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Hypoxic preconditioned bone marrow-derived mesenchymal stromal/stem cells enhance myoblast fusion and skeletal muscle regeneration

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Abstract

Background: The skeletal muscle reconstruction occurs thanks to unipotent stem cells, i.e., satellite cells. The satellite cells remain quiescent and localized between myofiber sarcolemma and basal lamina. They are activated in response to muscle injury, proliferate, differentiate into myoblasts, and recreate myofibers. Many stem and progenitor cells support skeletal muscle regeneration, which could be disturbed by extensive damage, sarcopenia, cachexia, or genetic diseases like dystrophy. Many lines of evidence showed that the level of oxygen regulates the course of cell proliferation and differentiation. Methods: In the present study, we analyzed hypoxic's impact on human and pig bone marrow-derived mesenchymal stromal cell (MSC) and mouse myoblast proliferation, differentiation, and fusion. Moreover, the influence of the transplantation of human bone marrow-derived MSCs cultured under hypoxic conditions on skeletal muscle regeneration was studied. Results: We showed that bone marrow-derived MSCs increased *VEGF* expression and improved myogenesis under hypoxic conditions *in vitro*. Transplantation of hypoxic preconditioned bone marrow-derived MSCs into injured muscles resulted in the improved cell engraftment and formation of new vessels. Conclusions: We suggested that SDF-1 and VEGF secreted by hypoxic preconditioned bone marrow-derived MSCs played an essential role in cell engraftment and angiogenesis. Importantly, hypoxic preconditioned bone marrow-derived MSCs more efficiently engrafted injured muscles, however, they did not undergo myogenic differentiation.

Introduction

Skeletal muscle regeneration is a complex process that allows restoration of skeletal muscle homeostasis lost due to the injury, such as intensive exercise, surgical procedures, and diseases. Skeletal muscle regeneration covers two distinct phases. The first one includes tissue degeneration, accompanied by inflammation, necrosis of damaged myofibers, and their phagocytosis by immune cells. The second one is regeneration, leading to new myofiber formation followed by their maturation, tissue reinnervation, and finally skeletal muscle functional recovery (1).

Muscle necrosis occurs when myofibers' integrity is severely disrupted what involves increased sarcolemma permeability, organelle dysfunction, and loss of myofiber architecture. Necrotic cell death and loss of plasmalemma integrity lead to the release of intracellular components that act as damage-associated molecular patterns (DAMPs) and trigger an inflammatory response (2, 3). Necrotic myofibers release many cytokines, growth factors, and chemoattractants. These signals activate tissue-resident and circulating inflammatory cells (4, 5). Neutrophils are the first to infiltrate the site of injury. These cells phagocytize damaged myofibers and release numerous factors which induce migration of local monocytes and their differentiation into macrophages (6-8). Two days after injury, macrophages become the predominant cell population present within damaged tissue (5, 9, 10). They can be divided into two distinct subpopulations – M1, also considered as pro-inflammatory macrophages, characterized by the presence of CD68, and responsible for phagocytosis of necrotic tissue and releasing pro-inflammatory factors, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL6, IL12, as well as nitric oxide (NO) and reactive oxygen species (ROS) (11-13). The second described population is M2, also called anti-inflammatory macrophages, characterized by the presence of CD163, releasing factors, like tumor growth factor β (TGF-β), IL4, IL10 or IL13, and for supporting myoblast differentiation, angiogenesis, and extracellular matrix (ECM) deposition (14, 15). The next phase of skeletal muscle repair covers myofiber regeneration which is possible due to satellite cells (SCs) – skeletal muscle-specific stem cells, characterized by a PAX7 transcription factor. These cells are tightly connected to the myofibers and located between basal lamina and sarcolemma. In healthy muscles, SCs remain quiescent, but after injury, they become activated, re-enter the cell cycle, start to proliferate, differentiate into myoblasts which further fuse to form myotubes. Finally, myotubes' maturation leads to new functional myofibers' formation (7, 16-18).

Quiescent SCs can quickly respond to changes in their niche and specific signals present in their microenvironment. Among the crucial factors causing SC activation and proliferation are mechanical disruptions of sarcolemma and action of growth factors released by inflammatory cells, endothelial cells, interstitial cells, such as fibroblasts, or released from the ECM by specialized proteases -

metalloproteinases (MMPs) (19, 20). Fibroblast growth factor (FGF) is one such factor. Its action via transient receptor potential channels (TRPC) leads to the translocation of nuclear factor of activated T-cells (NFATc) into the nucleus resulting in SC activation (21, 22). FGF action is also known to activate the p38 mitogen-activated protein kinase (p38 MAPK) pathway, which acts as a molecular switch during SC activation (23). Another ECM-derived factor crucial for SC activation is hepatocyte growth factor (HGF), which binding to hepatocyte growth factor receptor (HGFR/c-Met) promotes SC re-entry into the cell cycle (24). One of the signals produced by such cells as fibroblasts or myofibers is insulin-like growth factor 1 (IGF-1), which is well known to stimulate the PI3K/Akt/mTOR pathway and to downregulate the activity of transcription factor Forkhead box O (FOXO), what in consequence leads to downregulation of p27 cell cycle inhibitor and activation of SC cell cycle (25). Some of the previously mentioned factors are known to directly or indirectly regulate SC activation and proliferation. Among them are, for example, TNF-α activating nuclear factor kappa B (NF-κB) pathway, which leads to silencing Notch1, NO which stimulates MMP expression and ECM remodeling or IL6, which stimulates SC proliferation in signal transducer and activator of transcription 3 (STAT3)-dependent manner (19, 26-28). Finally, activated and proliferating SCs start to differentiate.

The myogenic differentiation of SCs is regulated by sequentially expressed transcription factors, called myogenic regulatory factors (MRFs). MRF family consists of MYF5, MYOD, myogenin, and MRF4 (29, 30). Quiescent SCs are characterized by the presence of paired box transcription factor 7 (PAX7). PAX7 and MYF5 are present in proliferating SCs and myoblasts. PAX7 regulates the expression of MYF5 and MYOD, while MYF5 presence enhances the expression of MYOD. MYOD is a critical factor of myogenic differentiation. It facilitates the transition from myoblast proliferation to the myocyte differentiation stage by inducing the myogenin and p21 and p57 cell cycle inhibitor expression (31, 32). Further, MYOD and myogenin trigger the expression of other genes essential for muscle cell function, such as MRF4, myosin heavy and light chains, muscle creatine kinase, or troponin (33). The expression of myogenin and MRF4 is accompanied by the downregulation of PAX7, MYF5, and MYOD. Some cells do not undergo differentiation but remain PAX7 positive, downregulate MYOD, and restore the SC population necessary for the next rounds of muscle regeneration (34-36). Those that differentiated fuse to each other to result in the formation of multinucleated myotubes and then myofibers (37, 38). Alternatively, differentiated myocytes can fuse with already existing myofibers during the regeneration of slightly damaged skeletal muscles (39). Finally, newly formed myotubes and myofibers undergo maturation to become fully functional. During maturation, myofibers grow, myofibers' proper contractility is restored, and neuro-muscular junctions are formed (40, 41).

In skeletal muscle diseases, sarcopenia, or cachexia, skeletal muscle regeneration is disturbed. Many populations of stem and progenitor cells are studied for potential therapeutic use. Two main strategies to support skeletal muscle regeneration are considered. First, the transplanted cells could participate in myofiber reconstruction; second, transplanted cells' secreted factors could support regeneration. One of the studied cells are bone marrow-derived stromal cells, also known as bone marrow-derived "mesenchymal" stem/stromal cells (bone marrow-derived MSCs). However, it should be noted that these cells do not present naïve myogenic potential (42). Bone marrow-derived MSCs are a heterogeneous population (43), typically isolated from bone marrow based on their ability to adhere to the culture plate's surface. It was proven that bone marrow-derived MSCs contain a population of cells that fulfill the rigorous criteria of stem cells (44). This subpopulation of bone marrow-derived MSCs present long-term expansion without phenotypic change, self-renewal probed during *in vivo* serial transplantations, and multipotency examined by *in vivo* differentiation assay at the single-cell level (44-48). CD146 appeared to be a handy marker to select and isolate stem cell subpopulations from bone marrow-derived MSCs (45). Human CD146+ bone marrow-derived MSCs were shown to be able to self-renew, differentiate into bone and bone marrow, a support organization of endothelial cells into functional blood vessels, and differentiate into chondrocytes and adipocytes (45, 49).

As we mentioned above, bone marrow-derived MSCs do not present naïve myogenic potential (45). These cells do not fuse in the absence of myoblasts and rarely fuse with myoblasts in co-cultures (45, 50-53). However, bone marrow-derived MSCs could follow myogenic differentiation as the result of reprogramming induced by 5-azacytidine treatment, overexpression of Notch intracellular domain (NICD), paired box transcription factor 3 (Pax3), or constitutively active β-catenin, or as a result of 3D co-culture with myofibers (54-59). It was also documented that bone marrow-derived MSCs could support skeletal muscle regeneration; however, these cells rarely participate in new myofiber formation (50, 59-64).

In the current study, we focused on the hypoxic effect on bone marrow-derived MSC and myoblast co-cultures. We also followed if cultured under hypoxic condition bone marrow-derived MSCs could more efficiently support skeletal muscle regeneration. The level of oxygen is an essential factor regulating gene transcription and cell fate. The level of O_2 during *in vitro* culture under hypoxic conditions (1-3%) is much more similar to the level present in the physiological bone marrow-derived MSC niche in the bone marrow (2-7%) than that observed under standard *in vitro* culture conditions. Accordingly, it was previously shown that bone marrow-derived MSCs cultured under hypoxic conditions induced their proliferation, migration, elevated colony-forming unit capabilities, increased ECM deposition, osteogenic and adipogenic potential, and angiogenic factors expression (65-71).

Moreover, preconditioning of bone marrow-derived MSC with hypoxic increased their ability to engraft injured tissues after transplantation. In the subacute murine limb ischemia model, hypoxic preconditioned bone marrow-derived MSCs injected into skeletal muscles engrafted this tissue more efficiently, induced neoangiogenesis, and improved blood flow (69). Similar results were observed after transplantation of hypoxic preconditioned bone marrow-derived MSCs to other ischemic tissues, including heart, brain, lung, and liver (70-74). We hypothesized that hypoxic preconditioning impacts the human bone marrow-derived MSC secretome. As a result, these cells could more efficiently engraft injured skeletal muscle, support myoblast fusion, and skeletal muscle regeneration. To follow this problem, we choose to investigate human and pig bone marrow-derived MSCs. We selected cells of two species as we previously showed that as far as MSCs are concerned, the cells' origin may determine their reaction to the same factors (75). Moreover, pig serves as a valuable model in preclinical research. We analyzed human and pig bone marrow-derived MSCs in vitro, co-culture with mouse myoblasts, and *in vivo* after their transplantation to mouse injured skeletal muscles.

Materials and Methods

Primary myoblasts, C2C12, and MSCs culture under normoxic and hypoxic conditions

Four different cell types were used during experiments. Human bone marrow-derived mesenchymal stromal cells (hMSCs) were obtained from Lonza (Lonza PT-2501). Fetal pig bone marrow-derived mesenchymal stromal cells (pMSCs) were kindly provided by dr. Joanna Wojtkiewicz from University of Warmia and Mazury in Olsztyn. pMSCs were isolated from transgenic pigs, which constitutively expressed green fluorescent protein (GFP). Both types of MSCs were cultured in Dulbecco's modified Eagle's medium (DMEM; ThermoFisher Scientific) containing glucose 4.5 g/l supplemented with 15% inactivated fetal bovine serum (FBSin; ThermoFisher Scientific) and 0,1% gentamycin solution (Sigma-Aldrich), further referred to as MSC medium (MSCmed). C2C12 cells (Sigma–Aldrich) were cultured in DMEM containing glucose 4.5 g/l, supplemented with 10% FBSin and 1% penicillin-streptomycin solution (ThermoFisher Scientific), further referred to as C2C12 medium (C2C12med). Mouse primary myoblasts (mPM) were isolated from *tibialis anterior* (TA), *soleus*, *extensor digitorum longus (*EDL), and *flexor digitorum brevis* (FDB) muscles of 2–3-month-old C57/BL6 male mice using Bischoff and Rosenblatt method (76, 77). Briefly, muscles were isolated from tendon to tendon, digested in 0.2% collagenase type I (Sigma-Aldrich) solution in DMEM. Then, single myofibers were collected in suspension, passed through a 21G syringe needle, and filtered through a 40 µm strainer. Primary myoblasts were cultured in DMEM containing glucose 1 g/l, supplemented with 10% horse serum (HS; ThermoFisher Scientific), 20% FBSin, 0.5% chicken embryo extract (CEE; ThermoFisher Scientific), and 1% penicillin-streptomycin solution, further referred to as PM medium (PMmed). All cell types were cultured in normoxic (37°C, 21% O_2, 5% CO_2) or hypoxic (37°C, 5% O_2, 5% CO_2).

