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Mesenchymal stromal cells reverse hypoxia-mediated suppression of α-smooth muscle actin expression in human dermal fibroblasts

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ABSTRACT

During wound healing, fibroblasts deposit extracellular matrix that guides angiogenesis and supports the migration and proliferation of cells that eventually form the scar. They also promote wound closure via differentiation into α-smooth muscle actin (SMA)-expressing myofibroblasts, which cause wound contraction. Low oxygen tension typical of chronic nonhealing wounds inhibits fibroblast collagen production and differentiation. It has been suggested that hypoxic mesenchymal stromal cells (MSCs) secrete factors that promote wound healing in animal models; however, it is unclear whether these factors are equally effective on the target cells in a hypoxic wound environment. Here we investigated the impact of MSC-derived soluble factors on the function of fibroblasts cultured in hypoxic fibroblast-populated collagen lattices (FPCLs). Hypoxia alone significantly decreased FPCL contraction and α-SMA expression. MSC-conditioned medium restored hypoxic FPCL contraction and α-SMA expression to levels similar to normoxic FPCLs. SB431542, an inhibitor of transforming growth factor-β_1 (TGF-β_1)-mediated signaling, blocked most of the MSC effect on FPCL contraction, while exogenous TGF-β_1 at levels similar to that secreted by MSCs reproduced the MSC effect. These results suggest that TGF-β_1 is a major paracrine signal secreted by MSCs that can restore fibroblast functions relevant to the wound healing process and that are impaired in hypoxia.

1. Introduction

Cell proliferation and migration are impaired in chronic wounds due, at least partially, to the prevailing hypoxic conditions, which are especially exacerbated on the lower extremities [1–3]. In skin, fibroblasts play a dominant role in the tissue repair process by synthesizing collagen as well as by promoting wound contraction and closure via differentiation into α-smooth muscle actin (SMA)-expressing myofibroblasts [2]. However, under hypoxic conditions, fibroblasts exhibit decreased proliferation and migration, impaired differentiation into α-SMA-expressing myofibroblasts, increased senescence, and impaired collagen secretion [1,3,4]. This ultimately leads to delayed wound contraction and closure [5–7].

Recent preclinical studies have shown that mesenchymal stromal cells (MSCs) enhance acute skin wound healing [8–11] in small animal models. In the context of chronic wounds, there are currently five clinical trials registered with clinicaltrials.gov involving the use of MSCs as a treatment for diabetic foot ulcers. So far published data from one of the studies showed that transplantation of MSCs temporarily improved microcirculation and wound healing in 18 out of 22 diabetic foot patients with critical limb ischemia [12]. Reports suggest that MSCs cultured in hypoxia quickly adapt to the low oxygen environment [13–15]. MSCs cultured in hypoxia exhibit an increase in proliferation and an increase in secretion of growth factors (e.g. VEGF and TGF-β) that play an important role in wound healing [13,15]. Studies also suggested that conditioned medium from MSCs cultured in hypoxia significantly increased the migration of dermal fibroblasts in vitro and wound healing in vivo [13,15]. However, there are no reports on how MSC secreted factors affect target cells exposed to prolonged hypoxia. In this study, we investigated the impact of soluble factors released by MSCs on the function of hypoxic fibroblasts cultured in collagen gels, used as models of skin dermis.

MSCs in suspension applied in vivo die or migrate away from the wound site within 7–14 days [10]. Application of MSC therapy requires consistent MSC numbers to be retained at the wound site,

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and treatment durations are likely to be on a time scale of weeks. MSCs have been successfully immobilized in alginate microcapsules, which have been shown to preserve their secretion of anti-inflammatory molecules, thus creating a continuous release device that can modulate local immune responses and promote tissue repair [16]. In this study, we modified the original immobilization technique to a flat sheet that would be more easily secured onto actual wounds. Using immobilized MSCs, we report that TGF-β_1 released by MSCs is a key factor that reverses many of the hypoxia-induced impairments in fibroblast function such as decreased differentiation into α-SMA-expressing myofibroblasts and ultimately wound contraction.

2. Materials and methods

2.1. Cell culture maintenance

Human bone-marrow derived MSCs were purchased from the Institute of Regenerative Medicine at Texas A&M at passage 1 and cultured as previously described [16]. Briefly, MSCs were cultured in α-minimal essential medium (α-MEM; Life Technologies, Grand Island, NY), containing no deoxyribo- or ribo-nucleotides, supplemented with 10% v/v fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA), 1% w/v penicillin-streptomycin (pen-strep; Life Technologies), 4 mM L-glutamine (Life Technologies) and 1 ng/ml basic fibroblast growth factor (bFGF; Life Technologies). Passage 3 cells were cultured until 70% confluency and immobilized in alginate sheets as described further below.

