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ORIGINAL PAPER

Effects of cobalt chloride on the stem cell marker expression and osteogenic differentiation of stem cells from human exfoliated deciduous teeth

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Abstract

Stem cells from human exfoliated deciduous teeth (SHEDs) are a promising source for tissue engineering and stem cell transplantation. However, long-term in vitro culture and expansion lead to the loss of stemness of SHEDs, compromising their therapeutic benefits. Hypoxia plays an essential role in controlling the stem cell behavior of mesenchymal stem cells (MSCs). Thus, this study aimed to investigate the effects of cobalt chloride ($CoCl_2$), a hypoxia-mimetic agent, on the stem cell marker expression and osteogenic differentiation of SHEDs. SHEDs were cultured with or without 50 or 100 μM $CoCl_2$. Their proliferation, apoptosis, stem cell marker expression, migration ability, and osteogenic differentiation were examined. Culture with 50 and 100 μM $CoCl_2$ increased the hypoxia-inducible factor-1 alpha (HIF-1α) protein levels in a dose-dependent manner in SHEDs without inducing significant cytotoxicity. This effect was accompanied by an increase in the proportion of STRO-1^+ cells. $CoCl_2$ significantly increased the expression of stem cell markers (OCT4, NANOG, SOX2, and c-Myc) in a dose-dependent manner. The migration ability was also promoted by $CoCl_2$ treatment. Furthermore, SHEDs cultured in osteogenic medium with $CoCl_2$ showed a dose-dependent reduction in alkaline phosphatase (ALP) activity and calcium deposition. The expression of osteogenic-related genes was also suppressed by $CoCl_2$, especially in the 100-μM $CoCl_2$ group. In conclusion, $CoCl_2$ increased the expression of stem cell markers and inhibited the osteogenic differentiation of SHEDs. These findings may provide evidence supporting the use of in vitro hypoxic environments mimicked by $CoCl_2$ in assisting the clinical application of SHEDs.

Keywords Cobalt chloride · Dental pulp · Deciduous teeth · Stem cells · Stemness · Osteogenic differentiation

Introduction

Stem cell-based therapies have increasingly become the ideal therapeutic approach to cure numerous degenerative diseases. Among the many types of cells that can be used, stem cells from human exfoliated deciduous teeth (SHEDs) have attracted significant attention. SHEDs are derived from the dental pulp of young patients and can differentiate into cells of multilineages, including osteogenic, chondrogenic, adipogenic, neural, hepatic, myogenic, and endothelial lineages (Miura et al. 2003; Rosa et al. 2016). Compared with human adult dental pulp stem cells (DPSCs) and human adult periodontal ligament stem cells (PDLSCs), SHEDs are more immature and present greater proliferation rates and better differentiation potential (Koyam et al. 2009; Miura et al. 2003). Moreover, because exfoliated deciduous teeth are usually discarded, SHEDs can be obtained less invasively with fewer ethical concerns than MSCs derived from other tissues (Huang et al. 2009). Therefore, SHEDs have been considered a promising cell source for tissue engineering and stem cell transplantation.

However, the clinical use of SHEDs for tissue engineering still faces many challenges. One of the challenges is the

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expansion of sufficient amounts of stem cells from clinically limited tissues. Therefore, long-term in vitro culture to generate the required cell numbers is needed, although this process results in replicative senescence and impaired proliferation (Bork et al. 2010). Thus, numerous attempts have been made to positively influence stem cell behavior and improve the efficiency of stem cell-based therapies.

Stem cells reside within a unique microenvironment called the stem cell niche, which is regulated by cellular and acellular factors (Moore and Lemischka 2006). Low oxygen tension is a critical environmental factor of the stem cell niche (Mohyeldin et al. 2010). In arterial blood, the oxygen tension is approximately 14%, while in a variety of other tissues, such as bone marrow and brain tissue, the oxygen tension ranges from 1 to 7% (Chow et al. 2001; Nombela-Arrieta and Silberstein 2014). Although dental pulp is a highly vascularized tissue, the oxygen concentration in dental pulp is low. A previous study found approximately 3% oxygen in the pulp tissue of rats (Yu et al. 2002). Moreover, many causes, such as trauma and caries, can lead to much lower oxygen tension in the pulp tissue (Rombouts et al. 2017).

However, current culture conditions contain much higher oxygen tension than physiologic conditions. It has been shown that ambient oxygen tension (20% oxygen) can lead to the loss of primitive stem cell characteristics by inducing premature senescence, DNA damage, chromosomal aberrations, and metabolic changes (Fehrer et al. 2007; Kim et al. 2016). Hypoxia has been demonstrated to play an essential role in the maintenance of stem cell properties such as self-renewal, survival, and multipotency. Culture under low oxygen concentrations enhanced the proliferation and expression of stem cell markers in MSCs (Berniakovich and Giorgio 2013; Kim et al. 2016). Low oxygen concentrations enhanced the expression of some pluripotency markers, trophic factors, and immunomodulatory factors as well as the secretome trophic effect in DPSCs (Ahmed et al. 2016). SHEDs were also able to maintain higher mRNA expression of the pluripotency markers within 7 days when cultured in hypoxic conditions (Werle et al. 2018).

Unfortunately, it is difficult to simulate physiologic hypoxia in the cultural environment in vitro. Hypoxic conditions achieved using the hypoxia chambers available in the laboratory are expensive and inconvenient. Moreover, it is sometimes difficult to control and maintain steady oxygen tension. Thus, creating hypoxia by chemical agents is a more attractive method.

Hypoxia-inducible factor-1 alpha (HIF-1α) has been shown to mediate the response to hypoxia. Under normoxia, HIF-1α is rapidly degraded because of the activation of prolyl-hydroxylases (PHDs). Under hypoxic conditions, PHDs are inhibited, which leads to the accumulation of HIF-1α and initiates the transcription of downstream genes involved in metabolism, erythropoiesis, and angiogenesis (Semenza 2012). $CoCl_2$, a widely used hypoxia-mimicking agent in vivo and in vitro, mimics hypoxia by preventing the degradation of HIF-1α (Ji et al. 2006). Previous studies found that $CoCl_2$ increased stem cell marker mRNA expression and inhibited osteogenic differentiation in two less primitive dental-derived cells, i.e., human dental pulp cells (hDPCs) (Laksana et al. 2017) and human periodontal ligament cells (hPDLCs) (Osathanon et al. 2015).

Although several studies have focused on the influence of hypoxia on dental-derived stem cell stemness, the impact of hypoxia on SHED behavior is still a matter of discussion. Moreover, information is not available on the influence of hypoxia induced by $CoCl_2$ on stem cell marker expression and osteogenic differentiation of SHEDs. In the present study, the effects of $CoCl_2$ on the proliferation, apoptosis, migration, stem cell marker expression, and osteogenic differentiation of SHEDs were investigated.

