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ORIGINAL RESEARCH

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Stemness maintenance of stem cells derived from human exfoliated deciduous teeth (SHED) in 3D spheroid formation through the TGF-β/Smad signaling pathway

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Abstract

Mesenchymal stem cells (MSCs) have shown great potential as important therapeutic tools for dental pulp tissue engineering, with the maintenance and enhancement of their stemness being crucial for successful therapeutic application *in vivo* and three-dimensional (3D) spheroid formation considered a reliable technique for enhancing their pluripotency. Human exfoliated deciduous tooth stem cells (SHED) were cultured in a low attachment plate to form aggregates for five days. Then, the resulting spheroids were analyzed for pluripotent marker expression, paracrine secretory function, proliferation, signaling pathways involved, and distribution of key proteins within the spheroids. The results indicated that 3D spheroid formation significantly increased the activation of the transforming growth factor beta (TGF-β)/Smad signaling pathway and upregulated the secretion and mRNA expression levels of TGF-β, which in turn enhanced the expression of pluripotency markers in SHED spheroids. The activation of the TGF-β/Smad signaling pathway through 3D spheroid formation was found to preserve the stemness properties of SHED. Thus, understanding the mechanisms behind pluripotency maintenance of SHED culture through 3D spheroid formation could have implications for the therapeutic application of MSCs in regenerative medicine and tissue engineering.

Keywords

3D spheroid culture; Stemness maintenance; Stem cells derived from human exfoliated deciduous teeth (SHED); TGF-β/Smad signaling

1. Introduction

Dental pulp, a highly organized soft connective tissue located within the mineralized structure of the tooth, plays a pivotal role in maintaining tooth homeostasis and serves as a vital organ for the tooth's viability [1]. Pulpitis is common in dental pulp, often caused by deep decay, dental trauma or advanced periodontal disease, and is traditionally treated by root canal therapy, which involves removing the affected pulp [2]. Recent studies have shown that mesenchymal stem cell (MSC)-based tissue regeneration is promising for engineering dental pulp tissue and restoring tooth viability [3, 4]. Further, in both nude mice ectopic and dogs or minipigs orthotopic regeneration models, the transplantation of stem cells from the apical papilla (SCAPs) or dental pulp stem cells (DPSCs) resulted in the regeneration of blood vessels, remodeling and dentin-pulp-like tissues [5–7].

In recent years, stem cells derived from human exfoliated deciduous teeth (SHED) have emerged as a promising candidate among MSCs for treating diseased dental organs [8, 9]. Compared to other MSC types, SHED has numerous advantages, including strong proliferative potential, higher cellular differentiation, minimal ethical concerns and less invasive procurement [10, 11]. The origin of SHED from the dental pulp of exfoliated deciduous teeth, which is in the developmental stage, is one of the reasons for its distinct characteristics. As exfoliated deciduous teeth are shed at a relatively early stage of tooth development, their pulp tissues may harbor stem cells possessing greater pluripotency, self-renewal ability and more effective differentiation than those derived from mature dental tissues or other adult MSCs. In addition, SHED possesses unique advantages, such as high levels of specific pluripotency marker expression and the ability to secrete a broad array of growth factors and cytokines [12–15]. Thus, SHED has been successfully applied therapeutically for repairing/regenerating both the local intraoral pulp-dentin complex and extraoral tissues. In nude mice models, the transplantation of tooth slices or hydroxyapatite/tricalcium phosphate (HA/TCP) containing SHED resulted in the formation of dentin-pulp-like tissues *in vivo*. In addition, the implantation of SHED with either recombinant human Collagen type I or PuraMatrix (peptide hydrogel) into the premolar roots of immunodeficient mice promoted

the regeneration of pulp-like tissues with new tubular dentin [3, 16–19]. Nevertheless, since many therapeutic applications have been tested in animal models, there is an urgent need for conducting extensive (pre-)clinical trials [1, 20].