Human dermal fibroblasts were donated from the W.M. Keck Center (Rutgers University, Piscataway, NJ) at passage 4. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Life Technologies) supplemented with 10% v/v FBS and 1% w/v pen-strep until confluence.

2.2. Immobilization of MSCs in alginate sheets

MSCs (5×10^5 cells) were mixed with 200 μL of 3% w/v sodium alginate (Sigma, St. Louis, MO). The cell suspension was poured into a 2×2 square, 0.05 cm deep, mold made of polydimethylsiloxane (PDMS), followed by immersion in a 500 mM calcium chloride solution for 10 min to cross-link the alginate thus forming a sheet. The sheets were then immersed in 0.05% w/v poly-L-lysine (83.3 kDa; Sigma) solution for 2 min at room temperature to increase sheet stability as described elsewhere [16]. MSC-conditioned medium (MSC-CM) to be used in the subsequent studies was made by culturing the alginate-embedded MSCs in low serum DMEM (0.1% v/v FBS and 1% w/v pen-strep) at a concentration of 5×10^5 cells/ml for 48 h at 37 °C in 5% CO_2 atmosphere balanced with air.

2.3. Fibroblast contraction assay

Human dermal fibroblasts (1×10^5 cells) were mixed with 600 μL type I collagen (BD Biosciences, San Jose, CA) at pH 7 and a concentration of 1.5 mg/ml and added to each well of a 24-well plate. Plates were incubated for 30 min at 37 °C to induce collagen gelation, thus forming fibroblast-populated collagen lattices (FPCLs) [17], and then cultured overnight in 1 ml of low serum DMEM. The FPCLs were then switched to treatment conditions, which consisted of 1 ml of one of the following: MSC-CM, low serum DMEM + 1 ng/ml of TGF-β1 (R&D, Minneapolis, MN), or low serum DMEM with no other supplements. The FPCLs were then cultured at 37 °C under normoxia (5% CO_2, 21% O_2, balance N_2) or hypoxia (5% CO_2, 1% O_2 balance N_2) for 48 h [18] in a gas controlled chamber. After 48 h and without changing the medium on the cells, the FPCLs were shortly removed from the chamber, manually detached from the well edges in order to initiate contraction, and returned to the chamber to continue hypoxia or normoxia. Top down images of the FPCLs were taken after 24 and 48 h to reveal their cross sectional areas using a digital camera, which were later quantified to calculate the extent of contraction.

For inhibition studies, FPCLs were treated for 1 h with 10 μM SB431542 (Sigma), a potent and specific inhibitor of TGF-β1 activin receptor-like kinase, or 0.38% dimethyl sulfoxide (DMSO) vehicle, before switching the cells to treatment conditions (MSC-CM, low serum DMEM + 1 ng/ml of TGF-β_1, or low serum DMEM supplemented with DMSO or SB431542) for 48 h. Contraction was induced and measured for 48 h as described above.

2.4. Cell viability determination

Viability of immobilized MSCs was measured weekly for 21 days while the cells were cultured in α-MEM with 10% v/v FBS, 1% w/v pen-strep, 4 mM L-glutamine and 1 ng/ml bFGF. Viability of fibroblasts in the FPCLs was determined after 96 h of culture in normoxia or hypoxia. The cells were incubated at 37 °C with 3 μM calcein-AM + 6 μM ethidium homodimer-1 (Life Technologies) for 30 min in basal medium and the nuclei counterstained with Hoechst 33342 (Life Technologies). The cells were washed 5 times with medium before imaging on an Olympus IX81 spinning disc confocal microscope with a 10× objective. Five serial 500 μm optical slices at 20 μm intervals were taken per experimental condition. Slidebook software (Intelligent Imaging Innovations, Denver, CO) was used to quantify the number of live (green fluorescence) and dead (red fluorescence) cells and calculate the percent viability as described elsewhere [19].

