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Improving stemness and functional features of mesenchymal stem cells from Wharton's jelly of a human umbilical cord by mimicking the native, low oxygen stem cell niche

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ABSTRACT

Introduction: Mesenchymal stem cells from Wharton's Jelly of a human umbilical cord (WJ-MSCs) are a potential tool in regenerative medicine based on their availability, proliferative potential and differentiation capacity. Since their physiological niche contains low oxygen levels, we investigated whether cultivation of WJ-MSCs at 3% O_2 affects their main features.

Methods: WJ-MSCs were cultured under 21% and 3% O_2. Proliferation rate was followed by short and long term proliferation assays, clonogenic capacity by CFU-F assay and cell cycle and death by flow cytometry. Differentiation capacity was investigated by histochemical staining after induced differentiation. Pluripotency and differentiation markers' expression was determined by RT-PCR. Migration capacity was followed by scratch assay and mobilization from collagen, and the activity of proteolytic enzymes by zymography. Specific inhibitors of MAPK and Wnt/β-catenin pathways were used to investigate underlying molecular mechanisms.

Results: Compared to standard 21% O_2, cultivation of WJ-MSCs at 3% O_2 did not influence their immunophenotype, while it modulated their differentiation process and enhanced their clonogenic and expansion capacity. 3% O_2 induced transient change in cell cycle and prevented cell death. The expression of *NANOG*, *OCT4A*, *OCT4B* and *SOX2* was increased at 3% O_2. Both cultivation and preculturing of WJ-MSCs at 3% O_2 increased their *in vitro* migratory capacity and enhanced the activity of proteolytic enzymes. ERK1/2 mediated WJ-MSCs' mobilization from collagen regardless of oxygen levels, while Wnt/β-catenin pathway was activated during migration and mobilization at standard conditions.

Conclusion: Culturing of WJ-MSCs under 3% O_2 should be considered a credible condition when investigating their properties and potential use.

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1. Introduction

The ability to self-renew and differentiate into cells of mesodermal origin, together with their immunomodulatory and paracrine properties, render mesenchymal stem cells (MSCs) convenient for tissue regeneration applications [1,2]. Perinatal tissues are, among others [1,3,4], often suggested as favorable sources of MSCs [5–8]. An umbilical cord (UC) represents a rich source of MSCs [9–12], and MSCs isolated from Wharton's Jelly (WJ), the stroma of the gelatinous connective tissue that surrounds the UC blood vessels [13], were shown to have immunosuppressive features, express genes characteristic for both embryonic and adult stem cells, and have higher self-renewal potential compared to BM-MSCs [14]. However, even with the great progress in this area, regulatory mechanisms of WJ-MSCs' functions and therapeutic potential are not completely elucidated.

In humans, stem cells reside in a specific microenvironment containing various signaling molecules and growth factors that influence their properties [15]. Oxygen level is an important regulatory factor of the stem cell niche. Physiological oxygen levels in tissues range from 1% to 14% [16], contrary to 21% O_2 in standard laboratory conditions. Previous research has shown that low oxygen levels (hypoxia) can regulate metabolism, primitiveness, differentiation and engraftment of hematopoietic stem cells [17–19] and its effect on MSCs' functional features has been investigated in MSCs from various sources [20], however, with inconsistent results.

Since different oxygen levels may affect MSCs' stemness, survival and differentiation, proper culturing conditions may direct the efforts towards more successful expansion of cells with preserved stem cell properties, which are relevant for their application. For this study, we have taken into account that O_2 concentrations ranging from 1% to 5% regulate the balance of MSC self-renewal/commitment and modulate the differentiation of committed mesenchymal progenitors [18], as well as the fact that the physiological O_2 level in the UC ranges between 2 and 3% [16]. Hence, we chose 3% O_2 as a concentration that reflects native microenvironment, which should be optimal for WJ-MSCs maintenance. The main goal of our study was to compare the effects of standard culture cultivation at 21% O_2 to the cultivation at 3% O_2, in order to obtain information whether O_2 levels can be used to manipulate WJ-MSCs' stem cell properties and migratory potential, hence their "quality" in terms of their cultivation prior to potential therapeutic use.

2. Materials and methods

2.1. Isolation and cultivation of WJ-MSCs

MSCs were isolated by the explant method as previously described [21] from human UCs provided from full-term deliveries at the Clinic for Gynecology and Obstetrics, Clinical Center of Serbia, Belgrade, after obtaining a written informed consent and in accordance with the local ethical committee standards. WJ-MSCs from passage 3 (P3) on were used. Cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 at 21% or 3% O_2 (Proox Culture Chamber with O_2 and CO_2 regulators BioSpherix, Ltd., Redfield, NY, USA). Growth medium (GM) consisted of high glucose Dulbecco's Modified Eagle's Medium, with 1% Penicillin/Streptomycin and 10% Fetal Bovine Serum (all from Capricorn Scientific). GM was changed every second day.

2.2. Immunophenotyping

Cells were cultured to confluence in GM under 21% or 3% O_2, and at P3 flow cytometry analysis was performed using a CyFlow CL (Partec, Münster, Germany) [21]. Cells were labeled with anti-human mouse monoclonal antibodies for CD105, CD29, (both from Invitrogen, Carlsbad, CA, USA), CD44H, CD90, and CD73 (all from R&D Systems, Minneapolis, MN, USA) as mesenchymal cell surface markers and CD34 (DAKO, Dako Cytomation, Glostrup, Denmark), CD45, and CD235a (both from R&D Systems) for hematopoietic cell surface markers, all phycoerythrin (PE)-conjugated, except CD45 which was conjugated with fluorescein isothiocyanate (FITC).

2.3. Multilineage differentiation

Cells were cultivated in GM until subconfluence when GM was changed with differentiation media (DM) [21]. Cells were then cultivated under 21% and 3% O_2 for three weeks, with the DM being changed three times a week. To determine osteogenic differentiation, cells were stained by BCIP/NBT (Sigma-Aldrich) for the alkaline phosphatase (ALP) activity and with alizarin red (Merck, Darmstadt, Germany) to identify calcium containing osteocytes. To determine adipogenic differentiation, cells were stained with oil red O (Merck) and with fluorescent probe Nile red (Santa Cruz Biotechnology, Texas, USA). To determine chondrogenic differentiation, cells were stained with safranin O for proteoglycans [21], and Alcian blue 8GX (both from Merck) [22]. Cells cultivated in GM served as control. The stained differentiated cells were photographed using a phase-contrast and an epi-fluorescent microscope (Olympus, Japan). The ALP activity was quantified by staining cells with p-nitrophenyl-phosphate (pNPP) and measuring optical absorbance at 405 nm using an automatic reader for microtiter plates (Labsystems Multiskan PLUS, Finland). The quantification of mineralization was assessed by dissolving Alizarin Red with 10% cetylpyridinium chloride (Sigma Aldrich) in 10 mM sodium phosphate for 30 min. The level of adipogenesis was determined by dissolving oil red O-stained lipid droplets in isopropanol. The absorbance for both assays was measured at 540 nm. To additionally quantify adipogenesis, Nile red was added into the medium along with Hoechst 33258 (Sigma Aldrich), both at the final concentration of 5 μg/ml. After 15 min at standard conditions, the medium was removed, cells were washed twice with PBS, and RIPA lysis buffer was added. The plate was incubated in dark at RT for 30 min. The cell lysates were then transferred to the black 96-well micro test plate and the fluorescence was measured on Wallac 1420 Victor2 Microplate reader (PerkinElmer, Finland) with the use of 531/615 filter sets for Nile red and 355/460 for Hoechst 33258. The mean fluorescent intensity of all replicates was calculated for both dyes and then Nile red:Hoechst ratio was established. For chondrogenesis quantification, Alcian blue stained monolayers were extracted with 6 M guanidine-HCl (Sigma Aldrich) for 2 h, and absorbance was read at 630 nm.

