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Long-term hypoxic atmosphere enhances the stemness, immunoregulatory functions, and therapeutic application of human umbilical cord mesenchymal stem cells

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Aims

Mesenchymal stem cells (MSCs) are usually cultured in a normoxic atmosphere (21%) in vitro, while the oxygen concentrations in human tissues and organs are 1% to 10% when the cells are transplanted in vivo. However, the impact of hypoxia on MSCs has not been deeply studied, especially its translational application.

Methods

In the present study, we investigated the characterizations of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) in hypoxic (1%) and normoxic (21%) atmospheres with a long-term culture from primary to 30 generations, respectively. The comparison between both atmospheres systematically analyzed the biological functions of MSCs, mainly including stemness maintenance, immune regulation, and resistance to chondrocyte apoptosis, and studied their joint function and anti-inflammatory effects in osteoarthritis (OA) rats constructed by collagenase II.

Results

We observed that long-term hypoxic culture surpassed normoxic atmosphere during hUC-MSCs culture in respect of promoting proliferation, anti-tumorigenicity, maintaining normal karyotype and stemness, inhibiting senescence, and improving immunoregulatory function and the role of anti-apoptosis in chondrocytes. Furthermore, we demonstrated that the transplantation of long-term hypoxic hUC-MSCs (Hy-MSCs) had a better therapeutic effect on OA rats compared with the hUC-MSCs cultured in the normoxic atmosphere (No-MSCs) in terms of the improved function and swelling recovery in the joints, and substantially inhibited the secretion of pro-inflammatory factors, which effectively alleviated cartilage damage by reducing the expression of matrix metallopeptidase 13 (MMP-13).

Conclusion

Our results demonstrate that Hy-MSCs possess immense potential for clinical applications via promoting stemness maintenance and enhancing immunoregulatory function.

Bone & Joint Research

Article focus

- Normoxic atmosphere still fails to achieve an anticipated effect in many diseases, due to causing senescence and dysfunction of long-term cultivation.
- We chose osteoarthritis (OA) with low-grade inflammation and transplanted human umbilical cord-derived mesenchymal stem cells (Hy-MSCs) to analyze their therapeutic effects in OA rats for the first time.

Key messages

- Mesenchymal stem cells (MSCs) were cultured continuously in hypoxic atmosphere until P30 generations to ensure their biological safety and promote the abilities of differentiation potential, anti-senescence, and stemness maintenance.
- We further proved the effects of Hy-MSCs on their immunoregulatory function, and their protection of chondrocytes.
- Hy-MSCs have a translational effect on OA treatment.

Strengths and limitations

- Long-term hypoxic cultivation facilitated hUC-MSCs attaining the better biological characteristics of MSCs.
- Hy-MSCs prevented matrix metallopeptidase 13 (MMP-13) expression to ameliorate cartilage injury by inhibiting pro-inflammatory factors in vitro and in vivo, therefore making them well-suited for clinical treatment of these diseases.
- The study lacked the molecular mechanism of Hy-MSC treatment.

Introduction

Currently, mesenchymal stem cells (MSCs) are the most extensively studied and widely used this type of cell in clinical practice worldwide.[1,2] MSCs exist in the interstitium of various tissues and organs, with abilities including self-renewal, multi-lineage differentiation, and immunoregulatory functions,[2,3] and not only participate in tissue repair, but also exert inhibitory effects in inflammatory reactions and contribute to the improvement of the microenvironment within the body.[4] In order to achieve the therapeutical effects clinically, many cultivation techniques for the preparation and expansion of stem cells aim to enhance their biological characteristics, therefore meeting clinical treatment requirements. For instance, many methods for expanding MSCs use platelet lysates as serum-free culture systems for cultivation instead of using fetal bovine serum (FBS),[5] thereby reducing the potential clinical adverse reactions associated with animal serum.

MSCs are mostly cultured in a normoxic atmosphere (21% O_2), but the oxygen concentration of the resident region of MSCs in the body is between 1% and 10%.[6] A normal oxygen atmosphere may be detrimental to the growth of MSCs or the maintenance of their biological functions, because long-term cultivation in a normoxic environment can cause the accumulation of reactive oxygen species (ROS) to damage MSCs.[7] It has been reported that the proliferative capacity of different hypoxic MSC populations is elevated compared with normoxic culture, which may be related to the enhanced glucose consumption and lactate production.[8] Drela et al[9] found that a physiological microenvironment (5% O_2) rejuvenated Wharton's jelly (WJ)-MSC culture toward less differentiated, more primitive, and faster-growing phenotypes. Kheirandish et al[10] confirmed that hypoxic preconditioning was an effective strategy for enhancing the proliferation capacity of human umbilical cord blood (hUB)-MSCs, and also triggered the expression of some neural genes. Additionally, Gómez-Leduc et al[11] demonstrated that oxygen tension is a key factor for chondrogenesis, and suggested that the 3D culture of UC-MSCs should begin in normoxia to obtain a more efficient chondrocyte differentiation before placing them in hypoxia for chondrocyte phenotype stabilization. Moreover, Zhao et al[12] reported that hypoxia enhanced the protective effect of adipose-derived stem cell (ADSC)-exosomes on lumbar facet joint osteoarthritis (OA) by protecting articular cartilage from degradation.

Mimicking a hypoxic environment in the body may protect MSCs from oxygen stress-mediated damage. Studies have applied 1% to 10% oxygen concentration as a pretreatment of one to 48 hours to analyze the impact of MSCs on their own biological functions and role in disease treatment.[13-15] Ishiuchi et al[13] applied 1% O_2 to pretreating MSCs for 24 hours and found a more substantial reduction in renal fibrosis and inflammation compared to MSCs exposed to 21% O_2. At both 1% and 20% O_2 levels, Gornostaeva et al[15] observed that MSCs strongly upregulated the immune regulatory molecules indoleamine 2,3-dioxygenase (IDO) and programmed death ligand-1 (PDL-L1) under interferon gamma (IFN-γ) and tumour necrosis factor alpha (TNF-α) stimulation, and IDO activity was not affected by the accumulation of L-kynurenine under hypoxic conditions. Hypoxia did not affect the ability of MSCs to inhibit the proliferation of $CD4^+$ and $CD8^+$ T cells stimulated by CD3/CD28.[14,15] Indeed, it is worth noting that the aforementioned studies primarily focused on MSCs cultured with serum and subjected to short-term hypoxia. Further research will contribute to a better understanding of the effects and potential applications of MSCs under long-term hypoxic conditions.

Due to the oxygen levels within human organs being usually much lower than those in standard culture conditions, MSCs used for therapeutic purposes may experience a transition from normoxic to hypoxic.[16] Therefore, it is necessary to comprehend the effects of long-term hypoxia on MSCs, including their biological functions and safety. For the translation to clinical application, the therapeutical merits of MSCs were valuated by transplanting them into an animal model. OA is a chronic inflammation caused by cartilage damage, which continuously aggravates cartilage lesions.[17] The biological function of MSCs may be suitable for treating OA, so we chose it for this rat model. Moreover, OA treatment can be achieved through joint cavity transplantation, which facilitates the analysis of the therapeutic effects of MSCs in the local area.[18] This study used normoxic culture as a control, while MSCs were continuously cultured in a serum-free system under long-term hypoxic atmosphere for 30 generations. The biological characteristics of Hy-MSCs were analyzed in vitro, and their translational value was verified in OA animals for the first time. Our study further clarifies that long-term hypoxic culture of MSCs may be the better option for clinical treatment, with great potential for clinical application which effectively enhances their therapeutic efficacy.

Methods

Preparation of hUC-MSCs in normoxic and hypoxic atmosphere

The hUC samples were collected and consent forms were signed by pregnant women who were healthy and had no hereditary diseases, and the experimental procedure was approved by the ethics committee of Guangdong Provincial Hospital of Traditional Chinese Medicine. The collected samples were tested for safety issues such as viral infection and genetic diseases. Culture cultivation of hUC-MSCs was based on a study by Wu et al.[19] Briefly, after hUC was washed with normal saline three times, Wharton's jelly was stripped and cut to about 1 mm^3. hUC-MSCs were cultured in serum-free medium, i.e. Minimum Essential Medium (MEM) alpha (Gibco, Thermo Fisher Scientific, USA) supplemented with 5% human platelet lysate (SEXTON, USA), 1× Insulin-Transferrin-Selenium-Ethanolamine (1× ITS X) (Gibco), 1% Non-Essential Amino Acids (NEAA) (Gibco), and 1% penicillin/streptomycin (Gibco). Normoxic hUC-MSCs (No-MSCs) and hypoxic hUC-MSCs (Hy-MSCs) were prepared by culturing tissue blocks under normoxic (21% O_2) and hypoxic (5% CO_2) conditions at 37°C in a common incubator (HERAcell150i; Thermo Fisher Scientific) and 5% CO_2, 1% O_2, and 37°C in a tri-gas incubator (HF100; HealForce, China), respectively. MSCs grew out from tissue blocks on the seventh day and subcultured to passage 1 (P1) on the 12th day, and the cells were digested by 0.25% trypsin (0.05% EDTA, Gibco) for passaging, and were passaged every three to four days until passage 30 (P30).