2.5. Cytokine secretion measurements

Immobilized MSCs were cultured either in α-MEM with 10% v/v FPS, 1% w/v pen-strep, 4 mM L-glutamine and 1 ng/ml bFGF, or in DMEM with 0.1% v/v FBS and 1% w/v pen-strep for 48 h under normoxic or hypoxic conditions at 37 °C. The cell supernatant was collected and assayed for interleukin-8 (IL-8), interleukin-6 (IL-6) and TGF-β_1 using commercial ELISA kits (BioLegend, San Diego, CA).

2.6. Western immunoblotting analysis

FPCLs were homogenized for cell extraction using a 2 ml Dounce tissue homogenizer (Sigma) and 1× RIPA Buffer (Thermo Scientific, Waltham, MA), in the presence of 1% protease inhibitor and 1% ethylene diamine tetraacetic acid (EDTA). Total protein content was determined using a bicinchoninic acid protein assay (Thermo Fisher). Equal amounts of total protein were separated by 10% SDS-PAGE gel (Bio-Rad, Hercules, CA) followed by blotting to nitrocellulose membrane (Bio-Rad). The membrane was blocked with 5% w/v non-fat milk (Bio-Rad) in Tris-buffered saline-Tween 20 for 2 h and then incubated with a polyclonal anti-α-SMA (Abcam, Cambridge, MA) and anti-hypoxia inducible factor (HIF)-1α (Abcam) at 4 °C overnight. After washing, the membrane was incubated with goat to rabbit secondary antibody for 1 h at room temperature. Signals were detected by staining the membrane with SuperSignal™ west pico chemiluminescent (Thermo Fisher) for 5 min and bands were digitized with a scanner.

2.7. Immunocytochemical staining

FPCLs were washed with phosphate buffer saline (PBS) and fixed with 4% paraformaldehyde for 15 min. After an additional wash with PBS, the cell membranes were permeabilized with 0.1% v/v Triton-X in PBS for 3 min before the cells were stained for F-actin

using Alexa fluor 488 phalloidin (Life Technologies), and the nucleus was counterstained using Hoechst 33342 for 20 min. The stained FPCLs were placed on glass slides and imaged on an Olympus IX8 spinning disc confocal microscope using 20× objective. Serial 500 μm optical slices at 20 μm intervals were taken of the FPCLs.

2.8. Statistical analysis

All numerical results are presented as means ± standard error of the mean (SEM). Statistical analysis of three or more independent experiments were assessed using two-tail Student's t-test or one-way analysis of variance (ANOVA) followed by Fischer post hoc analysis where $p < 0.05$ represents statistical significance.

3. Results

3.1. Immobilization of MSCs in alginate sheets

We immobilized MSCs in alginate flat sheets, the form in which the MSCs would ultimately be applied onto wounds (Fig. 1A). MSCs in alginate sheets remained over 90% viable for up to 21 days (Fig. 1B). The secretion pattern of immobilized MSCs was compared to that of MSCs cultured on standard tissue culture plastic, and there were no significant differences in secretion of IL-8, TGF-β_1 or IL-6 (Fig. 1C). We also compared MSC secretion in normoxia and hypoxia. While there was no significant difference in the secretion of IL-8 and IL-6, hypoxic MSCs secreted significantly more TGF-β_1 compared to normoxic MSCs (Fig. 1D).