The mesenchymal cell population in the craniofacial region during embryogenesis mainly originates from the neural crest of the ectoderm [21–23]. MSCs obtained from dental pulp tissues exhibit specific markers for embryonic stem cells, such as transcription factors SRY-box transcription factor 2 (SOX2), octamer-binding transcription factor 4 (OCT4) and (NANOG), and cell surface antigen Stage-specific embryonic antigen-4 (SSEA-4) [24, 25]. In addition, expressing pluripotency markers similar to embryonic stem cells (ESCs) could potentially enhance the effectiveness of stem cell therapy-based treatments [26]. However, the successful application of dental MSCs in regenerative medicine and tissue engineering heavily depends on the ability to regulate stemness properties and maintain pluripotency throughout multiple subcultures from the initial cells [27, 28]. Although the pluripotency of MSCs is maintained in the *in vivo* microenvironment through various mechanisms and factors such as cell-cell contacts, soluble growth factors and interactions with the extracellular matrix (ECM), these primitive stemness properties can gradually diminish over time during conventional monolayer tissue culture *in vitro* [29].

Cells in suspension can form three-dimensional (3D) spheroids, allowing for significant multiplanar interactions with the cell matrix, which create niches and microenvironments that influence cellular processes such as cell function and fate [30, 31]. MSCs cultured in spheroids exhibit a distinctive morphology, characterized by a spherical shape on the inside and an elongated shape on the outside. In contrast to conventional 2D monolayer cultures, they display reduced levels of extracellular matrix (ECM) and cytoskeletal molecules and a 75% reduction in individual cell volume [32, 33]. McBeath *et al.* [34] reported that MSCs with a small, rounded morphology were more likely to differentiate into an adipogenic lineage, thereby highlighting the importance of cellular morphology in determining cellular fates and phenotypes [35]. The formation of 3D spheroids is associated with several advantages, including the ability to study authentic *in vivo* cellular function and differentiation through *in vitro* cell culture [36, 37]. In addition, 3D cell cultures offer a rapid way to conduct experimental manipulations using molecular biology techniques and allow for superior imaging through microscopy compared to animal models [38]. Recent evidence suggests that MSCs cultured in 3D spheroids may enhance angiogenic, anti-inflammatory, multipotency and tissue regenerative effects both *in vivo* and *in vitro* as well as increasing the secretion of various factors, including cytokines, vascular endothelial growth factor (VEGF), leukemia inhibitory factor (LIF), stanniocalcin-1 (STC-1) and tumor necrosis factor-α stimulated gene/protein 6 (TSG-6) [12, 39, 40]. Microarray analysis has revealed a notable shift in gene expression profiles during 3D spheroid formation, marked by the upregulation of genes related to inflammation, angiogenesis, redox signaling, stress response and hypoxia [40, 41]. Moreover, MSCs cultured as 3D spheroids have shown enhanced therapeutic effects in diverse disease models, such as ischemic injury and wound healing, owing to the above-mentioned stem cell biological properties [42–44]. Thus, the primary objective of tissue engineering is to imitate the complex geometric and architectural characteristics of the body, including critical cell-to-cell interactions. In this regard, various techniques have been devised for regenerative medicine, among which the most widely used approach involves incorporating biological factors such as cells, proteins and genes into porous, degradable scaffold materials [45–47]. Despite the advantages of using scaffold materials, it is important to note that scaffold environments may not completely replicate the natural cell-to-cell and cell-to-ECM attachments observed *in vivo* tissues. Furthermore, there have been reports of infection and inflammation resulting from scaffold materials during *in vivo* degradation [48]. In 3D spheroid culture, the cells and their extracellular matrix (ECM) can self-assemble into 3D aggregations without a scaffold. Scaffold-free 3D cell culture techniques have gained substantial interest in stem cell research because they can simulate the *in vivo* microenvironment, improve the potency of MSCs, avoid the risk of infection and/or inflammation, and promote increased cell-to-cell interactions [49, 50]. Therefore, considering the current trends in cell biology and tissue engineering, investigating the stemness of SHED cells in 3D spheroids is a timely and relevant research topic.