Migration assay – scratch assay

Migration of pMSCs or hMSCs cultured either under hypoxic or normoxic was analyzed using scratch wound healing assay (78). Briefly, cells were cultured to obtain 90-100% confluence. Next, the cells were scratched from the plate using a plastic tip to create the "wound". The wound healing manifested by the ability of the cells to refill the created gap was observed. After 3.5h, 8h and 24h cells were fixed with cold methanol and stained using Giemsa-May Grünwald method. The pictures were taken, and the area of the scratch was calculated using GIMP 2.

Myoblast and bone marrow-derived MSC co-culture, fusion index, and hybrid myotube analysis

Co-cultures were obtained by seeding mPM in a 1:1 ratio with either pMSCs or hMSCs. Cells were cultured under normoxic or hypoxic conditions in MSCmed or PMmed for 5-7 days. C2C12 myoblasts ($3x10^4$ or $6x10^4$) were cultured in the absence of hMSCs or pMSCs or co-cultured with hMSCs or pMSCs in 3:2.5; 3:5; 3:7.5 ratio. Cells were cultured under normoxic or hypoxic conditions in C2C12med for 5-7 days. Further, cells were fixed, and fusion index or proportion of hybrid myotubes were estimated.

Fusion index of C2C12 or mPM cultured alone or in co-cultures either with pMSCs or hMSCs was calculated. Briefly, differentiated cells were fixed in cold methanol and stained according to the Giemsa-May Grünwald method. Images from 4 fields of view were collected, and nuclei number was counted. Fusion index was calculated as a percentage of nuclei present in myotubes compared to all visible cell nuclei.

Myotubes formed by either C2C12 or PM co-cultured either with pMSCs or hMSCs were visualized using skeletal myosin's immunolocalization. The participation of hMSCs in myotube formation was evaluated by visualization of human nuclei. pMSC contribution in hybrid myotubes formation was verified by the presence of GFP within myotubes.

Muscle injury and cell transplantation

Local Ethics Committee No. 1 in Warsaw, Poland, approved all procedures involving animals—permission number: 669/2018. To induce skeletal muscle injury, 2-3-month-old NOD SCID mice (Janvier Labs) were anesthetized, and their *Gastrocnemius* muscles were injected with 50 µl of 10 mM cardiotoxin (L8102, Latoxan). Further, 0.5 mln of hMSCs cultured in normoxic or hypoxic (for 48h) suspended in 20 µl of phosphate buffer saline (PBS) were injected into damaged *Gastrocnemius* muscle.

In contrast, the contralateral leg was injected with 0.9% NaCl solution (saline-treated muscles served as a control). After 14 days of regeneration, mice were sacrificed, muscles were isolated, and analyzed.

Immunocytochemistry and immunohistochemistry

Selected antigens were immunolocalized in *in vitro* cultured cells as well as in muscle cross-sections. *In vitro* cell cultures were fixed with 3% PFA, washed with PBS, and stored in 4°C. Dissected skeletal muscles were frozen in isopentane, cooled down with liquid nitrogen, transferred to -80°C, and cut into 10 µm sections using cryomicrotome (Microm HM, Thermo Fisher Scientific). Cryosections were fixed with 3% paraformaldehyde, washed with PBS, and stored in 4°C. Further fixed cells or cryosections were permeabilized with 0.1% Triton X-100 (Sigma-Aldrich) in PBS and incubated with 0.25% glycine (Sigma-Aldrich) in PBS. Non-specific binding of antibodies was blocked with 3% bovine serum albumin (BSA, Sigma-Aldrich) in PBS. Then cells or cryosections were incubated with primary antibodies diluted 1:100 in 3% BSA in PBS overnight. With appropriate secondary antibodies conjugated with fluorochromes diluted 1:200 in 1.5% BSA in PBS for 2h in room temperature. Next, samples were washed with PBS, and cell nuclei were visualized with Hoechst 33342 diluted 1:500 in 3% BSA in PBS. Finally, specimens were mounted with Fluorescent Mounting Medium (Dako Cytomation). Samples were analyzed using Axiovert 100M LSM 510 (Zeiss) and ZEN software. The following primary antibodies were used: rabbit anti-mouse skeletal myosin (M7523; Sigma-Aldrich), mouse anti-human nuclear antigen (ab191181; Abcam), rabbit anti-laminin (L9393; Sigma-Aldrich). The following secondary antibodies were used: donkey anti-mouse conjugated with AlexaFluor 488 (A21202, ThermoFisher Scientific) or AlexaFluor 594 (A21203, ThermoFisher Scientific), goat anti-rabbit conjugated with AlexaFluor 488 (A11008, ThermoFisher Scientific), or donkey anti-rabbit conjugated with AlexaFluor 594 (A21207, ThermoFisher Scientific).

Muscle histology

Dissected skeletal muscles were frozen in isopentane, cooled down with liquid nitrogen, transferred to -80°C, and cut into 10 µm sections using cryomicrotome (Microm HM, Thermo Fisher Scientific). Cryosections were fixed with 3% PFA, washed with PBS, and stored in 4°C. Samples were hydrated in PBS, incubated in Harris hematoxylin solution (Sigma-Aldrich), and washed in distilled water. Then, fixed sections were incubated in eosin Y solution (Sigma-Aldrich) and washed in distilled water. Specimens were mounted with UltraMount (Dako Cytomation) and analyzed using inverted light microscope Eclipse TE200 (Nikon) and ImageJ software (NIH).

Gene expression analysis

Total RNA was isolated from muscles, C2C12, mPM, pMSCs, and hMSCs cultured alone or in co-cultures, using High Pure Isolation Kit (Roche) and from dissected muscles using mirVana™ miRNA Isolation Kit (Thermo Fischer Scientific) and purified with Turbo DNA-free Kit (Thermo Fischer Scientific), according to the manufacturers' protocols. cDNA was obtained in reverse transcription reaction performed using RevertAid First Strand cDNA Synthesis Kit (ThermoFisher Scientific) according to manufacturer's protocol. The conditions of reverse transcription were as follows: 25°C for 10 min, 42°C for 60 min, 85°C for 5 min. Next, mRNA levels were examined using quantitative real-time PCR analysis (qPCR) with TaqMan assays (ThermoFisher Scientific) for the following genes: human: *CD9* (Hs00233521_m1)*, ADAM9* (Hs00177638_m1)*, CSPG4* (Hs00361541_g1), *PDFGRB* (Hs01019589_m1), *VWF* (Hs00169795_m1), *KDR* (Hs00911700_m1), *CDH15* (Hs00170504_m1), *MYOD1* (Hs02330075_g1)*, MYF5* (Hs00929416_g1)*, MYOG* (Hs01072232_m1), *MCAM* (Hs00174838_m1), *VCAM1* (Hs01003372_m1), *NES* (Hs04187831_g1), *CXCL12* (Hs03676656_mH), *VEGFA* (Hs05484830_s1), *WNT4* (Hs01573505_m1), *FAP* (Hs00990791_m1); pig: *SGCA* (Ss03821424_s1)*, ACTA1* (Ss04245853_m1), *DES* (Ss03378045_u1), *MYOG* (Ss03379073_u1); mouse*: Adam9* (Mm01218460_m1)*, Cd9* (Mm00514255_g1), *Cdh15* (Mm00483191_m1), *Ncam1* (Mm01149710_m1), *Vcam1* (Mm01320970_m1), *Pax7* (Mm01354484_m1), *Myf5* (Mm00435125_m1), *Myod1* (Mm00440387_m1), *Myog* (Mm00440387_m1), *Cxcl12* (Mm004485552_m1), *Vegfa* (Mm00437304_m1), *Vwf* (Mm00550376_m1), *Kdr* (Mm01222419_m1). *HPRT/Hprt* (Hs99999909_m1, Ss03382484_u1, Mm03024075_m1) was used as a reference gene for *in vitro* studies and *actin (ACTB*; Hs030233943_g1 and Mm01205647_g1) was used as reference gene for *in vivo* studies*.* All reactions were performed in duplicates. qPCR was performed with the TaqMan Gene Expression Master Mix (ThermoFisher Scientific) using LightCycler 480 (Roche) according to manufacturer's instruction. The conditions of qPCR were as follows: preincubation 2 min., 50°C; preincubation 10 min., 95°C; amplification (40 cycles) 15 s., 95°C, and 1 min., 60°C. Expression levels were calculated with $2^{-(\Delta CT)}$ formula.

Statistical analysis

At least three independent biological experiments were shown as mean with standard deviations with GraphPad Prism 7. The results were analyzed in GraphPad Prism7 with the One-Way ANOVA test and Bonferroni multi comparison test or t-Student test (Figure 5). The results were compared to cells cultured in MSCmed under normoxic conditions.

Results

The proliferation, migration, and fusion of human and pig bone marrow-derived MSCs, mouse primary myoblasts, and C2C12 under normoxic and hypoxic conditions

First, we analyzed the proliferation of human bone marrow-derived mesenchymal stromal cells (hMSC), fetal pig bone marrow-derived mesenchymal stromal cells (pMSC), as well as mouse primary myoblasts (mPM), which we used to set up the co-culture experiments (Figure 1A). Next, we performed similar analyzes of hMSC or pMSC co-cultured with mPM or mouse C2C12 myoblasts. The two types of myoblasts were analyzed because of differences between primary cultures and cell lines. All these experiments were carried out either under normoxic or hypoxic conditions. Cells and co-cultures were analyzed after 5-7 days of culture in the following media types: MSCmed and either C2C12med or PMmed. MSCmed and C2C12med allowed studying the cells cultured under proliferating conditions and PMmed under differentiating conditions. Analysis of mPMs showed that their number was significantly higher in cultures carried under hypoxic conditions, regardless of the medium used. Neither hypoxic nor the type of medium influenced the number of hMSCs or pMSCs. Co-culture of mPMs with either hMSCs or pMSCs, conducted under hypoxic conditions, also increased overall cell number (Figure 1A). No significant change in hMSC or pMSC migration was noticed comparing normoxic and hypoxic conditions (Figure 1B.).

Second, we compared the fusion index and proportion of hybrid myotubes formed due to the fusion between either hMSCs or pMSCs with either mPM or C2C12 myoblasts. Depending on the experiment set, the co-cultures were conducted in MSCmed, C2C12med, or PMmed, in each case under normoxic or hypoxic conditions (Figure 2). First, we analyzed the fusion index of mPM (Figure 2A) or C2C12 myoblasts (Figure 2B). We noticed that hypoxic conditions significantly decreased myoblast fusion. Next, fusion was considerably higher when mPM and C2C12 were cultured under hypoxic conditions in the presence of either hMSCs or pMSCs. In the case of co-cultures conducted under normoxic, the presence of hMSCs did not impact the fusion index of mPM nor C2C12. Interestingly, pMSCs had a negative impact on C2C12 myoblast fusion when cultured under normoxic conditions. The proportion of hybrid myotubes formed by mPM either with hMSCs or pMSCs significantly increased under hypoxic conditions (Figure 2C, D). The fusion of hMSCs or pMSCs and C2C12 differed depending on the culture medium, and hypoxic conditions did not increase hybrid myotubes' formation (Figure 2D, F).

The changes in expression of selected markers in human and pig bone marrow-derived MSCs and mouse primary myoblasts under normoxic and hypoxic conditions

To follow the changes in myogenic differentiation of cells cultured under normoxic and hypoxic conditions in two different types of media (MSCmed and PMmed), the expression of transcript encoding PAX7, myogenic regulatory factors (MRFs), cytoskeletal proteins, and adhesion proteins was examined in mouse, human and pig cells (Figure 3). The expression of mouse *Pax7, Myf5, Myod1,* and myogenin (*Myog*) significantly increased in mPM cultured under hypoxic conditions, regardless of the culture medium used (Figure 3A). The higher level of mRNAs encoding Pax7 and MRFs in cells cultured under hypoxic conditions corresponded to myoblasts' higher proliferation. It is well known that *Pax7, Myod1,* and *Myf5* are expressed in activated satellite cells, and *Myod1* and *Myf5* in proliferating cells (29). The expression of MRFs in hMSCs and pMSCs was barely detectable (Figure 3B, C). Human *MYF5*, *MYOD1, and MYOG* level increased in hMSCs cultured under hypoxic conditions but was still very low (Figure 3B).