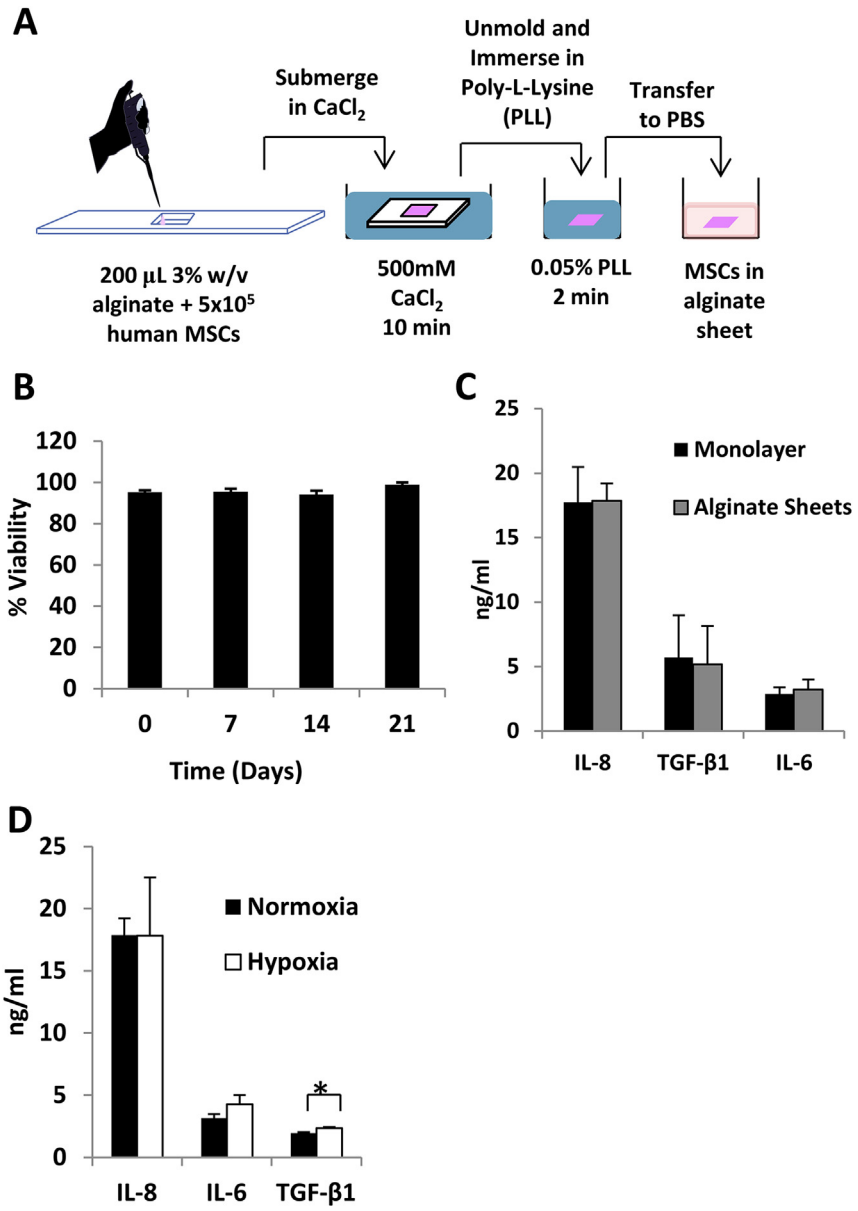

Fig. 1. Immobilization of mesenchymal stromal cells (MSCs) in alginate sheets does not affect viability or secretion but hypoxia differentially modulates MSC secretion. (A) MSCs were mixed with soluble alginate and polymerized using calcium chloride to form alginate sheets containing MSCs. (B) Immobilized MSCs were cultured for 21 days and percent cell viability was determined using live/dead fluorescent staining. Calcein AM^+ and $EthD\text{-}1^-$ MSCs in alginate sheets were considered viable. N = 3. (C) Accumulated IL-8, TGF-β1 and IL-6 secreted by MSCs (250, 000 cells/ml) after 48 h in monolayer on tissue culture plastic plates vs. immobilized in alginate sheets for 48 h. N = 6. (D) Accumulated of IL-8, TGF-β1 and IL-6 secreted by MSCs after 48 h in normoxia vs. hypoxia. N = 6. *:$p < 0.05$ compared to normoxia based on Student's t-test.

3.2. Hypoxia decreases fibroblast differentiation

Hypoxia has been shown to decrease the expression of α-SMA in dermal fibroblasts (7). We embedded fibroblasts in type I collagen to create FPCLs that simulate the dermal layer of the skin. Ensuing FPCL contraction was used as an in vitro wound contraction model. FPCLs were switched to 1% O_2 tension to replicate the hypoxic environment typical of chronic wounds. HIF-1α expression increased after 4 h in the hypoxia chamber compared to normoxic controls, which confirms that the cells were responding to the hypoxic stress (Fig. 2A). Hypoxic culture did not affect viability, as the cells in the FPCL remained over 90% viable after 96 h in culture (data not shown). However, FPCL gel area, which decreased to 70% initial area in the normoxic group (-hypoxia/-TGF-β_1/-MSC-CM), only decreased to 90% initial area in the hypoxic group (+hypoxia/-TGF-β_1/-MSC-CM) as shown in Fig. 2B. Consistent with this observation, the expression of α-SMA, a marker of differentiation into myofibroblasts, which are important mediators of ECM contraction, decreased compared to the normoxic control (Fig. 2C).

