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REGULAR ARTICLE

Differentiation potential of different regions-derived same donor human Wharton's jelly mesenchymal stem cells into functional smooth muscle-like cells

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Abstract

The present study evaluates the transdifferentiation potential of different region-derived same donor Wharton's jelly MSCs (WJMSCs) into functional smooth muscle-like cells (SMLCs). All regions showed baseline expression for early smooth muscle cell (SMC) markers (αSMA and SM22-α) whereas mid marker CALPONIN gradually reduced during in vitro culture expansion and late marker myosin heavy chain type-11 (MHY-11) was completely absent. Furthermore, WJMSCs were induced to SMLCs using DMEM containing 10% FBS supplemented with different concentrations/combinations of TGF-β1 and PDGF-BB under normoxia (20% O_2) condition. Three treatment groups namely group A: 2.5 ng/ml TGF-β1, group B: 5 ng/ml PDGF-BB and group C: 2.5 ng/ml TGF-β1 + 5 ng/ml PDGF-BB were used for the induction of WJMSCs into SMLCs. Cells were evaluated for SMC-specific marker expression at different time intervals. Finally, selection of the SMC-specific highly potent region along with the most suitable treatment group was done on the basis of highest outcome in terms of SMC-specific marker expression and functional competence of transdifferentiated cells. Among all regions, baby region-derived WJMSCs (B-WJMSCs) exhibited highest SMC marker expression and functional ability. To mimic the in vivo physiological conditions, hypoxic conditions (3% O_2) were used to evaluate the effect of low oxygen on the SMLC differentiation potential of selected WJMSCs using previously used same parameters. Annexin-V assay was performed to check the effect of cytokines and different oxygen concentrations, which revealed no significant differences. It was concluded that different induction conditions have different but positive effects on the functional SMLC differentiation ability of WJMSCs.

Keywords Transdifferentiation · Smooth muscle cells · Mesenchymal stem cells · Wharton's jelly · Electrophysiology

Abbreviations

WJMSCs	Wharton's jelly mesenchymal stem cells
SMLCs	smooth muscle-like cells
SMC	smooth muscle cell
α-SMA	alpha smooth muscle actin
SM22-α	alpha-smooth muscle 22

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MHY-11 myosin heavy chain 11

Introduction

Smooth muscle cells are present in many tissues and organs such as respiratory, cardiovascular, urinary, gastrointestinal and reproductive tracts and are also the main constituents of stromal cells in the vascular wall. During any deformity and in vivo pathological conditions, there is a transformation of contractile SMCs to synthetic SMCs with an increase in their proliferation rate, which ultimately leads to vascular disorders such as hypertension, aneurysm, atherosclerosis, restenosis etc. These disorders may be fatal, if remain unnoticed and untreated for longer duration. To get rid of these problems, use of stents, balloon angioplasty and bypass surgery have been common practice since the last decade. Other valuable alternatives include use of MSCs as therapeutic agents as they can be successfully differentiated into SMLCs. There is a presence of a fibroblast population or myofibroblasts in Wharton's jelly, which are basically the mesenchymal stem cells (Takechi et al. 1993; Corrao et al. 2013). WJMSCs have been shown to possess cytoplasmic α-smooth muscle actin microfilaments after the second trimester and also show positive expression for desmin and vimentin (Kobayashi et al. 1998). During the last 6 months of pregnancy, these cells undergo myofibroblast differentiation and provide protection to umbilical vessels against compression. However, after post-delivery and in vitro expansion, whether these cells retain their myofibroblast characteristics is still not clear and no such in vitro studies have been elucidated till today. Various researchers have tried to derive functional SMCs from embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) (Guo et al. 2013; Wanjare et al. 2013) but due to sample scarcity, ethical issues, high cost, and risks concerning teratoma formation, their use in clinical settings has been neglected. Therefore, to avoid such problems, postnatal tissue-derived MSCs have been given preference. The functional SMCs have been differentiated from various postnatal and adult MSC sources either by cellular reprogramming or by inducing them in muscle-specific culture medium enriched with growth factors (Ross et al. 2006; Wang et al. 2010; Moonen et al. 2010; Xu et al. 2013; Ghionzoli et al. 2013; Song et al. 2016). The cytokine TGF-β1 has been widely used as an efficient growth factor for inducing smooth muscle differentiation from MSCs. It has been shown that TGF-β1 alone or in combination with other cytokines such as PDGF and BMP-4 under different concentrations can promote the SMLC differentiation potential of various MSC sources such as bone marrow, adipose, peripheral blood, hair follicle, amniotic fluid, dental pulp tissue etc. (Ross et al. 2006; Wang et al. 2010; Moonen et al. 2010; Xu et al. 2013; Ghionzoli et al. 2013; Song et al. 2016). However, there is no report on the SMLC differentiation potential from Wharton's jelly especially derived from different regions of the same umbilical cord (UC). MSCs when induced under such cytokines have been shown to exhibit spindle morphology with a heap and valley like cell arrangement and increased expression of smooth muscle-specific early, mid and late markers. However, fewer studies have demonstrated the successful differentiation of MSCs by highlighting functional SMC properties in terms of collagen gel contraction ability and electrophysiological competence in comparison to their un-induced counterparts (Ross et al. 2006; Ghionzoli et al. 2013; Xu et al. 2013). In accordance with the myofibroblast nature and existence of baseline expression of early smooth muscle markers, the present study was conducted to select the SMC specific highly competent WJMSC part among different regions of whole human UC by using SMC-specific cytokine-enriched differentiation media. Fulfillment of all criteria such as morphological changes, expression of SMC markers both at the mRNA and protein level as well as functional properties were taken into consideration to confirm the successful SMLC differentiation. Furthermore, to mimic the in vivo physiological conditions and to evaluate the effect of low oxygen concentration on SMLC differentiation potential, selected B-WJMSCs were induced to SMLCs under hypoxic (3% O_2) conditions. In our previous study (Bharti et al. 2017), we already demonstrated the presence of valuable WJMSCs throughout the length of the whole umbilical cord and here we show the utility of WJMSCs as suitable candidates for SMLC differentiation. This is the first study of its kind that demonstrates the ability of different region-derived same donor WJMSCs to differentiate into functional SMLCs under different induction conditions.

Materials and methods

All chemicals were purchased from the Sigma chemical company (Sigma, St. Louis, MO, USA) and media from Gibco (Invitrogen, Burlington, ON, Canada), unless otherwise specified.

Isolation and expansion of WJMSCs

After obtaining informed donor's consent and approval from the Committee for Clinical Research at GNUH (GNUHIRB-2012-09-004), human umbilical cord samples were obtained from Gyeongsang National University Hospital. Isolation of WJMSCs was done as described previously (Bharti et al. 2017). Briefly, human umbilical cord ($n = 5$) samples obtained from patients undergoing full term cesarean/vaginal delivery were aseptically collected in sterile Dulbecco's phosphate buffered saline (DPBS) containing containers and transported to the laboratory within 2–3 h. Same donor-derived whole UC

was used to isolate WJMSCs and three different regions mainly towards the mother's side (mother region), center of the whole cord (central region) and the region towards the baby side (fetus) were selected and cut into 1–2 cm pieces and were properly washed 2–3 times with DPBS. After removing arteries, vein and the amnion layer, ~ 1 mm explants from all the three regions were separately attached onto coated and pre-incubated 6 cm plates that contained advanced Dulbecco's modified Eagle's medium (ADMEM) supplemented with 10% fetal bovine serum (FBS) (10% ADMEM). To assure firm attachment, explant-seeded plates were incubated at 37 °C for 1–2 h in an inverted position. Further, 1–2 ml of 10% ADMEM culture media was gently poured in properly attached explant-seeded plates and plates were kept inside a humidified 5% CO_2 incubator at 37 °C till the outgrowth of adherent cells was observed from the tissue explants. After 10–12 days, explants were removed and adherent cells were grown till 70–80% confluence. Cells were then harvested by using 0.25% (*w*/*v*) trypsin-EDTA (Gibco), followed by centrifugation at 300×*g* for 5 min. Harvested cells were further sub cultured up to third passage. The growth behavior of primary and passaged cells was assessed by using an inverted phase contrast microscope (Nikon DIAPHOT 300, Japan). Third passage cells were used in all the experimentation. Different region-derived WJMSCs were designated as M-WJMSCs (isolated from the mother region towards placenta), C-WJMSCs (isolated from the central region) and B-WJMSCs (isolated from the fetal region). All parts were collectively referred to as MBC-WJMSCs. Culture media was changed on every alternative day.

Pluripotency marker expression and multilineage differentiation potential

At third passage, MBC-WJMSCs were evaluated for the expression of pluripotency markers (Oct4, Sox2 and Nanog) at the protein level. Furthermore at 70% confluence, all region-derived WJMSCs were seeded into 6-well plates at a density of 7.5×10^4 cells/well and induced to adipogenic and osteogenic lineages by culturing under lineage-specific conditions for 21 days following previously published protocol (Shivakumar et al. 2015). Cells were induced to adipocyte lineage using culture medium consisting of 10% ADMEM supplemented with 1 mM dexamethasone, 100 mM indomethacin, 10 mM insulin and 500 mM isobutyl methyl xanthine. For osteogenic differentiation, culture medium consisted of 10% ADMEM supplemented with 0.1 mM dexamethasone, 10 mM glycerol-2-phosphate and 50 mM ascorbate-2-phosphate. After 21 days, successful differentiation was evaluated by staining with Oil red O, von Kossa and alizarin red in differentiated adipocytes and osteocytes, respectively. Untreated cells were also stained with respective differentiation specific stains. Media was changed every 2 days interval.