The transforming growth factor β (TGF-β) superfamily plays a crucial role in regulating numerous cellular processes in MSCs. The signaling process begins with TGF-β factors binding to a cell surface receptor complex consisting of two pairs of receptor serine/threonine kinases, specifically types I and II. The type II receptor then phosphorylates the type I receptor, which induces the signal by phosphorylating receptor-activated Smad (R-Smads) proteins, including Smad2/3 (TGF-β-responsive Smads) and Smad1/5/8 (bone morphogenetic protein (BMP)-responsive Smads). R-Smad proteins are primarily present in the cytoplasm [51]. Once activated, R-Smad proteins form a trimeric complex with Smad4 in the cytoplasm and nucleus, which then enters the nucleus to regulate gene expression. Studies have shown that TGF-β-responsive Smads can bind to the NANOG proximal promoter, and recent research indicates that TGF-β/Smad signaling enhances the activity of the NANOG promoter to sustain the stemness properties of human embryonic stem cells [52]. In addition to human ESCs, studies have reported that TGF-β/Smad signaling can regulate the expression of NANOG in mouse pluripotent stem cells derived from the epiblast layer of pre-gastrula stage mouse embryos (EpiSCs) [53].

Furthermore, MSCs cultured in 3D spheroid formation have been shown to preserve the epigenetic status of pluripotent genes [54]. Additionally, they synthesize significantly higher levels of TGF-β or BMP2 compared to monolayer cultures, leading to the activation of the TGF-β/Smad signaling pathway [55, 56]. Herein, we hypothesize that the TGF-β/Smad signaling pathway plays a crucial role in altering pluripotent gene expressions in MSCs cultured in 3D spheroids. Therefore, we investigated the impact of 3D spheroid formation on pluripotency and analyzed the function of the TGF-β/Smad signaling pathway in modifying the epigenetic status of pluripotent genes. By understanding the mechanisms behind the main-

tenance of pluripotency in SHED cultured in 3D spheroids, we hope to make significant contributions to the therapeutic applications of MSCs in regenerative medicine and tissue engineering.

2. Materials and methods

2.1 Cell isolation and culture

SHEDs were isolated from clinically extracted deciduous teeth of three healthy patients aged 6–9 years old. The isolation procedure was performed using a standard enzymatic digestion protocol, and the isolated SHEDs were characterized according to the method described by Miura *et al.* [57]. SHED was cultured in Eagle's minimum essential medium alpha modification (α-MEM; GIBCO, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA) and 1% penicillin-streptomycin solution (Invitrogen, Carlsbad, CA, USA) at 37 °C in 5% carbon dioxide (CO_2). The cells were sub-cultured at a ratio of 1:6 when they reached 80% confluence, and the culture medium was changed every 2–3 days. SHED of passages 4–5 were used for all experiments.

2.2 3D spheroid formation

SHED were cultured in a low attachment 6-well plate (Corning, Corning, NY, USA) at a density of 100,000 cells/well. For comparison, cells were also cultured in standard plastic dishes (Corning, Corning, NY, USA) as a control. The SHED spheroid colonies were harvested for further assays at 1, 3 and 5 days (n = 3). The number and size of spheroids were evaluated under an inverted microscope with 10× magnification at two-time points: 3 and 5 days. Three independent experiments were conducted, and for each observation, six randomly selected fields of a 6-well plate were used. The number of spheroids was counted, and the size of the spheroids was measured in each field. To assess cell proliferation, a cholecystokinin octapeptide (CCK-8)-based assay was performed (Dojindo Molecular Technologies, Inc. Kumamoto, Japan) following the manufacturer's protocol.

2.3 Enzyme-linked immunosorbent assay (ELISA)

The supernatant was collected from SHED cultured in spheroids or monolayers for 1, 3 and 5 days and stored at −80 °C for later experiments. To assess the levels of secreted TGF-β, the supernatants were centrifuged at 15,000 × g for 10 minutes at 4 °C, and a Human TGF-β ELISA kit (R&D Systems, Minneapolis, MN, USA) was used according to the manufacturer's instructions.