3.3. MSCs reverse hypoxia-mediated impairment in FPCL contraction via TGF-β_1

FPCLs exposed to MSC-CM (-hypoxia/-TGF-β_1/+MSC-CM) exhibited a significant increase in contraction compared to standard culture (-hypoxia/-TGF-β_1/-MSC-CM), as the remaining gel area decreased to 52% initial area in the former, compared to 70% in the latter (Fig. 3A). Furthermore, this effect persisted under hypoxic conditions where FPCLs in hypoxia alone (+hypoxia/-TGF-β_1/-MSC-CM) increased gel area to 90% initial area, and in the presence of MSC-CM, this area decreased to 65% (+hypoxia/-TGF-β_1/+MSC-CM). It is noteworthy that the latter value is similar to what was seen in the normal culture conditions (-hypoxia/-TGF-β_1/-MSC-CM); thus MSC-CM restored contraction of hypoxic gels to levels similar to normal culture conditions.

Among products known to be secreted by MSCs, TGF-β_1 was thought to be a plausible candidate to mediate this response since

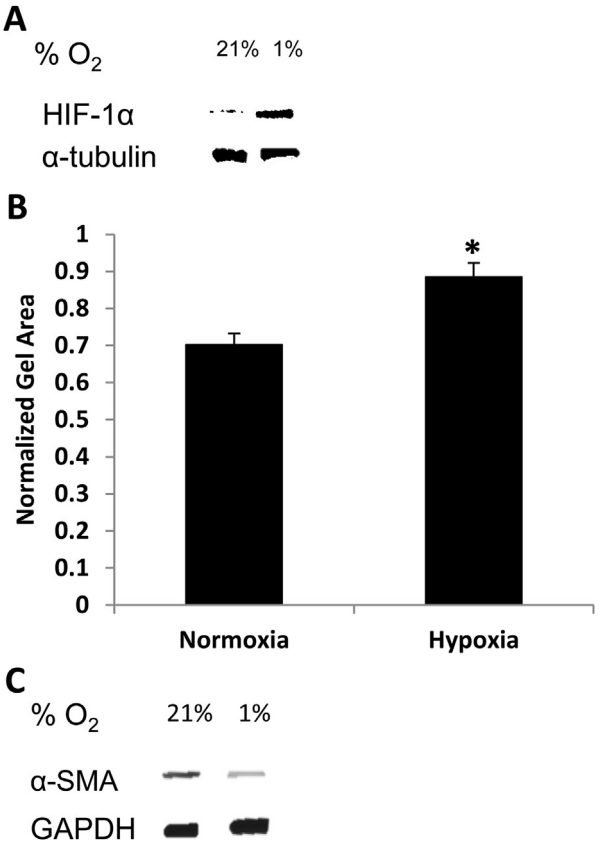

Fig. 2. Hypoxia induces HIF-1α while inhibiting α-SMA expression and FPCL contraction. Fibroblast-populated collagen lattices (FPCLs), each containing 1×10^5 normal human dermal fibroblasts seeded in 200 μL collagen type I (1.5 mg/ml), were cultured in low serum medium and exposed to normoxia (21% v/v O_2) or hypoxia (1% v/v O_2). (A) FPCLs were harvested after 4 h of incubation and analyzed by Western blot for HIF-1α and α-tubulin (B) FPCL area was measured after 48 h of incubation and normalized to initial area. N = 6. *:p < 0.01 based on Student's t-test. (C) FPCLs were harvested after 96 h of incubation and levels of α-SMA and GAPDH measured by Western blotting.