The level of mRNAs encoding adhesion proteins, such as VCAM, NCAM, CD9, ADAM9, and m-cadherin (*cdh15 or CDH15*) which are engaged in cell-cell adhesion and myoblast fusion (79-86) depended on the cell type and culture conditions used, i.e., medium and oxygen level (Figure 3A, B). Their expression was significantly higher in mPM cultured in PMmed than in MSCmed under normoxic. Moreover, we observed a significant decrease in *Cd9* expression in mPM cultured under hypoxic conditions, regardless of medium type (Figure 3A). It corresponds to less efficient fusion of myoblasts observed under hypoxic conditions (Figure 2A). Analysis of hMSCs documented a significant decrease in *ADAM9, CDH15*, and *CD9* expression levels when cells were cultured under hypoxic conditions, regardless of the medium used. In the case of pMSCs, the level of α-sarcoglycan (*SGCA*) and desmin (*DES*) transcripts, i.e., encoding proteins characteristic for muscle cells (87, 88), changed dependently on culture conditions, being the highest in cells cultured in PMmed under hypoxic conditions (Figure 3C).

Co-culture of hMSCs or pMSCs cells with mPMs or C2C12 myoblasts were analyzed to establish the changes in the expression of transcripts encoding MRFs, cytoskeletal, and adhesion proteins in human and pig cells cultured in the presence of myoblasts, under both normoxic or hypoxic conditions (Figure 4). Hypoxic increased hybrid myotube formation in co-cultures between either hMSCs or pMSCs and mPMs, in comparison to co-cultures conducted under normoxic. The level of human MRFs was significantly higher in hMSCs co-cultured with mPM than hMSCs cultured alone under all culture conditions tested (Figure 3B and 4A). Thus, the presence of myoblasts impacted the MRF expression in hMSCs. Moreover, we detected a higher expression level of human *MYF5* and *MYOD1* in co-cultures with mouse myoblasts carried in PMmed under normoxic conditions and in both types of the medium

under hypoxic conditions (Figure 4A). Also, the level of transcripts encoding human *CD9* and *CDH15* significantly increased in cells cultured under hypoxic conditions. Hypoxic and myoblasts' presence did not alter the *MYOG* expression in pMSCs, but the high level of *DES* (desmin) was noticed in cells cultured in MSCmed (Figure 4B).

The transplantation of human bone marrow-derived MSCs cultured under normoxic and hypoxic conditions into mouse injured skeletal muscles

As we demonstrated, human and pig bone marrow-derived MSCs influenced myoblasts' proliferation and fusion when cultured under hypoxic conditions. Since any significant differences between human and pig cells' impact on mouse myoblasts were found, we decided to inject human bone marrow-derived MSCs into injured mouse muscle. Before transplantation, we examined the expression of human progenitor cell (*MCAM/CD146*), pericyte (*PDGFRb, NG2*), endothelial (*VEGFR*), and fibroblast (*FAP*) markers in hMSCs cultured in MSCmed under normoxic and hypoxic conditions (Figure 5). CD146 was shown to be a marker of a subpopulation of human bone marrow-derived stem cells [88, 89]. We noticed that the expression of *MCAM/CD146* and *NG2* (CSPG4) increased under hypoxic conditions. However, the expression of FAP also increased. The level of *PDGFRb* and KDR (*VEGFR*) did not change significantly under hypoxic conditions. Then we analyzed the level of transcripts of secreted proteins engaged in cell mobilization and differentiation, such as SDF-1 (CXCL12), VEGF, VWF, and WNT4. We found that the level of *VEGF* increased in hMSCs cultured under hypoxic conditions.

Finally, hMSC cultured under normoxic conditions and hypoxic preconditioned hMSCs were transplanted into ctx injured skeletal muscle of SCID mice (Figure 6). The muscle mass, nerve area, and a number of newly formed myofibers did not differ between muscles that received hMSCs cultured under either normoxic or hypoxic conditions (Figure 6A, B). Importantly, the area of blood vessels was higher after hypoxic preconditioned hMSC transplantation. Moreover, a higher number of human cells was detected in mouse muscles injected with hypoxic preconditioned hMSCs (Figure 6A, C). These cells were found between myofibers (Figure 6C). However, the higer area of connective tissue in muscles injected with hMSCs cultured under hypoxic conditions compering to control muscles was noticed (Figure 6A). Then, we analyzed the level of mouse and human transcripts after cell transplantation. The level of mouse *Vwf* was lower in injured muscles than in intact muscle; however, it did not differ between muscles transplanted with hMSC cultured under either standard or hypoxic conditions (Figure 6D). Notably, only in mouse muscles injected with hypoxic preconditioned hMSCs the human transcripts such as laminin, *VCAM, MCAM, PDGFRb, CSPG4* (NG2), *FAP, CXCL12* (SDF-1), and *VEGF* were found (Figure 6E). Besides, the *WNT*, *MYH3, MYF5, MYOD1,* and *MYOG* transcripts were not detected. We

concluded that hypoxic preconditioned hMSCs efficiently engrafted injured muscle but did not follow myogenic differentiation based on obtained results.

Discussion

Under physiological conditions, the oxygen level in healthy resting human skeletal muscles equates from 3% to 4% (25-34 mmHg) (89). Moreover, during intense exercise, human skeletal muscle oxygenation decreases to 7.5 mmHg O_2 (90). In resting mouse muscle, the oxygenation reaches approximately 50 mmHg O_2 (90). All these values are significantly lower than oxygen pressure under standard cell culture, amounting to circa 142 mmHg (i.e., approx. 20% O2), thus being higher (hyperoxic) to cells than oxygen pressure *in vivo*. For this reason, cell cultures performed under hypoxic conditions (oxygen pressure below 50 mmHg, 2-6% O_2) are considered more physiological for bone marrow and muscle cells (90, 91). The level of oxygen influences many cellular processes regulating the activity of O_2-consuming enzymes such as cytochrome c oxidase, stearoyl-CoA desaturase, NADPH oxidases, prolyl hydroxylase, as well as influencing on ROS formation (90-93). One of the essential proteins responsible for cell reaction to O2 level changes is hypoxic-inducible factor 1 (HIF-1), which regulates hypoxic responsive genes (94). The O_2 level impacts inter alia, cell proliferation, migration, differentiation, viability, protein synthesis, and secretion (90).

In our study, we noticed an increase of primary myoblast proliferation when cultured under hypoxic conditions, which stays in agreement with other studies showing the higher proliferation of rat, human, and mouse primary myoblasts cultured in hypoxic (2-6% O_2) (95-98). The C2C12 proliferation also increased in co-cultures with mouse bone marrow-derived MSCs in VEGF dependent manner (99). Importantly, under hypoxic conditions, we observed the increased *VEGF* expression in human bone marrow-derived MSCs. However, neither human nor pig bone marrow-derived MSC proliferation or migration changed when comparing the cells cultured under normoxic or hypoxic conditions. Although no significant influence of hypoxic on rat bone marrow-derived MSC migration was found, it was shown that the oxygen concentration affected bone marrow-derived MSC response to chemokines, inflammatory cytokines, and growth factors (100). On the other hand, mouse bone marrow-derived MSCs migrated more efficiently, in transmembrane migration assay, in response to conditioned medium under hypoxic conditions (68). Human bone marrow-derived MSCs cultured under hypoxic conditions increased their migration rates and HGF receptor expression, i.e., c-Met (101). Thus, the influence of hypoxic on bone marrow-derived MSC migration abilities depends on many variables.

In the current study, we documented that primary myoblasts and C2C12 myoblasts fused less efficiently under hypoxic conditions. Other studies showed that the impaired fusion of mouse C2C12

myoblasts cultured under hypoxic conditions was also connected to myotube atrophy and a lower number of nuclei per myotube (102). Also, mouse primary and H-2K myoblast differentiation were less efficient under such conditions (103). Moreover, most of the studies described the inhibition of myogenic differentiation under hypoxic (91). The response of mouse satellite cells to oxygen level changes is regulated by HIF2A (89). Quiescent satellite cells residing within the niche are hypoxic and express HIF2A, which maintains their quiescence and self-renewal and blocks differentiation (89). We noticed that the expression of *Pax7* and MRFs, i.e., *MyoD, Myf5*, and *Myog*, increased in myoblasts under hypoxic conditions. The higher level of *Pax7* and MRF expression could be connected with the elevated proliferation of myoblasts in hypoxic. Moreover, hypoxic culture conditions were shown to activate Notch1, leading to a reduction in miR1 and miR206 expression and PAX7 upregulation (104). The observed decrease in primary myoblast fusion corresponds to the lower expression level of tetraspanin Cd9 in cells under hypoxic conditions, independent of culture medium. CD9 protein is engaged in myoblast fusion. Thus, its lower level could be connected with impaired fusion (82, 83, 105, 106).

Interestingly, we noticed that fusion of myoblasts changed in the presence or absence of bone marrow-derived MSCs. As long as the bone marrow-derived MSCs and myoblasts were co-cultured under standard culture conditions, the fusion did not change significantly comparing to myoblast cultures. Under hypoxic conditions, the presence of human or pig bone marrow-derived MSCs significantly improved myoblast fusion, i.e., enhanced myoblast differentiation. Simultaneously, the higher number of hybrid myotubes formed by mouse primary myoblasts and either human or pig bone marrow-derived MSCs were observed under hypoxic conditions. To find the mechanism determining such phenomenon, we examined the changes in human or pig MRFs' expression level, adhesion proteins, cytoskeletal proteins, and other myoblast markers, such as dystrophin or sarcoglycan, in bone marrow-derived MSCs and co-cultures of bone marrow-derived MSCs and myoblasts. The level of human or pig MRF mRNAs was very low or undetectable. It, however, increased under hypoxic conditions but only in human bone marrow-derived MSCs. The expression level of adhesion proteins engaged in cell fusion varied depending on cell culture medium and conditions. Then, we analyzed the level of secreted factors transcripts, such as *SDF-1, VEGF-1, WNT4,* and *VWF,* and found that under hypoxic conditions, expression of *VEGF* significantly increased in human bone marrow-derived MSCs. It was also documented that mouse bone marrow-derived MSCs overexpressed *Vegf* and secreted a higher VEGF level under hypoxic conditions (67, 68). Moreover, both mouse primary myoblasts and C2C12 myoblasts expressed VEGF and its receptors (107). VEGF enhanced C2C12 myoblast migration and prevented apoptosis (107) and myogenic differentiation, which resulted in the promotion of myotube hypertrophy of C2C12 cells, increased mitogenic activity, migration, and proliferation (99,

108). Thus, we concluded that an increase of VEGF secretion by bone marrow-derived MSCs, observed under hypoxic conditions, could account for improved myoblast proliferation and differentiation.

Finally, we transplanted hypoxic preconditioned human bone marrow-derived MSCs to injured skeletal muscles of SCID mice to study their influence on tissue regeneration. Bone marrow-derived MSCs cultured under hypoxic more efficiently engrafted the muscle. However, they were found only between myofibers. We were able to detect human lamina, *VCAM, NG2, CD146, PDGFR,* and *FAP* in muscles after hypoxic preconditioned bone marrow-derived MSC transplantation with more effective engraftment. However, we did not notice the presence of myofibers formed by human bone marrow-derived MSCs, and the expression of human *MYF5, MYOD, MYOG,* and *MYH3* was not detected. Thus, we concluded that hypoxic preconditioned bone marrow-derived MSCs more efficiently engrafted injured muscles but did not follow myogenic differentiation. Notably, the human *SDF-1* and *VEGF* transcripts were present in mouse muscle after hypoxic preconditioned bone marrow-derived MSC transplantation, and these factors could impact skeletal muscle regeneration. We suggested that VEGF, which is upregulated in hypoxic preconditioned bone marrow-derived MSCs, could play a vital role in cell engraftment after transplantation. Verma and coworkers showed that tissue-resident satellite cells expressed VEGF, which recruited endothelial cells (109). In this way, satellite cells induced capillary formation in their niche. We suggested that improved expression of VEGF in hypoxic preconditioned bone marrow-derived MSCs could induce vessel formation and support cell engraftment. A higher number of vessels was found in muscles after hypoxic preconditioned bone marrow-derived MSC transplantation. Reconstruction of vessel network is essential for muscle reconstruction, and the reduction of skeletal muscle network was described in dystrophic, ALS, or denervated muscles (110). Acute muscle damage led to disruption in the microvasculature, hypoxic, and activation of HIF-1α signaling – the main factor of hypoxic response (111). One of the HIF-1 target genes is *VEGF*, i.e., a well-described factor triggering angiogenesis also in skeletal muscles (111). VEGF and angiogenesis improved skeletal muscle regeneration and chronic skeletal muscle diseases (108, 111-113). Similarly, SDF-1 expression increased in injured muscles, presented a proangiogenic effect, and mobilized stem cells to injured muscles (114-118). The restoration of blood flow and vascular formation was also enhanced after intra-arterial injection of hypoxic preconditioned mouse bone marrow-derived MSCs to mice with hind limb ischemia (101). Hypoxic preconditioned mouse bone marrow-derived MSC transplantation increased WNT4 expression in skeletal muscles, and WNT4 was shown to induce bone marrow-derived MSC proliferation and migration well as endothelial cell migration and myoblast differentiation (101). Based on all the abovementioned results, we concluded that SDF-1 and VEGF secreted by hypoxic preconditioned bone marrow-derived MSCs increased new vessel formation during skeletal muscle reconstruction.