2.4 Real-time quantitative PCR (qRT-PCR) analysis

To analyze the gene expression, SHEDs were cultured in spheroids or monolayers for 1, 3 and 5 days before being harvested. To investigate the role of TGF-β/Smad signaling pathways in 3D spheroid formation, the specific inhibitor of Smad3 (SIS3, 2 μM) was added to the low attachment culture plate according to the manufacturer's instructions. Total RNA was extracted from spheroids and monolayers using TRIzol® reagent (Invitrogen, Burlington, ON, Canada). The expression levels of pluripotency markers, including OCT4, NANOG, SOX2 and TGF-β, were analyzed using real-time quantitative polymerase chain reaction (PCR). cDNA was synthesized from RNA using the PrimScriptTM II 1st strand cDNA Synthesis Kit (TaKaRa, Kyoto, Japan). Real-time PCR was performed to amplify the cDNA of target genes using the SYBR Premix Ex Taq II Kit (TaKaRa, Dalian, China) and primers listed in Table 1.

TABLE 1. Primer sequences used for the real-time quantitative PCR.

Target gene	Primer sequences
GAPDH	
Forward	5′-GGCATGGACTGTGGTCATGAG-3′
Reverse	5′-TGCACCACCAACTGCTTAGC-3′
OCT4	
Forward	5′-AGCAAAACCCGGAGGAGT-3′
Reverse	5′-CTATATGTGTCCGGCTACACC-3′
NANOG	
Forward	5′-CCTGTGATTTGTGGGCCTG-3′
Reverse	5′-GACAGTCTCCGTGTGAGGCAT-3′
SOX2	
Forward	5′-GCCGAGTGGAAACTTTTGTCG-3′
Reverse	5′-GGCAGCGTGTACTTATCCTTCT-3′
TGF-β	
Forward	5′-CTAATGGTGGAAACCCACAACG-3′
Reverse	5′-TATCGCCAGGAATTGTTGCTG-3′

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; OCT4: octamer-binding transcription factor 4; SOX2: SRY-box transcription factor 2; TGF-β: Transforming growth factor beta.

2.5 Western blot analysis

After culturing SHED in either spheroids or monolayers, they were washed with Dulbecco's phosphate-buffered saline (DPBS) and treated with radioimmunoprecipitation assay (RIPA) buffer (Thermo, Rockford, IL, USA) supplemented with a protease inhibitor cocktail (Thermo, Rockford, IL, USA). The supernatants were collected from the lysates after centrifugation at 12,000 × g for 15 minutes. Then, we used a PierceTM BCA Protein Assay Kit (Thermo, Janakpuri, DL, India) to determine the protein concentrations. Proteins were separated by Sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% nonfat dry milk (wt/vol) in 1% Tris Buffered Saline Tween-20 (TBST) solution. Antibodies against phospho-Smad3, Smad2, Smad3, Smad4 and β-actin obtained from Cell Signaling Technology were used for Western blotting. After overnight incubation with primary antibodies at 4

°C and extensive washing in TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Abcam, Cambridge, UK) for 1 hour. Proteins were detected by chemiluminescence using an ECL solution (ATTO Corporation, Tokyo, Japan).

2.6 Immunofluorescence

The spheroids were washed and fixed in 4% paraformaldehyde at 4 °C overnight on the 5th day of culture. Then, they were treated with 1× Polybutylene Succinate (PBS) containing 1% Triton X-100 (Sigma-Aldrich, Rehovot, Israel) and 10% FBS at room temperature for 1 hour. Next, primary antibodies against phospho-Smad3 and Smad3 (Abcam, Cambridge, UK) were added and incubated overnight at 4 °C. After washing with 1× PBS, samples were incubated with a secondary antibody, goat anti-rabbit IgG-Alexa-488-conjugated (Invitrogen, Eugene, OR, USA), in the dark for 2 hours at room temperature. Their nuclei were counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Santa Cruz Biotechnology, Santa Cruz, CA, USA), and images were captured using a Leica confocal microscopy system (version 2.61, Leica, Wetzlar, Germany).

2.7 Statistics

The analyses were performed using SPSS statistical software (version 20.0, IBM Corp., Armonk, NY, USA). The number of samples used in the experiments is indicated in each figure legend, and the data are shown as mean ± scanning electron microscopy (SEM). Statistical analyses were performed using repeated-measures one-way analysis of variance (ANOVA) with *post-hoc* Tukey's test for multiple comparisons. Paired *t*-tests were used to compare two sets of independent sample data. A *p*-value of less than 0.05 was considered statistically significant.

