALS AND METHODS

CELL CULTURE

Human mesenchymal stem cells (MSCs) were isolated and expanded as described before [Mehlhorn et al., 2006]. In brief, mononuclear cells (MNCs) were purified by density gradient centrifugation with Biocoll Separating Solution (Biochrom AG, Berlin, Germany) from human bone marrow. MNCs were seeded in culture flasks at a density of 5×10^5 cells/cm^2 in expansion medium (alpha-MEM, 10% FCS, 50 μg/ml gentamicin, 5 ng/ml bFGF) at 37°C, 5% CO_2. The medium was changed twice weekly, removing all nonadherent cells. Once adherent cells had grown to confluence, they were detached and reseeded at a density of approximately 2000 cells/cm^2 and cultivated for two further passages. In our experiments, we used MSCs from five different donors. MSCs were pooled, cultivated for two further passages in standard medium (alpha-MEM, 50 μg/ml gentamicin, 10% FCS) and cryoconserved.

CHARACTERIZATION OF HUMAN MSCs

Flow cytomertic analysis were performed for the characterization of surface protein expression pattern. Cell surface markers CD105, CD90, CD73, CD45, and CD14 were analyzed using phycoerythrin (PE)-labeled monoclonal antibodies (abcam, Cambridge, UK) according to previously published protocols [Dominici et al., 2006]. MSCs expressed CD105, CD90, and CD73, while CD45 and CD14 were not expressed.

Hif-1α ELISA

MSCs were seeded in triplicate in 6-well plates at a density of 2×10^5 cells per well. The next day, cells were infected with Hif-1α or GFP control adenovirus (Cell Biolabs, San Diego) at a multiplicity of infection (MOI) of 1. Both adenoviruses (Hif-1α encoding virus and GFP control virus) contain a green fluorescent protein (GFP) open reading frame as a marker gene under control of a constitutive active CMV promoter. After infection, cells were incubated for the indicated time periods. Medium was changed every third day without addition of new adenovirus. At the indicated time points, cell lysates were prepared and total protein was quantified by using the BCA protein assay kit (Thermo Scientific, Rockford). Hif-1α protein in cell lysates was measured by using the Hif-1α human ELISA Kit (abcam, Cambridge, UK) according to manufacturer's instructions. Hif-1α protein content was normalized to total protein content and expressed as ng Hif-1α per mg total protein. Shown are mean values and standard deviations.

CELL PROLIFERATION ASSAY

MSCs were infected with Hif-1α or GFP adenovirus at MOI=1 and incubated for 48 h. Thereafter, cells were trypsinized and seeded in triplicates into 48-well plates at a density of 4×10^3 cells per well. After attachment of cells, medium was replaced by alpha-MEM, 50 μg/ml gentamicin containing 2% FCS. Cells were then incubated for the indicated time points. Medium was changed after 3 days. Cell numbers were determined by using the CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega, Heidelberg, Germany) according to manufacturer's instructions.

APOPTOSIS ASSAY

Apoptosis was assessed by quantification of fragmented DNA by ELISA (Cell Death Detection ELISA kit, Roche Diagnostics, Germany). This assay allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fraction of apoptotic cells. MSCs were infected with Hif-1α or GFP adenovirus at MOI=1 and incubated for 2 days. Thereafter, cells were seeded at 5×10^4 cells per well in 12-well plates in alpha-MEM, 50 μg/ml gentamicin containing either 0% or 10% FCS and incubated for 48 h. Thereafter, cells were directly lysed in incubation buffer and oligonucleosomes were extracted from the cytoplasmic fraction of the cells by incubation at room temperature for 30 min with vigorous shaking. The extracts were centrifuged for 10 min at 12000 rpm and the resulting supernatants were incubated with peroxidase-labelled anti-DNA antibody and biotinylated anti-histone antibody in streptavidin-coated microtiter plates following the manufacturer's instructions. After washing, peroxidase substrate ABTS (2,2′-Azino-di[3-ethylbenzthiazolin-sulfonat]) was added to develop and visualize binding of mono- and oligonucleosomal DNA. Microtiter-plates were analyzed at 410 nm using a microtiter plate reader.

MIGRATION AND INVASION ASSAY

MSCs were infected with Hif-1α or GFP adenovirus at MOI = 1 and incubated for 2 days. MSC migration and invasion was analyzed in 12-well transwell cell culture chambers by using a transparent membrane with pores of 8 μm (BD Biosciences, Heidelberg, Germany). For invasion assays, the membrane was coated overnight at room temperature with 100 μl of growth factor reduced Matrigel (500 μg/ml; BD Biosciences, Heidelberg, Germany). 5×10^4 MSCs were seeded in the upper chamber of the transwell cell culture system in alpha-MEM, 50 μg/ml gentamicin without FCS. The chamber was

inserted in a 12-well culture dish containing either alpha-MEM, 50 μg/ml gentamicin, 10% FCS or alpha-MEM, 50 μg/ml gentamicin, 0% FCS, 50 ng/ml VEGF.