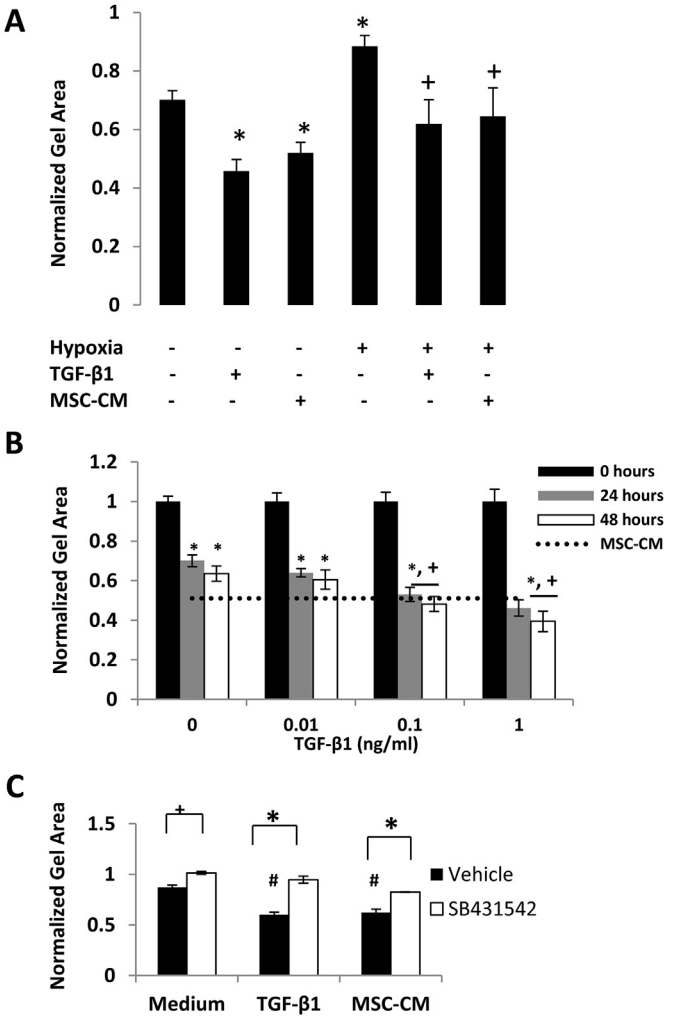

Fig. 3. MSC-CM and recombinant TGF-β_1 promote fibroblast-mediated contraction. (A) FPCLs were placed in low serum medium, or the same supplemented with 1 ng/ml TGF-β_1, or MSC-CM for 48 h and gel area was measured after 24 h of incubation. FPCL area was normalized to initial area. N = 6. *:p < 0.01 compared − hypoxia/-TGF-β_1/-MSC. +:p < 0.05 compared to +hypoxia/-TGF-β_1/-MSC. Statistical significance determined by ANOVA. (B) FPCLs were treated with increasing concentrations of recombinant TGF-β_1 for 48 h and the extent of contraction was measured after 24 and 48 h of incubation. FPCL area at each time point was normalized to initial area. N = 5. *:p < 0.0001 compared to 0 h time point. +:p < 0.01 compared to the group receiving 0 ng/ml TGF-β_1. Statistical significance determined by ANOVA. (C) FPCLs were treated with 10 μM SB431542 or vehicle for 1 h, and then switched to low serum medium, or the same supplemented with 1 ng/ml recombinant TGF-β_1, or MSC-CM. After 48 h incubation, contraction was induced and measured after 24 h. FPCL areas at that time were normalized to initial area. N = 4. *:p < 0.0001. +:p < 0.01. #:p < 0.0001 compared to group receiving medium with vehicle. Statistical significance determined by ANOVA.

TGF-β_1 is known to promote fibroblast differentiation into myofibroblasts [17,20]. Addition of exogenous TGF-β_1 alone to FPCLs caused a dose-dependent increase in contraction rate, and doses ranging from 0.1 to 1 ng/ml led to contraction rates similar to that seen with MSC-CM (Fig. 3B). We measured TGF- β_1 secretion by the MSCs cultured in low serum (0.1% FBS) medium, and found concentrations in the range of 0.6–1 ng/ml (data not shown); which is consistent with the contraction response induced by the MSC-CM (Fig. 3B).