3. Results

3.1 Spheroid formation of SHED

The SHED cells cultured in a low attachment 6-well plate began to form multicellular aggregates within 1 day, which then gradually became irregular spheroids (Fig. 1A). By day 3, various sizes of SHED spheroids were observed, and the number of spheroids increased gradually, while the number of suspended cells continuously decreased (Fig. 1B). On day 5, the SHED aggregates formed compact and round-shaped multicellular spheroids, with the number of spheroids plateauing and no obvious suspended cells in the medium (Fig. 1C). In the treatment group with SIS3, the cell morphology, number and size of spheroids were consistent with the cells in the normal medium (Fig. 1D–H).

3.2 TGF-β transcript and secretion levels and proliferation properties of SHED in spheroids and monolayers

3D spheroid formation of MSCs regulates their properties of stemness and multipotency. It was found that TGF-β was involved in targeting regulatory signaling pathways that control MSCs differentiation and lineage fate determination [58]. To test the relative mRNA expressions of TGF-β in SHED spheroids at different time points, the transcript levels of TGF-β were upregulated with time during the 5 days of spheroid culture (Fig. 2A). On the other hand, the mRNA levels of TGF-β decreased significantly over the course of 5 days of monolayer culture (Fig. 2B). Based on the changes in mRNA expressions, ELISA assay demonstrated that secreted TGF-β level was markedly increased in culture medium after 1, 3 and 5 days of 3D spheroid formation (Fig. 2C). The level of secreted TGF-β in the monolayer culture medium increased markedly on day 3 but decreased on day 5 (Fig. 2D). CCK-8 showed that cells in monolayers exhibited more robust proliferative capacity; statistical differences were observed from day 1 to day 5. However, cell proliferation of SHED in spheroids was inhibited in both groups with and without SIS3 (Fig. 2E).

3.3 Expression of pluripotent markers of SHED in spheroids and monolayers

To investigate the effects of 3D spheroid culture on the pluripotency of SHED, the expression levels of pluripotency genes were analyzed after 5 days of culture (Fig. 3A–C). The results showed that the expression patterns of OCT4, NANOG and SOX2 in SHED spheroids tended to increase on days 3 and 5 compared to day 1. Interestingly, the mRNA expression levels of OCT4, NANOG and SOX2 were significantly reduced in SHED spheroids treated with SIS3, indicating that the TGF-β/Smad signaling pathway mediated the maintenance of pluripotency markers in SHED spheroids. In contrast, the mRNA expression patterns of pluripotency genes in SHED cultured in monolayers for 5 days differed from those in SHED spheroids (Fig. 3D). Then, OCT4, NANOG and SOX2 mRNA levels of SHED in monolayers were examined by qRT-PCR. The results indicated that the mRNA expression levels of OCT4 did not change significantly, while NANOG mRNA levels were significantly decreased on days 3 and 5 compared to day 1. The mRNA expression levels of SOX2 were significantly reduced on day 3 but slightly upregulated on day 5 compared to day 1.

3.4 3D spheroid formation regulate the TGF-β/Smad signaling pathway

To investigate the potential involvement of the TGF-β/Smad signaling pathway in maintaining pluripotency of SHED in 3D spheroids, we performed Western blot analysis to assess the expression levels of Smad2, Smad3, phospho-Smad3 (P-Smad3) and Smad4. The results showed that the expression of P-Smad3 was significantly increased in SHED cultured for 5 days in 3D spheroids compared to the SIS3 treatment and 2D monolayer groups, indicating the initiation of the TGF-β/Smad signaling pathway in 3D spheroid formation. Similarly, protein expressions of Smad2 and Smad4 were significantly enhanced in the 3D spheroids group compared to the other two groups. However, the protein level of Smad3 was not changed (Fig. 4). These findings suggest that 3D spheroid formation of SHED activates the TGF-β/Smad signaling pathway, resulting in increased expression levels of P-Smad3, OCT4, NANOG and SOX2, which help to maintain