Cells were incubated in the transwell chamber for 24 h at 37°C, 5% CO_2. Thereafter, non-migrated cells on the upper surface of the membrane were removed, the lower side of the membrane was washed with PBS and fixed with 4% paraformaldehyde. For quantification, cell nuclei were stained with DAPI (Invitrogen, Darmstadt, Germany). The number of MSCs that migrated to the lower side of the membrane was determined microscopically by counting 5 regions of interest (ROI) per membrane. The assays were run in triplicates. Shown are mean values and standard deviations.

SENESCENCE ASSAY

MSCs were infected with Hif-1α or GFP adenovirus at MOI = 1 and incubated for 2 days. Thereafter, cells were seeded in triplicates into 6-well plates at a density of 5×10^4 cells per well and incubated for further 3 days in alpha-MEM, 50 μg/ml gentamicin, 0% FCS. Cells were then washed with phosphate-buffered saline (PBS) and fixed with Fixing Solution (Cellular Senescence Assay, Merck Millipore, Darmstadt, Germany) for 15 min at room temperature. After washing with PBS, the cells were incubated at 37°C overnight with senescence-associated galactosidase detection solution (Cellular Senescence Assay, Merck Millipore, Darmstadt, Germany). Thereafter, cells were washed twice with PBS and cells expressing senescence-associated galactosidase were counted microscopically in each well.

QUANTITATIVE REAL TIME RT-PCR

MSCs were infected with Hif-1α or GFP adenovirus at MOI=1 and incubated for 7 days. TaqMan RT-PCR was carried out as previously described. [Medhurst et al., 2000]. Total RNA was prepared using RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Total RNA (3 μg) was treated with 3 units of deoxyribonuclease I (DNase I) (Invitrogen, Karlsruhe, Germany) to digest genomic DNA contamination. Random-primed cDNA synthesis was performed using 3 μg of DNase I-treated total RNA and 50 units of StrataScript reverse transcriptase according to the manufacturer's instructions (Stratagene, La Jolla, CA). TaqMan PCR assays were performed in 384-well optical plates on a LightCycler (Roche, Mannheim, Germany) using Absolute QPCR ROX Mix (Abgene, Hamburg, Germany) according to the manufacturer's instructions. Oligonucleotide primers and probes for human GAPDH (GADPH forward: 5′-TGGGCTACACTGAGCACCAG-3′; GAPDH reverse: 5′-CAGCGTCAAAGGTGGAGGAG-3′; GAPDH probe: 5′-FAM- TCTCCTCTGACTTCAACAGCGACACCC-TAMRA-3′) human bFGF (bFGF forward: 5′-AATCAAAAGTTCGGCATGTAGCT-3′; bFGF reverse: 5′-CTGAGCA-GGGCAGATTTGC-3′; bFGF probe: 5′-FAM-TTTCTATGTCGTGGAAGCACCG-GATGG-TAMRA-3′) human VEGF (VEGF forward: 5′-GCGCAAGAAATCCCGGTATA-3′; VEGF reverse: 5′-TGCTTTCTCCGCTCTGAGC-3′; VEGF probe: 5′-FAM-CCTGGAGCGTTC-CCTGTGGGC-TAMRA-3′) human OSF-2 (OSF-2 forward: 5′-CCCAGCAGTTTTGCCCATT-3′; OSF-2 reverse: 5′-ATAGCGCTGCGTTGTGGTG-3′; OSF-2 probe: 5′-FAM-TGGCA-CTCTGGGCATCGTGGG-TAMRA-3′) were designed using Primer Express (Applied Biosystems, Forster City, CA) according to company guidelines. Oligonucleotide primers and TaqMan probes for BMP-2, ALP, RunX2, PDGFR-alpha and PDGFR-beta were purchased from Applied Biosystems (Forster City, CA). The thermal cycling conditions were 95°C for 15 min followed by 50 cycles at 95°C for 15 s and at 60°C for 1 min. Data were analyzed using the relative standard curve method, with each sample being normalized to GAPDH to correct for differences in RNA quality and quantity. Results from three experiments are expressed as mean arbitrary units and standard deviations.

ALIZARIN RED STAINING

MSCs were infected with Hif-1α or GFP adenovirus at MOI = 1 and incubated for 2 days. Thereafter, cells were trypsinized and seeded in triplicates into 24-well plates (6×10^4 cells per well). Cells were incubated either in normal growth medium (alpha-MEM, 50 μg/ml gentamicin, 10% FCS) or in osteogenic differentiation medium (DMEM, 50 μg/ml gentamicin, 10% FCS, 10 mM beta-glycerolphosphate, 0.1 μM dexamethasone, 50 μM ascorbic acid) for 21 days with medium changes every third day. Alizarin staining and quantification was performed as described [Guo et al., 2011]. In brief, after washing twice with PBS, cells were fixed with 70% EtOH for 30 min at RT. After rinsing again, 1ml of 40 mM Alizarin Red S solution (Merck Millipore, Darmstadt, Germany) was added. Thereafter, cells were washed 4 times with PBS and extracellular matrix calcification was visualized under a phase-contrast microscope. For the quantification of calcium deposition, cells were incubated with 20% methanol/10% acetic acid for 30 min at RT to release the Alizarin Red S staining. The eluates were collected, transferred to a 96-well plate and measured at 450 nm using a microtiter plate reader.