Materials and methods

Cell isolation, culture, and characterization

The entire study was approved by the Ethics Committee of Sun Yat-sen University. All individuals who participated in this study provided informed consent. SHEDs were isolated from healthy exfoliated deciduous teeth of children between 6 and 12 years old. Briefly, the pulp tissues were removed from the teeth and washed with sterile phosphate-buffered saline (PBS) supplemented with 1% penicillin/streptomycin (P/S; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA). The tissues were cut into approximately 1 × 1 mm pieces and enzymatically digested with 3 mg/ml type I collagenase (Sigma-Aldrich) and 4 mg/ml of dispase II (Roche) at 37 °C for 30 min. The cells were then cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 20% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (P/S; Gibco) at 37 °C in 5% CO_2. When confluent, cells were detached by 0.25% trypsin-EDTA (Gibco) and subcultured at a 1:3 ratio. The medium was changed every 3 days. Cells from passages 3–5 were used in this study.

The isolated cells were characterized using flow cytometry. The expression of mesenchymal cell markers (CD73, CD90, and CD105) and hematopoietic cell markers (CD34, CD45) were examined. Cells were stained with PE-conjugated anti-CD73 antibody (BD Biosciences Pharmingen), PerCP-CyTM5.5-conjugated anti-CD90 antibody (BD Biosciences Pharmingen), PE-conjugated anti-CD105 antibody (BD Biosciences Pharmingen), FITC-conjugated anti-CD34 antibody (BD Biosciences Pharmingen), and PE-conjugated anti-CD45 antibody (BD Biosciences Pharmingen). The stained cells were analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA).

To evaluate the differentiation capacity, osteogenic induction was performed. In brief, cells were cultured in osteogenic medium (OM), which was DMEM supplemented with 10% FBS, 10 mmol/l sodium β-glycerophosphate (Sigma-Aldrich), 10^{-8} mol/l dexamethasone (Sigma-Aldrich), and 50 mg/ml L-ascorbic acid (Sigma-Aldrich). After 14 days of culture, calcium deposits were assessed by Alizarin red staining. Cells cultured in basal medium served as controls.

Cell viability assay

The viability of SHEDs was evaluated using a Cell Counting Kit-8 (CCK-8; Dojindo, Tokyo, Japan). SHEDs were seeded into 96-well plates at a concentration of 5×10^3 cells/well and then treated with $CoCl_2$ at concentrations of 0, 50, and 100 μM. After culturing for 1, 3, 5, and 7 days, 100 μl of serum-free DMEM containing 10 μl of CCK-8 solution was added to each well. After incubation at 37 °C for 2 h, the optical density (OD) at 450 nm was detected with a microplate reader (Tecan, Infinite f200 PRO, Switzerland).

Flow cytometric analysis to quantify STRO-1^+ cells

The expression levels of STRO-1, a marker for mesenchymal progenitor populations, were analyzed using a CytoFLEX flow cytometer (Beckman Coulter, Brea, CA, USA) as previously described. SHEDs were treated with or without 50 or 100 μM $CoCl_2$ for 3 days. Then, the cells were harvested with trypsin-EDTA and resuspended in the wash buffer. After incubation with a Dylight 650 conjugated anti-STRO-1 antibody (Novus Biologicals, USA) in the dark at 4 °C for 1 h, the cells were washed twice with PBS and then analyzed by flow cytometry. The data were analyzed with FlowJo 10 (Treestar, Ashland, OR, USA).

Cell apoptosis assay

The rates of SHED apoptosis were determined with an Annexin V-FITC/PI double staining apoptosis detection kit (Nanjing Jiancheng Biotechnology Institute, Nanjing, China). SHEDs were seeded into 6-well plates at a concentration of 2×10^5 cells/well and then treated with or without 50 or 100 μM $CoCl_2$ for 3 days. The cells were collected, washed, and resuspended in 500-μl binding buffer. Next, 5 μl FITC-Annexin V and 5 μl PI were added to the cell suspension. The cells were incubated at room temperature for 10 min. Then, the samples were analyzed by flow cytometry. The data analysis was performed using CytExpert software.

Real-time quantitative polymerase chain reaction (qRT-PCR)

The expression of an antiapoptosis gene (e.g., Bcl-2), stemness genes (e.g., OCT4, NANOG, SOX2, and c-Myc), osteogenic genes (e.g., ALP, RUNX2, and COLI), hypoxia-inducible factor (HIF)-related genes (e.g., HIF-2α and VEGF), and β-catenin was detected by a qRT-PCR analysis. Briefly, SHEDs were seeded on 6-well plates at a density of 2×10^5/well and treated with or without 50 or 100 μM $CoCl_2$. To assess the expression of osteogenic genes, the cells were incubated in OM with or without 50 or 100 μM $CoCl_2$. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. One microgram of mRNA was converted into complementary DNA using the PrimeScript™ RT Master Mix (Takara, Japan). qRT-PCR was performed on a Light Cycler 480 Detection System (Roche, Sweden) with a SYBR PCR Master Mix kit (Roche, Indianapolis, IN, USA). β-Actin was used as an internal control. The primer sequences are shown in Table 1.

Western blot analysis

To confirm the hypoxia-mimetic effects, the expression of HIF-1α protein was detected by western blot analysis. In

Table 1 qRT-PCR primer sequences

Gene	Primer sequences (5′-3′)
Bcl-2	F: GAGGATTGTGGCCTTCTTTG R:GCCGGTTCAGGTACTCAGTC
OCT4	F:TGGGGGTTCTATTTGGGAAGG R:GATCTGCTGCAGTGTGGGT
NANOG	F:CCAGCCTTTACTCTTCCTACCA R:GCTGATTAGGCTCCAACCATAC
SOX2	F: GGATAAGTACACGCTGCCCG R: ATGTGCGCGTAACTGTCCAT
c-Myc	F: GCTGCTTAGACGCTGGATTT R:CCTCCTCGTCGCAGTAGAAA
ALP	F:CCTCCTCGGAAGACACTCTG R:GCAGTGAAGGGCTTCTTGTC
RUNX2	F: CCACTGAACCAAAAAGAAATCCC R: GAAAACAACACATAGCCAAACGC
COLI	F: CGATGGATTCCAGTTCGAGTATG R: TGTTCTTGCAGTGGTAGGTGATG
VEGF	F:CGCAGCTACTGCCATCCAAT R:GTGAGGTTTGATCCGCATAATCT
HIF-2α	F:GTCTCTCCACCCCATGTCTC R:GGTTCTTCATCCGTTTCCAC
β-Catenin	F:AAGTTCTTGGCTATTACGACA R:ACAGCACCTTCAGCACTCT
β-Actin	F:CATGTACGTTGCTATCCAGGC R:CTCCTTAATGTCACGCACGAT

parallel with the β-catenin gene analysis, the β-catenin protein levels were also detected. Briefly, SHEDs were washed twice in PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (KeyGen BioTECH, Nanjing, China) supplemented with 1 mmol/l protease inhibitor cocktail (CWBIO, Beijing, China). Whole cell lysates were separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; CWBIO) and transferred onto polyvinylidene fluoride (PDVF) membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% fat-free milk in TBST (10 mmol/l TrisHCl, 50 mmol/l NaCl, 0.25% Tween 20) for 1 h at room temperature and subsequently incubated with primary antibodies (anti-HIF-1α, anti-β-catenin, anti-β-Actin [1:1000; Cell Signaling Technology, Inc., Danvers, MA, USA]). The membranes were then treated with horseradish peroxidase-conjugated anti-rabbit secondary antibodies. Bands were detected with an enhanced chemiluminescence kit (Millipore).