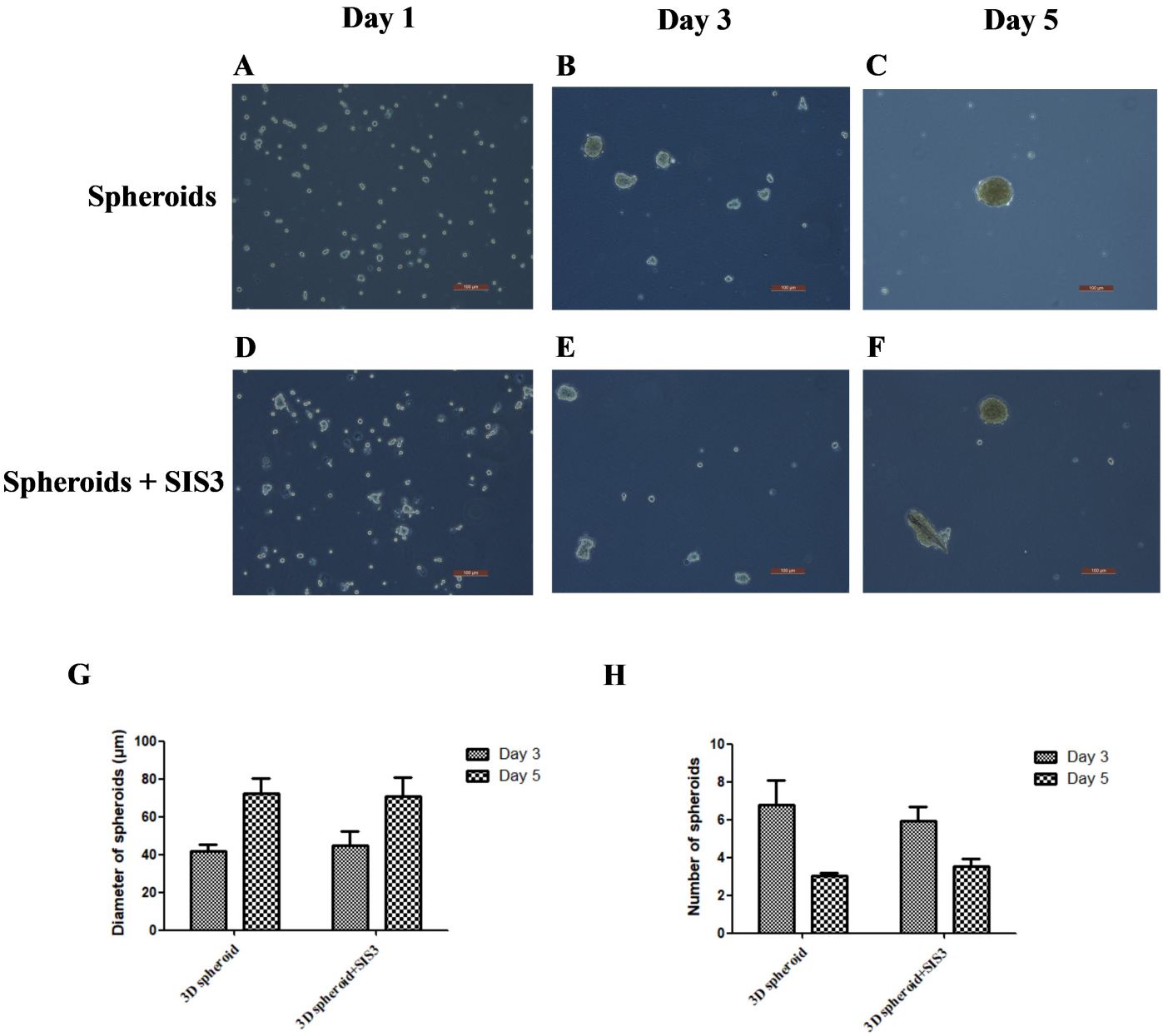

FIGURE 1. The morphologic characteristics of SHED spheroids. (A–C): Morphology of SHED spheroids cultured in 3D spheroid formation on days 1 (A), 3 (B) and 5 (C). (D–F): Appearance of SHED spheroids cultured in 3D spheroid formation with SIS3 on days 1 (D), 3 (E) and 5 (F). The scale bar represents 100 μm. The average diameter of spheroids increased on days 3 and 5 without statistically significant differences in the two different groups (G). Although a decrease in spheroid number was observed between days 3 and 5, no significant difference was observed between the groups of 3D spheroid formation with or without SIS3 (H). 3D: three-dimensional. SIS3: specific inhibitor of Smad3.

the stemness properties.

3.5 Representative immunostaining of Smads in SHED spheroids

To determine the impact of 3D spheroid formation on Smads, immunofluorescence assays were performed on spheroids with or without SIS3 treatment. The results showed that in both groups of spheroids, P-Smad3 was primarily present in the nucleus, indicating activation of the TGF-β/Smad signaling pathway. Treatment with SIS3 suppressed the nuclear accumulation of P-Smad3, while Smad3 proteins were predominantly present in the cytoplasm (Fig. 5). These findings suggest that 3D spheroid formation activates the TGF-β/Smad signaling pathway in SHED.

4. Discussion

MSCs can be used in regenerative medicine and tissue engineering as a cell-based therapy for treating various clinical conditions, including age-related orthopedic degenerative diseases and autoimmune and inflammatory diseases [59, 60]. MSCs expressing pluripotency markers of ESCs, such as OCT4, NANOG and SOX2, make them promising candidates