2.4. Cell growth assays

For short term proliferation assay, 1×10^4 cells/well were incubated at 21% and 3% O_2. After three, five and seven days of culture, cells were detached with Trypsin/EDTA (PAA Laboratories, Linz, Austria) and counted using Trypan blue (Biological industries). For determining population doubling time (PDT) cells were seeded at 2.5×10^5 cells/well and incubated at 21% or 3% O_2. After reaching confluence, cells were detached, counted and seeded again at the initial density. This process was repeated for 24 days. PDT was calculated according to the formula PDT = (T-T0) lg2/(lgNt-lgN0), where T0 and T are starting and ending time of culture, while N0 and Nt represent the cell number at the start and the end of each cultivation period, respectively.

2.5. Colony-forming units-fibroblastic (CFU–F) assay

Cells were seeded at densities 10, 50 and 100 cells/well in GM. After two weeks of culturing under 21% and 3% O_2, cells were fixed with ice-cold methanol and stained with 0.3% crystal violet (Carlo Erba reagents S.A.S.) in methanol. Visible colonies with more than 50 cells were counted. Colony forming efficiency was defined as the percentage of the ratio of the number of colonies to the number of cells seeded.

Table 1
Primer sequences and programs used for PCR analysis.

Gene	Sequence 5′-3′	Product lenght	Annealing temperature
Quantitative PCR			
ALP	F: CACCCACGTCGATTGCATCT R: TAGCCACGTTGGTGTTGAGC	211bp	60 °C
RUNX	F: GCCTAGGCGCATTTCAGA R: CTGAGAGTGGAAGGCCAGAG	66bp	60 °C
OCN	F: GGCGCTACCTGTATCAATGG R: TCAGCCAACTCGTCACAGTC	106bp	60 °C
PPARγ	F: CAGGAAAGACAACAGACAAATCA R: GGGGTGATGTGTTTGAACTTG	94bp	60 °C
LPL	F: TCAACTGGATGGAGGAGGAG R: GGGGCTTCTGCATACTCAAA	169bp	60 °C
SOX9	F: GCCAGGTGCTCAAAGGCTA R: TCTCGTTCAGAAGTCTCCAGAG	213bp	60 °C
COL2	F: ACACTGGGACTGTCCTCTG R: GTCCAGGGGCACCTTTTTCA	270bp	60 °C
GAPDH	F: GAAGGTGAAGGTCGGAGTC R: GAAGATGGTGATGGGATTTC	226bp	60 °C
Semiquantitative PCR			
OCT4-A	F: AGTGAGAGGCAACCTGGAGA R: GTGAAGTGAGGGCTCCCATA	270bp	55 °C
OCT4-B	F: TATGGGAGCCCTCACTTCAC R: CAAAAACCCTGGCACAAACT	194bp	55 °C
NANOG	F: CTCCATGAACATGCAACCTG R: CTCGCTGATTAGGCTCCAAC	209bp	55 °C
SOX2	F: ATGGGTTCGGTGGTCAAGT R: GGCGCCGTGGGAGATACATG	126bp	51 °C
HIF-1α	F: CAGAGCAGGAAAAGGAGTCA R: AGTAGCTGCATGATCGTCTG	234bp	52 °C
GAPDH	F: ACCACAGTCCATGCCATCAC R:TCCACCACCCTGTTGCTGTA	452bp	52 °C

2.6. PCR

mRNA was extracted using TRIzol (Ambion Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Two micrograms of RNA were used to obtain complementary DNA by the RevertAid™ H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). PCR products were obtained after 33–38 cycles of amplification using primer sequences and annealing temperatures listed in Table 1. The amplicons were resolved in 1.5% agarose (Lonza, Rockland, ME, USA) gel and stained with ethidium bromide (Sigma-Aldrich). GAPDH was amplified as a loading control. The intensity of the bands was quantified using NIH-Image J.

Quantitative real-time PCR (qRT-PCR) was performed on Mic qPCR cycler (BioMolecular systems, Australia) using primers listed in Table 1 and FastGreen kit (Applied Biosystems, California, USA). The relative gene expression was calculated by the comparative ΔΔCt method with GAPDH as a reference.

2.7. Cell cycle and cell death analysis

Cells were cultivated under 21% and 3% O_2 for 24, 48 and 72 h and detached with Trypsin/EDTA. For the cell cycle analysis, aliquots of 2×10^5 cells were washed with PBS, fixed with absolute ice-cold ethanol and resuspended in PBS solution containing 50 μg/ml propidium iodide (PI; Molecular Probes, Life Technologies, Eugene, OR, USA), 0.1% Triton X-100 (Serva Electrophoresis GmBh Heidelberg, Germany) and 0.1 mg/ml RNase (Thermo Scientific). For the cell death analysis, aliquots of 2×10^5 cells were resuspended in Annexin binding buffer containing Annexin V-FITC and PI (all from Molecular Probes, Life Technologies). The samples were analyzed on CyFlow CL flow cytometer (Partec, Münster, Germany).

2.8. Scratch assay

Cells were seeded in 24-well plates (5×10^4 cells/well) and allowed to grow until confluence when a scratch in the monolayer was made. After washing with PBS, cells were allowed to migrate for additional 24 h in GM under 21% and 3% O_2. In a separate experiment, cells were left to migrate at 21% and 3% O_2 for 24 h in the media supplemented with 25 μM of MEK1 and MEK2 inhibitor, PD-98059 (Tocris Bioscience, Bristol, UK), and 100 μM of the Wnt/β-catenin pathway inhibitor, PNU-74654 (Santa Cruz). Cells were then fixed with ice-cold methanol and stained with 0.1% Crystal violet. The cell migration into the scratch area was photographed using an inverted light microscope and quantified by TScratch software (Computational Science and Engineering Laboratory, Swiss Federal Institute of Technology, ETH Zurich, Switzerland).