Cell surface marker expression

Cells at third passage from three regions were evaluated for the expression of CD markers using flow cytometry (BD FACS Calibur; Becton Dickinson and Company, Franklin Lakes, NJ, USA) as described previously (Shivakumar et al. 2015). Briefly, cells were harvested at 80–90% confluence and fixed with 3.7% formaldehyde solution for 1 h followed by 2–3 times washing with DPBS. Cells at a density of 1×10^5 cells per marker were directly labeled with fluorescein isothiocyanate (FITC)-conjugated CD34 (BD Pharmingen, CA, USA, FITC Mouse Anti-Human CD34), CD45 (Santa Cruz Biotechnology, FITC Mouse Anti-Human CD45), CD90 (BD Pharmingen, FITC Mouse Anti-Human CD90) and unconjugated CD73 (Santa Cruz Biotechnologies, Mouse monoclonal) and CD105 (Santa Cruz Biotechnologies, Mouse monoclonal IgG2a), CD14 (Santa Cruz Biotechnologies, Mouse monoclonal), CD19 (Santa Cruz Biotechnologies, Mouse monoclonal), HLA-DR (Santa Cruz Biotechnologies, Mouse monoclonal) and vimentin (Santa Cruz Biotechnologies, Mouse monoclonal) for 30 min. The unconjugated primary antibodies were treated with secondary FITC-conjugated goat anti-mouse IgG (BD Pharmingen) for 30 min in the dark whereas Mouse IgG1 (BD Pharmingen) was used as an isotype matched negative control. All experiments were done in triplicate.

Evaluation of smooth muscle-specific marker expression in a time-dependent manner

To check whether WJMSCs isolated from different regions possess expression for smooth muscle specific markers, third passage WJMSCs were cultured in 6-well plates using 10% ADMEM. Cells were harvested at different time intervals, i.e., 1, 3, 5, 10 and 15 days followed by an assessment of SMC-specific early, mid and late marker proteins using Western blotting analysis.

Smooth muscle-like cell differentiation and selection of highly potent region and treatment group

MBC-WJMSCs were analyzed for their ability to differentiate into SMLCs. Briefly, at 70–80% confluence, WJMSCs at third passage were induced to SMLCs using three different treatment groups namely group A: 2.5 ng/ml TGF-β1, group B: 5 ng/ml PDGF-BB and group C: 2.5 ng/ml TGF-β1 + 5 ng/ml PDGF-BB. DMEM supplemented with 10% FBS was used as basal medium. Induced WJMSCs were harvested in a time-

dependent manner and evaluated for the SMC-specific marker expression. Morphological observations were made using an inverted phase contrast microscope (Nikon DIAPHOT 300, Japan). The treatment group with the highest SMC marker expression was selected and used for differentiation. Differentiated MBC-WJMSCs were evaluated for the SMC-specific marker expression at both the mRNA and protein level. The region with the best outcome in terms of highest SMC marker expression and functional properties was selected for further study.

Effect of hypoxia (3% O_2) on differentiation ability of selected WJMSCs

To mimic the in vivo physiological conditions, selected region-derived WJMSCs were induced to SMLCs under hypoxic conditions using a previously selected treatment group. Cells harvested at different time intervals were evaluated for the expression of SMC-specific early, mid and late markers both at the mRNA and protein level. Functional ability of the induced WJMSCs was evaluated by collagen gel contraction assay and electrophysiology.

Collagen gel contraction assay

Both the differentiated and undifferentiated cells from all the experimental groups cultured under different oxygen concentrations were evaluated for their ability to contract the collagen gel according to the previously published protocol (Ngo et al. 2006). Briefly, an aliquot of 1×10^5 cells was mixed with soluble rat tail collagen type 1 (Gibco, Life Technologies) containing NaOH (1 M; Sigma), DMEM 10× mixture, and acetic acid to create a cell collagen suspension. A total of 500 μl aliquot of cell collagen suspension was placed onto a 12-well tissue culture plate (BD Biosciences) and allowed to polymerize for 20–30 min at room temperature. Polymerized gels were dissociated using 200-μl pipette tip followed by addition of 500 μl DMEM for the growth of cells in a humidified 5% CO_2 incubator at 37 °C. The initial diameter of the lattice was measured before and after mechanical release of the cell collagen lattice for contractile force measurement for 12 h at regular intervals of time and the relative change in the lattice diameter was calculated to evaluate the extent of contraction between all the experimental groups. Release of cell-embedded lattices was performed after administration of 1 N KCl as an agonist in serum-free media. The extent of contraction was calculated by using the formula (Du–Dr) / Du × 100, where Du and Dr represent the diameter unreleased and released lattices, respectively. Each experiment was performed in triplicate.

Whole-cell voltage clamp recording

Ca^{2+} currents were recorded by the whole-cell patch-clamp technique using a patch clamp amplifier (Axopatch 200, Axon Instruments, Union City, CA, USA) at room temperature. The patch pipette (Kimax-51, Kimble Co., USA) had resistance ranging between 2 and 4 MΩ when filled with pipette solution. The bath solution contained (mM): 130 NaCl, 6 KCl, 0.3 Na-pyruvate, 1 $CaCl_2$, 1 $MgCl_2$ and 10 HEPES. The pH was adjusted to 7.4 with NaOH. The pipette solutions contained (mM) 125 CsCl, 25 TEA-Cl, 1 $MgCl_2$, 5 EGTA and 10 HEPES (pH 7.3). CsCl and TEA-Cl were used to block the K^+ component of the currents. The membrane potential was held at − 60 mV and depolarizing voltage pulses of 250 ms duration were applied to voltages between −40 and + 50 mV with intervals of 5 s. The recorded signal was filtered at 2 kHz and transferred to a computer using the Digidata 1322A interface (Axon Instruments). Acquired whole-cell currents were analyzed with pCLAMP (version 10.4, Axon Instruments) and Origin® (Microcal Software, Inc., Northampton, MA, USA) programs.

Evaluation of the effect of different oxygen concentrations and cytokine treatment on WJMSCs

Different oxygen concentrations and cytokine treatments may cause adverse effects and can enhance the apoptotic activity of WJMSCs. Therefore, both untreated and treated B-WJMSCs cultured under normoxic and hypoxic conditions were evaluated for apoptosis using Annexin-V assay as per the manufacturer instructions (BD Pharmingen, CA, USA).

Immunocytochemistry

For immunocytochemical staining, cells were initially fixed with 3.7% formaldehyde for 1 h and permeabilized with 0.25% Triton X-100 for 10 min at room temperature. After blocking with 1% bovine serum albumin (BSA) in DPBS for 1 h, the cells were incubated overnight with primary antibodies such as mouse monoclonal anti-alpha smooth muscle actin (α-SMA, 1:200, Abcam), rabbit monoclonal anti-calponin (Calponin, 1:200, Abcam), rabbit monoclonal anti-alpha smooth muscle-22 (SM-22, 1:200, Abcam), rabbit polyclonal anti-myosin heavy chain 11 (MHY-11, 1:200, Abcam) at 4 °C followed by incubation with goat anti rabbit IgG CFL 488 (1:3000, Santa Cruz Biotechnology) and donkey anti mouse IgG FITC (1:3000, Santa Cruz Biotechnology) secondary antibodies for 45 min at 37 °C, respectively. Cell nuclei were counterstained with 1 μg/ml 4′, 6-diamidino-2-phenylindole (DAPI) for 5 min and images were taken using a fluorescent microscope (Leica, Wetzlar, Germany). Control cells were stained with corresponding secondary antibodies to eliminate the background auto fluorescence.

Western blotting

Protease inhibitor containing RIPA buffer (Thermo Scientific, Rockford, IL, USA) was used to prepare protein lysate from control and differentiated cells from all the experimental groups. After evaluating protein concentration by using Microplate BCA Protein Assay kit (Pierce Biotechnology, Rockford, IL, USA), a total of 25 μg of each protein sample was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Mini Protean, BioRad, Hercules, CA, USA) and transferred onto polyvinylidene difluoride membranes (PVDF, Millipore, USA). Membranes were then incubated with primary antibodies such as rabbit anti-Sox-2 (1:200, Santa Cruz Biotechnology), goat anti-Nanog (1:200, Santa Cruz Biotechnology), goat anti-Oct-3/4 (1:200, Santa Cruz Biotechnology), mouse monoclonal anti-alpha smooth muscle actin (α-SMA, 1:200, Abcam), rabbit monoclonal anti-calponin (Calponin, 1:200, Abcam), rabbit monoclonal anti-alpha smooth muscle-22 (SM22-α, 1:200, Abcam), rabbit polyclonal anti-myosin heavy chain-11 (MHY-11, 1:200, Abcam) and rabbit anti-β actin (1:1000, Cell Signaling Technology) for overnight at 4 °C followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:10,000, Santa Cruz Biotechnology) and goat anti-mouse IgG (1:10,000, Santa Cruz Biotechnology), donkey anti-goat IgG (1:10,000, Santa Cruz Biotechnology) secondary antibodies at room temperature for 1 h. Immunoreactivity was detected by enhanced chemiluminescence (ECL; Supersignal, West Pico Chemiluminescent substrate, PIERCE, IL, USA) and exposed to X-ray films.