hUC-MSC proliferation and viability assay

When hUC-MSCs reached 80% to 90% confluence, the cells underwent subculturation with 8×10^3 cells/cm^2 until P30. The cell counts for each passage were determined (Countess II; Thermo Fisher Scientific), and the proliferative multiple was calculated by comparing with primary hUC-MSCs. hUC-MSC viability was determined at passage 5 (P5) using Cell Counting Kit-8 (CCK-8; GlpBio, USA), and the initial numbers of hUC-MSCs were 1.5×10^3 cells and 3.0×10^3 cells in the No-MSC and Hy-MSC groups, respectively. The optical density (OD) values were measured by microplate reader (Multiskan SkyHigh; Thermo Fisher Scientific) at 24 hours, 48 hours, 72 hours, and 96 hours, and MSC viability was calculated using the following formula:

$$[(experimental\ \ OD - blank\ \ OD)/(control\ \ OD - blank\ \ OD)] \times 100\%.$$

Flow cytometry assay for hUC-MSCs

Both No-MSCs and Hy-MSCs (1×10^6 cells/ml) were collected at P5 and P10, and were incubated with: fluorescein isothiocyanate (FITC)-conjugated antibodies against human CD34, CD44, and CD45; phycoerythrin (PE)-conjugated antibodies against human CD90, CD73, and CD105; and peridinin-chlorophyll-protein complex (PerCP)-conjugated antibodies against human HLA-DR (all antibodies from BioLegend, USA) at 4°C for 30 minutes in the dark. Appropriate isotype-matched antibodies were used as negative controls. Flow cytometry antibodies are listed in Supplementary Table i. Data from 10,000 viable cells were acquired by a FACSCalibur instrument (BD, USA) and analyzed by express software (CellQuest Pro, BD).

Colony formation assay in soft agar

Both No-MSCs and Hy-MSCs (P5) (1×10^5 per well) were resuspended in 0.35% low-melting-point agarose (Lonza, Switzerland) and seeded on top of a 0.6% agarose layer in a six-well plate. Cells were maintained in a 5% CO_2 incubator at 37°C for approximately 28 days, and the tumour cell line U251 was used as a control (BeNa biology, China). The colony formation was determined with an inverted microscope (CKX53; Olympus, Japan).

Tumorigenicity analysis in vivo

No-MSCs, Hy-MSCs, and human lung cancer cells (A549; BNCC) of 4×10^6 in 200 µl phosphate-buffered saline (PBS) were injected into subcutaneous tissue in the backs of eight-week-old immunodeficient mice (NOD/shi-scid/IL-2Rγnull (NOG), Charles River Laboratories, China). All the animal experiments in this study adhered to the ARRIVE guidelines, and we have included an ARRIVE checklist to show this. Subsequently, the mice were raised normally and tumour sizes were measured by calipers, which ensured tumour formation at the injection site one month later.

Karyotype analysis

Karyotype stability of No-MSC and Hy-MSC (P20) was analyzed using the G-banding technique, and the karyotype formula was described according to published clinical practice guidelines[20] and laboratory guidelines[21] for clinical chromosomal microarray testing. Briefly, when the confluence of MSCs in a six-well plate reached 60%, colchicine (50 ng/ml) was added and treated at 37°C for two hours. After cell digestion, hypotonic treatment, fixation, Giemsa staining, and drying, the slides were uploaded to the fully automatic scanning and analysis system for scanning films (GSL120; Leica Biosystems, Germany), which performed the chromosome karyotype analysis.

Differentiation of hUC-MSCs in vitro

Both No-MSCs and Hy-MSCs (P5) were cultured in six-well plates (5×10^4 cells per well). Media were changed every two days. Osteogenic, adipogenic, and chondrogenic stimulation was conducted according to the manufacturer's instructions (OriCell; Cyagen, China).

For microscopic evaluation, cells were fixed with 4% paraformaldehyde in PBS for ten minutes and stained for 20 minutes at room temperature with the following staining: adipogenesis was verified with Oil Red-O (Cyagen) staining; and matrix calcification, which is present following osteogenesis, was visualized with Alizarin Red (Cyagen). After embedding and slicing the chondrospheres at 21 days, they were stained with Alcian Blue (Cyagen) accordingly. All stained cells were visualized with an inverted microscope (CKX53).

All these induced cells were subjected to messenger RNA (mRNA) extraction, and subsequently gene expression was analyzed using quantitative real-time polymerase chain reaction (qRT-PCR).

Senescence-related β-galactosidase staining detection

No-MSC and Hy-MSC (P10) were seeded on six-well plates (2×10^5 cells per well). After 24 hours of culture, the cells were taken out and rinsed with PBS, fixed, and then rinsed with PBS. The reaction solution, which was prepared according to the

manufacturer's instructions, was incubated at 37°C overnight (Beyotime Biotechnology, China). The staining status of the cells was observed under a light microscope (CKX53).

Quantitative real-time PCR analysis

Total RNA was extracted from UC-MSCs using Trizol reagent (Thermo Fisher Scientific). The RNA concentration was measured by Nano 2000 (Thermo Fisher Scientific), and RNA (100 ng to 1 μg) was reverse transcribed into complementary DNA (cDNA) using M-MLV reverse transcriptase (Promega, China) according to the manufacturer's instructions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for normalization. RT-PCR was performed with qRT-PCR Mix (Sinogene, China) in a Real-Time system (Applied Biosystems, USA). The relative expression of mRNA was evaluated by the $2^{-\Delta\Delta Ct}$ method, and the sequences of primers for qRT-PCR are listed in Supplementary Table ii.

Measurement of telomerase activity

Telomerase activity of hUC-MSCs was measured using PCR enzyme-linked immunosorbent assay (ELISA) kit (Roche, Switzerland) following the manufacturer's protocols.[22] MSCs were harvested and lysed in 200 μl cold lysis buffer for 30 minutes and kept on ice for 30 minutes after being rinsed twice with ice-cold PBS. The supernatant was collected by centrifugation at 16,000 rpm for 20 minutes at 4°C, and the protein concentration was determined by the modified Coomassie protein assay (Pierce, Thermo Fisher Scientific). Subsequently, 10 μg of protein was added into 25 μl of reaction mixtures and 20 μl to 25 μl sterile DEPC-H_2O to a 50 μl total volume. Following primer elongation for 30 minutes at 25°C, which was then heated at 94°C for five minutes to deactivate the telomerase, the telomeric repeat amplification protocol (TRAP) assay was performed in a thermal cycler (94°C for 30 s, 50°C for 30 s, and 72°C for 90 s, to a total of 30 cycles). Afterwards, ELISA assay was conducted according to the manufacturer's instructions. Telomerase activity was represented by the difference in absorbance (ΔA) using the following formula: $\Delta A = A_{450} - A_{690}$.

hUC-MSC coculture with lymphocytes and the detection of its immunoregulatory ability

hUC-MSCs (P5) were cultured in six-well plates (5×10^4 cells/well) under hypoxic (1% O_2) and normoxic (21% O_2) atmosphere until reaching 60% to 70% confluence. The peripheral blood lymphocytes (PBLs; 1×10^6 cells) were isolated from five healthy donors who signed the informed consent and were added to the hUC-MSCs for co-culturing with RPMI 1640 medium containing 10% FBS. At day 3, the suspended cells were collected and then stained with FITC-CD3, PE-CD56/16, and PerCP-CD8 (all from BioLegend) at 4°C for 30 minutes in the dark. Other suspended cells were stained with FITC-CD25, PE-Foxp3, and PerCP-CD4. The expression of Foxp3 was detected using fixation buffer and intracellular staining perm wash buffer according to the manufacturer's instructions (BioLegend). PE labelled Mouse immunoglobulin 1 (IgG1), κ Isotype was used as the intracellular control. The percentages of T lymphocytes and regulatory T cells (Treg) were detected by flow cytometry with antibodies, which are listed in Supplementary Table i.

hUC-MSCs coculture with chondrocytes and the detection of its proliferative and anti-apoptosis ability

Chondrocyte line C28/I2 was cultured in transwell plates (2 × 6 wells, 2×10^5 cells/well; BNCC), and then No-MSCs and Hy-MSCs (P5) were cultured in chambers (0.8 μm, 1×10^5 cells), in 3 ml Dulbecco's Modified Eagle Medium (DMEM) with high glucose and 10% FBS. One group was used for counting the numbers of C28/I2 cells after trypsin digestion every three days by Countess II (Thermo Fisher Scientific). Another group was used for analyzing the mortality of the cells that were cultured in the death-inducing medium containing 3 ml DMEM/high glucose, 10% FBS, and 10 ng/ml interleukin-1 β (IL-1β). At day 6, the mortality of the chondrocyte C28/I2 line was detected by Annexin V and PI apoptosis kit (BioLegend), which was then detected by flow cytometry and analyzed by FlowJo software (BD, USA).