2.9. Cell mobilization from collagen gel

Type I collagen was extracted from rat tail tendons as previously described [23] and the solution containing 20% 5xDMEM, 10% FCS, 10% $NaHCO_3$, 0.4% 1 M NaOH, 2% distilled water, 40% collagen and 17.6% of cell suspension was made [24]. Cells were embedded in a single collagen drop at a density of 1×10^5 cells and 25 μl drops/well were plated in 24-well plates. After 1 h of incubation at 37 °C, the collagen drops jellified, GM was added and drops were incubated at 21% and 3% O_2 for 72 h. Additionally, the cell mobilization from collagen was followed at 21% and 3% O_2 in media containing 25 μM PD-98059 and 100 μM PNU-74654. After the incubation period, collagen drops were fixed with 3.7% formaldehyde in PBS and stained with 0.1% crystal violet. The cell mobilization from the collagen drops was monitored by phase-contrast microscopy.

2.10. Zymography assay

Cells were prepared for a scratch assay in the presence of 25 μM PD-98059 and 100 μM PNU-74654 inhibitors. The serum-free conditioned media were collected after the migration period and protein-normalized aliquots were subjected to zymography as described earlier [23] to determine the urokinase-type plasminogen activator (uPA) and matrix metalloproteinases (MMP) activity. The quantification of uPA and MMPs bands was performed by densitometry analysis using NIH-Image J.

2.11. Statistical analyses

All the experiments were repeated at least three times and the results were presented as mean ± standard error of the mean. Statistical comparisons were performed using student's two-tailed *t*-test, and one-way ANOVA with Holm-Sidak's multiple comparisons test, with significance level α set to 0.05 and p values < 0.05 considered significant. Data analyses and graphical representations were performed using GraphPad Prism 7 software (GraphPad, San Diego, CA, USA).

3. Results

3.1. 3% O_2 does not influence the immunophenotype of WJ-MSCs, while it modulates their differentiation

Both WJ-MSCs grown at 21% and 3% O_2 showed fibroblast-like shape (Fig. 1A) and displayed mesenchymal immunophenotype (Fig. 1B and C). Cells were positive for CD29, CD44H, CD73, CD90 and CD105 (over 93% of cell population) and negative for hematopoietic cell markers CD34, CD45 and CD235a.

After cultivation in DM, tri-lineage differentiation was confirmed in cells cultivated under both 21% and 3% O_2 (Fig. 2A). Higher activity of an early osteogenic marker ALP after one week and higher

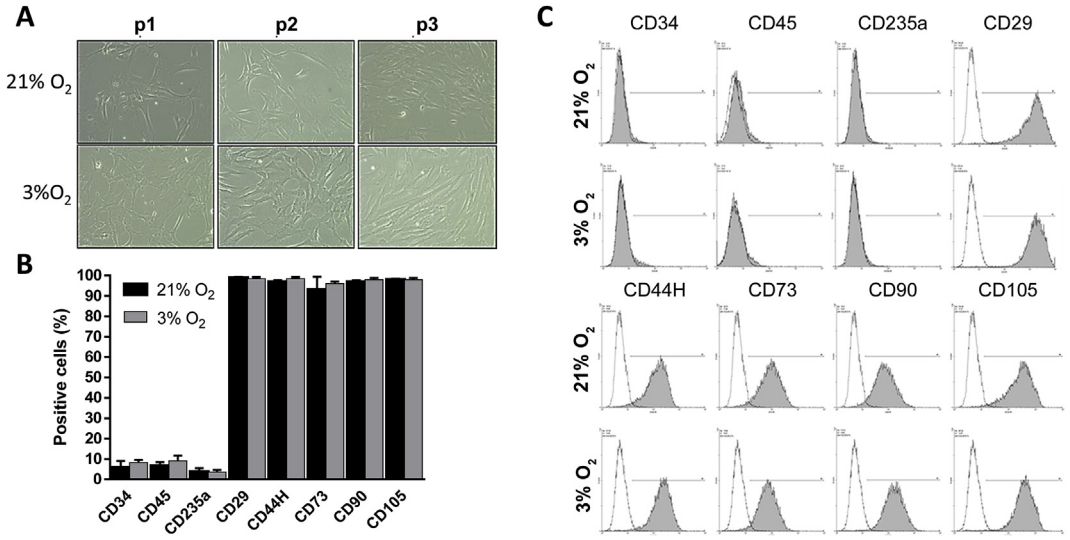

Fig. 1. Characterization of WJ-MSCs cultured under different oxygen levels. A) Morphology of WJ-MSCs grown at 21% and 3% O_2. Phase contrast micrographs of monolayer cultures at passage 1, 2 and 3 showing fibroblast-like morphology. B) Immunophenotype of WJ-MSCs at P3 grown under 21% and 3% O_2. The bar graph shows the percentage of cells positive for the CD marker expression. C) Representative flow cytometry histograms show the expression (grey areas) of selected molecules (CD45, CD34, CD235a, CD29, CD44H, CD73, CD90 and CD105) compared with isotype controls (white areas).

mineralization after three weeks of incubation in DM was observed in WJ-MSCs grown at 21% O_2 (Fig. 2B). Consistent with these findings, mRNA expression for osteogenesis relevant genes, *ALP*, *RUNX2* and *OCN* was also higher in WJ-MSCs grown under 21% O_2 (Fig. 2C). The number of lipid droplets was higher (Fig. 2B) and mRNA expression of *PPARγ* and *LPL* was increased in WJ-MSCs grown at 3% O_2 (Fig. 2C). Proteoglycan levels were higher in the cells grown at 3% O_2 (Fig. 2B). Inducing effect of 3% O_2 on chondrogenic differentiation was supported