RNA extraction, cDNA synthesis and qRT-PCR

Using the RNeasy mini kit (Qiagen, Valencia, CA, USA), total RNA was isolated following the manufacturer's protocol with the additional elimination of genomic DNA. To evaluate the concentration and purity of total RNA, optical density was measured at 260 nm and the 260 nm/280 nm ratio. From a total of 2 μg RNA, complementary DNA (cDNA) was prepared with the help of Omniscript RT kit (Qiagen) with oligo-dT primer and the reaction was carried out at 37 °C for 60 min. Real-time PCR was carried out on a Rotor gene Q (Qiagen) using Rotor Gene™ SYBR green PCR kit (Qiagen) to evaluate the expression of transcription factors and lineage specific marker genes. Briefly, a 25-μl reaction volume was formulated using a total of 50 ng cDNA mixed with 12.5 μl SYBR Green mix, 5.5 μl RNase free water and 1 μl each of forward and reverse primers at 400 nM concentration. In accordance with the manufacturer's instructions, PCR assay was performed with initial denaturation at 95 °C for 10 min, followed by 40 PCR cycles of 95 °C for 10 s, 60 °C for 6 s and 72 °C for 4 s, followed by a melting curve from 60 to 95 °C at 1 °C/s and then cooling at 40 °C for 30 s. Further, Rotor-Gene Q series software (Qiagen) was used to analyze CT values and melting curves of each sample. For the normalization of data, *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) was used as housekeeping gene. The PCR products were evaluated by 2% agarose gel electrophoresis. Gel images were analyzed using zoom browser EX5.7 software (Canon) and the relative level of gene expression was calculated by using $2^{-\Delta\Delta CT}$ method. The primers used are listed in Table 1. All experiments were carried out in triplicate.

Statistical analysis

All experimental groups were analyzed for their statistical differences by one-way ANOVA using SPSS 21.0. All data were presented as mean ± standard error and Tukey's test was performed for multiple comparison test. All experiments were done in triplicate. Values with $P < 0.05$ were considered as significant.

Results

Cell morphology and pluripotency marker expression

Cells isolated from all the experimental groups using explant method were shown to have fibroblastoid morphology without any noticeable differences. Adhered primary cells tend to become confluent within 4–5 days after removal of explant tissues and were further sub-cultured up to third passage.

The stemness of MBC-WJMSCs was confirmed by the expression of pluripotent markers OCT4, SOX2 and NANOG (Supplementary Fig. S1a).

Lineage-specific differentiation potential and immunophenotyping

MBC-WJMSCs were successfully differentiated into adipocyte and osteocyte lineages under lineage-specific induction conditions. All MSCs were shown to exhibit positive staining (Supplementary Fig. S1b). Intracellular lipid droplets developed during adipocyte differentiation showed positive results for Oil red O stain. The development of nodules in osteocyte-differentiated cells was confirmed by Alizarin red and von Kossa staining. No significant differences were observed among MBC-WJMSCs. Immunophenotyping was carried out by using flow cytometer. MBC-WJMSCs exhibited strong expression for the positive mesenchymal markers such as CD90, CD73, CD105 and vimentin and were found to be negative for CD45, CD34, CD14, CD19 and HLA-DR markers (Supplementary Fig. S1c). No significant differences were observed among the experimental groups.

Table 1 List of primers used in the gene expression profiling of WJMSCs by using RT-qPCR

Gene	Primer sequence	Product size (bp)	Accession No.
αSMA	F: ACTGGGACGACATGGAAAAG R: CATACATGGCTGGGACATTG	168	BC093052.1
SM22	F: AGCCTTCTTTCCCCAGACAT R: CACCAGCTTGCTCAGAATCA	216	D17409.1
CALP	F: TGACCCCAAGAACCATATCC R: CAGGGACATGGAGGAGTTGT	169	BC141833.1
MHY-11	F: CAGCCAGCATTAAGGAGGAG R: AAGTACCGCTCCCTCAGGTT	190	BC031040.1
GAPDH	F: AGTCAGCCGCATCTTCTTTT R: CCAATACGACCAAATCCGTT	97	NM_002046.5

Smooth muscle cell marker expression by un-induced MBC-WJMSCs

To check whether untreated WJMSCs spontaneously express SMC-specific markers, MBC-WJMSCs were evaluated for their SMC-specific marker expression in a time-dependent manner. All experimental groups showed expression for early markers (αSMA and SM22) that lasted till the 15th day of culture. However, untreated MSCs showed marginal expression for mid marker CALPONIN up to the 5th day of culture and gradually declined with extension in culture time. Time course protein expression of SMC markers did not show any significant differences for treatment days 3 and 5, whereas none of the untreated WJMSC group was found to be positive for late marker MHY-11 (Fig. 1a).

Transdifferentiation into SMLCs under different cytokine inductions and selection of best treatment group and highly potent WJMSC region

In order to induce SMLC differentiation, all experimental groups were treated with different cytokines (TGF-β1, PDGF-BB, TGF-β1 + PDGF-BB) using 10% DMEM. Furthermore, evaluations of the best treatment group as well as the SMC-specific highly potent WJMSC region were made on the basis of highest SMC-specific marker expression. MBC-WJMSCs were induced for 5 days with different treatment groups and were compared for their differentiation ability under different cytokine inductions. RT-qPCR results revealed that treatment group A (2.5 ng/ml TGF-β1) was able to significantly increase *α-SMA* expression in all the experimental groups where M-WJMSCs showed highest expression among all regions (Fig. 1b), whereas treatment groups A and C could increase *SM22-α* expression in mother and central region-derived WJMSCs without any significant differences. Interestingly, the highest *SM22-α* was found in the B-WJMSCs when induced with treatment group C in comparison to others (Fig. 1c). Both *α-SMA* and *SM22-α* are the early SMC markers and were expected to be equally expressed by all WJMSC regions under similar treatment conditions. However, the main reason behind their varied expression especially high *α-SMA* expression by M-WJMSCs and *SM22-α* by B-WJMSCs is not known and needs further research. MBC-WJMSCs showed mixed expression for mid marker *CALP*. Treatment groups A and C were found to increase mid marker expression in MBC-WJMSCs. Among all the experimental groups, highest mid marker expression was shown by B-WJMSCs when induced with treatment group C (Fig. 1d). For the expression of matured SMC late marker MHY-11, treatment group C showed highest expression among all treatment groups where M-WJMSCs and B-WJMSCs showed significantly higher expression in comparison to the C-WJMSCs (Fig. 1e). Treatment group B resulted in lowest expression (Fig. 1b–e) and therefore, its use was neglected. It is evident from previous studies that the expression of mid and late markers corresponds to the matured SMCs. Therefore, treatment group C was selected for further experimentation. It was also noted that B-WJMSCs when treated with a cocktail combination of TGF-β1 and PDGF-BB cytokines (treatment group C) showed highest expression for *SM22-α, CALP*. Moreover, higher *MHY-11* expression was observed in M-WJMSCs and B-WJMSCs without any significant differences (Fig. 1e). Time-dependent protein expression and morphological changes in the induced MBC-WJMSCs were also evaluated using treatment group C. A gradual change in the morphology in the form of heap and valley like structures with a spindle shape was observed that resembles SMLCs (Fig. 2). Protein expression results from the induced MBC-WJMSCs revealed that during the 1st day of differentiation, MBC-WJMSCs positively expressed early and mid-markers while lacking the expression of late marker MHY-11 (Fig. 1f). With the extension of the induction period, all regions showed expression for early, mid and late SMC markers. Among MBC-WJMSCs, B-WJMSCs showed highest SMC-specific marker expression (Fig. 1g) as well as