Rat OA model and hUC-MSC transplantation

All the animal experiments in this study adhered to the ARRIVE guidelines, and we have included an ARRIVE checklist to show this. A total of 20 healthy male and 20 healthy female three-month-old Sprague-Dawley (SD) rats, at a body weight of 200 to 250 g, were used in the experiments. The rat OA model was induced by injecting type II collagenase into the joint cavity of knees as follows:[23] rats were anaesthetized by isoflurane in a small animal anaesthesia machine, and then type II collagenase (20 μg/50 μl; Sigma-Aldrich) was injected into the joint cavity. The rats were randomly divided into four groups: I, Control group; II, Model group; III, No-MSCs; and IV, Hy-MSCs. hUC-MSCs were transplanted into the joint cavities with 5×10^5 cells each on the 7th, 14th, and 21st day after modelling, for a total of three times. After transplantation, the joint swellings were examined at seven, 14, 30, and 60 days, and the samples of rats were collected when euthanized on the 81st day. The modelling and treatment scheme are shown in Figure 1a.

Measurement of the joint swelling diameter in rat knees

The diameter of the right knee joint of rats was measured using an electronic digital caliper before and after modelling, and at one week, two weeks, one month, and two months after hUC-MSC transplantation. The swelling changes in rat knee joints were calculated and compared with each other.

H&E staining of joint tissues

On day 81, the right knee joints in each group of rats were collected after execution. The joint specimens were fixed with 4% neutral formaldehyde solution, decalcified with 15% ethylenediaminetetraacetic acid (EDTA) for two weeks, embedded in paraffin, and analyzed with haematoxylin and eosin (H&E) and Safranin O-Fast Green staining. The morphological changes of the joints were observed with a light microscope (BX63; Olympus), which evaluated the experimental rats with two scales before execution, including the Mankin scale[24] and Osteoarthritis Research Society International (OARSI) scale.[25] The degree of OA was evaluated by two investigators (QMH, ZXD) in a blinded manner following the standard of two scales. The interobserver variability among three observers (QMH, ZXD, YMH) for the Mankin system showed a good intraclass correlation coefficient (ICC > 0.92),

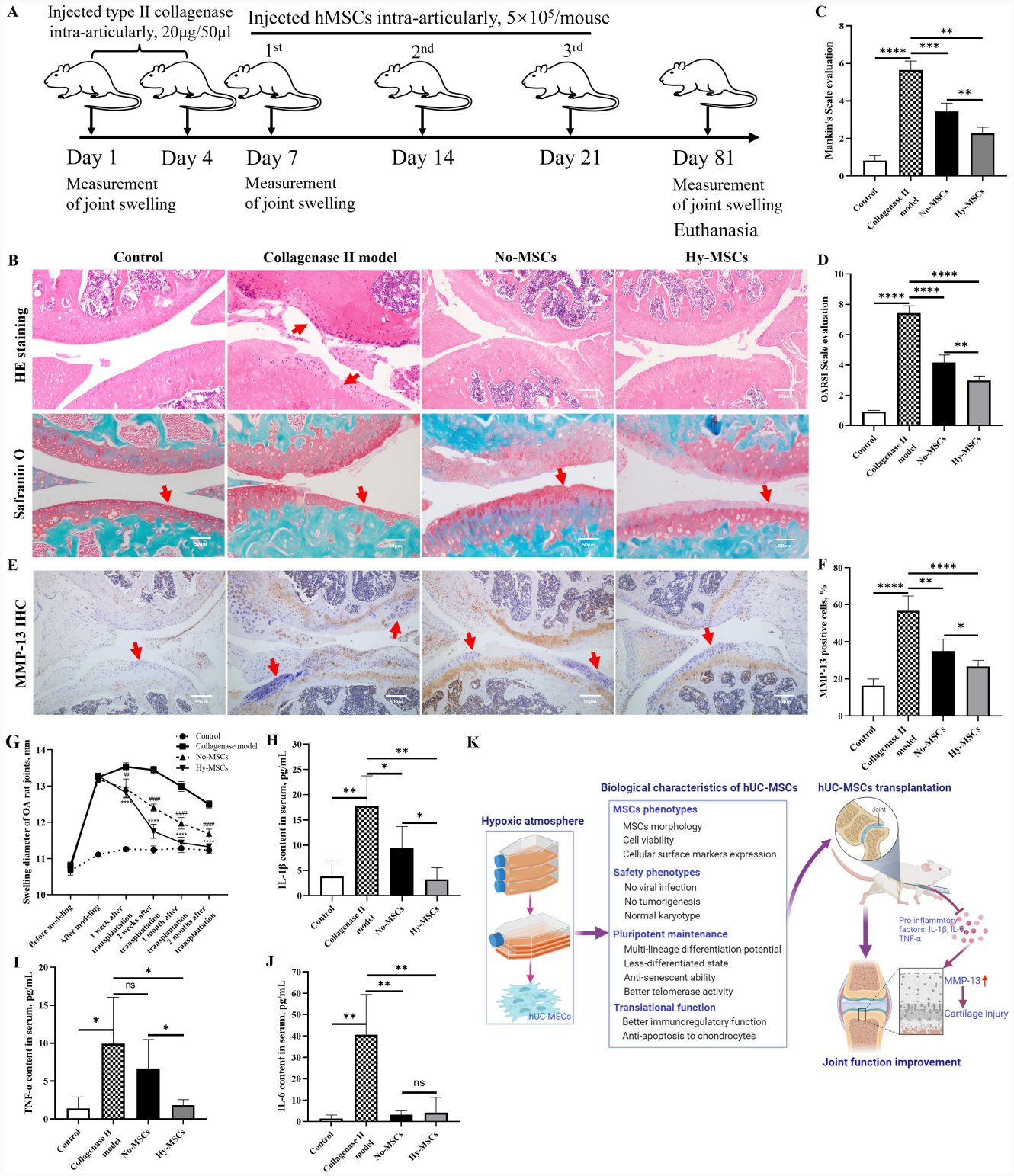

Fig. 1

Long-term hypoxic human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) better represented joint function repair and anti-inflammatory effects in osteoarthritis (OA) rats. a) The scheme of modelling and MSC transplantation in OA rat. b) The pathological analysis of joint tissues in OA rats by haematoxylin and eosin (H&E) and Safranin O staining, scale bar: 50 μm. c) and d) Analysis of joint function before and after hUC-MSC transplantation in OA rats using Mankin scale and Osteoarthritis Research Society International (OARSI) scale evaluation (n = 5). e) Matrix metallopeptidase 13 (MMP-13) analysis of joint tissues in OA rats by immunohistochemistry (IHC), scale bar: 50 μm. f) Statistical analysis of MMP-13 positive cells percentage in joint tissues of OA rats. g) Measurement of joint swelling diameter before and after hUC-MSC transplantation in OA rats, including one, two, three, and four weeks (n = 5), and comparison with OA model. h) to j) Measurement and statistical analysis of pro-inflammatory factor, i.e. interleukin (IL)-1β, tumour necrosis factor alpha (TNF-α), and IL-6 (n = 5). k) Overview on biological characteristics and translational potential of hUC-MSCs in hypoxic atmosphere. Data are presented as mean (SD). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; one-way analysis of variance (ANOVA). Hy-MSCs, hypoxic hUC-MSCs; No-MSCs, hUC-MSCs cultured with normoxic atmosphere; ns, non-significant.