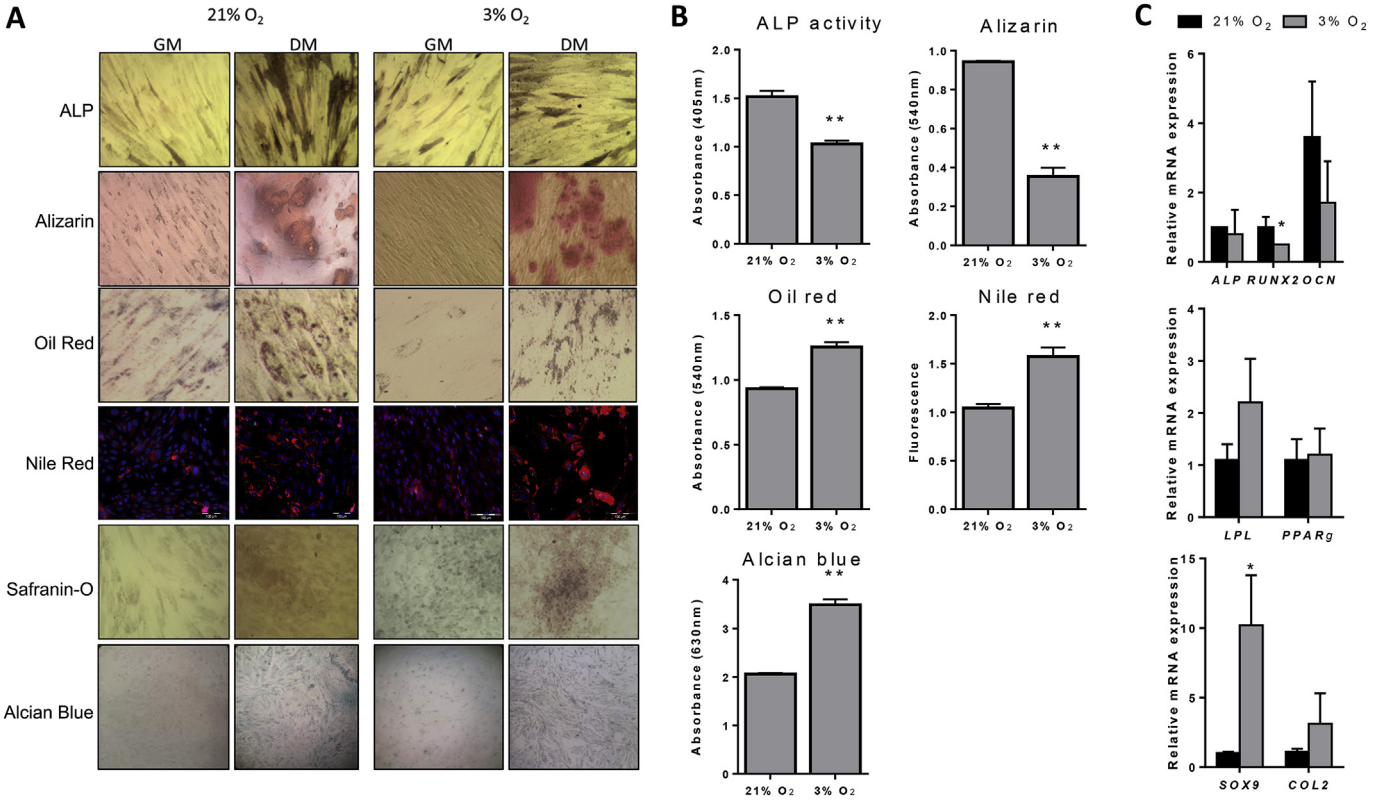

Fig. 2. Differentiation potential of WJ-MSCs cultivated under 21% and 3% O_2. A) Histochemical staining of cells after induced differentiation. Osteogenic differentiation was confirmed by positive staining for alkaline phosphatase (ALP) activity and staining of mineralization with alizarin red; adipogenic differentiation was detected by Oil Red O and Nile red staining of intracytoplasmic lipid droplets; chondrogenic differentiation of MSCs was detected by safranin-O and Alcian blue staining of proteoglycans; B) Quantitative analysis of ALP, Alizarin, Oil red, Nile red and Alcian blue. C) Q-PCR analysis of *ALP*, *RUNX2*, *OCN*, *LPL*, *PPARγ*, *SOX9* and *COL2* mRNA expression in WJ-MSCs cultivated under 21% and 3% O_2 in specific differentiation media for 7 or 14 days. The results are expressed as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, 21% vs 3% O_2. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

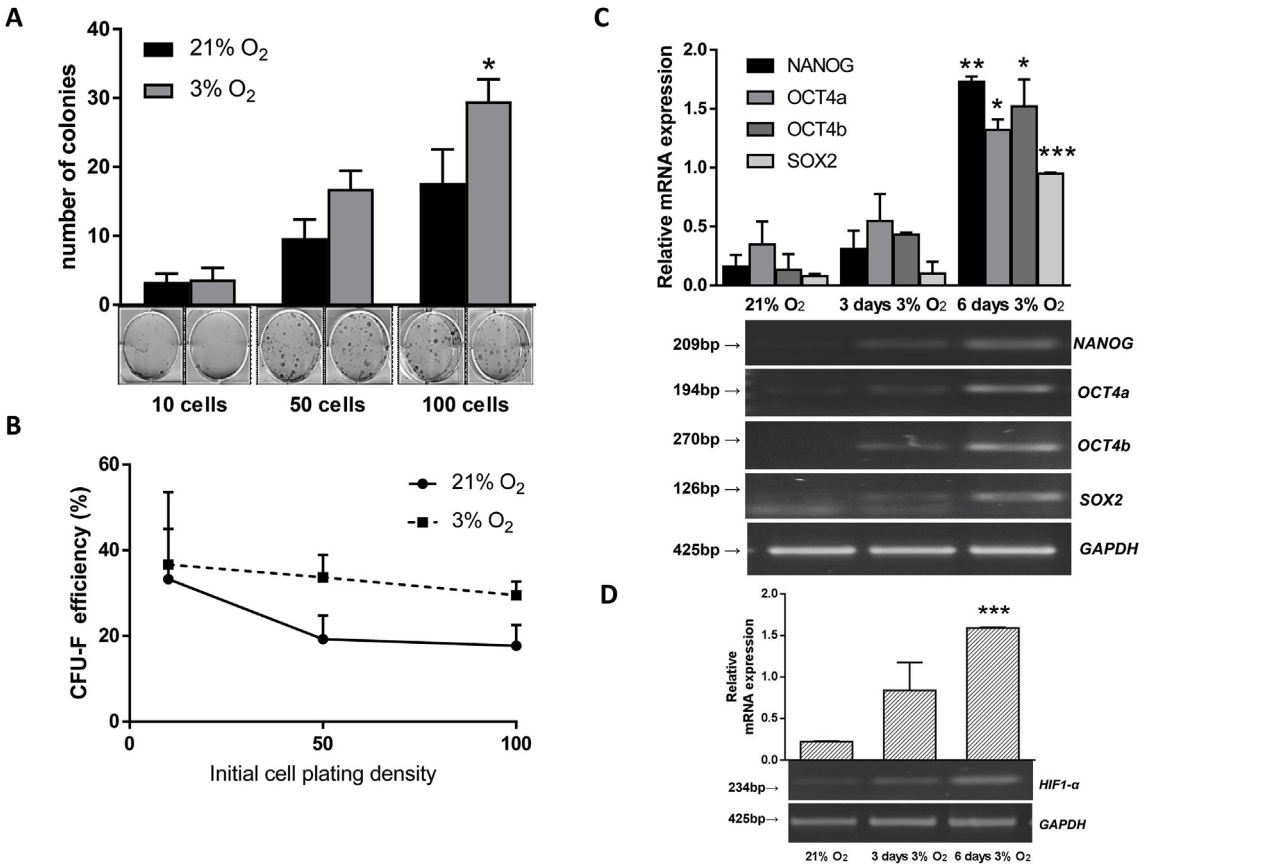

Fig. 3. Effect of 3% O_2 on clonogenic potential and multipotency of WJ-MSCs. A) The clonogenic capacity of WJ-MSCs cultivated under 21% and 3% O_2 evaluated by a number of colonies per initial plating number of cells. MSCs were cultivated at low density (10, 50 or 100 cells/well) in growth medium for 14 days and stained for CFU-F with crystal violet. B) Colony-forming efficiency after expanding WJ-MSCs at different initial plating densities under 21% and 3% O_2 is defined as the percentage of the ratio of the number of colonies to the number of cells seeded. C) RT-PCR analysis of pluripotency genes. The plot shows *NANOG*, *OCT4a*, *OCT4b and SOX2* mRNA expression relative to GAPDH expression. Representative RT-PCR bands are shown below. D) RT-PCR analysis of HIF1-α expression relative to GAPDH expression. The results are expressed as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, 21% vs 3% O_2.

by higher mRNA expression of chondrogenic markers *SOX9* and *COL2* at 3% O_2 (Fig. 2C). Based on the aforementioned results, 3% O_2 decreases osteogenic differentiation and stimulates adipogenic and chondrogenic differentiation of WJ-MSCs.