Wound healing assay

SHEDs were seeded in 6-well plates at a density of 2×10^5 cells/well. When the cells formed a confluent monolayer, wounds were produced by scratching with a 200-μl pipette tip across the center of the plate. The medium was changed to FBS-free medium with or without 50 or 100 μM $CoCl_2$. After 24 h, the wells were photographed under an inverted microscope (Zeiss, Oberkochen, Germany). The width of the scratches was compared, and the number of cells migrating into the scratches was counted by an experimenter blinded to the experimental conditions.

Transwell assay

Transwell assays were conducted using 24-well transwell plates (pore size 8 μm, polycarbonate membrane, Corning, catalog no. 3422). SHEDs were seeded into the upper chamber of inserts (4×10^4 cells/well) containing FBS-free medium with or without 50 or 100 μM $CoCl_2$. DMEM containing 10% FBS was added to the lower chambers of the inserts. After incubation for 24 h, the cells remaining on the upper side of the filter were removed with cotton swabs, and the filters were fixed in formaldehyde for 30 min and then stained with 0.1% crystal violet for 15 min. Cells that migrated to the lower surface were observed under a microscope. Five random fields (× 100) of cells were counted by an experimenter blinded to the experimental conditions. Each assay was repeated three times.

Osteogenic differentiation

SHEDs were seeded on 6-well plates at a density of 2×10^5/well. When the cells reached confluency, they were treated with OM with or without 50 or 100 μM $CoCl_2$ for 7–14 days. The OM was replaced every 3 days. On day 7 and day 14, ALP activity was detected using an Alkaline Phosphatase Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. On day 14, the cells were fixed in 4% paraformaldehyde and stained in Alizarin red solution (Cyagen Biosciences Inc., Guangzhou, China) to detect calcium deposits. Furthermore, calcium deposits were quantified by destaining the deposits with 10% cetylpyridinium chloride solution for 30 min. The absorbance was measured at 562 nm by a microplate reader. To assess the expression of osteogenic genes, total RNA was extracted using TRIzol reagent (Invitrogen) on day 7, and a qRT-PCR assay was performed.

Statistical analyses

All the data are shown as the mean ± standard deviation from triplicate independent experiments. Statistical analyses were performed using one-way analysis of variance (ANOVA) and the Bonferroni method was used for multiple comparisons. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using SPSS 20.0 software (SPSS Inc., Chicago, IL, USA).

Results

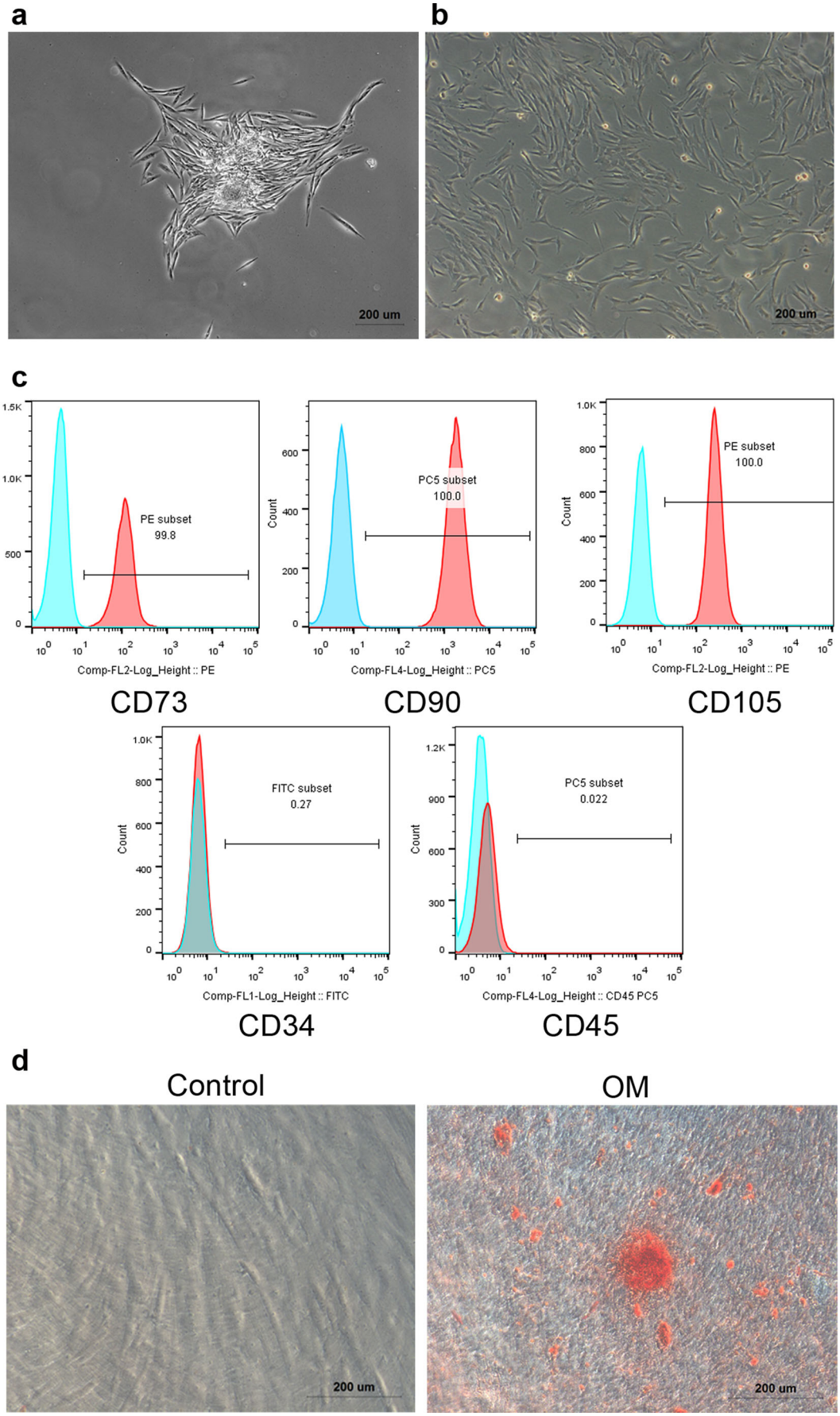
Characterization of SHEDs

SHEDs showed a typical fibroblastic morphology in primary culture (Fig. 1a) and after being passaged (Fig. 1b). SHEDs were positive for mesenchymal cell markers (CD73, CD90, and CD105), but negative for hematopoietic cell markers (CD34, CD45) (Fig. 1c). When SHEDs were cultured under osteogenic culture conditions, we observed calcium deposits (Fig. 1d), confirming that the isolated SHEDs had the ability to differentiate into osteogenic lineages. These data suggest that SHEDs exhibit mesenchymal stem cell characteristics.