STATISTICAL ANALYSIS

Values were analyzed for statistical significance by using an unpaired Student's *t*-test. Statistical significance was defined when $P < 0.05$.

RESULTS

Human MSCs were isolated from bone marrow and expanded. After two passages, MSCs were characterized via their surface marker expression according to the declaration of the *Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy* [Dominici et al., 2006]. MSCs were positive for CD73, CD90, and CD105, while CD45 and CD14 were not expressed (Fig. 1A). In order to overexpress Hif-1α in human MSCs, we have infected the cells with recombinant Hif-1α adenovirus or control GFP adenovirus. As evidenced by phase contrast and fluorescence microscopy, more than 90% of the cells were GFP-positive when infected with the Hif-1α or GFP adenovirus at a multiplicity of infection (MOI) of 1 (Fig. 1B).

In order to confirm Hif-1α protein overexpression in response to transduction of the cells with Hif-1α adenovirus, Hif-1α expression has been quantified in cell lysates by ELISA. As shown in Figure 1C, Hif-1α expression is dramatically higher at every time point in Hif-1α adenovirus infected MSCs as compared to GFP adenovirus

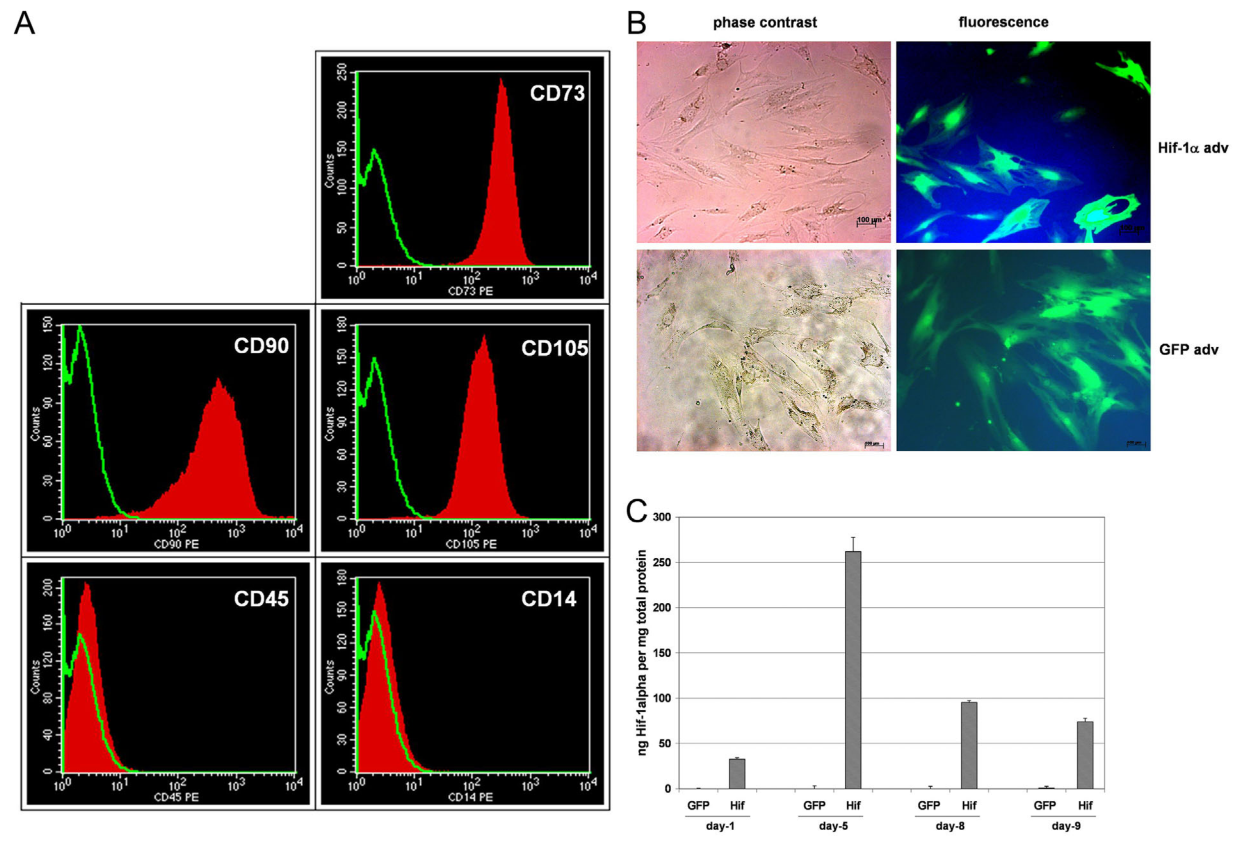

Fig. 1. Characterization of MSCs in terms of MSC marker gene expression (A), transduction efficiency (B) and Hif-1α protein levels (C). MSCs were analyzed by flow cytometric analysis for the presence of the classical MSC markers CD73, CD90, and CD105 and the absence of the hematopoietic markers CD45 and CD14 (A). Transduction efficiency of adenoviral infection was quantitated by determination of the ratio of GFP-positive cells in relation to total cell numbers 48 h after MSCs have been infected with Hif-1α or GFP control adenovirus at a MOI = 1. Shown are representative phase contrast and fluorescence images (B). Hif-1α protein levels in MSCs transduced with Hif-1α or GFP adenovirus were determined by ELISA. MSCs were transduced with GFP adenovirus or Hif-1α adenovirus and incubated for the indicated time periods. Hif-1α was measured by ELISA and Hif-1α protein was normalized to total protein. Shown are mean values ± standard deviations from three independent experiments (C).

infected cells, where Hif-1α was barely detectable. In Hif-1α infected cells, Hif-1α expression reaches its maximum at day 5 and then continuously declines until day 9.