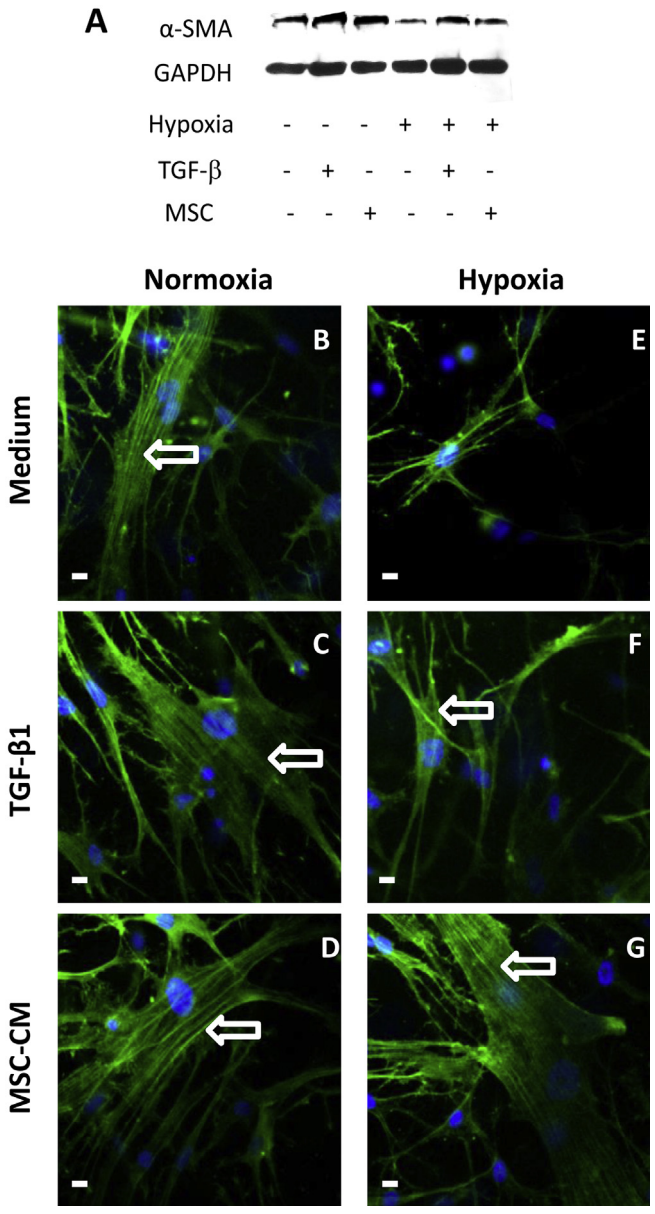

Fig. 4. MSC-CM and recombinant TGF-β_1 promote the differentiation of hypoxic fibroblasts. FPCLs were placed in low serum medium, or the same supplemented with TGF-β_1 (1 ng/ml), or MSC-CM, and incubated for 96 h under normoxia or hypoxia for 96 h. (A) Levels of α-SMA and GAPDH protein as determined by Western blotting. (B–G) The morphology of F-actin stress fibers in the fibroblasts was assessed by imaging FPCLs stained with FITC-conjugated phalloidin. Arrows indicate F-actin cytoskeleton. (B–D) Fibroblasts cultured under normoxic conditions have an elaborate F-actin network that is elongated and prominent. (E) Fibroblasts cultured in hypoxia with no other treatment are spindled shape without the elaborate F-actin network (F) Fibroblasts cultured in hypoxia and treated with 1 ng/ml TGF-β1 have larger stress fibers that are loosely organized (G) Fibroblasts cultured in hypoxia and treated with MSC-CM have large compact linear bundles that are aligned. Scale bar = 10 μm.

To verify whether TGF-β_1 released by MSCs promotes FPCL contraction, FPCLs were incubated with MSC-CM in the presence of the TGF-β_1 inhibitor SB431542. In the presence of the inhibitor, FPCL remaining gel area increased from 62% in the MSC-CM + vehicle group to 83% initial in the MSC-CM + SB431542 group. Similarly, the inhibitor caused the gel area to remain at 95% initial with 1 ng/ml TGF-β_1, while 1 ng/ml TGF-β_1 alone decreased gel area to 60% (Fig. 3C). Taken together, these data suggest that TGF-β_1 is a major MSC-derived factor that promotes FPCL contraction.

3.4. MSCs promote myofibroblast differentiation in FPCLs

Next we investigated whether MSC-CM promoted fibroblast expression of α-SMA, since exogenous TGF-β_1 is known to increase α-SMA expression in FPCLs [4]. α-SMA protein levels in FPCLs were indeed increased by both MSC-CM and TGF-β_1 (Fig. 4A). Furthermore, fibroblasts rich in cytoskeletal F-actin also use the F-actin stress fibers for wound contraction [17]. Fibroblasts in the collagen lattices were stained for F-actin using FITC-conjugated phalloidin. Fibroblasts cultured under normoxic conditions have an elaborate cytoskeleton network (Fig. 4B–D). Under normoxia and in plain medium, we observed well-developed stress fibers (Fig. 4B); however, under hypoxia with no other treatment (+hypoxia/-TGF-β1/-MSC-CM), fibroblasts were spindle shaped and did not have an elaborate F-actin network (Fig. 4E). Under normoxic conditions, there were no differences in the F-actin cytoskeleton when fibroblasts were treated with 1 ng/ml TGF-β1 (Fig. 4C) or MSC-CM (Fig. 4D). The stress fibers were prominent and elongated. In contrast, fibroblasts cultured under hypoxia and treated with MSC-CM had more compact linear bundles that are aligned and coursing one direction (Fig. 4G) whereas cells treated with 1 ng/ml TGF-β1 have more loosely organized stress fibers that are less compact (Fig. 4F).