$CoCl_2$ did not induce significant cytotoxicity in SHEDs

SHED viability was evaluated by CCK-8 assay. The cells were cultured in culture medium with or without 50 or 100 μM $CoCl_2$ for 1, 3, 5, and 7 days. The results showed that the viability of cells was suppressed by 50 and 100 μM

Fig. 1 Characterization of SHEDs. **a** Primary culture of SHEDs under a microscope (× 50). **b** 3rd passage of SHEDs under a microscope (× 50). **c** Flow cytometry analysis of surface markers of SHEDs. SHEDs were positive for mesenchymal cell markers (CD73, CD90, and CD105), but negative for hematopoietic cell markers (CD34, CD45). **d** SHEDs were cultured in the absence (control) or in the presence of OM for 14 days and then stained with Alizarin red


a
200 um
b
200 um
c
PE subset
99.8
Count
Comp-FL2-Log_Height :: PE
CD73
PC5 subset
100.0
Comp-FL4-Log_Height :: PC5
CD90
PE subset
100.0
Comp-FL2-Log_Height :: PE
CD105
FITC subset
0.27
Comp-FL1-Log_Height :: FITC
CD34
PC5 subset
0.022
Comp-FL4-Log_Height :: CD45 PC5
CD45
d
Control
OM
200 um
200 um

$CoCl_2$ at the start of culture. However, there was no significant difference between the control and the $CoCl_2$-treated cells on days 3, 5, and 7 (Fig. 2a). Next, because SHEDs likely do not include a homogeneous cell population, the proportion of progenitor cells was investigated by analyzing the percentage of the cells expressing STRO-1, a mesenchymal progenitor cell marker. The flow cytometry results showed that the proportion of STRO-1^+ cells was significantly higher in the cells cultured with $CoCl_2$ (Fig. 2b). The rate of apoptotic cells was also determined by flow cytometry. The results showed that after 3 days, $CoCl_2$ did not increase the rate of apoptotic cells (Fig. 2c). Moreover, the mRNA expression of the antiapoptosis gene Bcl-2 was significantly increased in the $CoCl_2$-treated groups compared with that of the control group (Fig. 2d). These data revealed that 50 or 100 μM $CoCl_2$ did not induce cytotoxicity in SHEDs.

$CoCl_2$ increased stem cell marker expression

The effects of $CoCl_2$ on mRNA expression of the stem cell markers OCT4, NANOG, SOX2, and c-Myc after the cells were cultured with or without 50 or 100 μM $CoCl_2$ for 3 days were evaluated. The results revealed that $CoCl_2$ significantly induced the mRNA expression of OCT4 and NANOG compared with the control in a dose-dependent manner. SOX2 and c-Myc mRNA were significantly higher in cells treated with 100 μM $CoCl_2$. However, a significant increase in the expression of SOX2 and c-Myc was not observed between the 50 μM $CoCl_2$ group and the control group (Fig. 3).

$CoCl_2$ induced hypoxia-inducible factor-related genes and β-catenin in SHEDs

After treatment with 50 μM or 100 μM $CoCl_2$, the expression of HIF-1α in SHEDs was determined by western blotting (Fig. 4a). The level of HIF-1α protein was enhanced in response to $CoCl_2$ treatment in a dose-dependent manner, confirming its hypoxic effect. The mRNA expression of VEGF, a downstream gene of HIF-1α, was enhanced after 100 μM $CoCl_2$ treatment, indicating activation of the HIF-1α pathway (Fig. 4b). Similarly, HIF-2α, a homologous protein of HIF-1α, also showed a significant increase in mRNA expression in the 100 μM $CoCl_2$ group (Fig. 4c). Next, we examined the expression of β-catenin, which is important in stem cell proliferation and survival. Interestingly, we found that β-catenin mRNA was increased in the SHEDs treated with 100 μM $CoCl_2$ (Fig. 4d), and β-catenin protein levels were increased upon the $CoCl_2$ treatment in a dose-dependent manner (Fig. 4e).

$CoCl_2$ enhanced SHED migration

To detect the effects of $CoCl_2$ on the migration of SHEDs, scratch wound healing and transwell assays were conducted. The wound healing assay results showed that 50 and 100 μM $CoCl_2$ both significantly increased the number of SHED cells migrating toward the wound at 24 h (Fig. 5a, b). Similarly, the transwell assay results showed that the migration capability of the $CoCl_2$-treated groups increased significantly compared with that of the control group (Fig. 5c, d). However, there was no significant difference between the 50 μM $CoCl_2$ group and the 100 μM $CoCl_2$ group. Taken together, these results indicate that $CoCl_2$ can promote SHED migration in vitro.

$CoCl_2$ inhibited osteogenic differentiation

To examine the effect of $CoCl_2$ on osteogenic differentiation, SHEDs were cultured in OM with or without 50 or 100 μM $CoCl_2$. $CoCl_2$ caused a dose-dependent decrease in ALP activity at both 7 and 14 days (Fig. 6a). Consistent with the ALP activity results, SHEDs cultured with $CoCl_2$ exhibited a diminished capacity to form calcium deposits at 14 days (Fig. 6b). Furthermore, the mRNA expression of osteogenic markers was assessed by RT-qPCR. Consistent with the above results, the mRNA expression levels of ALP, RUNX2, and COLI were all significantly reduced in SHEDs cultured with $CoCl_2$ (Fig. 6c). These findings suggest that $CoCl_2$ inhibited the osteogenic differentiation of SHEDs.

Discussion

High-quality stem cells are necessary for tissue regeneration and stem cell therapies. However, long-term in vitro culture leads to a loss of cell stemness, which strongly limits the clinical use of MSCs. Therefore, maintaining the stemness of MSCs, particularly with regard to their proliferation, migration, and differentiation, is important before allowing these cells to enter the clinical testing stage. Many researchers have developed various strategies, including preconditioning, genetic modification, and optimization of MSC culture conditions, to improve cell behavior in vitro culture (Hu and Li 2018; Yu et al. 2016; Zhao et al. 2016). Among these strategies, the incubation of cells under hypoxic conditions is one available method to induce stem cell proliferation and improve the behavior of several types of MSCs. Creating hypoxia by adding chemical agents, such as $CoCl_2$, to the culture medium is an attractive option.