In order to investigate the effect of Hif-1α overexpression on cell proliferation, MSCs were infected with Hif-1α or GFP adenovirus for 2 days. Thereafter, cells were replated and incubated under low-serum conditions (2% FCS) for 1, 3, and 7 days. As shown in Figure 2A, Hif-1α overexpression significantly increased MSC proliferation at day 3 (Hif: 1.189 ± 0.05 vs. GFP: 0.927 ± 0.109) and day 7 (Hif: 0.941 ± 0,0186 vs. GFP: 0.676 ± 0.079), demonstrating a growth stimulatory effect of Hif-1α in human primary MSCs.

Next, we intended to explore whether Hif-1α may be able to modulate low-serum induced apoptosis of MSCs. As shown in Figure 2B, serum reduction from 10% to 0% results, as expected, in increased apoptosis in GFP as well as in Hif-1α transduced cells. When cells have been growing under normal serum conditions of 10% FCS, Hif-1α overexpression significantly reduced the apoptosis rate (Hif: 0.2503 ± 0.069 vs. GFP: 0.4563 ± 0.059). The same was true, when cells have been growing under serum-starved conditions (Hif: 0.6261 ± 0.15 vs. GFP: 1.0504 ± 0.3137). This result suggests that overexpression of Hif-1α supports MSC survival.

However, cell senescence as evidenced by the expression of senescence-associated galactosidase was not altered in Hif-1α overexpressing MSCs (Fig. 2C), suggesting that Hif-1α had no effect on replication-induced senescence and MSC aging.

Furthermore, we investigated migration and invasion of Hif-1α transduced MSCs towards FCS (10%) and VEGF (50 ng/ml) (Fig. 3). Hif-1α overexpression significantly enhanced migration of MSCs in comparison to GFP adenovirus infected cells in response to FCS (Hif: 21.71 ± 9.59 vs. GFP: 7.13 ± 2.44) or VEGF treatment (Hif: 13.53 ± 6.71 vs. GFP: 7.20 ± 3.5) in a transwell migration assay (Fig. 3A). However, invasion of MCS through a matrigel monolayer in response to FCS or VEGF was not affected by Hif-1α overexpression (Fig. 3B).

Quantitative real-time PCR was performed in order to evaluate the effect of Hif-1α on angiogenic and osteogenic marker gene expression (Fig. 4). As shown in Figure 4A, Hif-1α overexpression significantly increased the expression of all tested angiogenesis-related markers, namely VEGF (Hif: 0.9025 ± vs. GFP: 0.6828 ± 0.052), bFGF (Hif: 1.584 ± 0.349 vs. GFP: 0.626 ± 0.111), PDGFR-alpha (Hif: 3.494 ± 0.312 vs. GFP: 2.461 ± 0.18) as well as PDGFR-beta (Hif: 1.1 ± 0.102 vs. GFP: 0.69 ± 0.041). The effect was