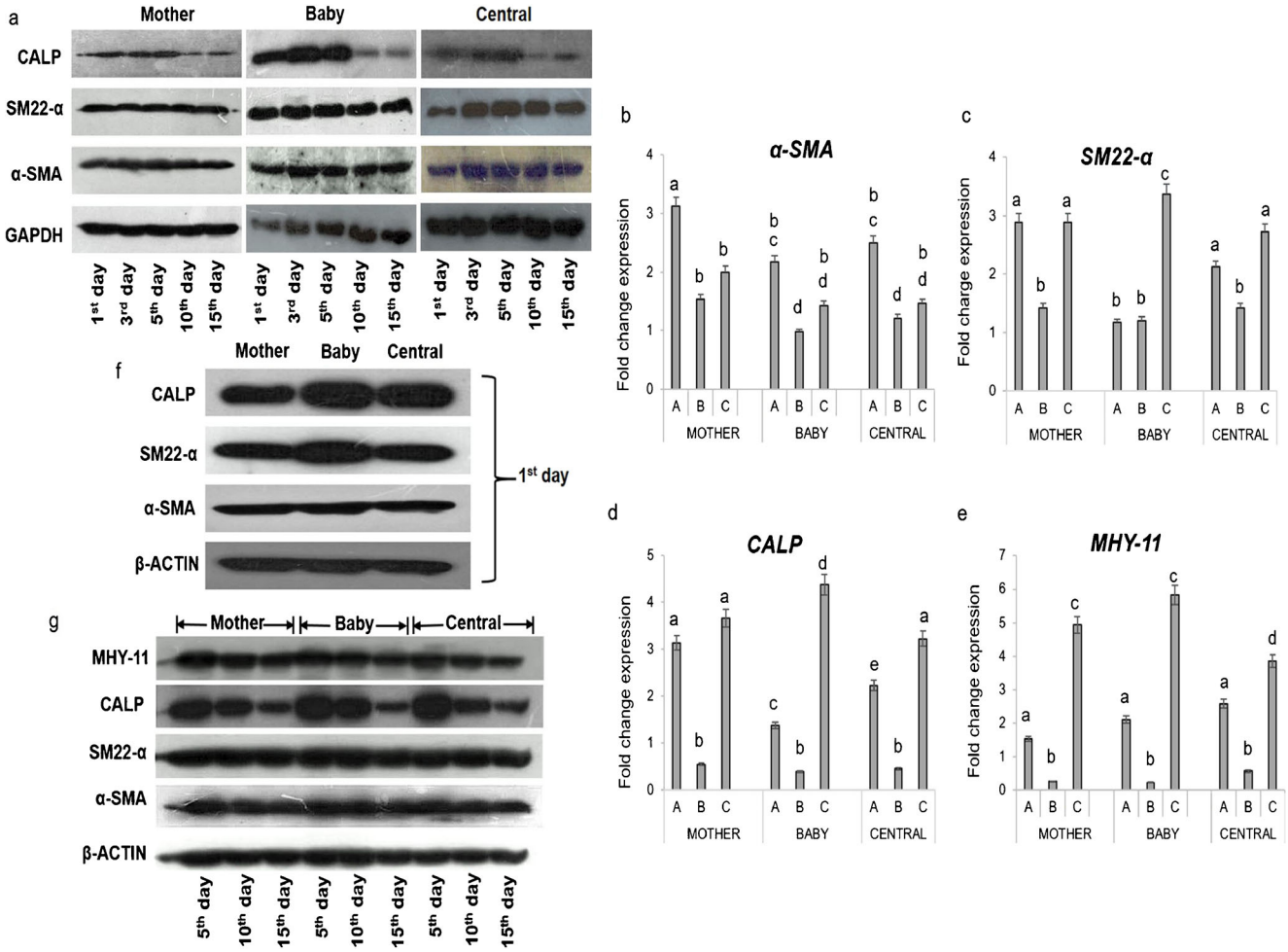

Fig. 1 SMC specific marker expression from untretaed and treated cells at mRNA and protein level. **a** Time-dependent SMC-specific protein marker expression from untreated MBC-WJMSCs. Western blot images of SMC-specific early (α-SMA and SM22-α) and mid (CALP) markers from untreated MBC-WJMSCs during 1, 3, 5, 10 and 15 days of culturing at passage 3. **b**–**e** SMC-specific marker gene expression under different cytokine inductions and selection of highly potent WJMSC region. Relative mRNA levels of SMC specific early (*α-SMA & SM22-α*), mid (*CALP*) and late (*MHY-11*) marker genes in MBC-WJMSCs induced upto 5 days with three treatment groups: (A) 2.5 ng/ml TGF-β1, (B) 5 ng/ml PDGF-BB and (C) 2.5 ng/ml TGF-β1 + 5 ng/ml PDGF-BB. Significant differences were considered when $P < 0.05$. **f** SMC-specific protein marker expression in induced WJMSCs. All the three Wharton's jelly regions were positively expressed for early (αSMA & SM22-α) and mid-marker (CALP) at the 1st day of induction without the expression of late marker MHY-11. **g** With the elongation in the induction period, MBC-WJMSCs showed positive expression for all the SMC protein markers including MHY-11, where B-WJMSCs showed highest expression for SMC markers in comparison to mother and central region-derived WJMSCs

enhanced electrophysiological competence; therefore, it was selected for further experimentation (Fig. 4d–d").

Immunocytochemical analysis of induced MBC-WJMSCs

Immunocytochemical analysis of induced MBC-WJMSCs revealed that under SMC-specific cytokine treatment, WJMSCs have the potential to undergo SMLC differentiation irrespective of their location in the UC. Induced WJMSCs showed a strong expression for early and mid-markers (Fig. 3a–i"). However, induced MBC-WJMSCs were found to be less efficient for translating fully into MHY-11 positive cells and resulted in low cellular density by MHY-11 positive cells. Therefore, a comparatively less number of induced cells were shown to have expression for late marker (MHY-11) (Fig. 3j–l"). A slight increase in the expression level of MHY-11 positive cells was shown by hypoxia-induced cells, which may be due to the changed (low) oxygen concentration that favored the SMLC differentiation ability (Fig. 7d–d"). However, the exact mechanism is not known and needs further research.

Gel contraction by induced cells

MBC-WJMSCs (un-induced/induced) were tested for their ability to contract gels under the influence of muscarinic receptor KCl. WJMSCs induced with cytokine-enriched differentiation media (treatment group C) were shown to contract

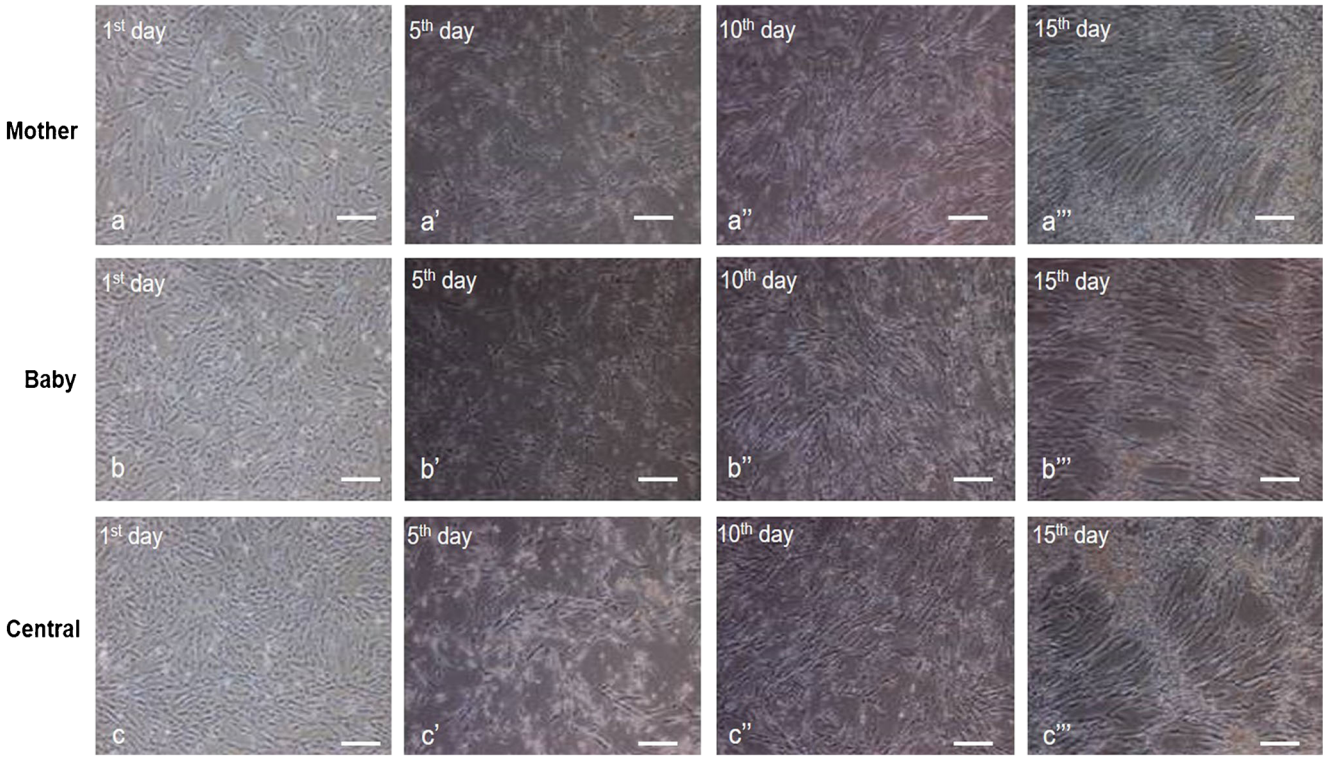

Fig. 2 Time-dependent morphological changes in the induced WJMSCs. Phase contrast microscopic images of induced MBC-WJMSCs at different time intervals; scale bar = 100 μm

the gels upon stimulation. Readings at different time intervals were taken, which showed a gradual decrease in the gel size (Fig. 4a, b"', c). After 3 h, no significant differences were observed in the gel area. Interestingly, a small amount of gel contraction was also shown by untreated MBC-WJMSCs.