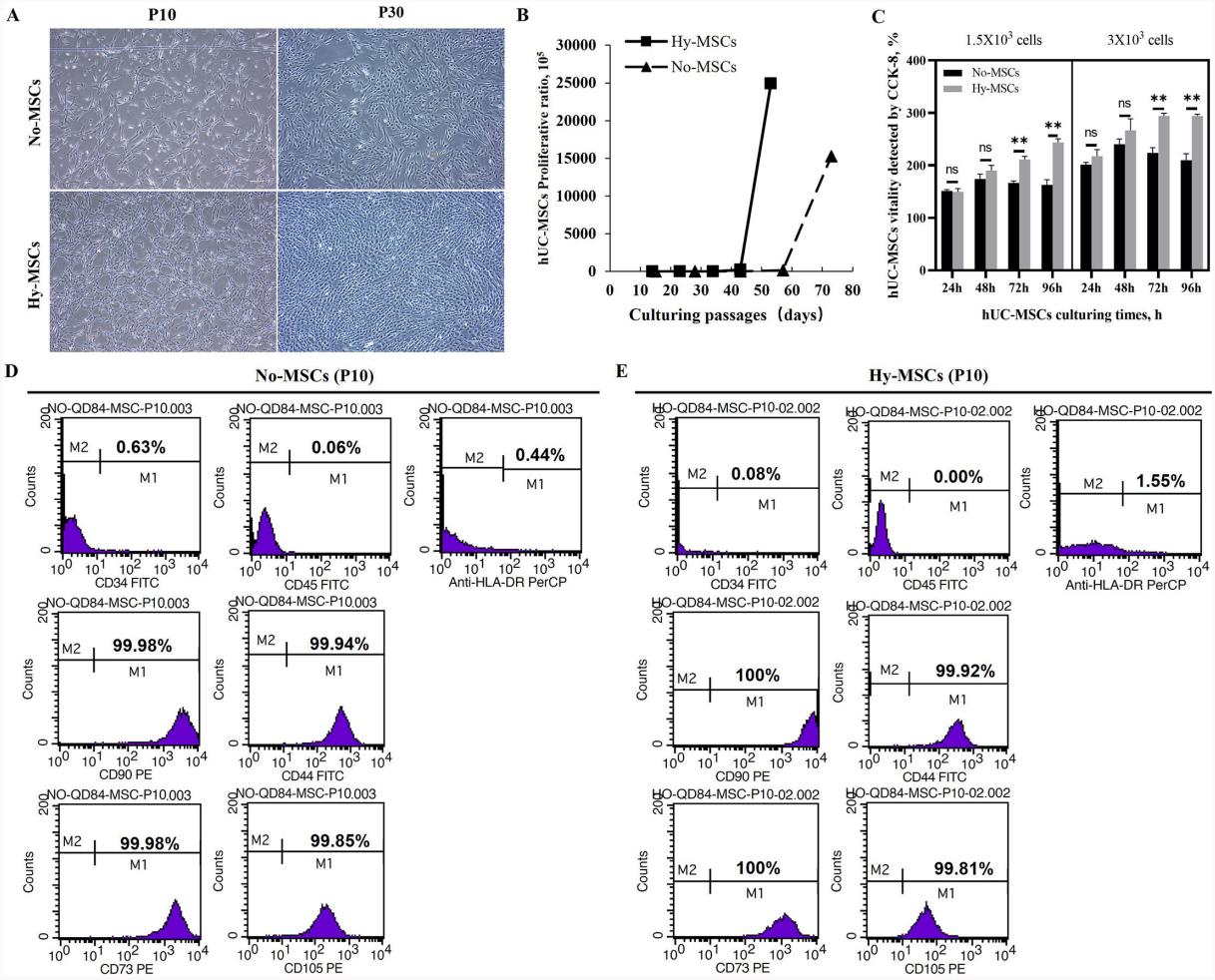

Fig. 2

Proliferative ability and phenotypes of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) in long-term normoxic and hypoxic atmospheres. a) The growth morphology of hUC-MSCs in normoxic and hypoxic atmospheres. Scale bar: 50 μm. b) Proliferative times of hUC-MSCs were detected by cell count continuously. c) Proliferative viability of hUC-MSCs (left: 1.5 × 10^3 cells; right: 3 × 10^3 cells) was detected by Cell Counting Kit-8 (CCK-8) assay. d) and e) Phenotypic assay of hUC-MSCs was detected by flow cytometry. Data are presented as mean (SD). **p < 0.01, one-way analysis of variance (ANOVA). Hy-MSCs, hypoxic hUC-MSCs; No-MSCs, hUC-MSCs cultured with normoxic atmosphere; ns, non-significant.

as well as for the OARSI system (ICC > 0.89). Repeat scoring by investigators showed very good agreement (ICC > 0.94 for Mankin and OARSI systems). The Mankin score provided grades from 0 to 4: Grade 0: normal cartilage; Grade 1: minimal articular damage; Grade 2: articular cartilage damage affecting up to 30% of the articular surface; Grade 3: loss of up to 50% of the articular cartilage; and Grade 4: severe loss of cartilage affecting more than 50% of the articular surface. The histopathology OARSI system provided grades from 0 to 6: Grade 0: normal articular cartilage; Grade 1: intact surface; Grade 2: surface discontinuity; Grade 3: vertical fissures extending into mid zone; Grade 4: erosion; Grade 5: denudation; and Grade 6: deformation.

MMP-13 immunohistochemical analysis of joint tissues

The joint specimens were used to detect the expression level on matrix metallopeptidase 13 (MMP-13) by immunohistochemistry. After dehydration, the paraffin sections were sealed with a horse serum and then incubated with rabbit polyclonal anti-MMP-13 antibody (1:100 dilution; Santa Cruz Biotechnology, USA). Then, they were incubated with goat anti-rabbit IgG secondary antibody (1:200 dilution; HUABIO, China), which resulted in the formation of the chromogenic reagent colour. The number of positively stained cells per specimen was calculated, and four groups were taken from each mice group.

Measurement of inflammatory factors in serum by ELISA

The levels of total serum inflammatory factors, including IL-1β, TNF-α, and IL-6, were quantitatively measured using a commercially available ELISA, following the manufacturer's instructions (Jingmei, China).

Statistical analysis

The experiment used GraphPad Prism 8.0 software for statistical difference analysis (GraphPad Software, USA), and all data were expressed as mean (SD). Independent-samples *t*-test was used to compare differences between any two groups, and the data of the diversity samples were analyzed using one-way analysis of variance (ANOVA). P-values < 0.05 were considered to be statistically significant.

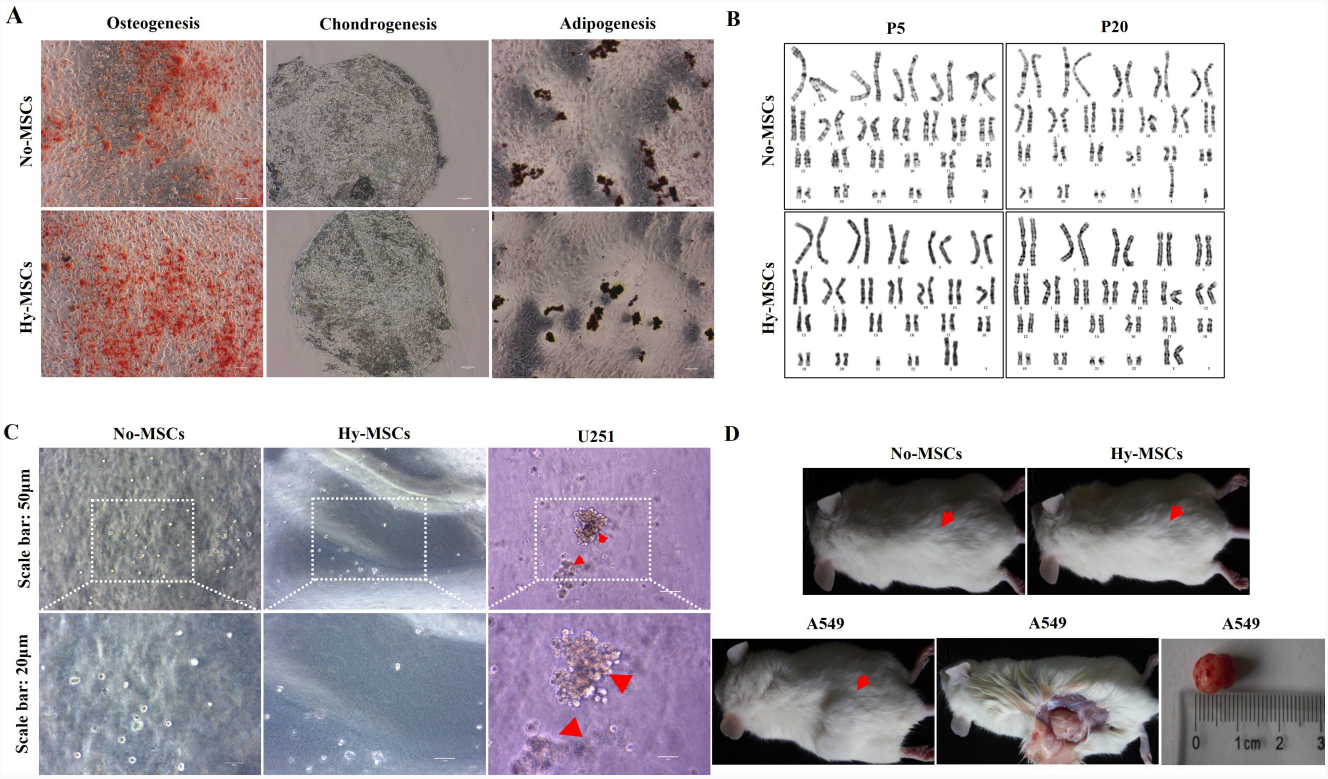

Fig. 3

The multilineage differentiation, tumorigenicity, and karyotypes of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) in long-term hypoxic atmosphere. a) The differentiative potential of both hUC-MSCs cultured with normoxic atmosphere (No-MSCs) (P5) and hypoxic hUC-MSCs (Hy-MSCs) (P5) was shown through staining by Alizarin Red, Oil Red-O, and Alcian Blue, respectively, including in osteoblasts, chondrocytes, and adipocytes. Scale bar: 50 μm. b) The karyotypes of hUC-MSCs (P5 and P20) were measured by G-banding assay. c) Both No-MSCs and Hy-MSCs (P5) were analyzed for tumour formation in soft agar cloning experiments, and glioma cell line (U251) was used as a control. d) Both No-MSCs and Hy-MSCs (P5) were analyzed for tumour formation in immunodeficient mice (NOG), and lung cancer cell line (A549) was used as a control. The experiments were repeated three times independently, and the data of one representative experiment were shown.