3.2. 3% O_2 increases clonogenic potential and pluripotency marker expression in WJ-MSCs

CFU-F assay showed a higher number of colonies formed by WJ-MSCs cultured at 3% O_2 compared to the cells grown at 21% O_2 (Fig. 3A). Also, MSCs' efficacy to form CFU-F was inversely correlated to the number of cells seeded and was higher at 3% O_2 (Fig. 3B). These results suggest that 3% O_2 increases the clonogenic potential of WJ-MSCs.

WJ-MSCs were cultivated at different oxygen levels for three and six days, when RNA was isolated and expression of pluripotency marker genes *NANOG*, *OCT4a*, *OCT4b* and *SOX2* was analyzed. We observed that expression of mRNA for all four genes increased when WJ-MSCs were grown under 3% O_2 for both time intervals, with statistically significant effect after six days (Fig. 3C). As expected, a higher expression of *HIF1-α* was measured in WJ-MSCs cultivated under 3% O_2 for six days compared to 21% O_2 (Fig. 3D).

3.3. 3% O_2 increases WJ-MSCs' yield and protects the cells from apoptosis without influencing cell cycle

WJ-MSCs grown under 3% O_2 had a higher yield (Fig. 4A) and proliferated faster, as their average PDT was 33.8 h, while PDT of the cells grown under 21% O_2 was 41.4 h (Fig. 4B). A similar low percentage of the apoptotic cells (approximately 8%) was observed after 24 h and 48 h of cultivation at both oxygen levels. However, after 72 h of incubation, a significantly lower number of apoptotic cells (and necrotic cells, although not significantly) was present when WJ-MSCs were grown at 3% O_2 compared to the cells grown under 21% O_2 (Fig. 4C).

Flow cytometry analyses of WJ-MSCs' cell cycle under different oxygen levels showed a transient increase in the percentage of the cells in G0/G1 phase after 24 h of cultivation at 3% O_2 (Fig. 4D and E). After 72 h, the percentage of the cells in G0/G1, S, and G2/M phase, grown either under 3% or 21% O_2 was not significantly altered. These results show that 3% O_2 does not change the cell cycle profile of WJ-MSCs.

3.4. 3% O_2 stimulates migration of WJ-MSCs

In the first experimental setting, WJ-MSCs were grown to the confluence at 21% O_2 and then allowed to migrate at 21% or 3% O_2 for 24 h (Fig. 5A). In the second experimental setting, WJ-MSCs were grown to confluence under 21% and 3% O_2 and then allowed to migrate at 21% O_2 (Fig. 5B). A significantly larger number of cells migrated into the scratch area when the oxygen level was changed from 21% to 3%, and also, WJ-MSCs pre-grown at 3% O_2 migrated faster compared to cells pre-grown at 21% O_2. No difference was observed in the cells' proliferation rate under different O_2 conditions after 24 h of incubation (data not shown), therefore the aforementioned results can be