SHEDs represent an ideal source for tissue engineering and stem cell transplantation. Although they share many similar characteristics with DPSCs, SHEDs are unique due to their higher proliferative rates, stronger differentiation capacities, easily accessible source, and fewer ethical concerns (Koyam

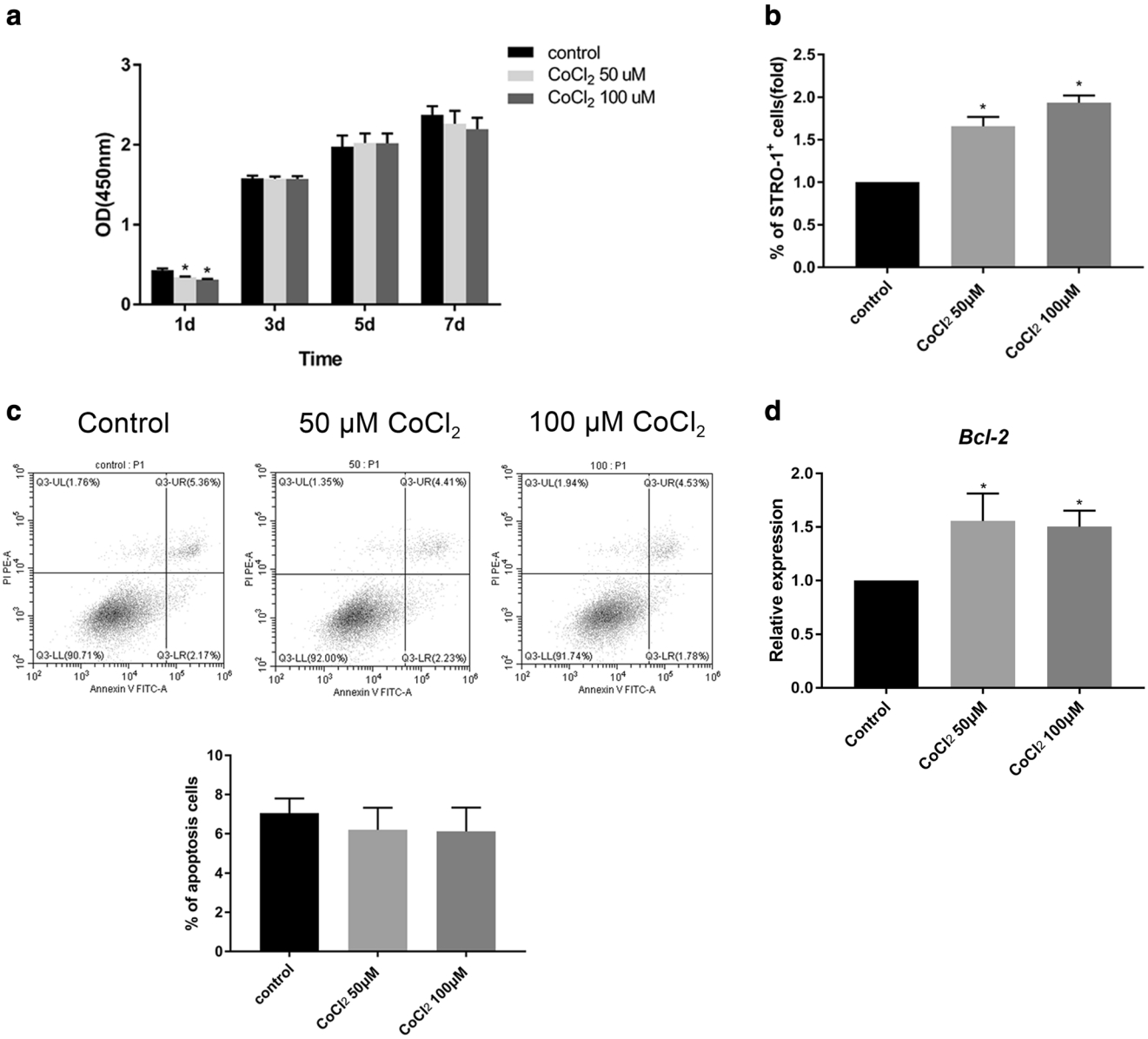


Fig. 2 The cytotoxicity of $CoCl_2$ was measured. **a** Cell viability of SHEDs was determined using a CCK-8 assay after incubation with or without 50 or 100 μM $CoCl_2$ for 1, 3, 5, and 7 days. The absorbance was measured at 450 nm. **b** The percentage of STRO-1^+ cells in SHEDs after incubation with or without 50 or 100 μM $CoCl_2$ for 3 days. The data are shown as relative fold changes compared with the control. **c** Apoptosis of SHEDs was determined with Annexin V-FITC/PI double staining after incubation with or without 50 or 100 μM $CoCl_2$ for 3 days. Flow cytometric contour plots and the percentage of apoptotic cells are shown. **d** The mRNA expression of Bcl-2 was examined by qRT-PCR. The data are shown as relative gene expression compared with the internal control. Bar graphs represent the mean ± SD of triplicate independent experiments.*$P < 0.05$ vs. control

et al. 2009; Miura et al. 2003). These specific properties lead to diverse responses to hypoxia. To date, the effect of hypoxia on the behavior of SHEDs is still unclear. Thus, the aim of this study was to investigate the effects of hypoxia induced by $CoCl_2$ on the stem cell marker expression and osteogenic differentiation of SHEDs. Our studies showed that $CoCl_2$ induced the expression of stem cell markers, promoted migration, and inhibited the osteogenic differentiation of SHEDs.

Our study demonstrated that $CoCl_2$ treatment activated HIF-1α pathways in SHEDs. Therefore, $CoCl_2$ effectively mimicked the hypoxic conditions in SHEDs. However, $CoCl_2$ is a chemical agent. To prevent potential toxicity induced by $CoCl_2$, we performed a careful cytotoxicity test. Our CCK-8 assay showed that 50 or 100 μM $CoCl_2$ inhibited SHED proliferation at the start of treatment. Nevertheless, there was no significant difference among these groups for treatments longer than 1 day. One possible explanation for this phenomenon is that an initial period of adaptation exists, as there is evidence that MSCs exposed to 5% O_2 initially show lower proliferation but grow fast thereafter (Grayson et al.