Results

Hypoxic atmosphere enhances proliferation capacity of hUC-MSCs and maintains elementary features of MSCs

Our results showed that there was no distinct difference in cell morphology between No-MSCs and Hy-MSCs in each generation, in which the cells showed a spindle shape or irregular triangle. When hUC-MSCs were cultured to over P20, the cells became larger and wider, they were spindle shaped, and their proliferation rates slowed down. However, the status of Hy-MSCs was much better than that of No-MSCs (Figure 2a). In addition, it could take 53 and 72 days for amplifying to 2.49×10^9 and 1.53×10^9 cells of P30 from the initial 3 to 5×10^6 hUC-MSCs on day 9 to day 11 of P0 in hypoxic and normoxic atmospheres, respectively (Figure 2b), suggesting that long-term hypoxia enhanced the proliferation of hUC-MSCs. Moreover, our results show that the viability of Hy-MSCs was higher than that of No-MSCs by CCK-8 detection (Figure 2c), suggesting that a hypoxic atmosphere is favourable for the survival of MSCs compared with normoxic conditions. Furthermore, both No-MSCs and Hy-MSCs highly expressed CD90, CD44, CD73, and CD105, and had low or no expressions of CD34, CD45, and HLA-DR (Figure 2e). These results indicate that a hypoxic atmosphere promotes the proliferation of MSCs and maintains their elementary features.

Hy-MSCs possess multi-directional differentiation and maintain stable chromosome karyotypes without tumorigenicity

The multidirectional differentiational abilities including osteogenesis, chondrogenesis, and adipogenesis of hUC-MSCs were determined at normoxic and hypoxic atmosphere in vitro. These induced osteoblasts had generated mineralized calcium nodules, stained with Alizarin Red S and appearing deep red in colour on day 17 (Figure 3a). hUC-MSCs successfully differentiated into chondrospheres at day 28, and then the sections of chondrospheres were coloured blue by Alcian Blue staining (Figure 3a). They could be induced to differentiate into adipocytes for 24 days, which were fat droplets and orange red (Figure 3a). The results showed that MSCs are able to differentiate into osteocytes, chondrocytes, and adipocytes in both normoxic and hypoxic conditions. In addition, G-banding assay showed that there were no abnormalities or lack of chromosomes in both karyotypes of No-MSCs and Hy-MSCs at P5 and P20, suggesting that long-term hypoxic culturation does not affect their karyotypes of MSCs (Figure 3b). Next, we conducted in vivo and in vitro experiments to clarify the role of MSCs in tumorigenicity. Our results showed that there were no colony formations in soft agar in vitro and also no tumorigenesis in NOG mice in vivo for both No-MSCs and Hy-MSCs at P10, whereas U251 and A549 cells formed colonies in the soft agar and produced tumours in NOG mice, respectively (Figure 3d). These results indicate

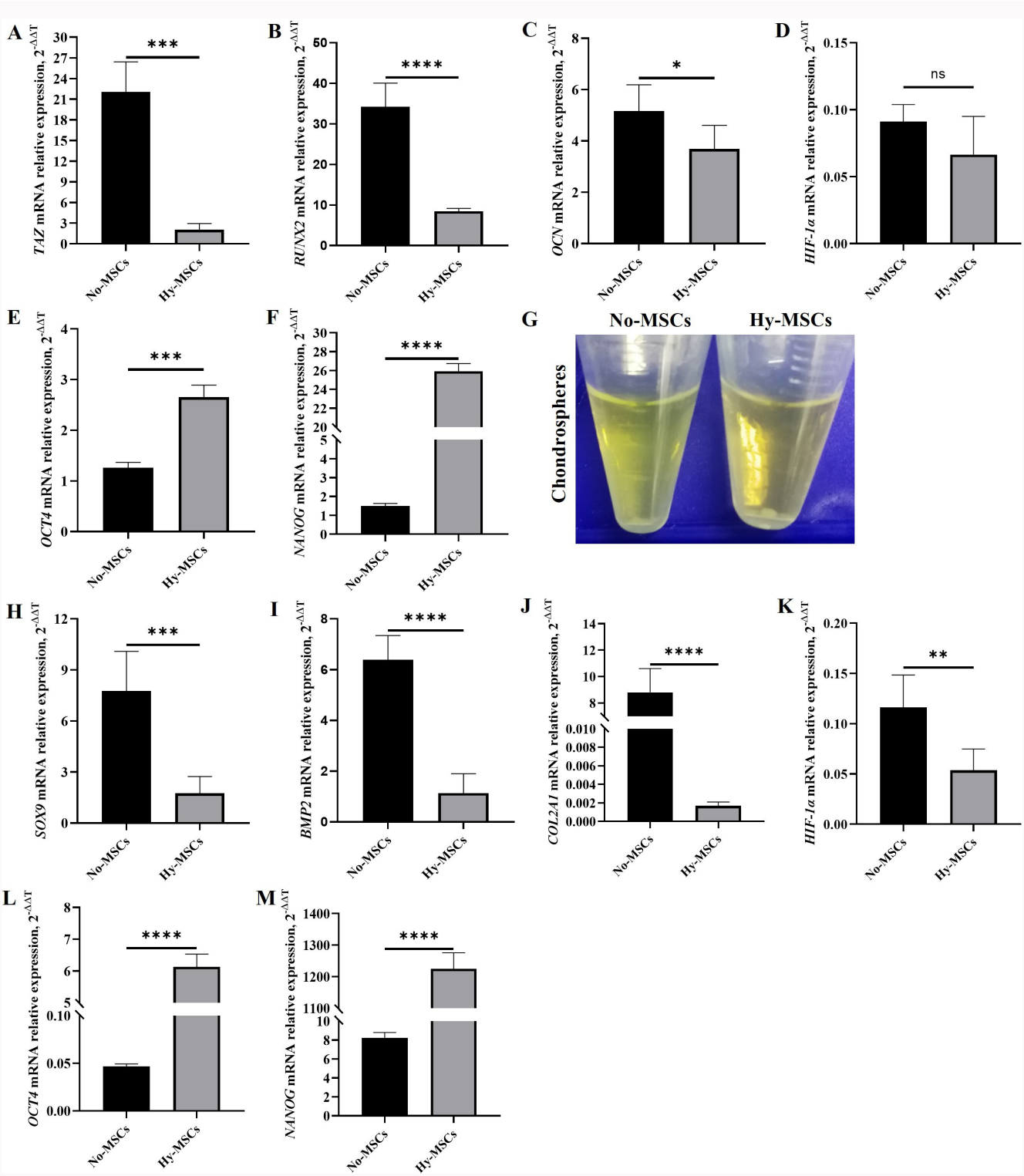

Fig. 4

The pluripotency and ability to low-differentiation of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) in a long-term hypoxic atmosphere. a) to c) The expression of specific genes of osteoblast differentiation was detected by quantitative real-time polymerase chain reaction (qRT-PCR) assay, i.e. *TAZ, Runx2,* and *OCN*. d) *HIF-1α* messenger RNA (mRNA) expression was detected in the osteoblast differentiation of hUC-MSCs. e) and f) The expression of stemness genes was measured in the osteoblast differentiation of hUC-MSCs, including *OCT-4* and *NANOG*. g) The chondrospheres were formed in hUC-MSC chondrogenic differentiation at day 4. h) to j) The expression of related genes was detected in chondroblast differentiation, i.e. *Sox-9, BMP2* and *COL2A1*. k) *HIF-1α* mRNA expression was detected in the chondroblast differentiation of hUC-MSCs. l) to m) The expression of stemness genes was measured in the chondroblast differentiation of hUC-MSCs, including *OCT-4* and *NANOG*. Data are presented as mean (SD). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001; one-way analysis of variance (ANOVA).

that MSCs with hypoxic culturation maintain the abilities of the multidirectional differentiational potentials and stable karyotypes without the risk of tumorigenicity, suggesting that Hy-MSCs are safe after long-term culture cultivation.