4. Discussion

While MSCs are multipotent cells [8–11], some of their therapeutic properties that appear most promising involve their secretion of immunomodulatory molecules with beneficial effects in cases of acute inflammation (e.g. spinal cord injury) as well as chronic inflammation (e.g. inflammatory bowel disease, diabetic foot ulcers) [12,21]. Several studies have suggested that MSCs may promote skin wound healing also via their secreted products [22]. Since skin wounds are associated with hypoxic conditions, we asked the question whether hypoxia alters the response of the wound cells targeted by the MSC-derived products. Among the soluble factors secreted by MSCs that promote wound healing, we focused on TGF-β_1, a potent regulator of fibroblast activity and all phases of wound healing [6,17,20,23]. TGF-β_1 enhances the proliferation and migration of fibroblasts into the wound bed and induces the secretion of collagen and other components of the new ECM [17,24]. Furthermore, it has been suggested that TGF-β_1 induces α-SMA expression in myofibroblasts, which enables them to generate the strong contractile forces needed to cause wound contraction [17,20].

While hypoxia alone decreased both α-SMA expression in human dermal fibroblasts, as well as FPCL contraction, we report for the first time that human bone marrow-derived MSCs could overcome these changes. MSCs enhanced FPCL contraction by roughly the same magnitude under normoxia vs. hypoxia. This is interesting in as much as hypoxia could deplete ATP stores and in turn slow down the rate of any energy-requiring process [25], such as cell-mediated contraction of the FPCLs. Since MSC-CM, as well as TGF-β_1, could increase FPCL contraction in spite of the hypoxia, this suggests that cellular energy production was not limiting this

process. Thus, local administration of MSCs or their secreted products may be a suitable alternative to reverse the deleterious effects of hypoxia. Furthermore, TGF-β_1 secretion by MSCs was increased under hypoxic conditions; although the increase was 1.25 fold (Fig. 1D), it is conceivable that this could also increase the potency of the MSCs in the chronic wound environment.

Much of the effects of MSCs could be reproduced when using recombinant TGF-β_1 at levels secreted by MSCs. A specific inhibitor of the TGF-β_1 pathway, SB431542, when used in presence of MSC-CM, significantly decreased FPCL contraction. Taken together, these data suggest that MSCs affect fibroblast differentiation to myofibroblasts via a TGF-β_1-mediated mechanism. It has been reported that TGF-β_1 induces α-SMA-expression in part by the SMAD signaling pathway [20,26,27]. When active TGF-β_1 binds to the TGF-βRII, it phosphorylates and forms a heteromeric receptor complex with TGFβRI. The activated complex then phosphorylates SMAD2 and SMAD3, which binds to SMAD4, followed by translocation into the nucleus where the complex increases gene transcription via DNA transcription factors [23,26–28]. Another member of the SMAD family, SMAD7, is on the other hand an inhibitor of TGF-β_1 signaling [27,28] which is induced by hypoxia via HIF-1α expression. SMAD7 interacts with E3 ubiquitin ligases and recruits them to the TGF-β receptor (TGF-βR) complexes, which then degrade the phosphorylated TGF-βRI and subsequently decrease SMAD2 and SMAD3 phosphorylation. SB431542 prevents TGF-βRI phosphorylation, and thus effectively mimics the effect of hypoxia-induced increase SMAD7. Therefore, it is plausible that hypoxia may increase SMAD7, thus TGF-β_1 signaling, as shown by a decrease in FPCL contraction α-SMA expression under hypoxic conditions.

Other than activated HIF-1α, inflammatory mediators such as tumor necrosis factor-α (TNF-α) have been reported to upregulate SMAD7 expression [26]. Chronic wounds exhibit prolonged high levels of inflammatory proteins [4–6]. MSCs are known to be immunomodulatory and through paracrine signaling decrease TNF-α secretion by inflammatory cells [11,29]. While MSCs cannot change local oxygen tension in tissue, they may benefit the wound healing process by secreting TGF-β_1 on the one hand, and decreasing the levels of pro-inflammatory mediators that inhibit the TGF-β_1 signaling pathway on the other hand.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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