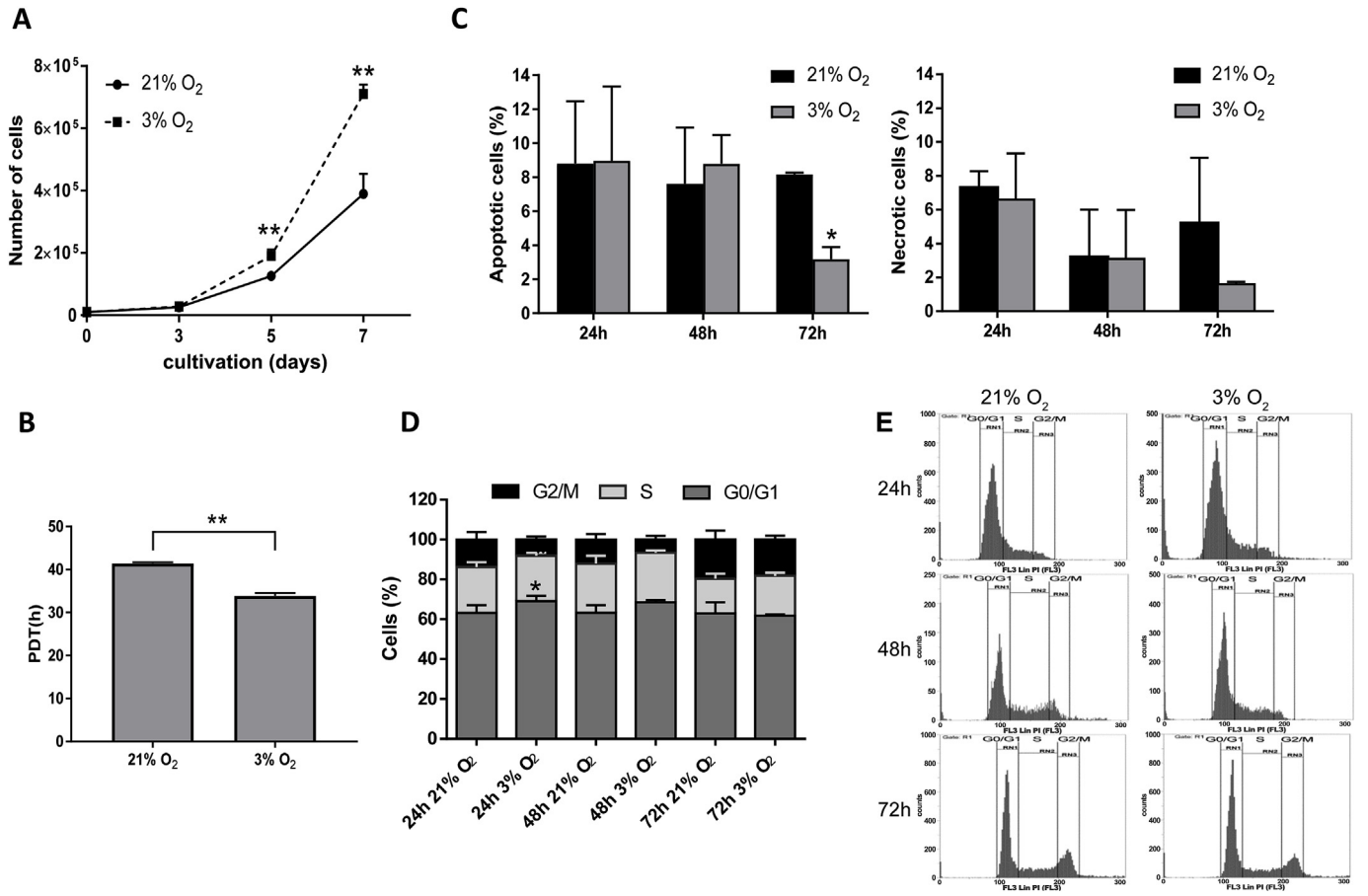

Fig. 4. Effect of 3% O_2 on growth kinetics of WJ-MSCs. A) The short-term proliferation rate of WJ-MSCs was assessed after three, five and seven days of culturing at 21% and 3% O_2. B) Population doubling times of MSCs were calculated after 24 days in long-term cultures under 21% and 3% O_2. C) Cell death analysis under 21% and 3% O_2 was investigated by flow cytometry. Bars represent the percentage of apoptotic and necrotic cells under both experimental conditions for indicated time points. D) The effect of 3% O_2 on the cell cycle profile of WJ-MSCs grown at different time-points. The graph represents the cell percentage in G0/G1, S and G2/M phase of the cell cycle. E) Representative flow cytometry histograms show cell cycle profile under both experimental conditions for indicated time points. The results are expressed as mean ± SEM (n = 3); *p < 0.05, **p < 0.01, ***p < 0.001, 21% vs 3% O_2.

accredited solely to cells' migratory capacity.

To examine if different oxygen levels affect the mobilization capacity of WJ-MSCs from collagen, cells were embedded in collagen drops and incubated at 21% and 3% O_2 for 72 h (Fig. 5C). In addition, cells were preconditioned at both O_2 concentrations for 72 h and afterward embedded in collagen drops and cultivated at 21% O_2 (Fig. 5D). The lower oxygen level slightly increased WJ-MSCs' mobilization from the collagen drops. In addition, the cells preconditioned at 3% O_2 had a slightly higher capacity to migrate out of collagen drops than the cells grown at 21% O_2, however, the difference observed was not statistically significant. Collectively, these results indicate that 3% O_2 improves the migratory and mobilization capacity of WJ-MSCs, and this trend was preserved when cells were re-cultivated at 21% O_2.

3.5. Mechanisms involved in migration and mobilization of WJ-MSCs under 3% O_2

To investigate the underlying signaling pathways in migration and mobilization of WJ-MSCs, inhibitors of MAPKK (PD-98059) and Wnt/β-catenin (PNU-74654) were used. A slight reduction in cells' migration was observed in the presence of both inhibitors under 3% O_2 while only PNU-74654 significantly reduced migration under 21% O_2 (Fig. 6A). PD-98059 significantly reduced mobilization of WJ-MSCs from the collagen drops under both oxygen levels and PNU-74654 only under 21% O_2 (Fig. 6B).

We next investigated the activity of extracellular matrix (ECM) proteolytic enzymes in WJ-MSCs under different O_2 levels. Zymography analysis showed that the activity of both uPA and MMP-2 (MMP-9 was not detected) was higher in the cells grown at 3% O_2 compared to the cells grown at 21% O_2, although statistical significance was not observed for MMP-2 (Fig. 6C). Both inhibitors significantly reduced the activity of uPA, while the reduced activity of MMP-2 was not statistically significant. The inhibitory effect of PD-98059 and PNU-74654 was more pronounced in WJ-MSCs cultivated at 3% O_2.

4. Discussion

The experimental question or expected therapy outcome should define the cultivation conditions for MSCs. On the one hand, conditions can be tailored in a way to mimic in vivo microenvironment, allowing a physiologically more relevant setting for research. On the other hand, when applied for therapy, MSCs inevitably enter a low oxygen environment to which they must react and adapt [25]. Therefore, changing *in vitro* conditions gives us a possibility to predict the cells' behavior in vivo or to prime MSCs for a specific need [25,26]. This study investigated whether WJ-MSCs exhibit different functional properties when cultivated at 3% O_2 instead of standard 21% O_2, with the aim to mimic the cells' natural microenvironment and analyze their features important for potential use.

WJ-MSCs' morphology and immunophenotype were maintained under different oxygen levels, in accordance with previous reports in WJ-MSCs cultivated at 2% O_2 [27,28] and 5% O_2 [29], compared to the