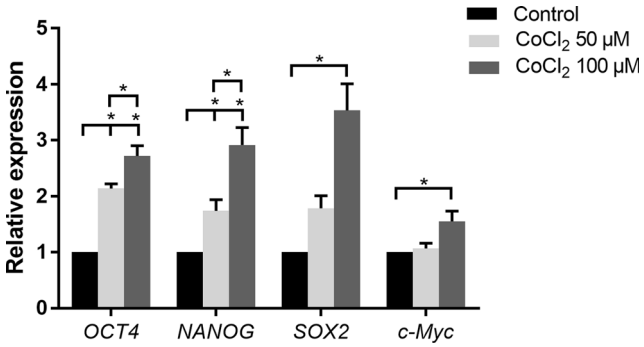


Fig. 3 Effects of $CoCl_2$ on stem cell marker expression of SHEDs. The mRNA expression of OCT4, NANOG, SOX2, and c-Myc was assessed by qRT-PCR. The data are shown as relative gene expression compared with the internal control. Bar graphs represent the mean ± SD of triplicate independent experiments. *$P < 0.05$ vs. control

2010). Other studies have also shown that MSCs establish redox homeostasis and, thus, adapt to hypoxic conditions (Boyette et al. 2014).

Hypoxia has been shown to have dual effects on MSC apoptosis. Severe and prolonged hypoxia may induce apoptosis, whereas acute and mild hypoxia induce adaptation and survival (Greijer and van der Wall 2004). Although previous studies have reported that dental-derived cells such as DPSCs and hPDLCs have high cell viability when cultured in 100 μM $CoCl_2$ (Osathanon et al. 2015; Teti et al. 2018), contradictory findings have also been reported, namely, that 100 μM $CoCl_2$ induced cell death (Laksana et al. 2017). These conflicting results may be due to variations in cell types and experimental conditions. In the present study, we showed that $CoCl_2$ did not induce negative cell growth or apoptosis, consistent with previous studies on MSCs in which hypoxia (1% O_2) reduced cell apoptosis compared with normoxia (Kim et al. 2016).

Previous reports have indicated that stem cells isolated from the pulp may not be a single-cell type but rather a heterogeneous stem cell population (Sloan and Waddington 2010). STRO-1 is an early MSC marker to identify clonogenic stromal cell progenitors (Kawashima 2012). Previous studies found that STRO-1^+ cells possess higher proliferation and multipotentiality than cells negative for this MSC marker (Xuechao et al. 2009; Yang et al. 2007). Moreover, the STRO-1 expression ranges from 0.02 to 9.56% in human pulp stem cell cultures and increases under hypoxic condition (Sakdee et al. 2009; Werle et al. 2016). Our study showed that

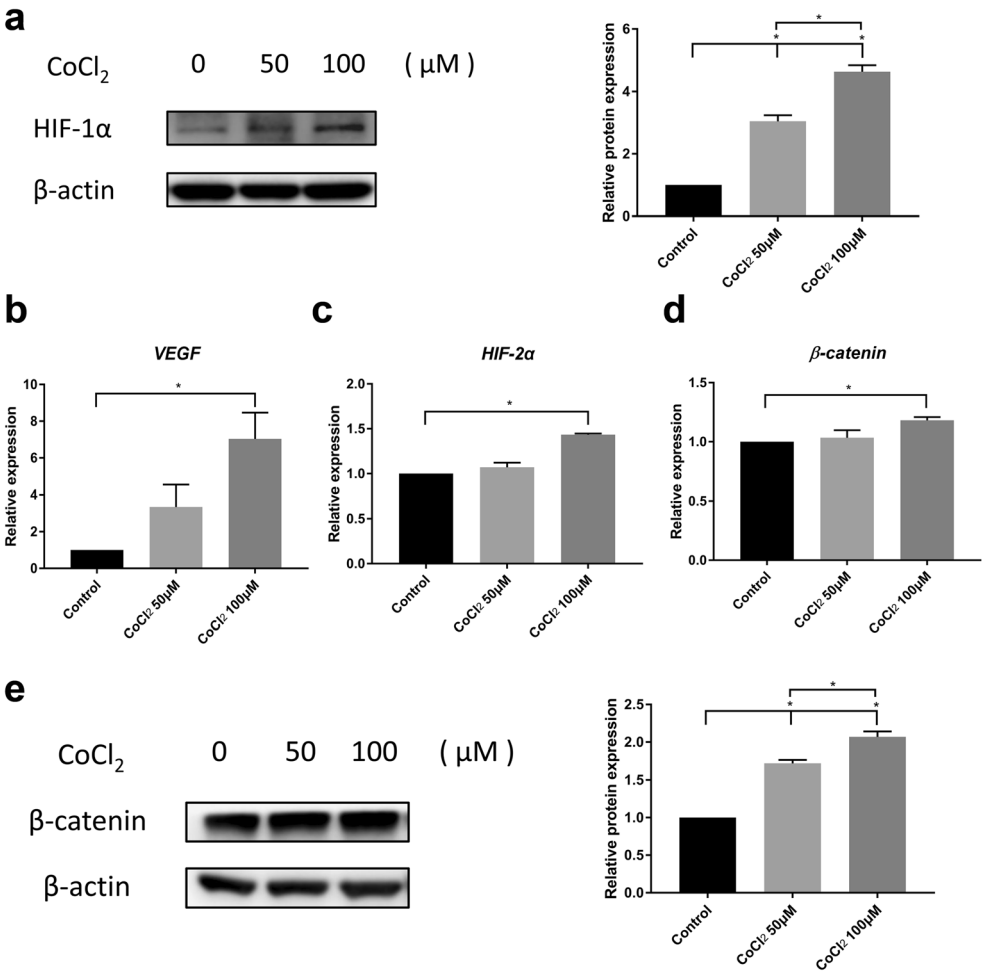


Fig. 4 $CoCl_2$ induced HIF-related genes and β-catenin in SHEDs. **a** Western blots of HIF-1α protein levels in SHEDs treated with 50 or 100 μM $CoCl_2$ for 12 h. β-Actin levels are shown as loading controls. **b**–**d** The mRNA expression of HIF-2α, VEGF, and β-catenin was evaluated in SHEDs cultured with or without $CoCl_2$ (50 μM and 100 μM) for 3 days. **e** The relative protein expression of β-catenin in SHEDs. β-Actin levels are shown as loading controls. Bar graphs represent the mean ± SD of triplicate independent experiments. *$P < 0.05$ vs. control