Conclusions

The hypoxic induced proliferation of myoblasts but delayed their differentiation, decreased transcripts encoding CD9, and increased PAX7 and MRFs transcripts expression. The bone marrow-derived MSCs significantly improved myoblast proliferation and differentiation in co-cultures under hypoxic conditions *in vitro* in VEGF dependent manner. Moreover, bone marrow-derived MSCs are more frequently fused *in vitro* with myoblasts under hypoxic conditions. Hypoxic preconditioning of bone marrow-derived MSCs increased the level of VEGF expression. Hypoxic preconditioned bone marrow-derived MSCs more efficiently engrafted injured muscles *in vivo* but did not follow myogenic differentiation. Transplantation of hypoxic preconditioned bone marrow-derived MSCs into injured muscles increased muscle mass and new vessels' formation in SDF-1 and VEGF dependent manner.

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Author s' contributions

Conceptualization, MJ and EB; Methodology, EB; KA and IG; Investigation, BM; JG; AG; MK; AMR; IK; WS; KJI and PW; Data Curation, EB; BM; KA; IG; Writing—Original Draft Preparation, EB; BM; KA; IG; Writing—Review and Editing, EB; BM; KA; IG; MAC; MJ and PW; Visualization, IG and KA; Supervision, EB; MAC and MJ; Project Administration, WS; Funding Acquisition, MJ.

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Abbreviations

ACTA1 – actin α1, skeletal muscle
ACTB – actin β
ADAM9 - A Disintegrin And Metalloproteinase Domain 9
Akt – protein kinase B
BSA – bovine serum albumin
CD – cluster of differentiation
CDH15 – M-cadherin
CEE – chicken embryo extract
CSPG4/NG2 – neural-glial antigen 2

CXCL12/SDF1 – C-X-C chemokine 12/stromal derived factor 1
DAMP – damage-associated molecular pattern
DES - desmin
DMEM – Dulbecco's modified Eagle's medium
ECM – extracellular matrix
EDL – extensor digitorum longus
FAP – fibroblast activation protein
FBSin – fetal bovine serum inactivated
FDB – flexor digitorum brevis
FGF – fibroblast growth factor
FOXO – forkhead box O
GFP – green fluorescent protein
HGF – hepatocyte growth factor
HGFR/c-Met – hepatocyte growth factor receptor
HIF – hypoxic-inducible factor
hMSC – human mesenchymal stem/stromal cell
HPRT – Hypoxanthine phosphoribosyltransferase
HS – horse serum
IGF-1 – insulin - like growth factor 1
IL – interleukin
KDR/VEGFR – vascular endothelial growth factor receptor
MAPK – mitogen-activated protein kinase
MCAM/CD146 – melanoma cell adhesion molecule
MMP – metalloproteinase
mPM – mouse primary myoblasts
MRF – myogenic regulatory factor
MRF4 – myogenic regulatory factor 4
MSC – mesenchymal stem/stromal cell
MSCmed – mesenchymal stromal cells medium
mTOR – mammalian target of rapamycin
MYF5 – myogenic factor 5
MYH3 – Myosin heavy chain 3, embryonic
MYOD – myoblast determination protein
MYOG – myogenin
NADPH - Nicotinamide adenine dinucleotide phosphate (reduced)
NCAM1 – neural cell adhesion molecule 1
NES – nestin
NFATc – nucelar factor of activated T-cells, cytoplasmic
NF-κB - nuclear factor kappa B
NICD - Notch intracellular domain
NO – nitric oxide
Pax3 – paired box transcription factor 3
Pax7 – paired box transcription factor 7
PBS – phosphate buffer saline
PDFGRB – Platelet-derived growth factor receptor β
PFA – paraformaldehyde
PI3K – phosphoinositide 3-kinase
PMmed – primary myoblasts medium
pMSC – pig mesenchymal stem/stromal cell
ROS – reactive oxygen species
SC – satellite cell
SCID – severe combined immunodeficiency
SGCA – α sarcoglycan
STAT3 – signal transducer and activator of transcription 3
TA – tibialis anterior
TGF-β – tumor growth factor β
TNF-α – tumor necrosis factor α

TRPC – transient receptor potential cation channel
VCAM – vascular cell adhesion molecule
VEGF – vascular endothelial growth factor
VWF – von Willebrand factor
WNT – wingless/integrated

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Figure legend

Figure 1. Cell proliferation and migration under normoxic and hypoxic conditions. A - The number of mouse primary myoblasts (mPM), human bone marrow-derived mesenchymal stromal cells (hMSC), pig bone marrow-derived mesenchymal stromal cells (pMSC), cells in co-cultures of hMSC and mPM, and cells in co-cultures of pMSC and mPM, cultured in two types of medium: MSCmed and PMmed, under normoxic (NORM) or hypoxic (HYPO) conditions. B – the invaded area measured in scratch wound healing assay of hMSC and pMSC cultured in MSCmed under normoxic and hypoxic conditions. C – the scratch wound healing assay of hMSC and pMSC cultured in MSCmed under normoxic and hypoxic conditions. P-value: * < 0.05; ** < 0.01; ***<0.001; ****<0.0001.

Figure 2. The fusion index and hybrid myotubes presence in either human bone marrow-derived mesenchymal stromal cells (hMSC) or pig bone marrow-derived mesenchymal stem cells (pMSC) co-culture with myoblasts: mouse primary myoblasts (mPM) or C2C12 myoblasts. A – the fusion index of mPM and hMSC or pMSC co-cultured in MSCmed or PMmed under normoxic (NORM) or hypoxic (HYPO) conditions. B – the fusion index of C2C12 myoblasts and hMSC or pMSC co-cultured in 3:2.5; 3:5; 3:7.5 ratio, in C2C12med, under normoxic (NORM) or hypoxic (HYPO) conditions. C – the percentage of hybrid myotubes in mPM and hMSC or pMSC co-cultures in MSCmed or PMmed under normoxic (NORM) or hypoxic (HYPO) conditions. D – the immunolocalization of hybrid myotubes in mPM and hMSC co-cultures, red – skeletal myosin, blue – cell nuclei, green human cell nuclei. E – the percentage of hybrid myotubes in C2C12 and hMSC or pMSC co-cultures in C2C12med under normoxic (NORM) or hypoxic (HYPO) conditions. F – the immunolocalization of hybrid myotubes in C2C12 and hMSC co-cultures, red – skeletal myosin, blue – cell nuclei, green human cell nuclei. P-value: * < 0.05; ** < 0.01; ***<0.001; ****<0.0001.

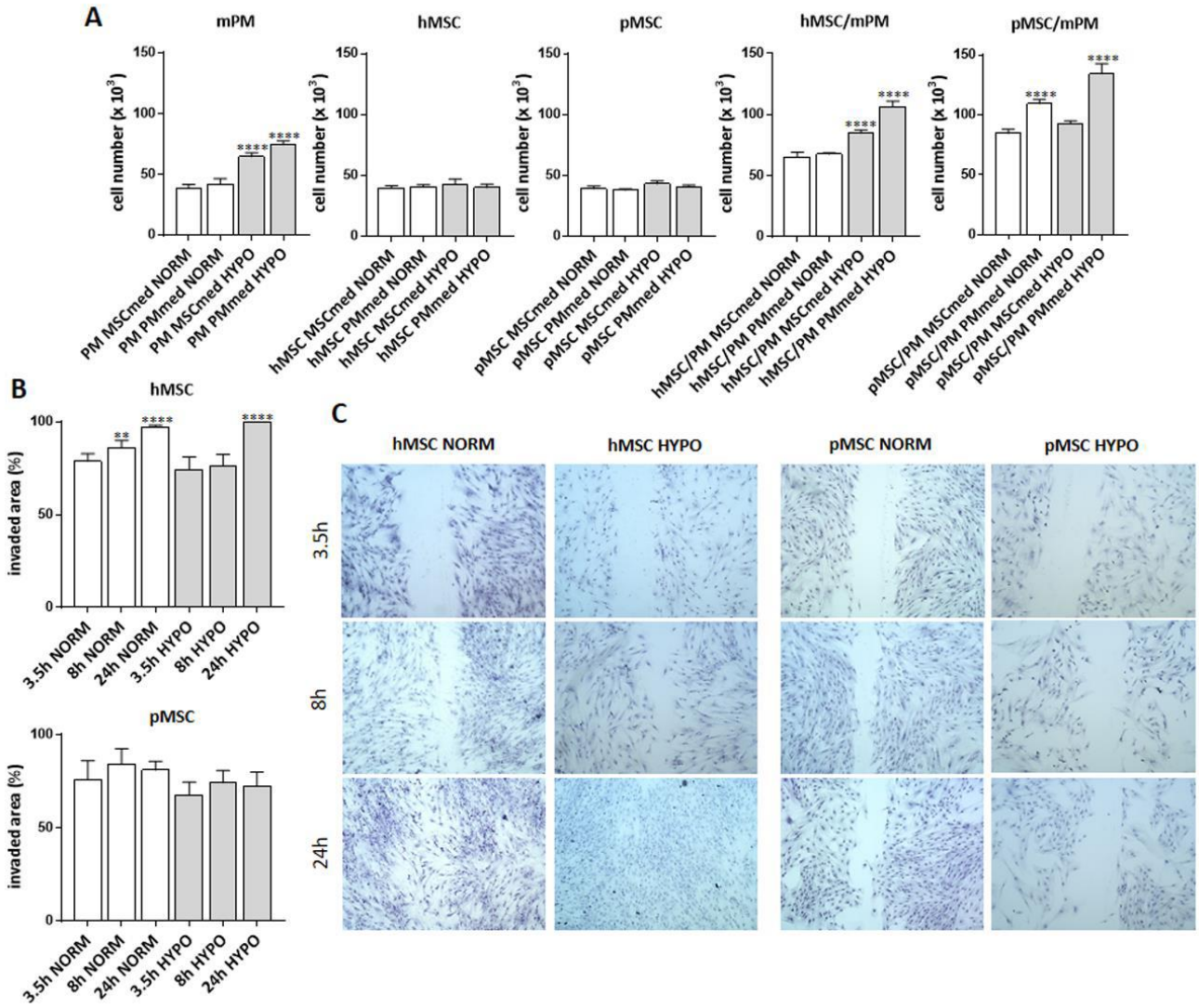
Figure 3. The expression of selected markers in cell cultures. A - the level of transcripts encoding PAX7, MYF5, MYOD1, myogenin (MYOG), VCAM1, NCAM1, CD9, ADAM9, m-cadherin (CDH15) in mouse primary myoblasts (mPM) cultured in PMmed or MSCsmed under normoxic (NORM) or hypoxic (HYPO) conditions. B – the level of transcripts encoding MYF5, MYOD1, myogenin (MYOG), VCAM1, CD9, ADAM9, m-cadherin (CDH15), nestin (NES) in human bone marrow-derived mesenchymal stromal cells (hMSC) cultured in PMmed or MSCsmed under normoxic (NORM) or hypoxic (HYPO) conditions. C - the level of transcripts encoding myogenin (MYOG), α-sarcoglycan (SGCA), desmin (DES), and actin α1 (ACTA1) in pig bone marrow-derived mesenchymal stem cells (pMSC) cultured in PMmed or MSCsmed under normoxic (NORM) or hypoxic (HYPO) conditions. P-value: * < 0.05; ** < 0.01; ***<0.001; ****<0.0001.

Figure 4. The expression of selected markers in cell co-cultures. A - the level of human transcripts encoding MYF5, MYOD1, myogenin (MYOG), VCAM1, CD9, ADAM9, m-cadherin (CDH15), nestin (NES) in co-cultures of mouse primary myoblasts (mPM) and human bone marrow-derived mesenchymal stromal cells (hMSC) in PMmed or MSCsmed under normoxic (NORM) or hypoxic (HYPO) conditions. B – the level of pig transcripts encoding myogenin (MYOG) SGCA, desmin (DES), and actin α1 (ACTA1) in mPM and pig bone marrow-derived mesenchymal stem cells (pMSC) co-cultured in PMmed or MSCsmed under normoxic (NORM) or hypoxic (HYPO) conditions. P-value: * < 0.05; ** < 0.01; ***<0.001; ****<0.0001.

Figure 5. The level of selected markers expression in human bone marrow-derived mesenchymal stromal cells (hMSC) cultured in MSCsmed under normoxic (NORM) or hypoxic (HYPO) conditions. P value: * < 0.05; ** < 0.01; ***<0.001; ****<0.0001.