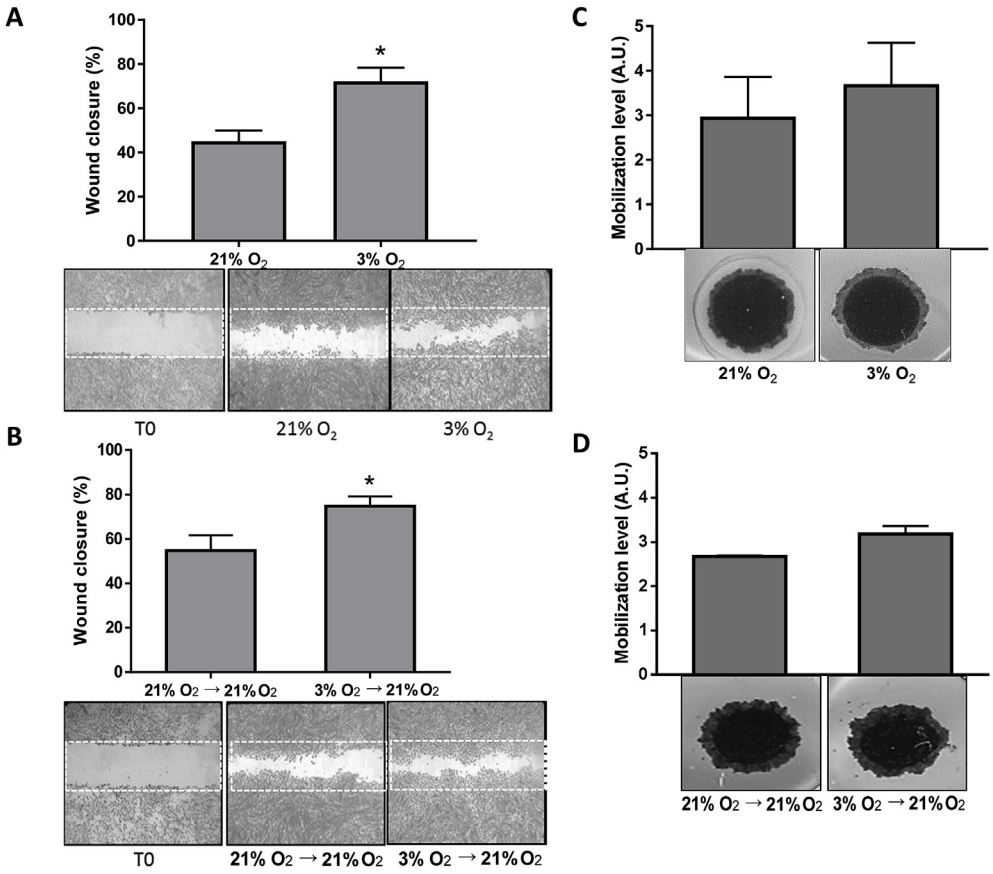

Fig. 5. Effect of 3% O_2 on the migratory potential of WJ-MSCs. A) Migration of WJ-MSCs was analyzed by scratch assay: a scratch was made in confluent cell monolayer and cells were cultivated under 21% and 3% O_2 for 24 h. Bars represent the percentage of the scratch area covered with migrating cells. Magnification ×40. B). Migration potential of WJ-MSCs after pre-cultivation under 21% and 3% O_2. Cells were cultivated under 21% and 3% O_2 for three days, and then a scratch was made in the confluent monolayer of cells. Migration was followed under standard 21% O_2 for 24 h and presented as a bar graph. Magnification ×40. C) Mobilization of WJ-MSCs from collagen gel. WJ-MSCs embedded in collagen were incubated under 21% and 3% O_2 for three days. After fixing and staining the samples were photographed. Bars represent the area of migrating cells. Drop magnification ×40. D) Mobilization of WJ-MSCs from collagen gel after pre-cultivation under 21% and 3% O_2. Cells were cultivated under 21% and 3% O_2 for three days, after which they were embedded in collagen and cultivated at standard 21% O_2 for three days. After fixing and staining the samples were photographed. Drop magnification ×40. A.U. -arbitrary unit

The results are expressed as mean ± SEM (n = 3); *p < 0.05, 21% vs 3% O_2.

cells cultivated at 21% O_2. Previously shown reduction of CD73, CD90 and CD105 expression levels in WJ-MSCs cultured at 2% O_2 [30] could be attributed to the different passage number at which immunophenotype was analyzed or to the variability of the cells derived from different donors.

We showed that WJ-MSCs display tri-lineage differentiation at both O_2 concentrations, similar to earlier studies performed at 2% and 5% O_2 [27,29]. The quantitative analysis of differentiation, supported by mRNA expression for differentiation relevant genes, showed that 3% O_2 decreases osteogenic differentiation while stimulating adipogenic and osteogenic differentiation of WJ-MSCs. Indeed, it was previously published only that 5% O_2 increases WJ-MSCs chondrogenesis [31]. To the best of our knowledge, this is the first study that made a comprehensive analysis of the effect of different oxygen levels on differentiation potential of WJ-MSCs. The estimation of MSCs' differentiation potential under low oxygen level can predict the cells' behavior after application and determine whether such cells should be used for a specific treatment (e.g. WJ-MSCs characterized by our protocol might be good candidates for cartilage regeneration), as suggested recently [26]. Hypoxia was shown to induce production of TGF-β by MSCs [32,33], which can lead to autocrine regulation of chondrogenic and osteogenic differentiation. Therefore, further studies should be aimed at exploring autocrine mechanisms of MSCs which are activated by low oxygen levels. This would be of importance not only to elucidate the impact WJ-MSCs' secretome has on differentiation but also to clarify an important therapeutic feature of WJ-MSCs, as already shown [34–36].

Further on, we confirmed that 3% O_2 stimulates proliferation of WJ-MSCs and shortens their duplication time, as previously shown for the cells grown at 2%, 2.5% and 5% O_2 [27,28,31,37,38]. Besides, 3% O_2 increased the clonogenic potential of WJ-MSCs which is consistent with the observations made at 5% O_2 [29,31]. Self-renewal and pluripotency of the embryonic stem cells are regulated at the molecular level by transcription factors *OCT4*, *NANOG* and *SOX2* [39]. Nevertheless, it is proposed that these genes, often termed as embryonal/pluripotency markers, play an important role in regulating stemness of adult MSCs, although with conflicting results [40]. Culturing of WJ-MSCs at 3% O_2 for six days significantly enhanced the expression of *OCT4*, *NANOG* and *SOX2*, in agreement with a study where WJ-MSCs were cultured at 5% O_2 for two and four weeks [28]. On the other hand, Reppel et al. failed to detect *NANOG* and *SOX2* in WJ-MSCs while they noticed that *OCT4* was similarly expressed under both conditions, 5% and 21% O_2 [31]. Also, low oxygen levels were shown to stimulate the expression of stemness-related genes in WJ-MSCs grown on chitosan films [41]. Our findings suggest that 3% O_2 enhances WJ-MSCs multipotency through stimulation of their self-renewal and elevation of pluripotency marker expression.

To the best of our knowledge, this is the first study that examined the effect of low oxygen level on WJ-MSCs cell cycle profile. Our results showed that culturing WJ-MSCs at 3% O_2 does not notably alter their cell cycle. Regarding cell viability, less than 10% of apoptotic cells were present at both O_2 levels. After 72 h, a significantly lower percentage of apoptotic and necrotic cells was observed in the cells grown at 3% O_2, indicating that 3% O_2 protects WJ-MSCs from apoptosis after 72 h of cultivation, reducing it by 68%. Majumdar et al. showed that 2% O_2 does not affect cell survival since there was no significant difference in a number of alive cells in these groups [30]. Therefore, for WJ-MSCs, 3% O_2 does not modulate cell cycle, yet it modulates apoptosis.

Efficient homing and migration toward lesion sites are among the most important features of MSCs that nominate them as therapeutically relevant [42]. MSCs mobilization and migration is, at least in part, influenced by local tissue oxygen in pathological situations when the blood supply is deranged [43]. We demonstrated that WJ-MSCs grown at 3% O_2 have a higher migration ability compared to the cells grown at 21% O_2. It was previously shown that 2% O_2 leads to increased (although not significantly) motility of WJ-MSCs [44], as well as that 3% O_2 stimulates BM-MSCs' motility [45]. Moreover, WJ-MSCs pre-