Ca^{2+} currents recorded in smooth muscle cells differentiated from WJMSCs

Under normoxic conditions, both the treated and untreated MBC-WJMSCs were cultured onto the cover slip coated with poly-L-lysine to record whole-cell currents. Inward currents were elicited in response to step depolarization in a condition that blocks K^+ currents. The inward currents were compared between differentiated (induced with treatment group C) and undifferentiated cells from all three WJMSC regions (data not shown). Compared to undifferentiated cells, differentiated cells showed large inward currents in the bath solution containing 10 mM Ca^{2+} (Fig. 4d). Among differentiated cells, the inward currents were frequently recorded in smooth muscle cells differentiated from B-WJMSCs (mother region 13.5 ± 6.5%, $n = 87$; central region 11.1 ± 5.1%, $n = 73$; baby region 25.2 ± 8.9%, $n = 63$). The inward currents were activated from − 20 mV and maximally activated at 0 mV. Further depolarization beyond 0 mV reduced the amplitude of the inward current. The maximum peaks of the inward currents in differentiated cells at 0 mV were 238 ± 46.4 pA, whereas we could not detect the inward currents in undifferentiated cells.

To identify the effect of extracellular Ca^{2+} concentration on the inward currents recorded in differentiated cells, we changed the Ca^{2+} concentration 1 to 10 mM. The inward currents were sensitive to the extracellular Ca^{2+} concentration ($[Ca^{2+}]$). The inward currents were very small or not recorded at 1 mM $[Ca^{2+}]$. When $[Ca^{2+}]$ was increased from 5 to 10 mM, the inward currents significantly increased from 50.6 ± 16.3 to 238 ± 46.4 pA as shown in Fig. 4(d') ($p < 0.05$). The inward currents were further characterized by their sensitivity to Ca^{2+} channel blockers. The currents were inhibited by treatment with 100 μM $NiCl_2$, a T-type Ca^{2+} channel blocker and 10 μM verapamil, a L-type Ca^{2+} channel blocker (95 ± 3% by $NiCl_2$ and 96 ± 4% by verapamil) (Fig. 4d"). These results indicate that the inward currents are carried via Ca^{2+} channels. Significantly higher inward currents were recorded in hypoxia-induced differentiated B-WJMSCs in comparison to MSCs differentiated under normoxic conditions (Fig. 6f).

Effect of hypoxia on the SMLC differentiation potential of B-WJMSCs

To study the effect of low oxygen concentration on the SMLC differentiation, B-WJMSCs were induced under

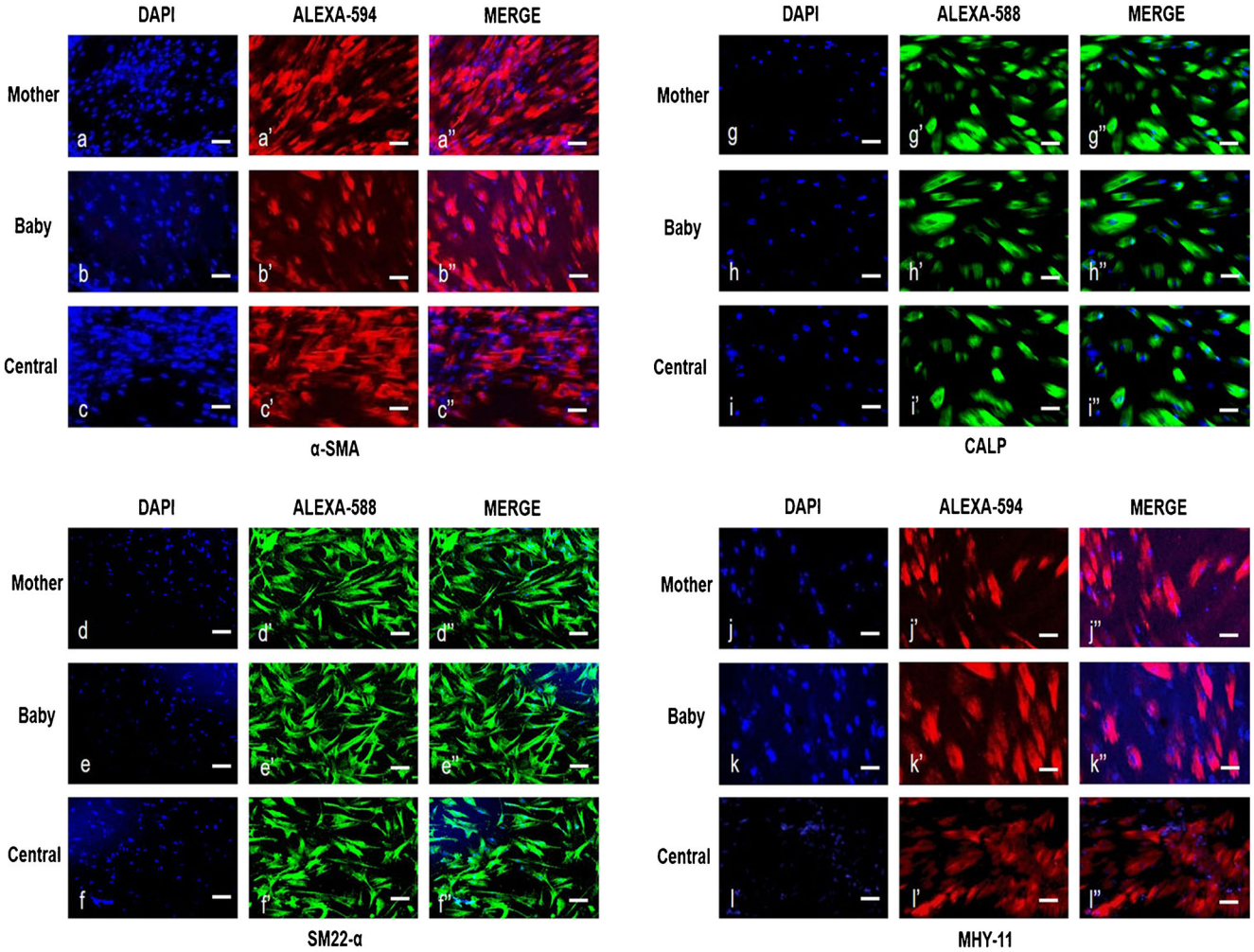

Fig. 3 Immunocytochemical analysis of induced WJMSCs. **a**–**i**" MBC-WJMSCs were induced to SMLCs using cytokines enriched differentiation media for 15 days and were analyzed for SMC-specific early, mid and late marker expression. All WJMSC groups showed positive expression for early (αSMA and SM22-α), mid (CALP) and late (MHY-11) markers without any significant differences; scale bar = 100 μm

hypoxic conditions. Under hypoxic conditions, B-WJMSCs followed the same expression pattern for cell surface marker expression as depicted under normoxic conditions. Hypoxia-induced B-WJMSCs were shown to have strong positive expression for CD73, CD90, CD105, and Vimentin whereas lack the expression of CD43, CD45, CD14, CD19 and HLA-DR (Fig. 5a–i). Induction time and treatment parameters were kept the same as used under normoxic conditions. Cytokine treatment under hypoxic conditions gradually altered the morphology from fibroblastoid to spindle like cells with heap and valley like arrangements (Fig. 5j–j"'). Hypoxia enhanced the expression level of SMC-specific early, mid and late markers without increasing the rate of apoptosis. Annexin-V assay revealed no significant differences among the apoptosis rate of untreated and treated B-WJMSCs cultured under different oxygen concentrations (Fig. 5k–n). Furthermore, induced B-WJMSCs showed a time-dependent increased expression of SMC-specific markers both at the mRNA and protein level in which the majority of the markers showed high expression during the 5th and 15th day of induction (Fig. 6a–e). Hypoxic conditions were also found to elevate the inward current recordings in induced B-WJMSCs when compared with the normoxia-induced B-WJMSCs (Fig. 6f). These results demonstrated that hypoxic conditions provide a conductive environment for SMC differentiation. Immunocytochemical analysis of induced B-WJMSCs under hypoxic conditions was also shown to exhibit SMC-specific marker expression (Fig. 7a-d"). Successful differentiation was further confirmed by the ability of induced B-WJMSCs to contract the collagen gels under the influence of KCl (Fig. 7e–e"', f). These results show that our protocol is safer and able to promote SMLC differentiation in WJMSCs. However, the basic mechanism behind the increase in SMC marker expression under hypoxic