Figure 6. The skeletal muscle regeneration after human bone marrow mesenchymal stromal cells (hMSC) transplantation. The hMSCs were cultured under normoxic (NORM) or hypoxic (HYPO) conditions and injected into cardiotoxin (CTX) injured muscles. A – the skeletal muscle weight, the area of connective tissue, blood vessels, nerves, and frequency of new myofibers in intact muscles or muscles injured (CTX) and transplanted with hMSC cultured either under normoxic (NORM) or hypoxic (HYPO) conditions. B – histology of intact muscles or in muscles injured (CTX) and transplanted with hMSC cultured under normoxic (NORM) or hypoxic (HYPO) conditions. C - localization of transplanted cells (blue – nuclei, red – laminin, green – human nuclei) in intact muscles or muscles injured (CTX) and transplanted with hMSC cultured under normoxic (NORM) or hypoxic (HYPO) conditions. D – the expression of selected mouse transcripts in intact muscles or muscles injured (CTX) and transplanted with hMSC cultured under normoxic (NORM) or hypoxic (HYPO) conditions. E – the expression of selected human transcripts in intact muscles or muscles injured (CTX) and transplanted with hMSC cultured under normoxic (NORM) or hypoxic (HYPO) conditions. P-value: * < 0.05; ** < 0.01; ***<0.001; ****<0.0001.

Fig. 1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mPM
hMSC
pMSC
hMSC/mPM
pMSC/mPM
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PM PMmed NORM
PM MSCmed HYPO
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hMSC MSCmed NORM
hMSC PMmed NORM
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pMSC/PM MSCmed NORM
pMSC/PM PMmed NORM
pMSC/PM MSCmed HYPO
pMSC/PM PMmed HYPO
B
hMSC
pMSC
invaded area (%)
3.5h NORM
8h NORM
24h NORM
3.5h HYPO
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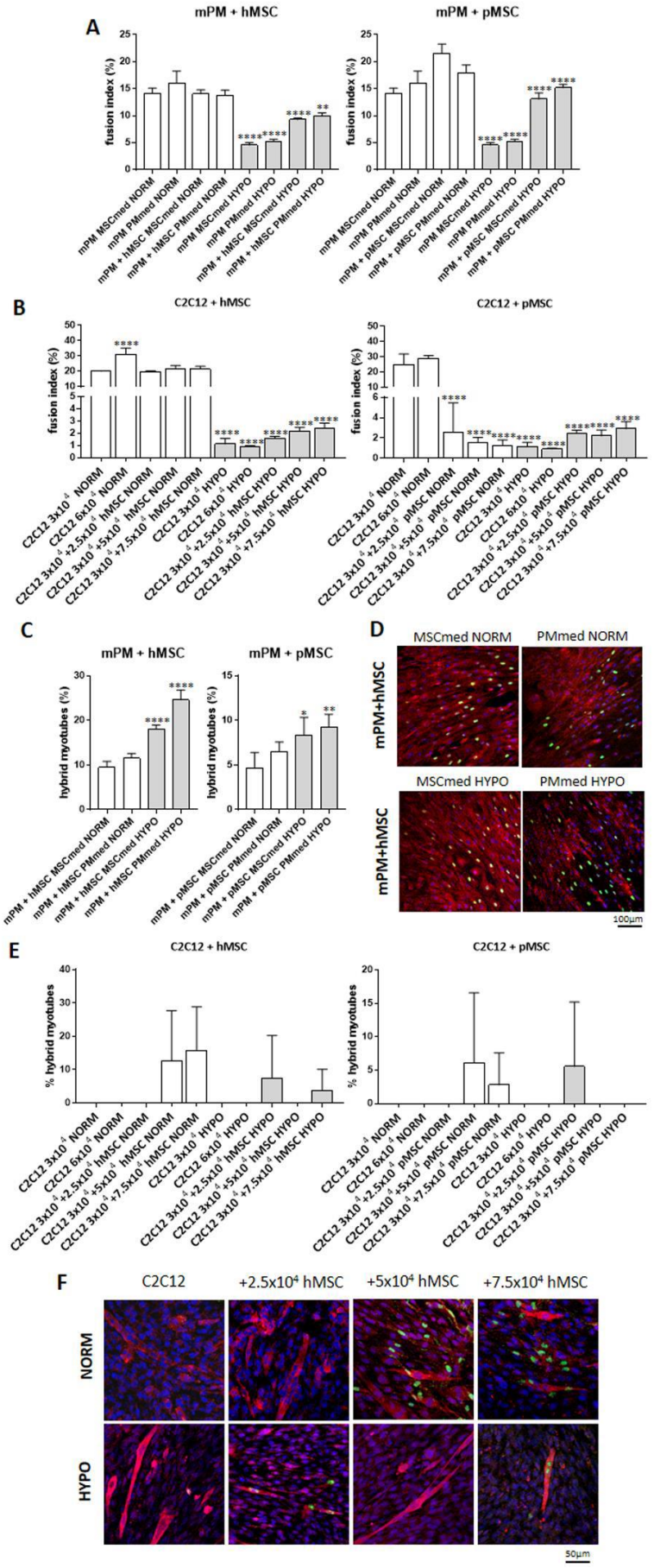
pMSC HYPO
3.5h
8h
24h

Fig. 2


A
mPM + hMSC
mPM + pMSC
fusion index (%)
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mPM PMmed NORM
mPM + hMSC MSCmed NORM
mPM + hMSC PMmed NORM
mPM MSCmed HYPO
mPM PMmed HYPO
mPM + hMSC MSCmed HYPO
mPM + hMSC PMmed HYPO
mPM + pMSC MSCmed NORM
mPM + pMSC PMmed NORM
mPM + pMSC MSCmed HYPO
mPM + pMSC PMmed HYPO
B
C2C12 + hMSC
C2C12 + pMSC
fusion index (%)
C2C12 3x10⁴ NORM
C2C12 6x10⁴ NORM
C2C12 3x10⁴ +2.5x10⁴ hMSC NORM
C2C12 3x10⁴ +5x10⁴ hMSC NORM
C2C12 3x10⁴ +7.5x10⁴ hMSC NORM
C2C12 3x10⁴ HYPO
C2C12 6x10⁴ HYPO
C2C12 3x10⁴ +2.5x10⁴ hMSC HYPO
C2C12 3x10⁴ +5x10⁴ hMSC HYPO
C2C12 3x10⁴ +7.5x10⁴ hMSC HYPO
C2C12 3x10⁴ +2.5x10⁴ pMSC NORM
C2C12 3x10⁴ +5x10⁴ pMSC NORM
C2C12 3x10⁴ +7.5x10⁴ pMSC NORM
C2C12 3x10⁴ +2.5x10⁴ pMSC HYPO
C2C12 3x10⁴ +5x10⁴ pMSC HYPO
C2C12 3x10⁴ +7.5x10⁴ pMSC HYPO
C
mPM + hMSC
mPM + pMSC
hybrid myotubes (%)
mPM + hMSC MSCmed NORM
mPM + hMSC PMmed NORM
mPM + hMSC MSCmed HYPO
mPM + hMSC PMmed HYPO
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D
MSCmed NORM
PMmed NORM
MSCmed HYPO
PMmed HYPO
mPM+hMSC
mPM+hMSC
100µm
E
C2C12 + hMSC
C2C12 + pMSC
% hybrid myotubes
C2C12 3x10⁴ NORM
C2C12 6x10⁴ NORM
C2C12 3x10⁴ +2.5x10⁴ hMSC NORM
C2C12 3x10⁴ +5x10⁴ hMSC NORM
C2C12 3x10⁴ +7.5x10⁴ hMSC NORM
C2C12 3x10⁴ HYPO
C2C12 6x10⁴ HYPO
C2C12 3x10⁴ +2.5x10⁴ hMSC HYPO
C2C12 3x10⁴ +5x10⁴ hMSC HYPO
C2C12 3x10⁴ +7.5x10⁴ hMSC HYPO
C2C12 3x10⁴ +2.5x10⁴ pMSC NORM
C2C12 3x10⁴ +5x10⁴ pMSC NORM
C2C12 3x10⁴ +7.5x10⁴ pMSC NORM
C2C12 3x10⁴ +2.5x10⁴ pMSC HYPO
C2C12 3x10⁴ +5x10⁴ pMSC HYPO
C2C12 3x10⁴ +7.5x10⁴ pMSC HYPO
F
C2C12
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+5x10⁴ hMSC
+7.5x10⁴ hMSC
NORM
HYPO
50µm

Fig. 3

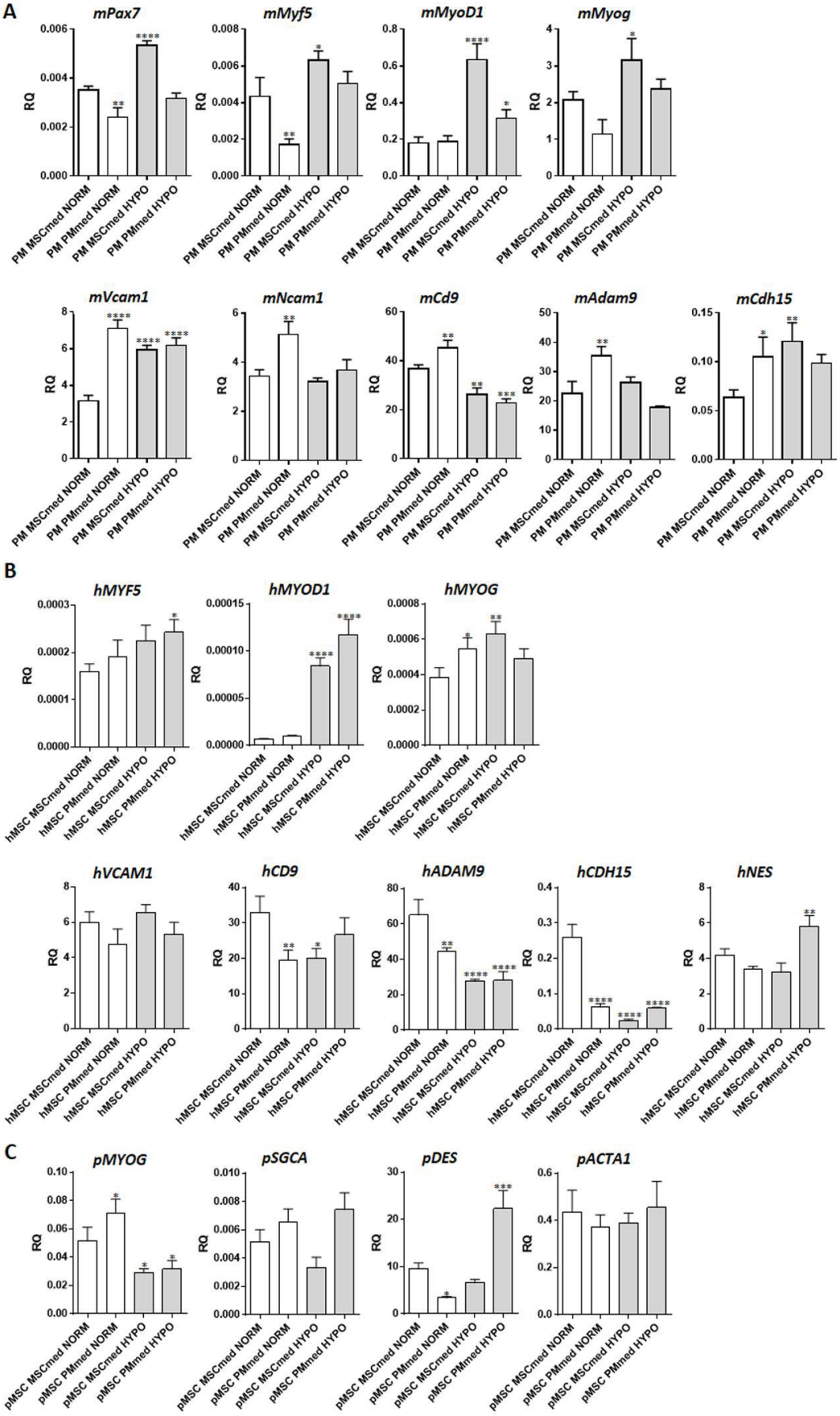
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Fig. 4