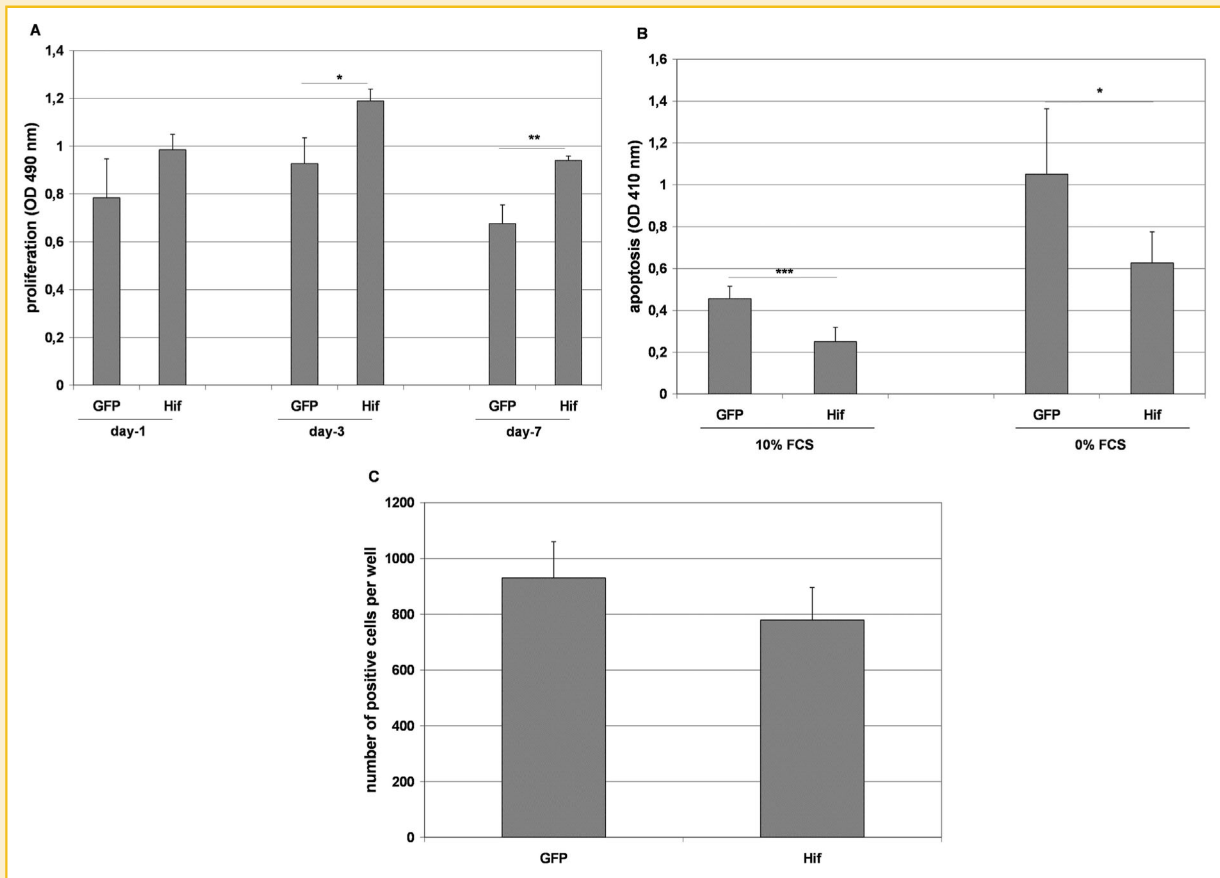

Fig. 2. Effect of Hif-1a overexpression on proliferation (A), apoptosis (B) and senescense (C). MSCs were transduced with GFP or Hif-1α adenovirus. Two days after transduction, cells were used for experiments. For proliferation experiments, cells were seeded into 48-well plates and incubated for the indicated time periods in alpha-MEM, 2% FCS. Cell numbers were measured by MTS assay. Shown are mean values of optical density at 490 nm ± standard deviation from three independent experiments (A). For measurement of low-serum induced apoptosis, cells were seeded into 12-well plates and incubated for 48 h either in alpha-MEM, 10% FCS or alpha-MEM without FCS. Apoptosis was measured by quantification of fragmented DNA by ELISA. Shown are mean values of optical density at 410 nm ± standard deviation from three independent experiments (B). For determination of senescence, cells were seeded into 6-well plates and incubated for 3 days in alpha-MEM, 0% FCS. Cells expressing senescence-associated galactosidase were counted microscopically. Shown are mean values ± standard deviations from three independent experiments (C). Asterisks indicate statistically significant differences between the indicated groups. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

most pronounced in respect to bFGF, where Hif-1α overexpression provoked a nearly threefold increase. In contrast, various classical markers for osteogenesis such as ALP and OSF-2 were unaffected in their expression, whereas BMP-2 (0.2022 ± 0.019 vs. GFP: 0.684 ± 0.028) and RunX2 (Hif: 0.816 ± 0.07 vs. GFP: 1.021 ± 0.061) mRNA expression was suppressed in response to Hif-1α infection (Fig. 4B).

In order to investigate, whether Hif-1α overexpression may have a direct effect on the osteogenic differentiation capacity of MSCs, alizarin red stainings were performed to visualize extracellular calcium deposition. In this context, we have seen that calcium deposition was unaffected by Hif overexpression irrespective of whether MSCs have been growing in normal growth medium or in osteogenic differentiation medium (containing beta-glycerolphosphate, dexamethasone, and ascorbic acid) for 21 days (Fig. 5A). The results from the stainings were confirmed by quantification of extracellular calcium deposition by releasing the alizarin red stain and measuring the optical density of the eluates (Fig. 5B).