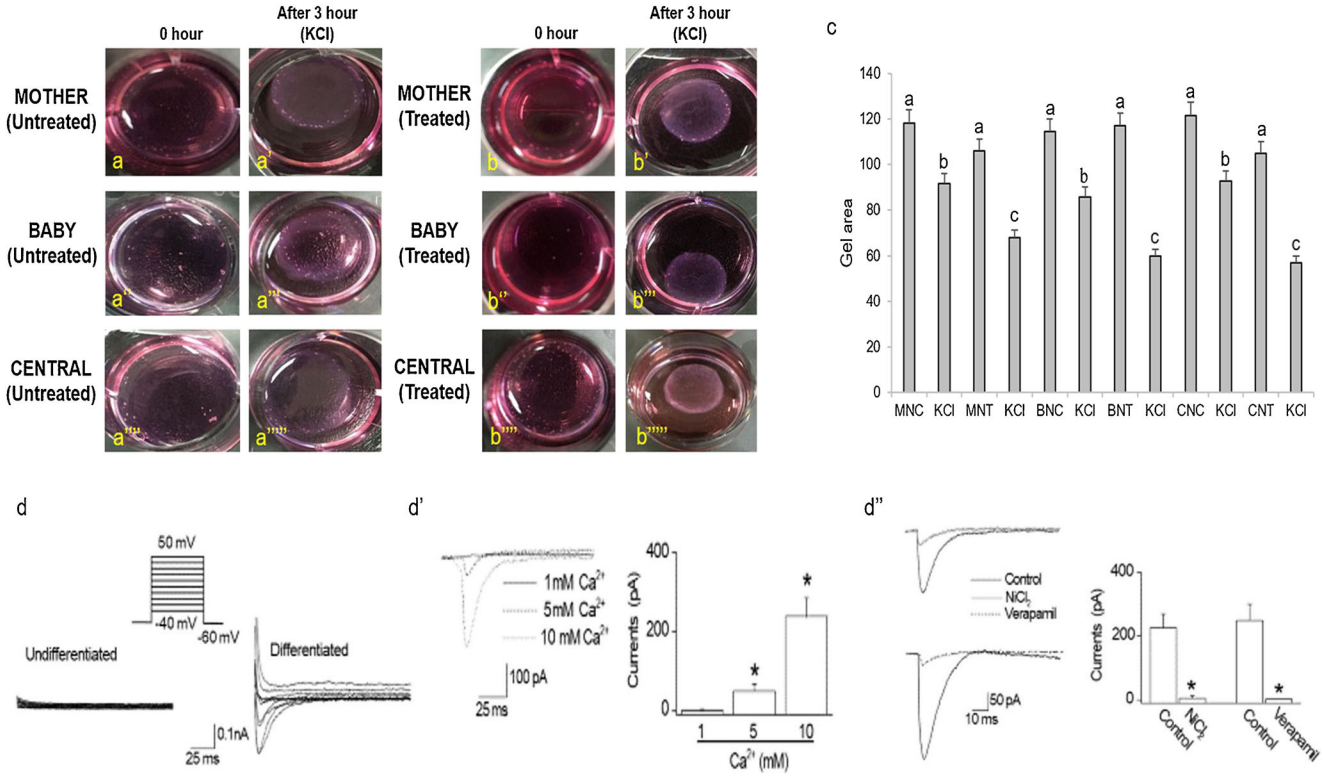

Fig. 4 Collagen gel contraction ability of induced WJMSCs. **a-b**"""' Untreated and treated MBC-WJMSCs were compared before and after contractile stimulation using 60 mM KCl for 3 h. **c** In collagen gel lattice assay, the contractile ability of both untreated and treated MBC-WJMSCs was measured after KCl (60 mM) stimulation. ImageJ software was used to measure the gel area before and after stimulation. MNC-Mother untreated cells in normoxia; MNT-Mother treated cells in normoxia; BNC-Baby untreated cells in normoxia; BNT-Baby treated cells in normoxia; CNC-Central untreated cells in normoxia; CNT-Central treated cells in normoxia; KCl-KCl treated cells. **d–d**" Inward currents recorded in smooth muscle cells differentiated from B-WJMSCs. **d** Representative traces of inward whole-cell currents in response to voltage-step depolarization in undifferentiated and differentiated cells. The currents were recorded in the bath solution containing 10 mM Ca^{2+}. **d'** Effect of external Ca^{2+} concentration on the inward currents. The amplitude of currents was measured at 0 mV. **d**" Effects of $NiCl_2$ and verapamil on the inward currents. All peak amplitudes were obtained by 0 mV step depolarization in a bath solution containing 10 mM Ca^{2+}. Significant differences were considered when $p < 0.05$

conditions is still not clear and needs further investigation.

Discussion

SMCs play an important role in the proper functioning of the organs where they reside. Under pathological conditions and any deformities in the SMCs, occurrence of some problems such as bladder dysfunction, cardiovascular diseases, gastrointestinal diseases and other disorders including aneurysm, atherosclerosis, restenosis etc. takes place that may become fatal if not treated properly. These disorders can be treated by using stents, balloon angioplasty or bypass surgery. Other possible curative measures can be vascular tissue engineering using primary human SMCs, which can be obtained either from autologous or allogeneic sources. But due to low replicative capacity and scarce availability, primary human SMCs are not preferred (Rodríguez et al. 2006). MSC-based cellular therapy has been proven as an efficient alternative to get rid of such problems. Recent studies have shown that MSCs obtained from various sources such as bone marrow, adipose, amniotic fluid, dental and hair follicle can be successfully transdifferentiated into SMCs by inducing them in SMC-specific cytokines (Ross et al. 2006; Rodríguez et al. 2006; Ghionzoli et al. 2013; Xu et al. 2013; Song et al. 2016). Therefore, selection of the suitable MSC source is expedient. The selected source should be easily available and its isolation

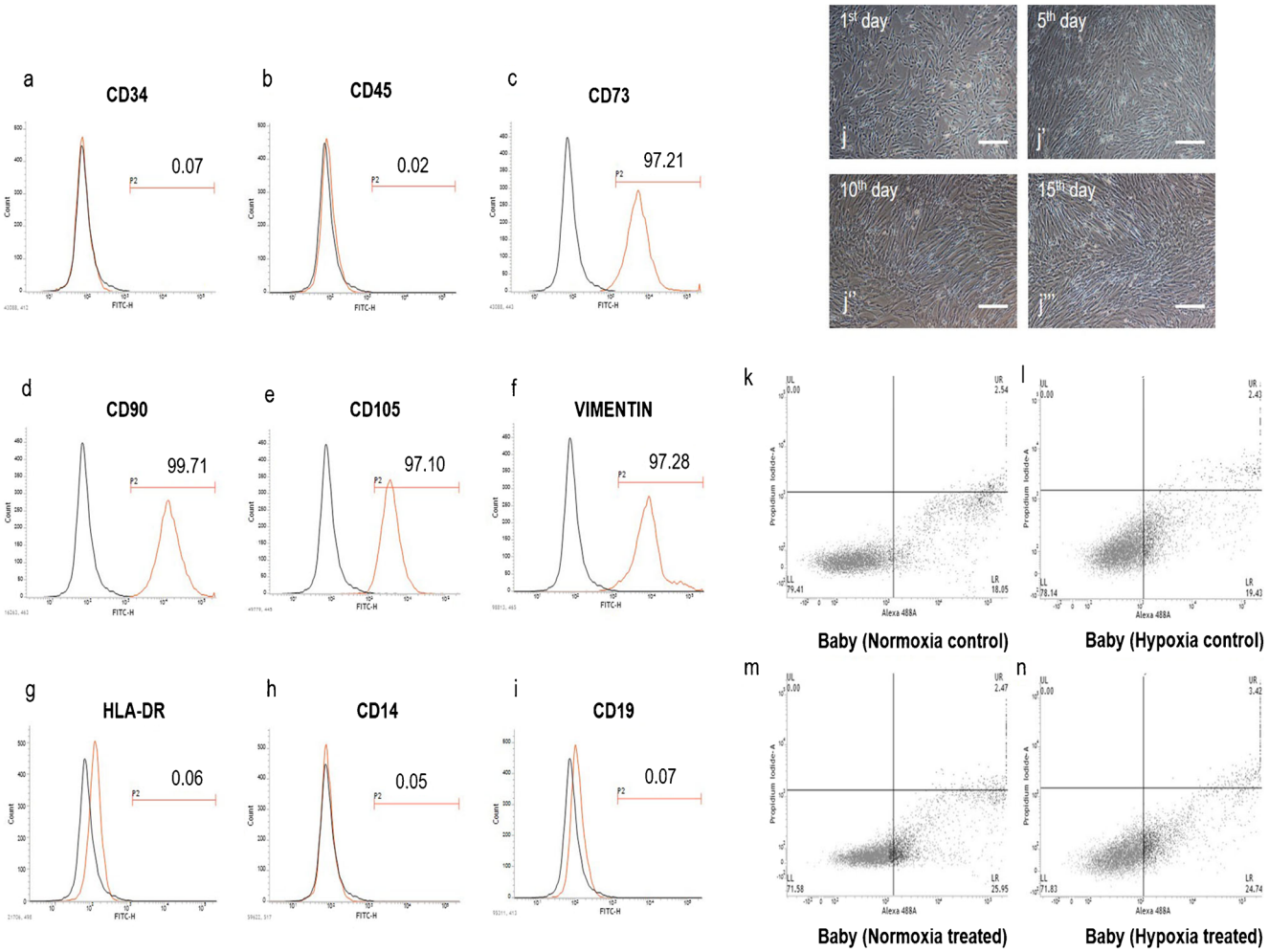

Fig. 5 Cell surface marker expression and morphological alterations in B-WJMSCs under hypoxic conditions. **a**–**i** Untreated B-WJMSCs when cultured under hypoxic conditions (3% O_2) were shown to have strong positive expression for CD73, CD90, CD105 and vimentin whereas lack expression for CD34, CD45, CD14, CD19 and HLA-DR. **j**–**j**"' Cytokine treatment under hypoxic conditions gradually altered the morphology from fibroblastoid to spindle like cells with heap and valley like arrangement; scale bar 100 μm. **k**–**n** Evaluation of apoptosis rate from untreated and treated B-WJMSCs under normoxic and hypoxic conditions. Annexin-V assay revealed that both untreated and treated B-WJMSCs cultured under normoxic and hypoxic conditions did not exhibit an increase in the apoptosis rate without any significant differences