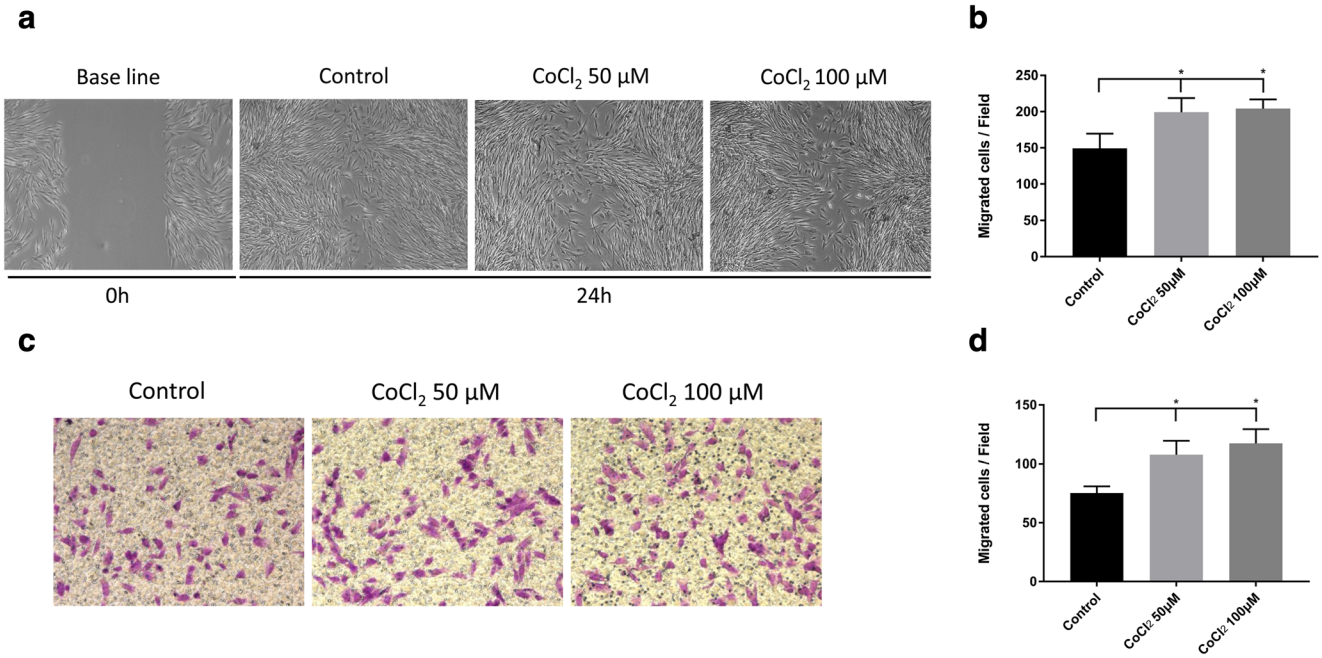


Fig. 5 Effects of $CoCl_2$ on SHED migration. **a** Representative image of SHED scratch wound healing with or without 50 or 100 μM $CoCl_2$ treatment (× 100). Cellular migration was monitored for 24 h. **b** Quantification of cells migrating into the scratches in the scratch wounding healing assay. **c** Representative image of migrating SHEDs treated with or without 50 or 100 μM $CoCl_2$ in the transwell assay (× 100). **d** Quantification of the migrated cells in the transwell assay. *$P < 0.05$ vs. control

$CoCl_2$ increased the expression of STRO-1, which is consistent with previous reports and suggests that $CoCl_2$ might enhance the proportion of progenitor cells among isolated SHEDs in this study.

OCT4, NANOG, and SOX2 are stemness-related genes essential for the major properties of stem cells, and they are critical in maintaining a pluripotent state and self-renewal. Previous studies have shown that the overexpression of OCT4, NANOG, and SOX2 promoted proliferation and prevented spontaneous differentiation, whereas the downregulation of these genes led to a loss of differentiation and senescence (Basu-Roy et al. 2010; Han et al. 2014; Tsai et al. 2012). c-Myc is a transcription factor that generates induced pluripotent stem cells and has a unique role in cell proliferation and differentiation (Liu et al. 2013). Our results showed that $CoCl_2$ upregulated the gene expression of OCT4, NANOG, SOX2, and c-Myc. This finding suggests that the $CoCl_2$ treatment might have positive effects on the stemness of SHEDs via the activation of OCT4-, NANOG-, SOX2- and c-Myc-related signaling pathways.

SHEDs, capable of multilineage differentiation, are a unique type of MSCs. Previous studies have shown that hypoxia can affect the osteogenic differentiation of MSCs, although the reported results are conflicting. Although 5% of oxygen promoted the mineralization of bone marrow mesenchymal stem cells (BMSCs) (Sheehy et al. 2012), a recent study showed that 2% of oxygen inhibited the osteogenic differentiation of BMSCs (Zhang et al. 2017). This discrepancy may be due to the variations in oxygen concentration. The duration of hypoxia has also been suggested to affect the osteogenic differentiation of MSCs (Boyette et al. 2014). In the present study, osteogenic-related gene expression, ALP activity, and calcium deposition were all reduced under hypoxic conditions mimicked by $CoCl_2$. This result corresponds with previous studies that reported the inhibitory effect of $CoCl_2$ on osteogenic differentiation in hDPCs (Laksana et al. 2017) and hPDLCs (Osathanon et al. 2015). However, it was found that $CoCl_2$ upregulated osteogenic gene expression in BMSCs (Yu et al. 2018). Differences in cell types may contribute to such disparities. Nevertheless, it is probable that $CoCl_2$ inhibit the osteogenic differentiation of SHEDs without maintaining their stemness for example by differentiation to the other lineages. Therefore, further experiments are needed to verify the effect of hypoxia on the multilineage differentiation of SHEDs.

The migration capacity of MSCs together with stemness is critical for repairing damaged tissues. The stromal-derived factor-1 alpha (SDF-1α)/CXCR4 axis plays a critical role in regulating the trafficking of MSCs toward the target tissue (Cencioni et al. 2012). Previous studies have suggested that hypoxia and hypoxia-mimetic agents, such as $CoCl_2$ or deferoxamine (DFX), may increase the migration of MSCs by increasing the expression of CXCR4 (Li et al. 2017; Wang et al. 2017). Our study also found that $CoCl_2$ treatment enhanced SHED migration. Our results imply that SHEDs treated with