DISCUSSION

It is widely accepted that Hif signaling is important for coupling angiogenesis and osteogenesis in vivo [Towler, 2007; Riddle et al., 2009; Sun and Wei, 2009]. However, it is sometimes difficult to discriminate between cell-autonomous and non-cell-autonomous effects resulting in augmented bone formation in vivo. A non-cell-autonomous effect is defined through the action of a molecule or protein that is produced in a specific cell type and is then transferred to other cells to promote genetic reprogramming, whereas cell-autonomous effects are caused by the molecule or protein inside the producing cell. A classical non-autonomous activity is represented by the fact that hypoxia-induced stabilization of Hif-1α results in increased expression and secretion of pro-angiogenic factors such as VEGF. VEGF binds to its receptors on endothelial cells in the surrounding vasculature thereby inducing migration and proliferation of ECs, which results in ingrowth of blood vessels into the bone tissue. These newly formed vessels support nutrition and

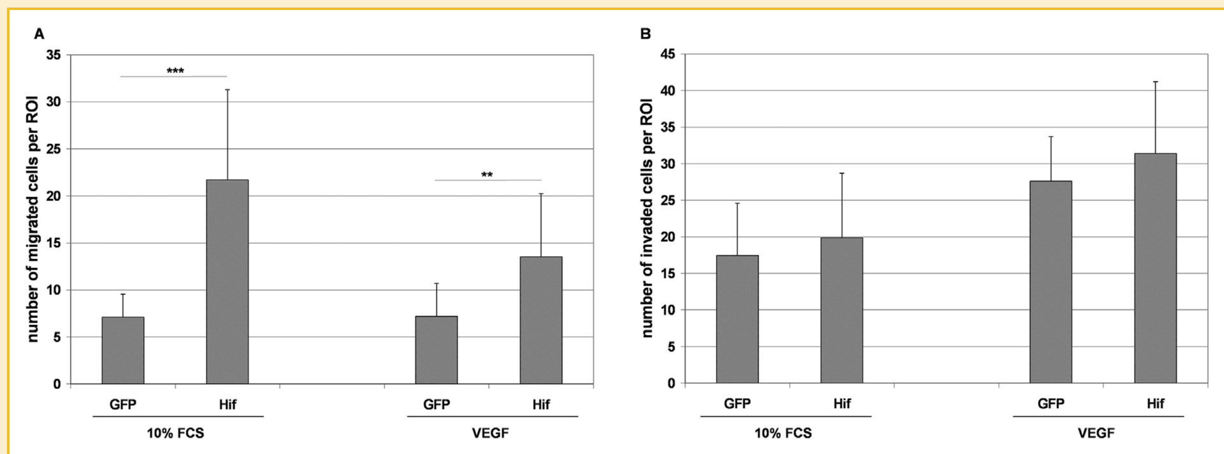

Fig. 3. Migration (A) and invasion (B) capability of GFP and Hif-1α transduced MSCs. MSCs were transduced with GFP or Hif-1α adenovirus. Two days after transduction, cells were seeded on transparent membranes with pores of 8 μm in 12-well plates in alpha-MEM without FCS. For invasion assays (B), membranes were pre-coated with growth factor reduced Matrigel. The lower chamber was filled with alpha-MEM, 10% FCS or alpha-MEM, 0% FCS, VEGF (50 ng/ml). After 24 h, cells which had migrated through the membrane were stained with DAPI and counted. Data are expressed as mean ± standard deviation (n = 15). Asterisks indicate statistically significant differences between the indicated groups. $^{**}P < 0.005$, $^{***}P < 0.0005$.

oxygenation of osteoblasts and osteoprogenitor cells in the bone. In total, the increased blood supply is directly associated with new bone formation during bone development and fracture repair.

In this study, we investigated cell-autonomous effects of Hif-1α overexpression in MSCs. Overexpression was achieved using a recombinant HIf-1α expressing adenovirus. In time course experiments, we have seen a robust and long-lasting overexpression of Hif-1α at the protein level in comparison to mock-infected MSCs. Nine days after infection, Hif-1α levels were still about 115-times higher in Hif-1α infected cells in comparison to GFP infected MSCs. Hif-1α overexpression resulted in increased MSC proliferation. This result is discrepant to studies from Holzwarth et al. [2010] demon-] demonstrating that hypoxic MSCs show increased Hif-1α expression, which was associated with a decreased proliferation rate and accumulation of cells in G1 phase. However, other reports have demonstrated increased proliferation of MSCs in response to hypoxia [Grayson et al., 2007; Peng et al., 2015]. These controversial results may be explained by donor-specific differences or by the origin of the tissues (adipose tissue, bone marrow, cord blood), from which the MSCs have been isolated, because it is known from the literature that MSCs isolated from different tissues may differ in their proliferation capacity and in their differentiation plasticity. This is especially evident if MSCs from postnatal cord blood are compared with adult MSCs from bone marrow or adipose tissue, where age-related impairment of cell functions associated with proliferation, senescence and stem cell plasticity can be observed.