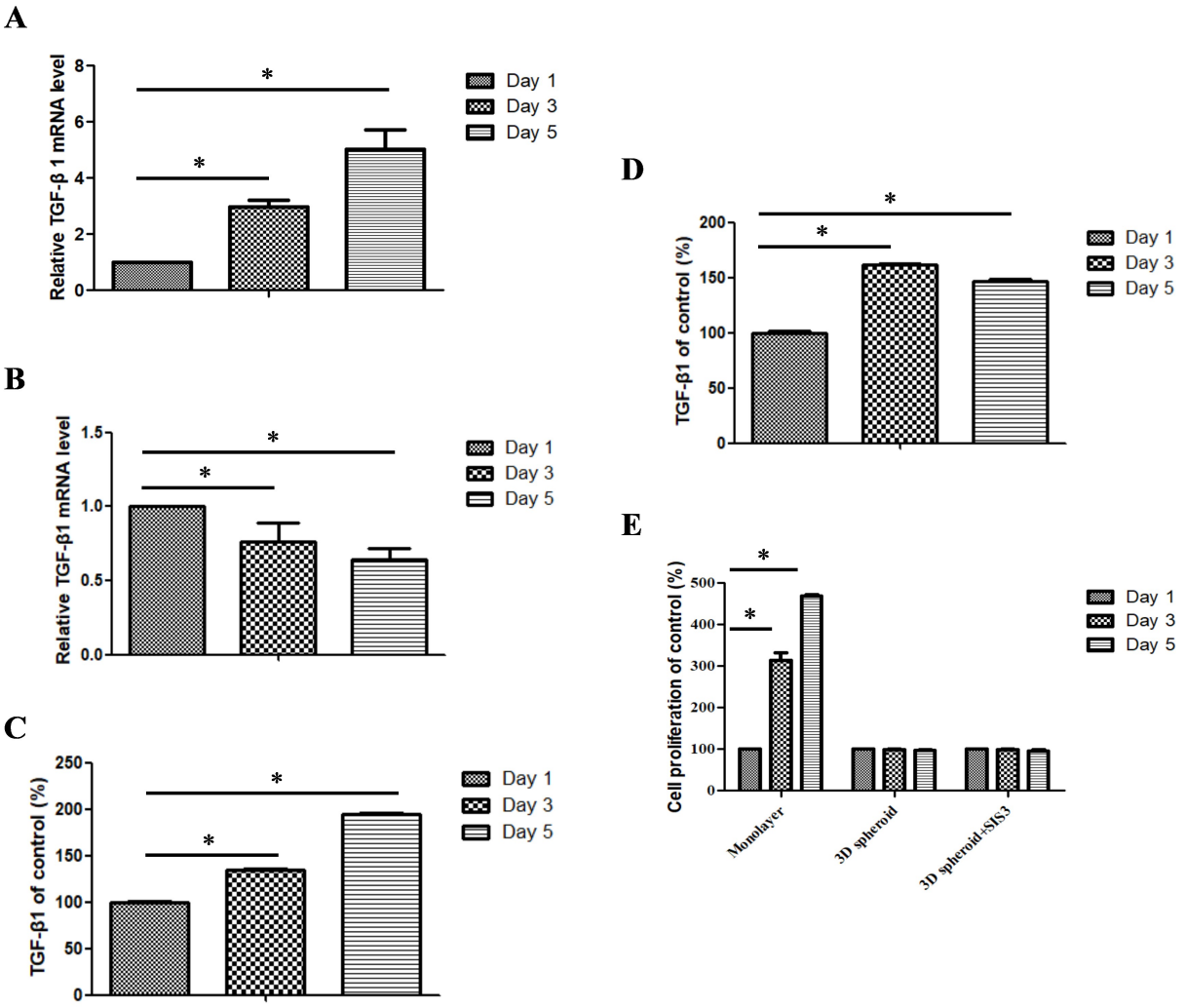

FIGURE 2. Relative mRNA expressions of TGF-β, soluble TGF-β level and cell proliferation of SHED in spheroids and monolayers. (A) the mRNA levels of TGF-β were markedly increased with time during the 5 days of 3D spheroid culture. (B) the mRNA levels of TGF-β were significantly decreased with time during the 5 days of monolayer culture. (C) ELISA experiments were conducted to target soluble TGF-β1 in culture medium after 1, 3 and 5 days of 3D spheroid formation (n = 9, 3 independent experiments, each with 3 technical repeats). (D) soluble TGF-β1 in the culture medium was measured *via* ELISA experiments on days 1, 3 and 5 of monolayer culture. (E) the proliferation of SHED was evaluated by CCK-8 assay on days 1, 3 and 5 in both monolayers and spheroids with or without SIS3 treatment (*$p < 0.05$). TGF-β: transforming growth factor β.

for clinical applications in tissue regeneration medicine [61]. However, the *ex vivo* expansion of MSCs can lead to the loss of stem cell properties and a rapid decline in pluripotent gene expression [62].

Recent research suggests that the formation of 3D spheroids enhances and maintains the stemness properties of MSCs [29]. In this study, SHED were cultured in a low attachment 6-well plate and formed compact, round-shaped multicellular aggregates 5 days after seeding. The expression of pluripotent genes (OCT4, SOX2 and NANOG) in SHED increased