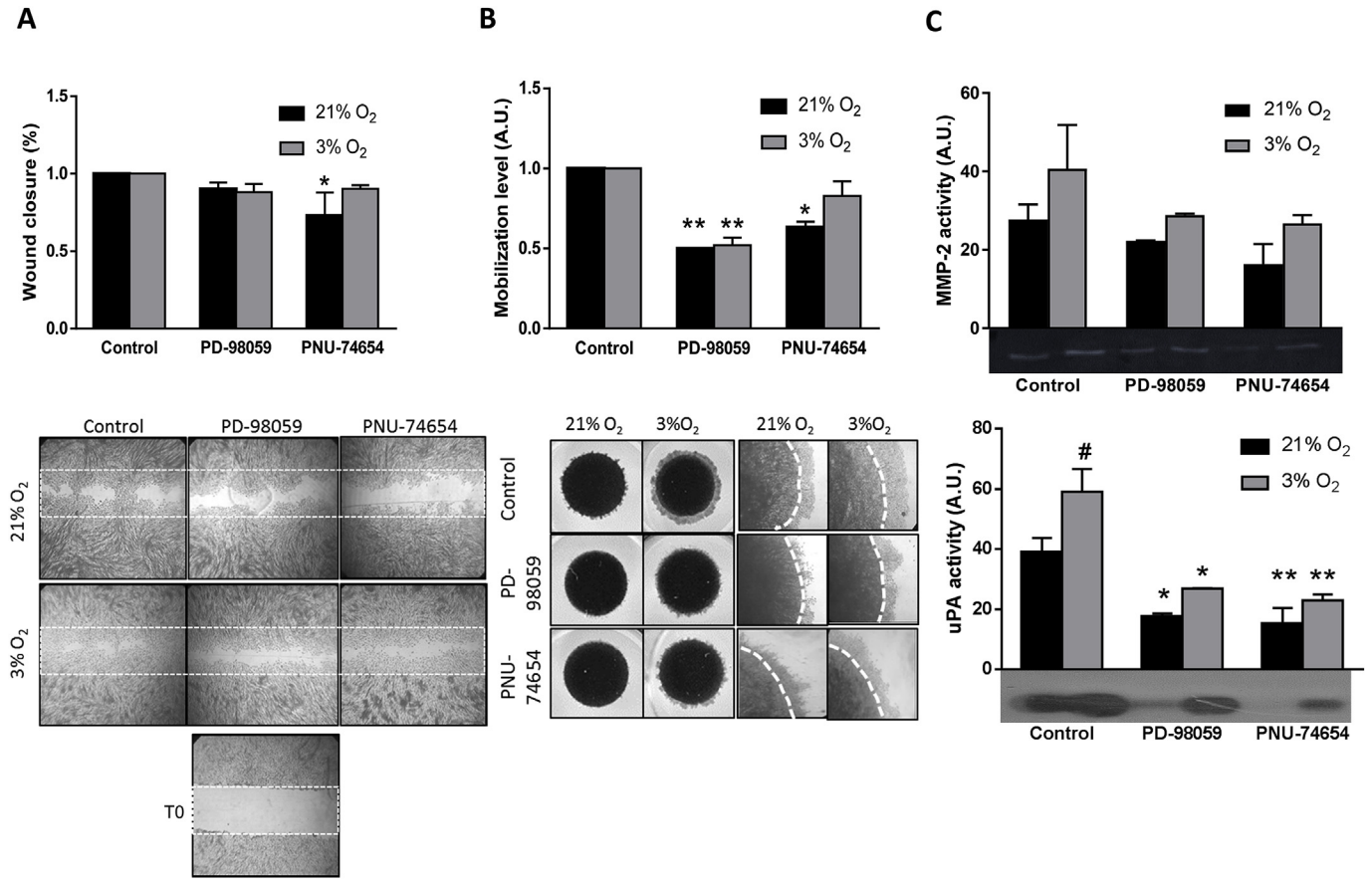

Fig. 6. Mechanisms involved in migration and mobilization of WJ-MSCs under 3% O_2. A) WJ-MSCs were subjected to scratch assay under 21% and 3% O_2 in the presence of MAPK and Wnt/β-catenin pathway inhibitors (PD-98059 and PNU-74654). Bar graphs represent the percentage of the scratch area covered with migrating cells. Value 1 was given to control (untreated cells). Representative images of migrating cells were taken using light microscopy. Magnification ×40. B) WJ-MSCs embedded into collagen were cultivated under 21% and 3% O_2 in the presence of inhibitors. After fixing and staining, drops were photographed with magnification ×40 and ×100. A value 1 was given to control (untreated cells). C) Effect of inhibitors on the activity of proteolytic enzymes urokinase-type plasminogen activator (uPA) and matrix metalloproteinase 2 (MMP-2) during migration was determined by zymography. After the scratch was made, WJ-MSCs were cultivated in serum-free conditions under 21% and 3% O_2 in the presence of inhibitors. The results are expressed as mean ± SEM (n = 3). *p < 0.05, **p < 0.01, presence vs absence of inhibitors; #p < 0.05 21% O_2 vs 3% O_2; A.U. -arbitrary unit.

cultivated at 3% O_2 exhibited a significantly higher migratory capacity than those pre-cultivated at 21% O_2. Similarly, increased cell migration was reported for human BM-MSCs preconditioned at 3% O_2 [46]. These results suggest that pre-cultivation of WJ-MSCs at 3% O_2 prior to administration could be one of the approaches for their potential use in cell therapy. Given that MSCs migrate into an injury site across the ECM [47], we examined the ability of WJ-MSCs to migrate from collagen drops under different oxygen levels. Our data showed, for the first time, that WJ-MSCs mobility level was higher when cells were both cultivated and pre-cultivated at 3%.

Several signaling pathways are involved in stimulation/modulation of MSCs' migration, among which are MAPK and Wnt signaling [48,49]. We demonstrated that mobilization of WJ-MSCs from the collagen drops was significantly reduced in the presence of MAPK inhibitor, PD-98059 at both 21% and 3% O_2. These results suggest that ERK1/2 is not involved in WJ-MSCs migration whereas it mediates WJ-MSCs mobilization from collagen regardless of oxygen level conditions. On the other hand, given that significant decrease in both migratory processes in the presence of Wnt/β-catenin inhibitor PNU-74654 was noticed only under 21% O_2, we conclude that this pathway is activated in WJ-MSCs during migration and mobilization at standard conditions. So far, it was observed only in BM-MSCs that 3% O_2-induced migration involves activation of ERK1/2 [45], hence our results provide important insights about the involvement of signaling pathways in migration and mobilization of WJ-MSCs under both 3% and 21% O_2. It is well known that cell migration involves remodeling of the ECM, associated with activation of proteolytic enzymes such as uPA and MMP [50,51]. Our analyses showed that 3% O_2 enhances uPA and MMP-2 activity during WJ-MSCs migration. However, in the presence of PD-98059 and PNU-74654, the activity of uPA was significantly reduced under both oxygen levels. These observations correlate with foregoing findings considering that both inhibitors reduce WJ-MSCs mobility for which remodeling of ECM is needed. It can be assumed that ERK1/2 and β-catenin are involved in the mobility of WJ-MSCs and that their role may be carried out through activation of uPA, hence further research will be directed to elucidate this hypothesis.

In summary, our findings show that cultivation of WJ-MSCs at 3% O_2 does not influence their immunophenotype but affects their differentiation potential. Furthermore, the cultivation at 3% O_2 improves their clonogenic and expansion capacity, augments the expression of pluripotency-associated markers and enhances the cells' migration potential. These findings offer a better insight into the intrinsic properties of WJ-MSCs and suggest that the cultivation of WJ-MSCs under 3% O_2 could enhance their therapeutic potential.

Author contributions

HO performed all the experiments, data analyses and wrote the manuscript; JK designed the experiments described, interpreted the data and wrote the manuscript; DT helped with the sample collection

process, cell culture and manuscript preparation; SM helped with the flow cytometry analyses and data analyses; IO and TK participated in the cell culture and PCR analyses; VI and AJ interpreted the data and helped with the manuscript preparation; MT provided the umbilical cords; DB principal investigator, supervised the entire project involving the manuscript preparation and gave the final approval. All authors approved the final manuscript.

Declarations of interest

None.

Acknowledgments

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