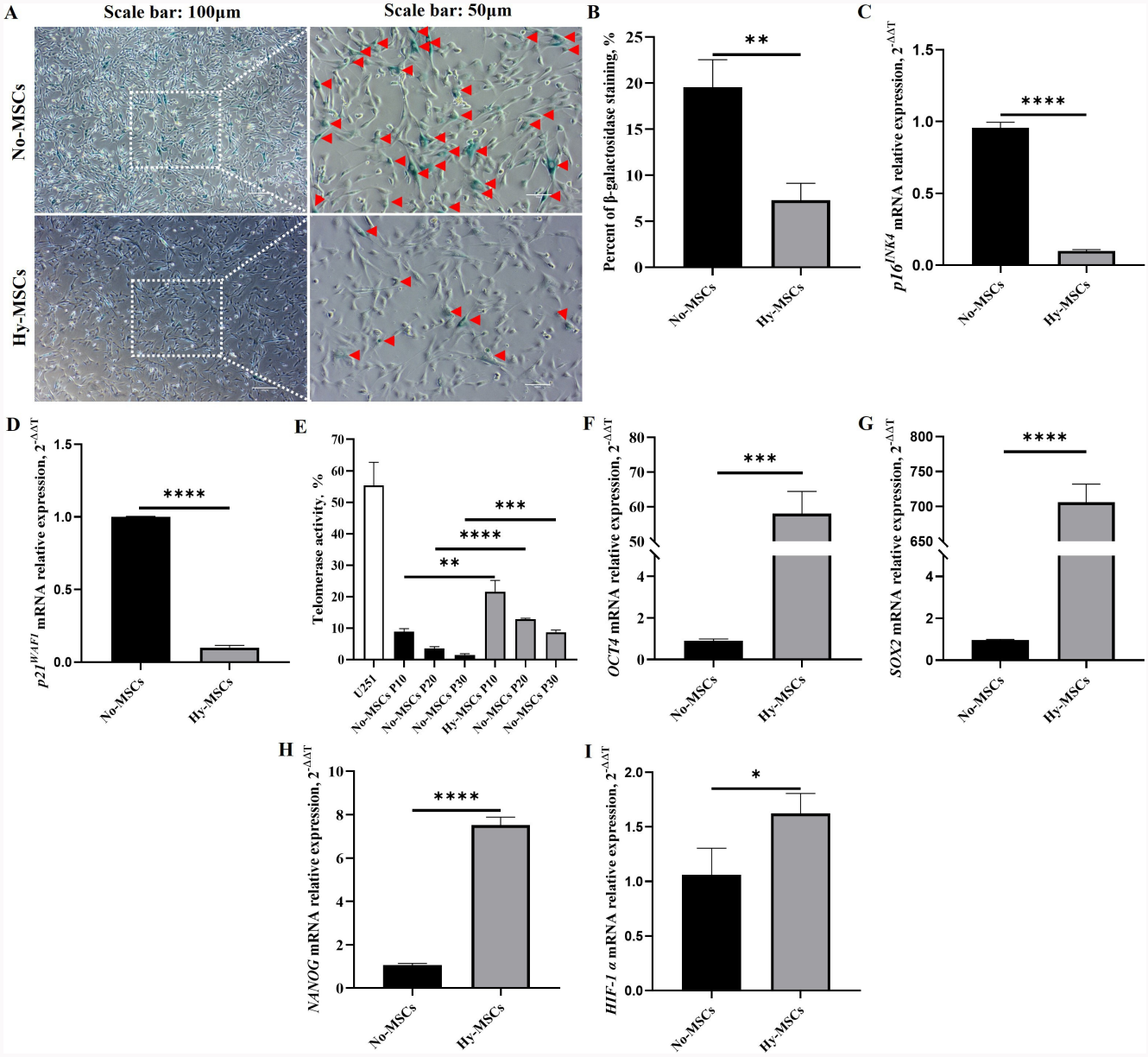

Fig. 5

The senescence level and stemness characteristics of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) in a long-term hypoxic atmosphere. a) The senescence level of both hUC-MSCs cultured with normoxic atmosphere (No-MSCs) and hypoxic hUC-MSCs (Hy-MSCs) were stained by β-galactosidase staining (P10). Scale bars: 100 μm and 50 μm. b) The positive β-galactosidase staining cells were counted and compared between No-MSCs and Hy-MSCs. c) and d) The expression of senescence genes of MSCs was detected by quantitative real-time polymerase chain reaction (qRT-PCR) assay, i.e. $p16^{INK4a}$ and $p21^{WAF1}$. e) Telomerase activity of hUC-MSCs (P10, P20, and P30) was analyzed at normoxic and hypoxic atmospheres by PCR-ELISA assay. f) to h) The expression of stemness genes of hUC-MSCs was detected by qRT-PCR assay, i.e. *OCT-4, SOX2,* and *NANOG*. i) The expression of *HIF-1α* was tested in both No-MSCs and Hy-MSCs. Data are presented as mean (SD). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, one-way analysis of variance (ANOVA). ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

The multidirectional differentiation potential of Hy-MSCs declared the stemness maintenance

The expression of specific genes in osteoblasts of Hy-MSCs was significantly lower than that of No-MSCs, including tafazzin (*TAZ*, Figure 4a), RUNT-related transcription factor 2 (*Runx2*, Figure 4b), and osteocalcin (*OCN*, Figure 4c), whereas hypoxia inducible factor-1 alpha (*HIF-1α*) showed no significant difference (Figure 4d). Moreover, the expression of the stemness genes was notably higher in Hy-MSCs than in No-MSCs, namely Octamer-binding transcription factor 4 (*OCT-4*) and Nanog homeobox (*NANOG*) (Figure 4f). When hUC-MSCs induced chondrogenesis, Hy-MSCs were more likely to form chondrospheres at day 4 (Figure 4g). Additionally, we unexpectedly observed that the gene expression of chondroblasts in Hy-MSCs was markedly lower than that in No-MSCs, including SRY-related high mobility group-box 9 (*Sox-9*), bone morphogenetic protein 2 (*BMP2*), and collagen type II A1 (*COL2A1*) (Figure 4k). *HIF-1α* mRNA expression was also higher in chondrogenic differentiation of Hy-MSCs (Figure 4l), and the expression of the stemness genes was significantly higher than that of No-MSCs in chondrogenic differentiation, including *OCT-4* and *NANOG* (Figure 4n). Similarly, the differentiational genes were down-regulated in Hy-MSCs by comparing with No-MSCs