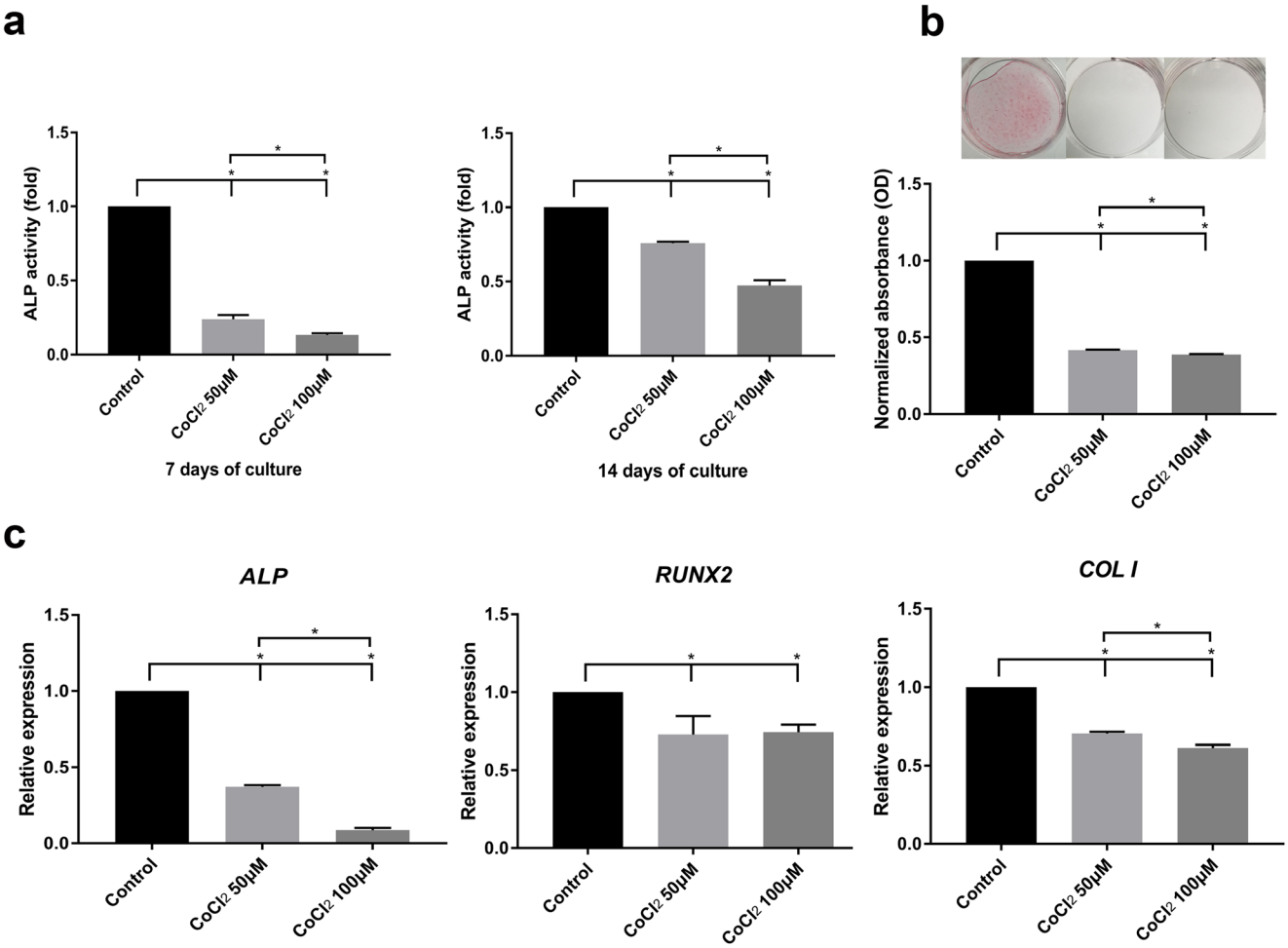


Fig. 6 $CoCl_2$ decreased the osteogenic differentiation of SHEDs. **a** SHEDs cultured in osteogenic medium with 50 μM or 100 μM $CoCl_2$ for 7 or 14 days showed an inhibition of ALP activity. **b** Deposition of calcium exhibited by Alizarin red staining. SHEDs cultured in OM with either 50 or 100 μM $CoCl_2$ for 14 days showed reduced mineral deposition. Data were expressed as the fold change compared with the control. **c** After 7 days of osteogenic induction, the mRNA expression of ALP, RUNX2, and COLI was examined by RT-qPCR. The data are shown as relative gene expression compared with the internal control. Bar graphs represent the mean ± SD of triplicate independent experiments. $^*P < 0.05$ vs. control

$CoCl_2$ might better migrate and home to the injured area, and thus, $CoCl_2$ may improve the therapeutic effect of SHEDs in stem cell-based therapies.

Previous studies have shown that hypoxia can induce pluripotency and modulate reprogramming in somatic cells by stabilizing HIF-1α (López-Iglesias et al. 2015). Consistent with previous data, our findings showed that $CoCl_2$ induced higher expression of HIF-1α protein. We also found that 100 μM $CoCl_2$ significantly increased the mRNA expression of HIF-2α, a homologous protein of HIF-1α. Both HIF-1α and HIF-2α have been reported to induce the expression of genes controlling cell proliferation, self-renewal, and pluripotency (Cummins 2012). Moreover, hypoxia has been shown to stimulate the activation of other signaling pathways involved in stemness preservation, such as the Wnt/β-catenin, Notch, and Hedgehog signaling pathways (Landor and Lendahl 2017; Zhao et al. 2018). In the current study, we observed the upregulation of β-catenin. The Wnt/β-catenin signaling pathway regulates the transcription of an array of genes related to cellular proliferation and survival (cyclin D1, c-Myc, etc.) (Kahn 2014). The variable activation level of Wnt signaling under hypoxic conditions induced multiple cell changes (Li et al. 2016). In our study, the increased expression of HIF-related genes and β-catenin by $CoCl_2$ treatment might partially explain the effect of $CoCl_2$ on SHEDs, although the associations between hypoxia and these signaling pathways remain to be elucidated.

In our study, we observed that the mRNA expression levels of Bcl-2 and c-Myc were both increased in SHEDs cultured with $CoCl_2$. Because of the well-known role of c-Myc and Bcl-2 in the regulation of apoptosis and tumorigenesis (Alarcon et al. 1996), safety concerns remain regarding the effect of increased expression of c-Myc and Bcl-2, which may stimulate cancer/tumor development, whereas cancer-

suppressing effects of Bcl-2 have also been reported (Slape et al. 2012). Moreover, tumor formation by MSCs may be affected by many factors, such as the methods used for cell culture and differentiation, the site of transplantation, and the host background (Kyoko et al. 2009). Therefore, firm conclusions cannot be reached based on our study. However, this finding should be taken into consideration in the application of $CoCl_2$ to maintain stem cells during in vitro expansion.

In conclusion, $CoCl_2$ treatment increased the expression of stem cell markers and inhibited the osteogenic differentiation of SHEDs. The findings presented here may provide beneficial evidence for the use of the in vitro hypoxic environment mimicked by $CoCl_2$ in assisting the clinical application of SHEDs. The use of $CoCl_2$ in maintaining the undifferentiated state of SHEDs in the laboratory as well as the mechanism underlying the effect of $CoCl_2$ on the stemness and differentiation of SHEDs needs further investigation.

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Compliance with ethical standards

The entire study was approved by the Ethics Committee of Sun Yat-sen University. All individuals who participated in this study provided informed consent.

Conflict of interest The authors declare that they have no conflict of interest.

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