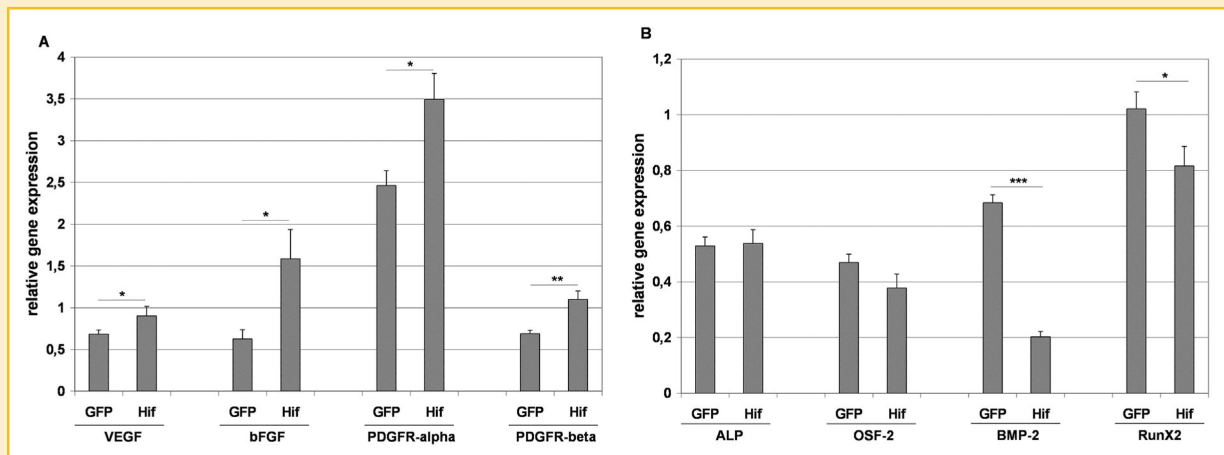

Fig. 4. Expression of angiogenic (A) and osteogenic (B) genes in GFP or Hif-1α transduced MSCs. MSCs were transduced with GFP or Hif-1α adenovirus and incubated for 7 days. mRNA levels were determined by TaqMan analysis and expression levels were normalized to GAPDH expression. Shown are mean values ± standard deviations from three independent experiments. Asterisks indicate statistically significant differences between the indicated groups. $^{*}P < 0.05$; $^{**}P < 0.005$; $^{***}P < 0.0005$.

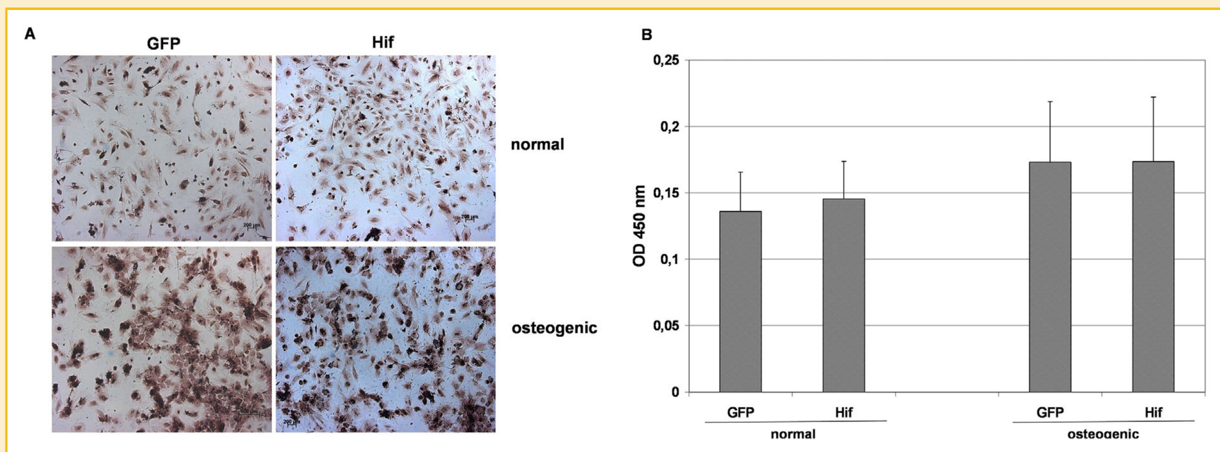

Fig. 5. Extracellular calcium deposition of GFP and Hif-1α transduced MSCs. MSCs were transduced with GFP or Hif-1α adenovirus. Two days after transduction, cells were seeded into 24-well plates and incubated for further 21 days either in normal growth medium or in osteogenic differentiation medium. Thereafter, cells were stained with alizarin-red. (A) microscopic pictures of alizarin-red stained cells. (B) colorimetric quantification of alizarin-red staining. Shown are mean values ± standard deviations from three independent staining experiments.