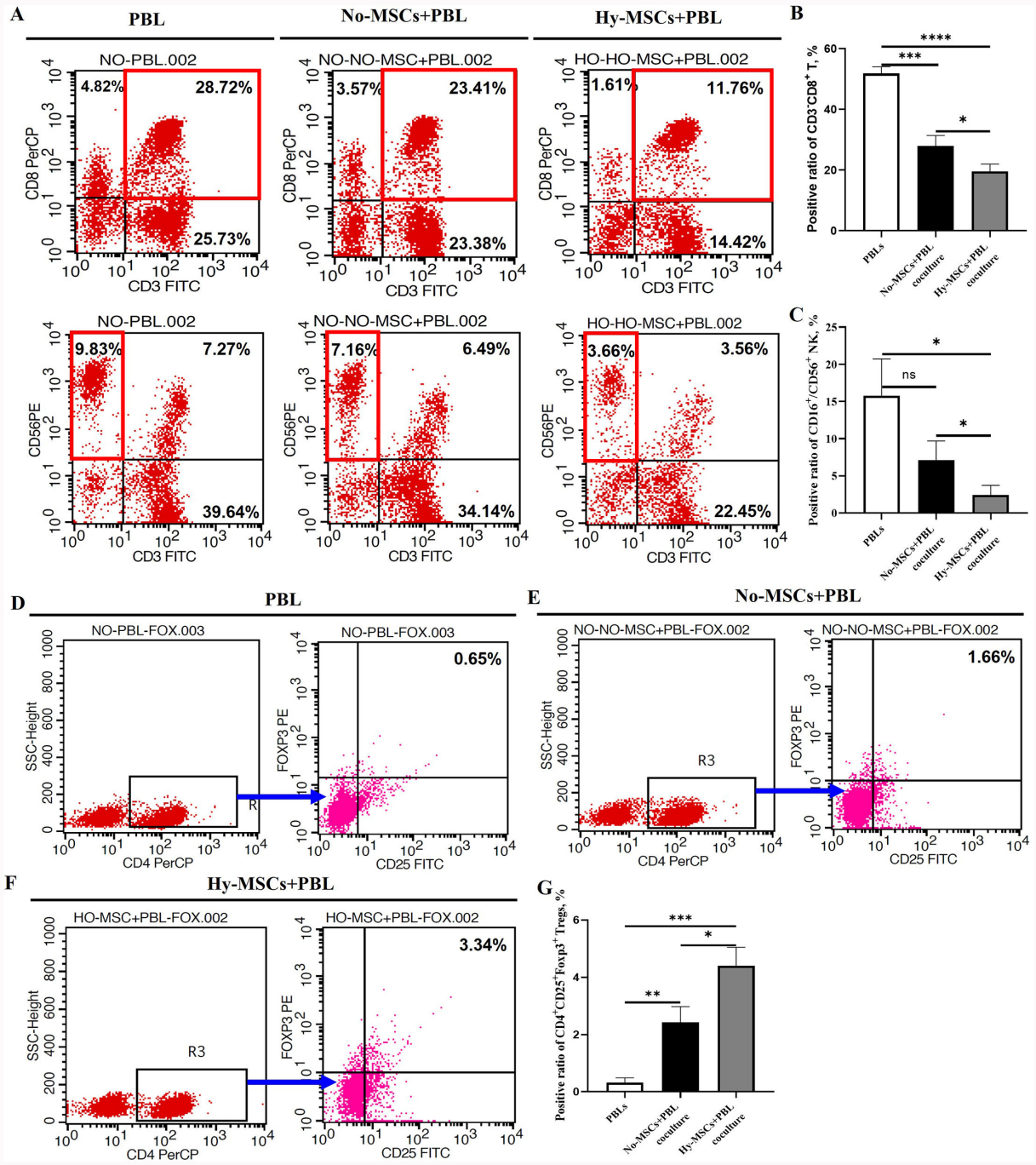

Fig. 6

Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) promoted the abilites of immunoregulation and proliferation and inhibited apoptosis of chondrocytes in a hypoxic atmosphere. a) The phenotypes of peripheral blood lymphocytes (PBLs) were detected by flow cytometry analysis. b) and c) Statistical analysis of different lymphocyte subsets, including CD3+ CD8+ T cells and CD3-CD56/16+ natural killer (NK) cells (n = 4). d) to f) The production of regulatory T (Treg) cells was measured after coculturing with both hUC-MSCs cultured with normoxic atmosphere (No-MSCs) and hypoxic hUC-MSCs (Hy-MSCs). g) Statistical analysis of Treg cells percentage, i.e. CD4+ CD25+ Foxp3+ Treg (n = 4). Data are presented as mean (SD). *p < 0.05, **p < 0.01, ***p < 0.001; one-way analysis of variance (ANOVA). ns, non-significant.

in adipogenesis (peroxisome proliferator-activated receptor-gamma 2 (*PPARγ-2*) and adipose differentiation related protein (*ADRP*); Supplementary Figures aa and ab), and the stemness genes were up-regulated in Hy-MSCs (*OCT-4* and *NANOG*; Supplementary Figures ac and ad). Although MSCs could differentiate into osteoblasts, chondroblasts, and adipocytes in hypoxia, Hy-MSCs might inhibit the expression of promoting differentiation genes and sustain the undifferentiated state.

Hypoxic culture cultivation suppresses senescence and sustains the stemness of MSCs

The senescence of hUC-MSCs occurs due to the continuous subculture. Therefore, cell senescence was examined in hypoxic and normaxic conditions, and the results showed that the number of β-galactosidase positive cells in the Hy-MSCs group was much lower than those in the No-MSCs group (Figure 5b). In addition, the expression of the senescence genes such as $p16^{INK4a}$ and $p21^{WAF1}$ in Hy-MSCs was also

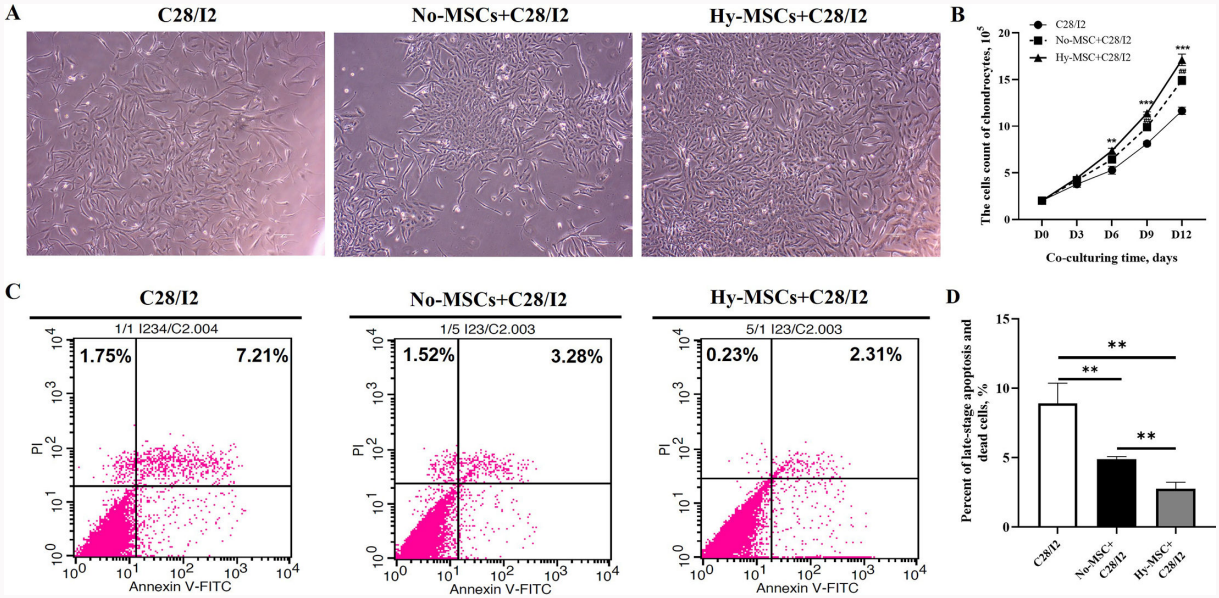

Fig. 7

Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) better inhibited apoptosis of chondrocytes in a hypoxic atmosphere. a) The growth state of chondrocytes after co-culturing with hUC-MSCs cultured with normoxic atmosphere (No-MSCs) and hypoxic hUC-MSCs (Hy-MSCs) for three days. b) The statistical analysis of the chondrocytes count after co-culturing with No-MSCs and Hy-MSCs by comparing with control C28/I2. c) Flow cytometry detection of apoptosis levels on chondrocytes after co-culturing with No-MSCs and Hy-MSCs for three days. d) The comparative analysis of late-stage and death apoptosis levels on chondrocytes after co-culturing with No-MSCs and Hy-MSCs. Data are presented as mean (SD). *p < 0.05, **p < 0.01, ***p < 0.001, one-way analysis of variance (ANOVA).

much less than that of No-MSCs (Figure 5d), suggesting that long-term hypoxic culture cultivation might favour maintaining the stemness of MSCs. Moreover, we also determined the telomerase activities of hUC-MSCs at P10, P20, and P30 due to the activities of the enzyme as referring to the senescence of the cells, and our results showed that the telomerase activities of Hy-MSCs in each passage were much higher than those of No-MSCs (Figure 5e). Furthermore, we also examined the expression of the stemness genes, namely *OCT-4, SOX2,* and *NANOG*, by qRT-PCR assay, and the results showed that the expression of these genes was obviously higher in Hy-MSCs than in No-MSCs (Figure 5h). Meanwhile, we also observed that Hy-MSCs had a higher expression of *HIF-1α* than No-MSCs (Figure 5), suggesting that Hy-MSC-mediated anti-senescence and stemness maintenance might be related to the function of *HIF-1α*.

Hy-MSCs possess a remarkable immunomodulatory capacity

Immunomodulatory ability is one of the fundamental features of MSCs. We examined the effects of hUC-MSCs on the functions of immune cells, and the results showed that both Hy-MSCs and No-MSCs significantly reduced the levels of immune cells including $CD3^+CD8^+$ T cells and $CD3^-CD56/16^+$ NK cells (Figure 6c), and facilitated $CD4^+CD25^+Foxp3^+$ Treg cellWe have corrected it. proliferation (Figure 6g). In comparison, these effects of Hy-MSCs were stronger than those of No-MSCs. Our results indicate that Hy-MSCs have a better immunomodulatory ability, suggesting that long-term hypoxia is beneficial for MSC cultivation.

Hy-MSCs inhibit chondrocyte apoptosis induced by pro-inflammatory cytokines

The proliferative capability of chondrocytes was also measured through co-culturing with hUC-MSCs, and the results showed that although both No-MSCs and Hy-MSCs promoted the growth of chondrocytes (Figure 7a), Hy-MSCs revealed a stronger ability to promote the proliferation of chondrocytes compared with No-MSCs (Figure 7b). It has been reported that the inflammatory cytokine IL-1β could cause ferroptosis of chondrocytes,[26] and IL-1β-induced chondrocyte apoptosis was examined in this study, while we observed that both Hy-MSCs and No-MSCs alleviated IL-1β-induced chondrocyte apoptosis (Figure 7c), and the inhibitory effect of Hy-MSCs was stronger than that of No-MSCs (Figure 7d). Taken together, these results indicate that Hy-MSCs significantly promote the proliferation of chondrocytes and inhibit apoptosis caused by inflammatory factors.