should not pose any invasive or ethical issues. High cell yield multi differentiation potential, low PDT, and immunomodulatory properties also come under the selection criteria for any MSC source to be used as a therapeutic agent. Human WJMSCs have been shown to exhibit all these properties and are considered to be a valuable source in comparison to other sources (Subramanian et al. 2015). Our recent report also exhibited such mesenchymal properties from WJMSCs isolated from different regions of the same umbilical cord (Bharti et al. 2017). Wharton's jelly is a gelatinous substance present throughout the length of the whole umbilical cord; It not only acts as a reservoir of valuable MSCs but also protects the underlying vessels. Previous reports have advocated the presence of myofibroblast-like cells in Wharton's jelly, which are positively expressed for SMC markers such as α-SMA and DESMIN (Takechi et al. 1993; Kobayashi et al. 1998; Corrao et al. 2013). However, after their expansion under in vitro conditions, up to what extent these cells retain their myofibroblast nature is still unknown. Therefore, the present study was conducted to explore the smooth muscle-specific nature of different region-derived WJMSCs under in vitro conditions. WJMSCs from all the regions displayed mesenchymal characteristics as per the ISCT guidelines (Dominici et al. 2006). All WJMSC groups were found to possess fibroblastoid morphology, positively expressed pluripotency markers and were successfully differentiated into adipocyte and osteocyte lineages. These results are in accordance with previously published reports (Shivakumar et al. 2016; Bharti et al. 2017) and hence prove the stemness of isolated cells. We further analyzed the expression of SMC-specific markers in MBC-WJMSCs in a time-dependent manner. Our results revealed the presence of strong, stable and consistent expression of early markers (α-SMA and SM22-α) throughout the culture period, which are in accordance with previous studies

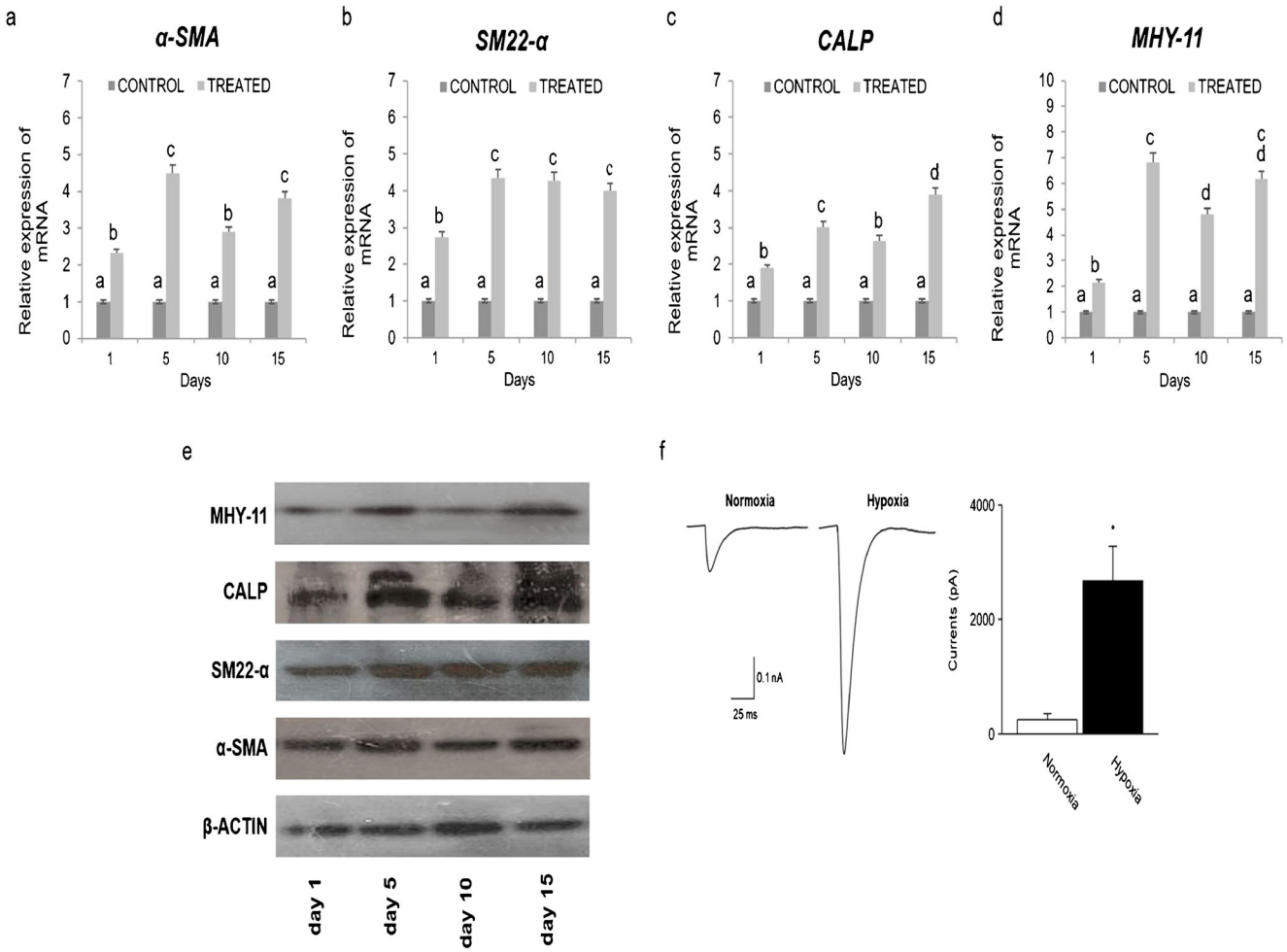

Fig. 6 Evaluation of SMC-specific marker expression of induced B-WJMSCs under hypoxic conditions and their functional competence. **a**–**d** Under hypoxic conditions, induced B-WJMSCs were shown to exhibit SMC-specific early, mid and late marker expression in a time-dependent manner. Significant differences were considered when $P < 0.05$. **e** Induced B-WJMSCs followed the same pattern (RT-qPCR) for protein expression as evaluated by Western blotting. **f** Electrophysiology of B-WJMSCs induced under normoxic and hypoxic conditions. B-WJMSC differentiated under hypoxic condition showed significantly larger inward currents than under normoxic condition. The currents were recorded by 0 mV step depolarization in a bath solution containing 10 mM Ca^{2+}

(Takechi et al. 1993; Kobayashi et al. 1998; Corrao et al. 2013). Expression of the mid marker CALP was found to be present till 5 days of culture, which gradually declined with culture expansion. However, there was no expression of late marker MHY-11. These results indicate that there may be presence of contractile SMC properties in WJMSCs under in situ conditions providing a myofibroblast and flexible nature that helps Wharton's jelly to provide protection to the underlying vessels. When these cells are isolated and cultured in vitro they tend to shift their contractile nature to synthetic with the loss of mid and late SMC markers with expansion in cultures. Therefore, we tried to differentiate MBC-WJMSCs into functional SMLCs by inducing them under lineage specific cytokine treatments. Many studies have previously reported the use of TGF-β1 in different concentrations alone or in combination with other cytokines such as PDGF-BB, BMP-4 etc. to transdifferentiate adult tissue derived MSCs into functional SMCs (Ross et al. 2006; Wang et al. 2010; Moonen et al. 2010; Ghionzoli et al. 2013; Xu et al. 2013; Song et al. 2015). In most of the studies, TGF-β1 was shown to be an important differentiation regulator that can promote SMC-specific marker expression alone or in combination with other cytokines. Therefore, we selected TGF-β1 and PDGF-BB for SMLC differentiation in our study. Cytokines used in three different treatment combinations resulted in a different pattern of lineage-specific marker expression in MBC-WJMSCs. TGF-β1 when treated alone promoted the expression of the early SMC marker α-SMA to the highest level. Further, TGF-β1 when treated alone or in combination with PDGF-BB promoted the SM22-α and CALP expression without any significant differences among mother and central region-derived WJMSCs. PDGF-BB when treated alone showed very less or negligible increase in SMC marker expression; therefore, its use was neglected. However,