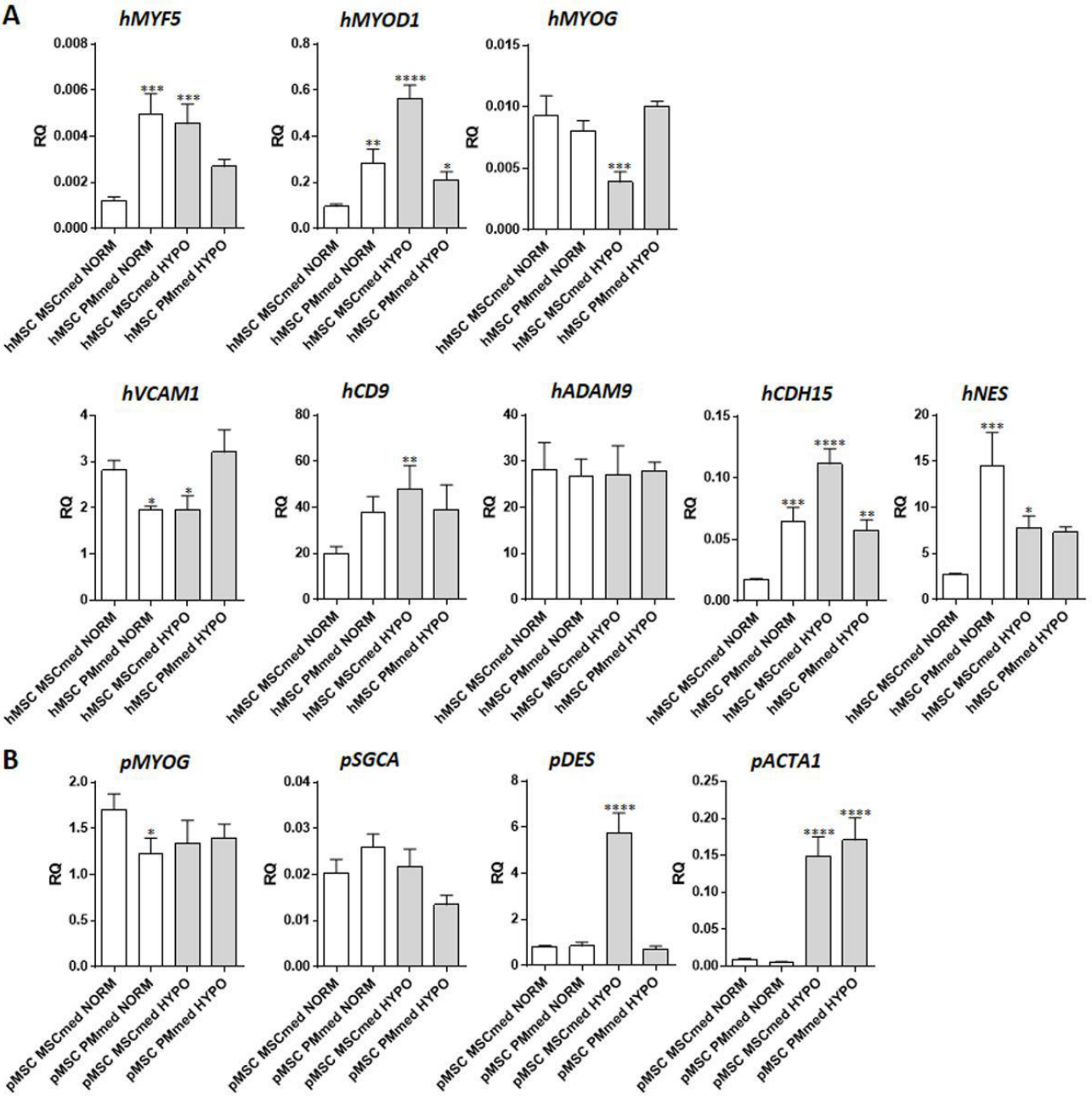
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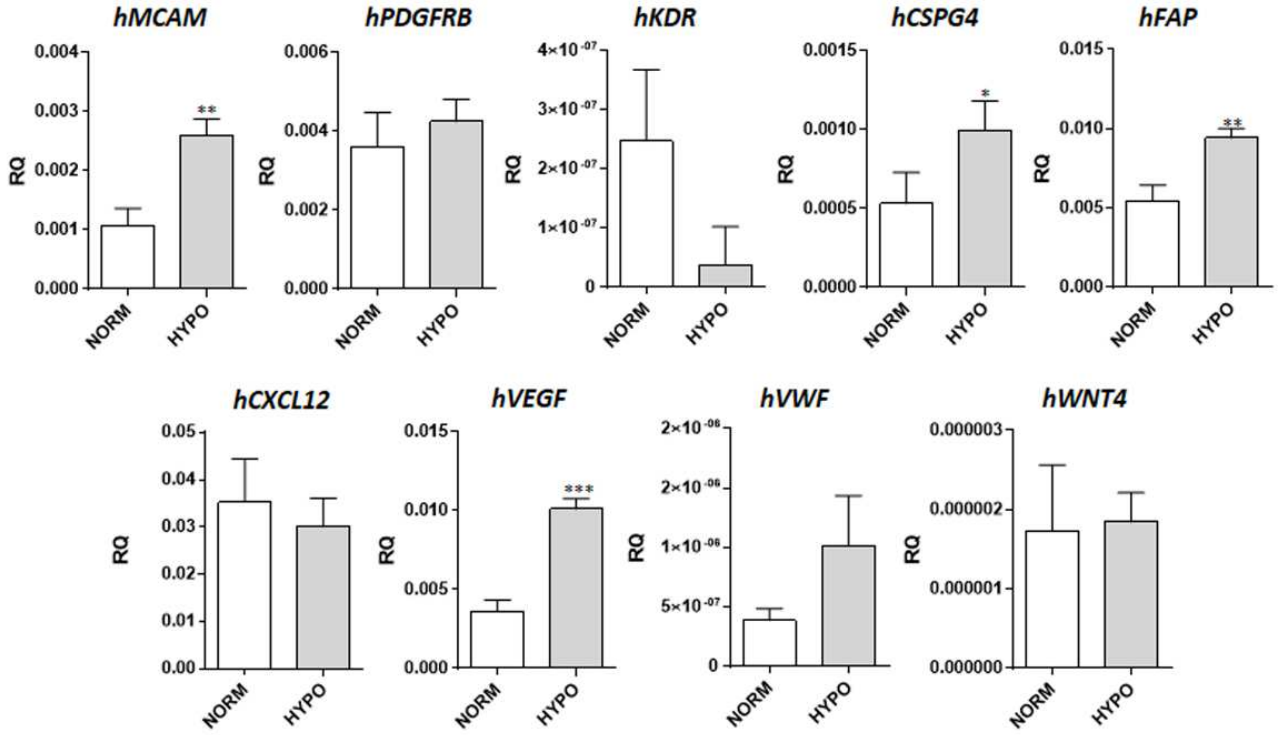
Fig. 5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NORM
HYPO
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*
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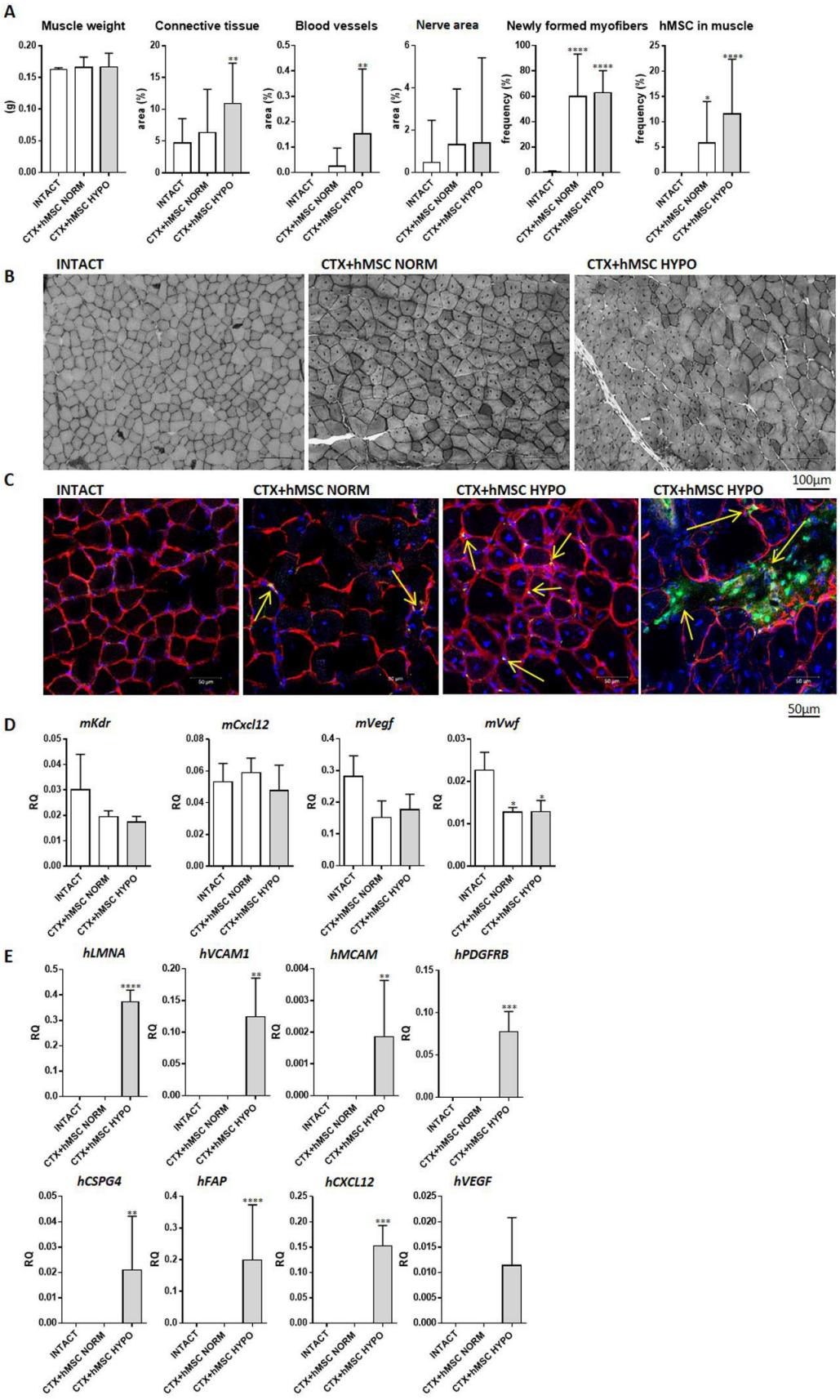
Fig. 6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Muscle weight
Connective tissue
Blood vessels
Nerve area
Newly formed myofibers
hMSC in muscle
(g)
area (%)
frequency (%)
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CTX+hMSC NORM
CTX+hMSC HYPO
B
INTACT
CTX+hMSC NORM
CTX+hMSC HYPO
C
INTACT
CTX+hMSC NORM
CTX+hMSC HYPO
CTX+hMSC HYPO
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D
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Figures