Hy-MSC transplantation revealed the greater preventative effect in OA rats

To further clarify the role of Hy-MSC transplantation in the treatment of diseases, we conducted an experiment for treating OA in rats, the procedure for which is shown in Figure 1a. Our results showed that both Hy-MSCs and No-MSCs significantly reduced Mankin scale and OARSI scale compared with collagenase II-induced OA models in rats (Figure 1c), among which the preventative effect of Hy-MSC transplantation was remarkably stronger than that of the No-MSCs group. Our H&E and Safranin O staining results showed that the collagenase II-induced injury of knee joint tissues was markedly improved in both No-MSC and Hy-MSC transplantation groups, evidenced by having a more regular cartilage surface, fewer cracks, a small amount of chondrocyte

proliferation, and an evident reduction in synovial swelling, congestion, and oedema. Furthermore, the infiltration of inflammatory cells and proliferative capillaries was also significantly reduced in the MSC transplantation groups compared with the model group (Figure 1d). IL-1β induced expression of MMP-13 caused cartilage damage in human chondrocytes, which resulted in OA.[27] MMP-13 immunohistochemical results suggested that the collagenase II model group was the most evident MMP-13 expression among the joint tissues of all groups, wherein hUC-MSC transplantation reduced MMP-13 expression (Figure 1e). Moreover, the MMP-13 positive percentage in the Hy-MSCs group was greatly reduced and significantly lower than in the collagenase II model and No-MSCs groups (Figure 1f). In addition, we observed that although the joint swelling gradually alleviated after modelling, both No-MSC and Hy-MSC transplantation significantly decreased the diameters of joint swelling from the first week until the second month after transplantation (Figure 1g). Furthermore, we extracted serum from the experimental rats to measure the secretion of inflammatory factors. We observed that serum levels of inflammatory factors such as IL-1β, TNF-α, and IL-6 were significantly increased in the collagenase II model group, whereas both No-MSC and Hy-MSC transplantations significantly reduced the secretions of these inflammatory factors (Figure 1j). It is noteworthy that the preventative effect of Hy-MSC transplantation on OA was more efficient than that of No-MSC transplantation.

Discussion

Due to possessing the advantages of self-renewal and homing ability, multi-directional differentiation potentials[28] and immunoregulatory function,[29] MSC-based cell therapy has attracted extensive attention both experimentally and clinically. However, there are still many issues on the clinical application of MSCs, including their cultivation and preparation in vitro, which require further in-depth research. From the early culture system containing FBS to the current serum-free culture system, the quality of MSCs has been continuously improved to better meet the needs of clinical applications.

Currently, MSCs can be isolated from many tissues of the body, such as hUCs, bone marrow, fat, skin, and dental pulp, which exist in a hypoxic environment with 1% to 10% of physiological oxygen concentration.[30] Since hypoxia is a status for cell growth and survival, one of the solutions is short-term pretreatment of stem cells with hypoxia to enhance their therapeutic effects.[13,31,32] Basciano et al[33] observed that undifferentiation and multipotency were increased in bone marrow MSCs (BM-MSCs) with long-term hypoxia (5% O_2) for up to P2 compared with normal culture conditions. Park et al[34] also found that hypoxic conditions (5% O_2) improved the proliferation of hUC-MSCs and increased the initial yield when subcultured from the primary passage to P5, and maintained the stemness and anti-apoptotic effect. Although hypoxia stimulation improved the culture of MSCs, the detailed analysis regarding the impact of long-term hypoxia on the biological function and therapy of MSCs is not enough. In the present study, we applied a free-serum culturing system to systemically investigate the characteristics of hUC-MSCs from P0 to P30 in the hypoxic atmosphere (1% O_2), and observed that Hy-MSCs had better morphology and proliferative ability than No-MSCs, and similar MSC phenotypes and the potential for multilineage differentiation into adipocytes, osteocytes, chondrocytes, and pancreatic β-like cells, which meet the criteria of the International Society for Cell and Gene Therapy.[35]

Safety is one of the most important issues for hUC-MSC-based cell therapy clinically.[36,37] In the present study, we demonstrated that Hy-MSCs did not exhibit tumorigenesis both in vitro and in vivo, and had normal karyotype over to P20, despite their ability to rapidly proliferate. The multidirectional differentiation potential is main biological function of MSCs, and the expression of all related genes was lower in the differentiated cells of Hy-MSCs compared with No-MSCs. The results were possibly related with the high expression of HIF-1α in No-MSCs, which facilitated the expression of these differentiation genes. Furthermore, the stemness genes were highly expressed in the differentiated cells of Hy-MSCs, so these results indicate that Hy-MSCs preserve the less differentiated state, which is consistent with other studies.[9,33,38] The physiological microenvironment (5% O_2) rejuvenated MSC culture towards less differentiated, more naïve, and faster-growing phenotypes,[9,33] and the cultivation of WJ-MSCs (3% O_2) did not change the cellular phenotype,[38] while it modulated their differentiation process and enhanced the clonogenic and expansion capacity.

The clinical dosage of MSCs is generally between 10^7 and 10^9 cells,[39] so preparing sufficient numbers of MSCs is very important in clinical therapy.[40] In order to produce sufficient numbers of MSCs from a single donor, continuous and long-term culture is necessary. However, long-term culture inevitably induces MSC senescence, resulting in the reduction of their multipotency and therapeutic efficacy and the activation of an inflammatory response.[41] We continuously passaged MSCs and confirmed that the senescent level of long-term hypoxic MSCs was significantly lower than that of No-MSCs, and they had better telomerase activity, inhibiting the expression of senescent genes and promoting the expression of stemness genes, indicating that long-term hypoxia has a preservative effect on the stemness of MSCs. Therefore, we further validated the superior stemness maintenance of Hy-MSCs.

Immunoregulation is another major function of MSCs, which have good therapeutic effects on autoimmune, allergic, and inflammatory diseases.[29] We analyzed the regulatory effect of MSCs by co-culturing with PBLs, which inhibited the number of $CD3^+CD8^+$ T and NK cells and promoted the proliferation of Treg cells. Additionally, we found that hUC-MSCs promoted the proliferation of chondrocytes by co-culturing with both, and Hy-MSCs promoted the proliferation of chondrocytes more significantly than No-MSCs. Yao et al[26] confirmed that chondrocyte ferroptosis can induce the occurrence of OA in mice model experiments. From the pathogenesis, the inflammatory and iron overload environments are two important factors that induce ferroptosis, and IL-1β can serve as a typical inducer to cause cytotoxicity in chondrocytes, accumulation of reactive oxygen species (ROS) and lipid peroxides, and expression of ferroptosis-related proteins. Therefore, we chose IL-1β as the inducer of chondrocyte ferroptosis, and observed that hUC-MSCs inhibited the mortality of chondrocytes, which was more evident in Hy-MSCs than in No-MSCs. These data indicate that Hy-MSCs not only enhance the growth of chondrocytes, but

also increase the protection of chondrocytes, and thus are potentially suitable for treating diseases of cartilage lesions.

OA is a common geriatric disease with a global incidence of 250 million patients, while China alone has nearly 130 million patients.[42] Nowadays, OA has the highest disability rate, with 53% of patients aged 50 years and above.[43] Since there are not any specific drugs for treating OA clinically, there is an urgent need to seek a drug that can alleviate the symptoms, but there also needs to be a permanent cure.[44,45] Therefore, we selected OA as a disease model, as it is a disease of the cartilage damage caused by inflammation of the joint due to continuous friction. The hUC-MSC transplantation alleviated the symptoms of OA rats; in particular, the preventative effect of Hy-MSCs was significantly better than that of No-MSCs. The mechanism of this preventative effect may be related to hUC-MSCs inhibiting the secretion of pro-inflammatory factors and improving the destructive effect of metalloproteinases on cartilage. Pro-inflammatory factors including IL-1β, IL-6, and TNF-α induce the degradation of cartilage collagen by mediating MMP-13, and can activate the activity of metalloproteinases.[26,46] The dual capability of Hy-MSCs allows them to maintain stemness but also regulate inflammation, making them an optimal method for OA treatment.

In conclusion, these results indicate that long-term Hy-MSCs exhibit better proliferative ability, stemness maintenance, anti-senescent feature, and immunoregulatory function, and also have no safety problems by culturing MSCs in serum-free medium. In addition, Hy-MSC transplantation promotes joint function improvement by inhibiting MMP-13 expression caused by pro-inflammatory factors in an OA rat model (Figure 1k). Therefore, Hy-MSCs can be translated into agents to treat these related diseases clinically.

Supplementary material

Tables showing flow cytometry antibodies and primers used in the study. Figure showing the related gene expressions which were analyzed in adipogenic differentiation of human umbilical cord-derived mesenchymal stem cells (hUC-MSCs). An ARRIVE checklist is also included to show that the ARRIVE guidelines were adhered to in this study.

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Data sharing

The data that support the findings for this study are available to other researchers from the corresponding author upon reasonable request.

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Ethical review statement

All of the protocols of animal experiments followed the principle of the Laboratory Animal Welfare Act, and were approved by the Animal Ethics Committee of Guangdong College of Traditional Chinese Medicine (permit number: 2021028).

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