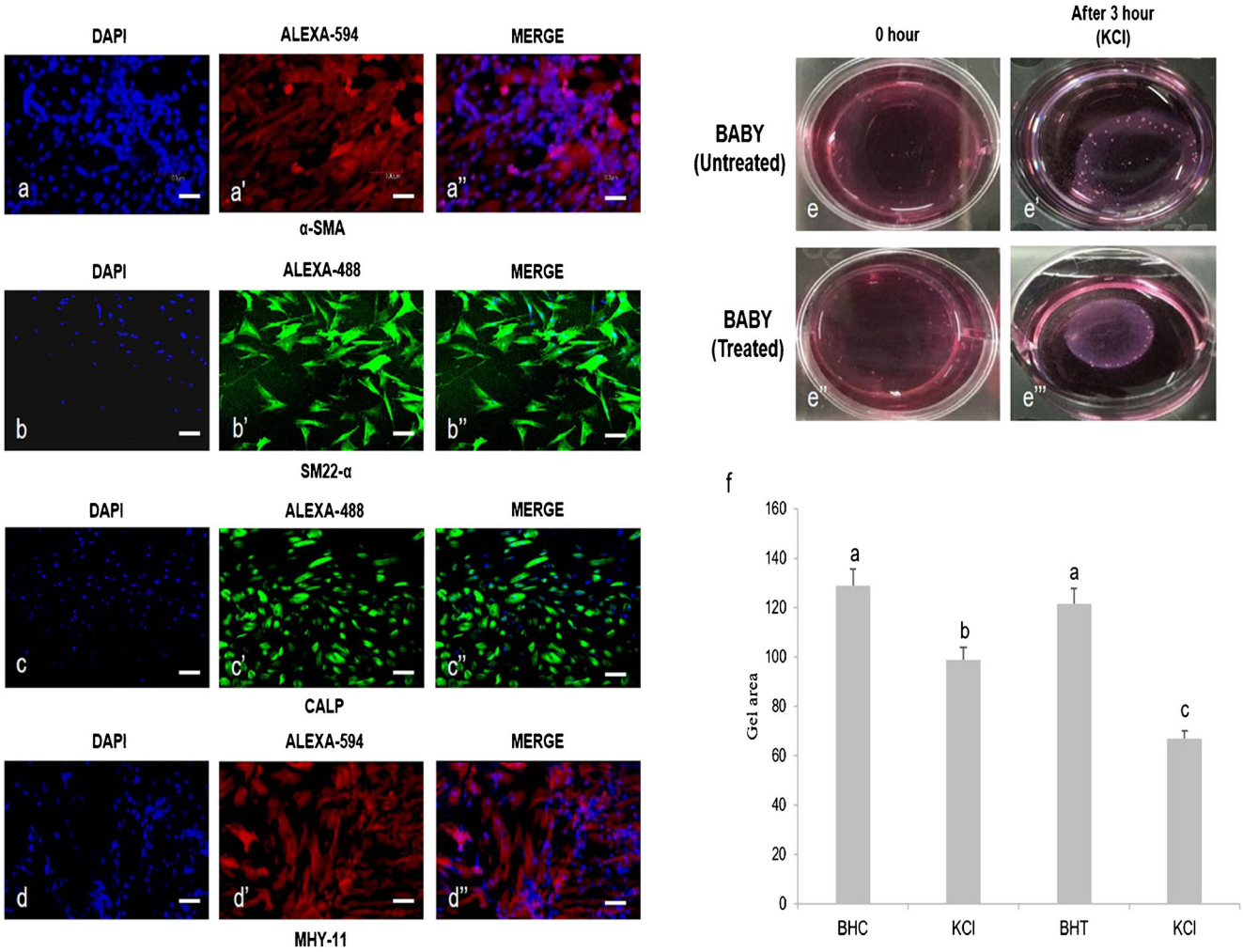

Fig. 7 Immunocytochemical analysis of cytokine-induced B-WJMSCs under hypoxic conditions. **a**–**d**" Under hypoxic conditions, cytokine-induced B-WJMSCs showed positive expression of all early, mid and late markers. **e**–**e**"' Collagen gel contraction ability of induced B-WJMSCs under hypoxic conditions. Untreated and treated B-WJMSCs were compared before and after contractile stimulation using 60 mM KCl for 3 h. **f** In collagen gel lattice assay, the contractile ability of both untreated and treated B-WJMSCs was measured after KCl (60 mM) stimulation. ImageJ software was used to measure the gel area before and after stimulation. Induced B-WJMSCs showed a significantly higher reduction in the gel area as compared to the untreated cells. BHC-Baby untreated cells in hypoxia; BHT-Baby treated cells in hypoxia; KCl-KCl treated cells

B-WJMSCs showed highest expression for SM22-α and CALP under combined cytokine treatments. Combined treatment of both cytokines could efficiently promote the late and matured marker expression in MBC-WJMSCs. Considering these observations, it was concluded that combined treatment of TGF-β1 and PDGF-BB can efficiently promote the expression of SM22-α, CALP and MHY-11 markers in MBC-WJMSCs. However, not all the induced cells from any of the WJMSC region could efficiently translate into MHY-11 positive cells and therefore only a low number of cells could be positively stained for this matured marker in comparison to other early and mid SMC markers. A possible reason for this low cell density may be the inefficiency of the induced cells to fully translate into MHY-11 positive cells at the protein level and further research is needed to understand the basic mechanism governing this phenomenon. Our results are in accordance with previously published reports where researchers showed a successful transdifferentiation of different source-derived MSCs using a combination of TGF-β1and PDGF-BB at different concentrations (Ross et al. 2006; Moonen et al. 2010; Wanjare et al. 2013; Ghionzoli et al. 2013; Xu et al. 2013).

Once the SMC-specific marker expression is obtained through in vitro differentiated MSCs, evaluation of their functional competence is necessary to justify their usage in cell-based therapies during pathological conditions. Contractility holds key characteristics of matured SMCs, which is helpful in controlling the appropriate blood pressure by altering the luminal diameter by contraction and relaxation (Andersson and Arner 2004; Ngo et al. 2006). Previous studies have demonstrated the contraction ability of differentiated cells using collagen gel contraction

assays employing various protocols (Ngo et al. 2006; Ghionzoli et al. 2013; Xu et al. 2017). Therefore, induced MBC-WJMSCs were evaluated for their ability to contract the collagen gels under the influence of externally applied agents. When treated with KCl, both undifferentiated and differentiated WJMSCs could successfully contract the collagen gels. The contraction of gels by untreated cells supports the presence of the myofibroblast nature of MBC-WJMSCs, which could be due to the presence of SMC-specific markers that were found to be retained even after their in vitro expansion. However, differentiated MBC-WJMSCs displayed a significantly higher gel contraction than undifferentiated cells. This enhanced contraction ability by differentiated cells is possibly due to the increased SMC marker expression after cytokine treatment that might have changed their synthetic phenotype to the contractile type. Ion channels present in the smooth muscle membranes also constitute the major factors in the regulation of muscle contraction. In this study, we focused on evaluating whether cytokine-induced WJMSCs could elicit inward currents based on the presence of Ca^{2+}. Electrophysiological observations revealed that both the L and T type of calcium channels are responsible for these inward currents. Inhibition of the current by using L and T type Ca^{2+} blockers further confirmed that inward currents were due to the activity of these calcium ions. Among differentiated cells, the B-WJMSCs displayed frequent records of these inward currents. Hypoxic conditions were shown to enhance the differentiation ability of WJMSCs and more inward currents were recorded in WJMSCs differentiated under hypoxic conditions than differentiated under normoxic conditions.

In the present study, WJMSCs derived from all the three regions showed a gradual change in morphology from fibroblastoid to heap and valley like arranged spindle cells that resemble SMLCs (Rodríguez et al. 2006). Moreover, MBC-WJMSCs showed positive expression for early, mid and late markers in a time-dependent manner. Cytokine induction gradually promoted the marker expression and showed highest expression in B-WJMSCs. Overall, B-WJMSCs exhibited highest SMC marker expression and electrophysiological properties under the combined treatment of both cytokines when compared to M-WJMSCs and C-WJMSCs. One possible reason may be due to the presence of highly potent MSCs in the baby region, which responded at a higher rate for SMC-specific lineage differentiation. Collectively, our comparative study between three region-derived WJMSCs showed the presence of high stemness properties in WJMSCs from the baby region when compared to the central and mother region WJMSCs of the same human umbilical cord. However, the basic mechanism behind the SMC-specific highly potent nature of B-WJMSCs is not known and needs further research.

Conclusion

In conclusion, all region-derived WJMSCs exhibit mesenchymal stem cell properties as per the ISCT guidelines. Positive expression for early and mid SMC-specific markers exhibited by in vitro propagated WJMSCs (untreated) advocates their synthetic SMC profile that can be transformed into contractile SMCs under the influence of SMC-specific differentiation promoters, i.e., TGF-β1 and PDGF-BB. Under normoxic conditions, RT-PCR results revealed enhanced expression for SMC-specific early, mid and late markers especially when MBC-WJMSCs were induced with TGF-β1 and TGF-β1 + PDGF-BB cytokines. Likewise, induced MBC-WJMSCs were also found to have positive expression. Furthermore, desired morphological alterations were also observed among all the experimental groups. However, no such developments were shown by PDGF-BB-induced MSCs; therefore, its use was neglected. Comparative analysis between all WJMSC groups and different treatments under normoxic conditions confirmed the ability of MBC-WJMSCs towards SMLC differentiation especially when treated either with TGF-β1 alone or TGF-β1 + PDGF-BB. Moreover, B-WJMSCs and and TGF-β1 + PDGF-BB treatment group were shown to have more potency and a tendency to induce to SMLCs. Furthermore, hypoxia conditions not only led to increased differentiation ability both at the mRNA and protein level but also promoted the functional competence in terms of increased inward currents without posing any harm to MSCs. Finally, it was concluded that irrespective of the isolation region, there is a presence of highly potent WJMSCs throughout the whole UC that can be transdifferentiated into SMLCs under SMC lineage-specific cytokines. Additionally, the different oxygen levels and treatment groups mentioned above have a different but positive effect on the SMLC differentiation ability of WJMSCs.

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Compliance with ethical standards

Competing interest The authors declare that they have no competing interest.

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