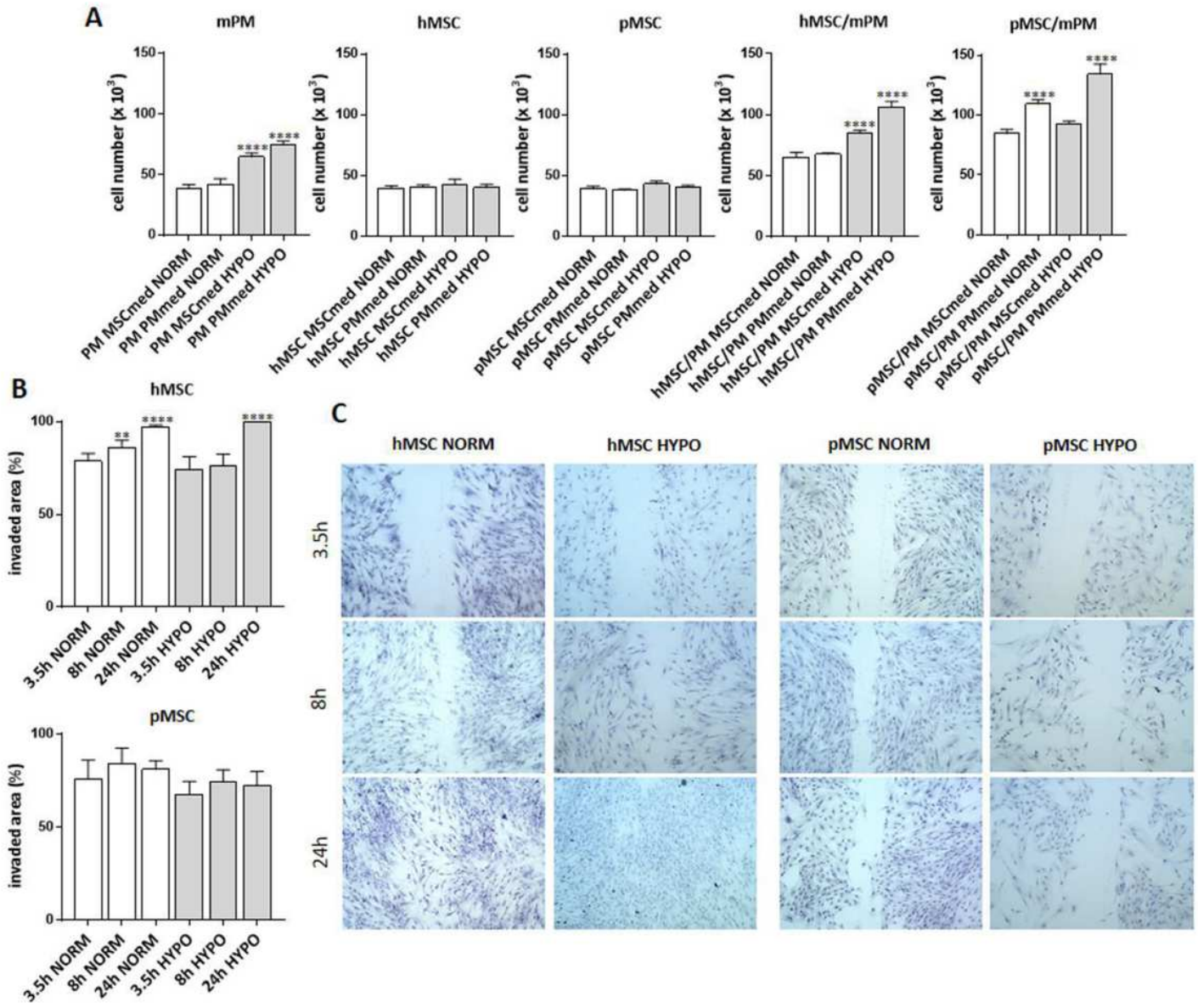

Figure 1

Cell proliferation and migration under normoxic and hypoxic conditions. A - The number of mouse primary myoblasts (mPM), human bone marrow-derived mesenchymal stromal cells (hMSC), pig bone marrow-derived mesenchymal stromal cells (pMSC), cells in co-cultures of hMSC and mPM, and cells in co-cultures of pMSC and mPM, cultured in two types of medium: MSCmed and PMmed, under normoxic (NORM) or hypoxic (HYPO) conditions. B – the invaded area measured in scratch wound healing assay of hMSC and pMSC cultured in MSCmed under normoxic and hypoxic conditions. C – the scratch wound healing assay of hMSC and pMSC cultured in MSCmed under normoxic and hypoxic conditions. P-value: * < 0.05; ** < 0.01; ***<0.001; ****<0.0001.

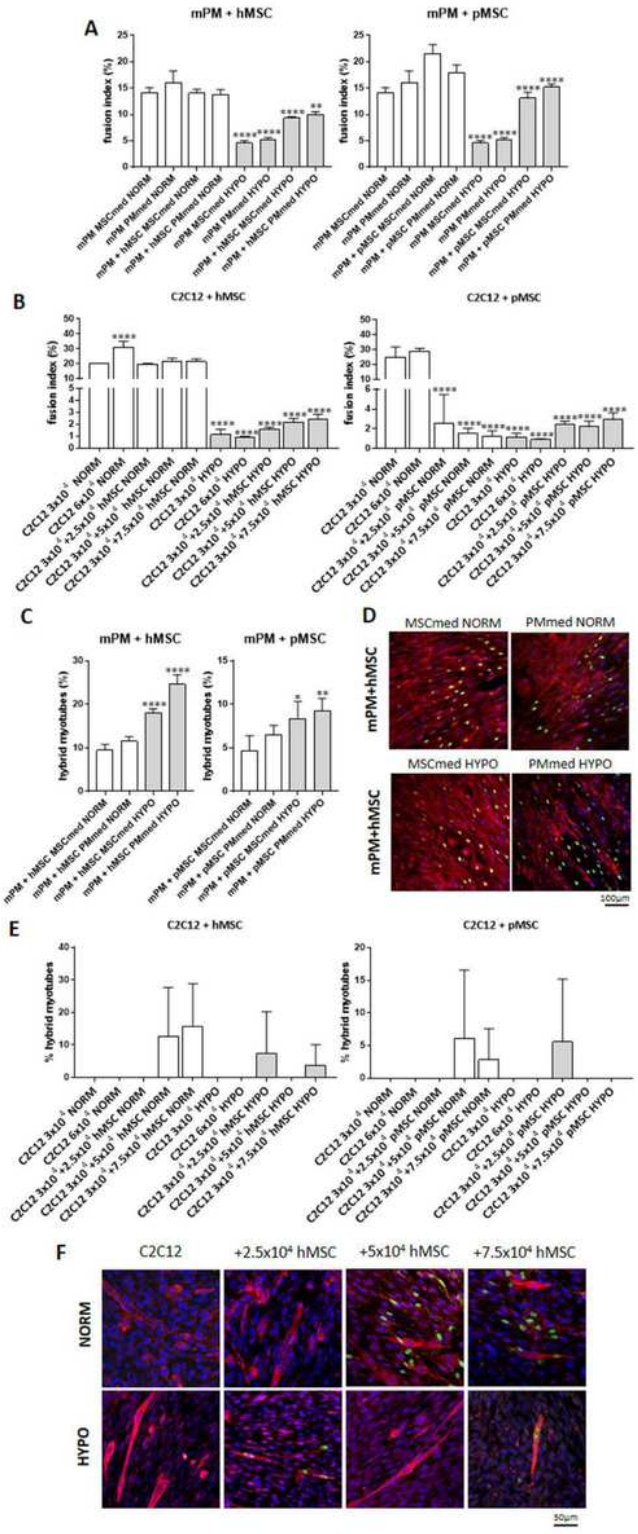

Figure 2

The fusion index and hybrid myotubes presence in either human bone marrow-derived mesenchymal stromal cells (hMSC) or pig bone marrow-derived mesenchymal stem cells (pMSC) co-culture with myoblasts: mouse primary myoblasts (mPM) or C2C12 myoblasts. A – the fusion index of mPM and hMSC or pMSC co-cultured in MSCmed or PMmed under normoxic (NORM) or hypoxic (HYPO) conditions. B – the fusion index of C2C12 myoblasts and hMSC or pMSC co-cultured in 3:2.5; 3:5; 3:7.5

ratio, in C2C12med, under normoxic (NORM) or hypoxic (HYPO) conditions. C – the percentage of hybrid myotubes in mPM and hMSC or pMSC co-cultures in MSCmed or PMmed under normoxic (NORM) or hypoxic (HYPO) conditions. D – the immunolocalization of hybrid myotubes in mPM and hMSC co-cultures, red – skeletal myosin, blue – cell nuclei, green human cell nuclei. E – the percentage of hybrid myotubes in C2C12 and hMSC or pMSC co-cultures in C2C12med under normoxic (NORM) or hypoxic (HYPO) conditions. F – the immunolocalization of hybrid myotubes in C2C12 and hMSC co-cultures, red – skeletal myosin, blue – cell nuclei, green human cell nuclei. P-value: * < 0.05; ** < 0.01; ***<0.001; **** <0.0001.

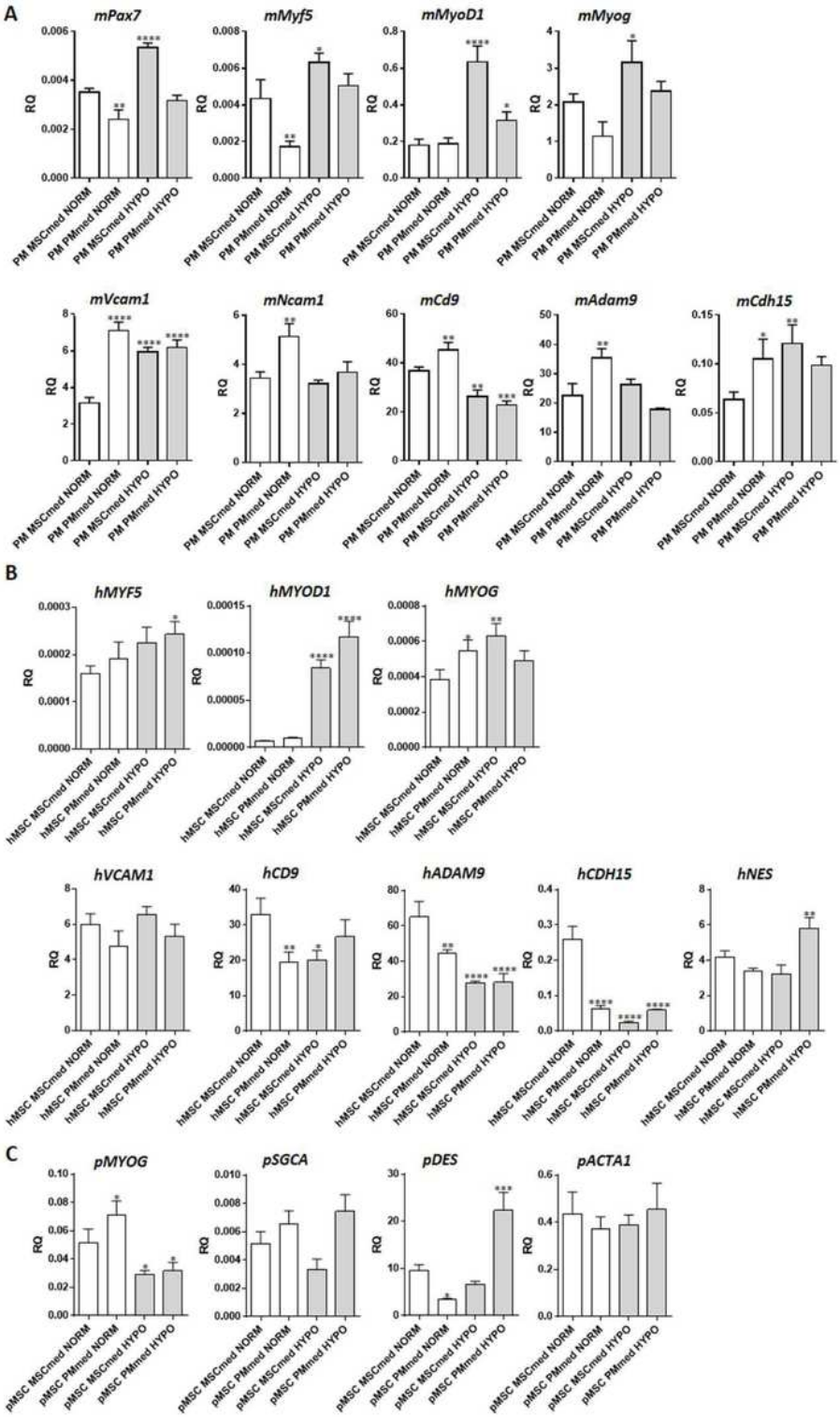

Figure 3

The expression of selected markers in cell cultures. A - the level of transcripts encoding PAX7, MYF5, MYOD1, myogenin (MYOG), VCAM1, NCAM1, CD9, ADAM9, m-cadherin (CDH15) in mouse primary myoblasts (mPM) cultured in PMmed or MSCsmed under normoxic (NORM) or hypoxic (HYPO) conditions. B – the level of transcripts encoding MYF5, MYOD1, myogenin (MYOG), VCAM1, CD9, ADAM9, m-cadherin (CDH15), nestin (NES) in human bone marrow-derived mesenchymal stromal cells

(hMSC) cultured in PMmed or MSCsmed under normoxic (NORM) or hypoxic (HYPO) conditions. C - the level of transcripts encoding myogenin (MYOG), α-sarcoglycan (SGCA), desmin (DES), and actin α1 (ACTA1) in pig bone marrow-derived mesenchymal stem cells (pMSC) cultured in PMmed or MSCsmed under normoxic (NORM) or hypoxic (HYPO) conditions. P-value: * < 0.05; ** < 0.01; ***<0.001; **** <0.0001.