In general, controversy still remains in the literature for some parameters that have been investigated in our study. For example, we have seen that Hif-1α overexpression resulted in increased migration of MSCs towards serum and VEGF. Increased migration of MSCs in response to Hif-1α overexpression and/or hypoxia was also demonstrated by other groups [Okuyama et al., 2006; Cerrada et al., 2013], whereas Raheja et al. [2011] reported that hypoxia decreases MSC migration through a Hif-1α and RhoA-mediated pathway. Similarly, we have investigated expression of osteogenic genes and found that these genes were either unaffected by Hif-1α overexpression (ALP, OSF-2) or decreased in their expression (BMP-2, RunX2). Decreased expression of osteogenic genes (BMP-2, RunX2) in response to hypoxia was also reported by Yang et al. [2011], whereas Zou et al. [2011] reported an increased expression of osteogenic markers ALP, BMP-2, RunX2, OCN, OPN and BSP. We have also investigated extracellular calcium deposition by alizarin-red staining, as a biological end point of osteogenic differentiation of MSCs in in vitro experiments. In this context, we have not been able to see a notable difference between Hif-1α and GFP infected cells. However, Zou et al reported an increased extracellular calcium deposition in response to Hif-1α overexpression. Similarly, Wagegg et al. [2012] have shown that hypoxia promoted osteogenesis and suppressed adipogenic differentiation of MSCs in a Hif-1α dependent manner.

Another parameter that was not altered in response to Hif-1α overexpression in our study was the senescence of MSCs, measured by the determination of senescence-associated beta galactosidase expression. Although it was reported that hypoxia is able to decrease senescence of MSCs [Tsai et al., 2011], one might speculate that not all hypoxic effects are solely mediated via Hif-1α. The complexity of hypoxia response is much higher and is represented by the fact that there exist multiple families of Hif-1 related genes [Lee et al., 2004] as well as Hif-independent pathways. In this context, it was shown that hypoxia signaling can also occur in a Hif-independent manner involving mammalian target of rapamycin (mTOR) kinase and activation of the unfolded protein response (UPR) [Wouters and Koritzinsky, 2008]. Therefore, it is important to keep in mind that hypoxic responses are not necessarily mediated by Hif. This may explain the controversy in the literature that still exists in the context of hypoxia-induced cellular responses. To our knowledge, a direct effect of Hif-1α overexpression on MSC senescence was not investigated so far.

We also investigated the effect of Hif-1α on MSC apoptosis. Cultivation of cells in the absence of FCS led to a strong increase in the apoptosis rate in relation to MSCs grown in the presence of 10% FCS. However, we have seen that Hif-1α overexpression significantly reduced MSC apoptosis under both growth conditions. A protective role of Hif-1α was also reported under hypoxic and oxidative stress conditions [Kiani et al., 2013].

It is well known that hypoxia induces the expression of pro-angiogenic factors such as VEGF (Minchenko et al., 1994). Therefore, we have analyzed the expression of angiogenesis-related genes in Hif-1α overexpressing MSCs, demonstrating, as expected, an upregulation of VEGF, as already reported [Zou et al., 2011; Razban et al., 2012]. In the study of Razban and colleagues, it was also shown that the supernatant of Hif-1α overexpressing MSCs caused enhanced migration of endothelial cells in a wound healing assay, suggesting that Hif-1α activation in MSCs led to the secretion of factors increasing the migratory potential of endothelial cells. It is tempting to speculate that VEGF may be one of these pro-angiogenic factors that are induced in MSCs by Hif-1α overexpression. Interestingly, we have also observed an inducing effect of Hif-1α on bFGF expression, as well as on the expression of the growth factor receptors PDGF receptor alpha and beta. As already mentioned, Hif-1α overexpression significantly elevated the proliferation of MSCs. bFGF does not only represent an angiogenic growth factor, but is also a strong mitogen for MSCs [Hebert et al., 2009]. Therefore, it is tempting to speculate that the increased proliferation rate of MSCs in response to Hif-1a adenoviral infection may be mediated, at least in part, by autocrine-acting bFGF.

CONCLUSIONS

In summary, our experiments revealed multifarious effects of Hif-1α overexpression on cell-autonomous parameters. Hif-1α overexpression supported proliferation, migration, cell survival and expression of angiogenic genes in MSCs, whereas expression of the osteogenic genes BMP-2 and Runx2 was inhibited. Other parameters such as senescence, extracellular calcium deposition and cell invasion were not modulated. In general, overexpression of Hif-1α may represent a therapeutic option to improve cellular functions of MSCs. However, we have seen that expression of the osteogenic markers Runx2 and BMP-2 were decreased after Hif-1α adenoviral infection, whereas extracellular calcification was not modulated. These results suggest that although some cellular functions of MSCs are improved, osteogenic differentiation of MSCs is not ameliorated, or in light of decreased Runx2 and BMP-2 expression, may even be impaired. In future experiments we will investigate whether implantation of Hif-1α genetically modified MSCs in critical-sized bone defects may improve vascularization and bone regeneration.

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