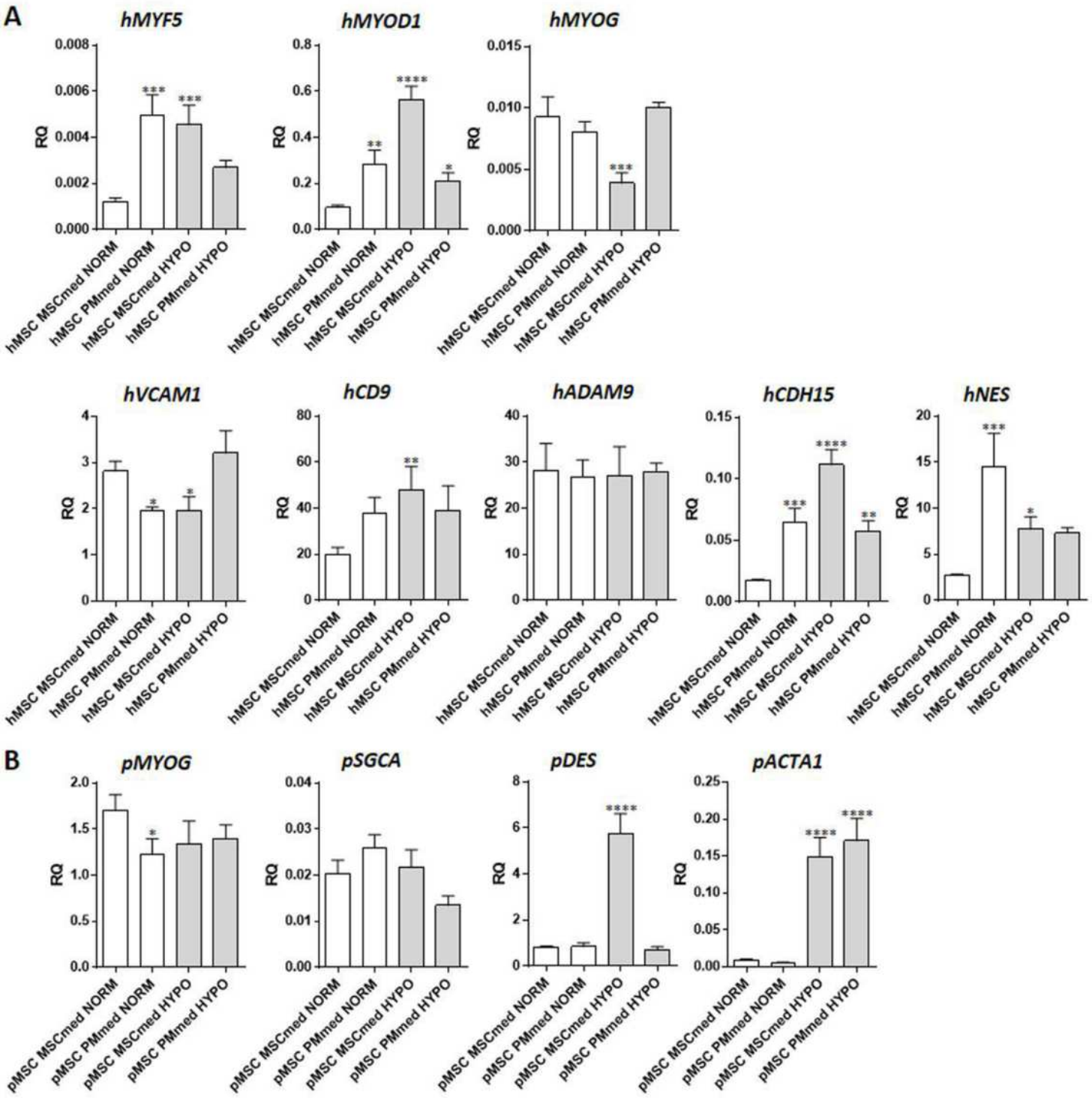

Figure 4

The expression of selected markers in cell co-cultures. A - the level of human transcripts encoding MYF5, MYOD1, myogenin (MYOG), VCAM1, CD9, ADAM9, m-cadherin (CDH15), nestin (NES) in co-cultures of mouse primary myoblasts (mPM) and human bone marrow-derived mesenchymal stromal cells (hMSC) in PMmed or MSCsmed under normoxic (NORM) or hypoxic (HYPO) conditions. B – the level of pig transcripts encoding myogenin (MYOG) SGCA, desmin (DES), and actin α1 (ACTA1) in mPM and pig bone marrow-derived mesenchymal stem cells (pMSC) co-cultured in PMmed or MSCsmed under normoxic (NORM) or hypoxic (HYPO) conditions. P-value: * < 0.05; ** < 0.01; ***<0.001; ****<0.0001.

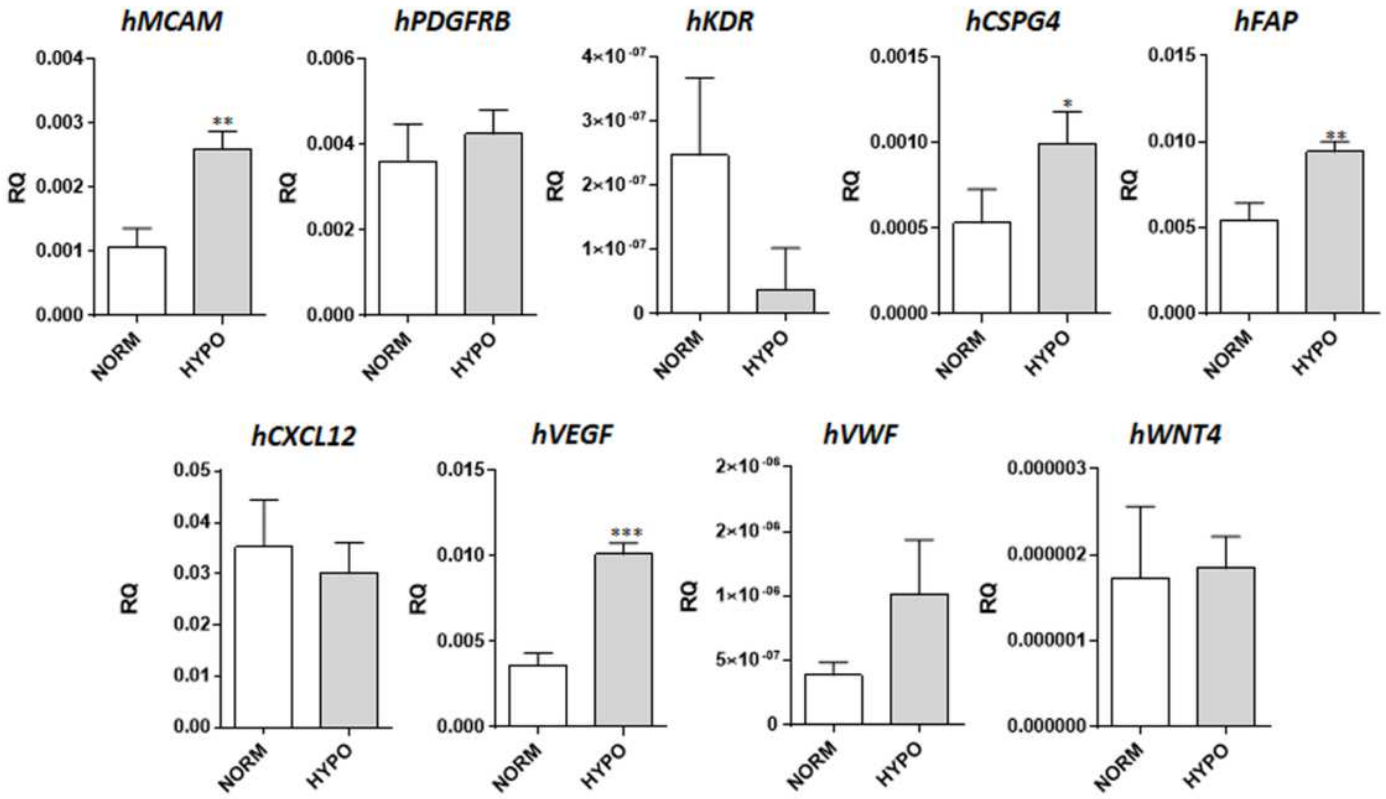

Figure 5

The level of selected markers expression in human bone marrow-derived mesenchymal stromal cells (hMSC) cultured in MSCsmed under normoxic (NORM) or hypoxic (HYPO) conditions. P value: * < 0.05; ** < 0.01; ***<0.001; ****<0.0001.

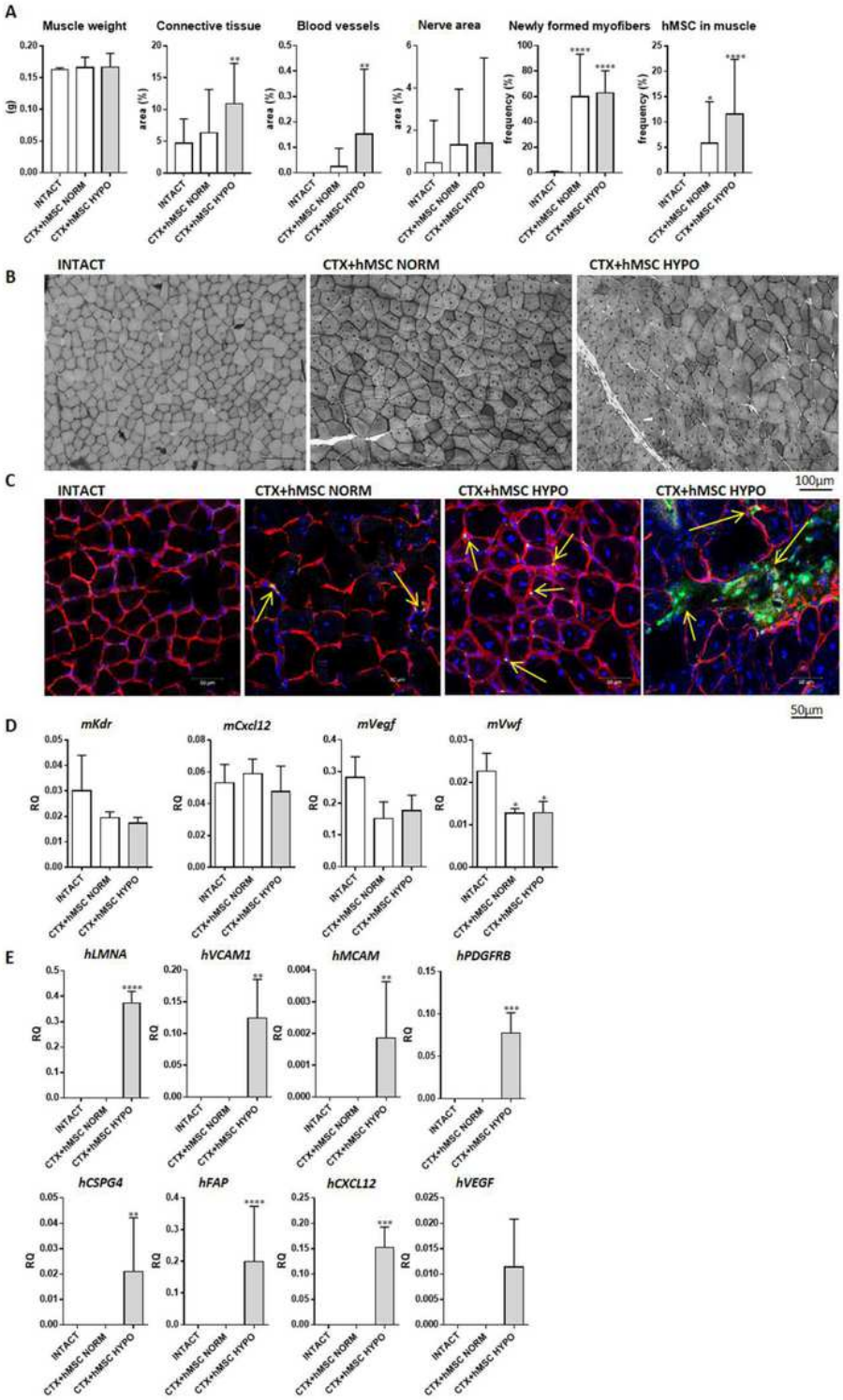

Figure 6

The skeletal muscle regeneration after human bone marrow mesenchymal stromal cells (hMSC) transplantation. The hMSCs were cultured under normoxic (NORM) or hypoxic (HYPO) conditions and injected into cardiotoxin (CTX) injured muscles. A – the skeletal muscle weight, the area of connective tissue, blood vessels, nerves, and frequency of new myofibers in intact muscles or muscles injured (CTX) and transplanted with hMSC cultured either under normoxic (NORM) or hypoxic (HYPO) conditions. B –

histology of intact muscles or in muscles injured (CTX) and transplanted with hMSC cultured under normoxic (NORM) or hypoxic (HYPO) conditions. C - localization of transplanted cells (blue – nuclei, red – laminin, green – human nuclei) in intact muscles or muscles injured (CTX) and transplanted with hMSC cultured under normoxic (NORM) or hypoxic (HYPO) conditions. D – the expression of selected mouse transcripts in intact muscles or muscles injured (CTX) and transplanted with hMSC cultured under normoxic (NORM) or hypoxic (HYPO) conditions. E – the expression of selected human transcripts in intact muscles or muscles injured (CTX) and transplanted with hMSC cultured under normoxic (NORM) or hypoxic (HYPO) conditions. P-value: * < 0.05; ** < 0.01; ***<0.001; ****<0.0001.