over time as they were cultured in spheroids for 5 days. Although the exact molecular mechanisms responsible for the upregulation of pluripotent genes in SHED spheroids are not fully understood and remain an area of active research, several theories explain the alterations in the epigenetic status of pluripotent genes in SHED under 3D spheroid culture conditions. Due to reduced oxygen and nutrient supply, the inside of spheroids may become apoptotic and hypoxic [49, 63]. Yamamoto *et al.* [64] reported that hypoxic and apoptotic cells were present in the center of 3D spheroid aggregations. Hypoxia-inducible factors (HIFs) are key transcription factors of hypoxia-associated genes. Studies have shown that spheroids from mouse MSCs have higher expression of hypoxia-inducible factor-2α (HIF-2α), which plays a critical role in influencing the pluripotency of MSCs [63, 65]. The hypoxic microenvironment in the inner layers of 3D spheroids has been suggested to promote stemness properties by restoring MSCs to a more primitive state [66]. Furthermore, hypoxia has been shown to stimulate the release of extracellular vesicles by human MSCs [67, 68]. For instance, MSC spheroids exhibited higher levels of 370, miR-433 and miR-489, which are responsible for maintaining the quiescent state of adult stem cells [54, 69, 70]. Additionally, SHED spheroids were formed on non-adherent culture conditions that provide lower cytoskeletal

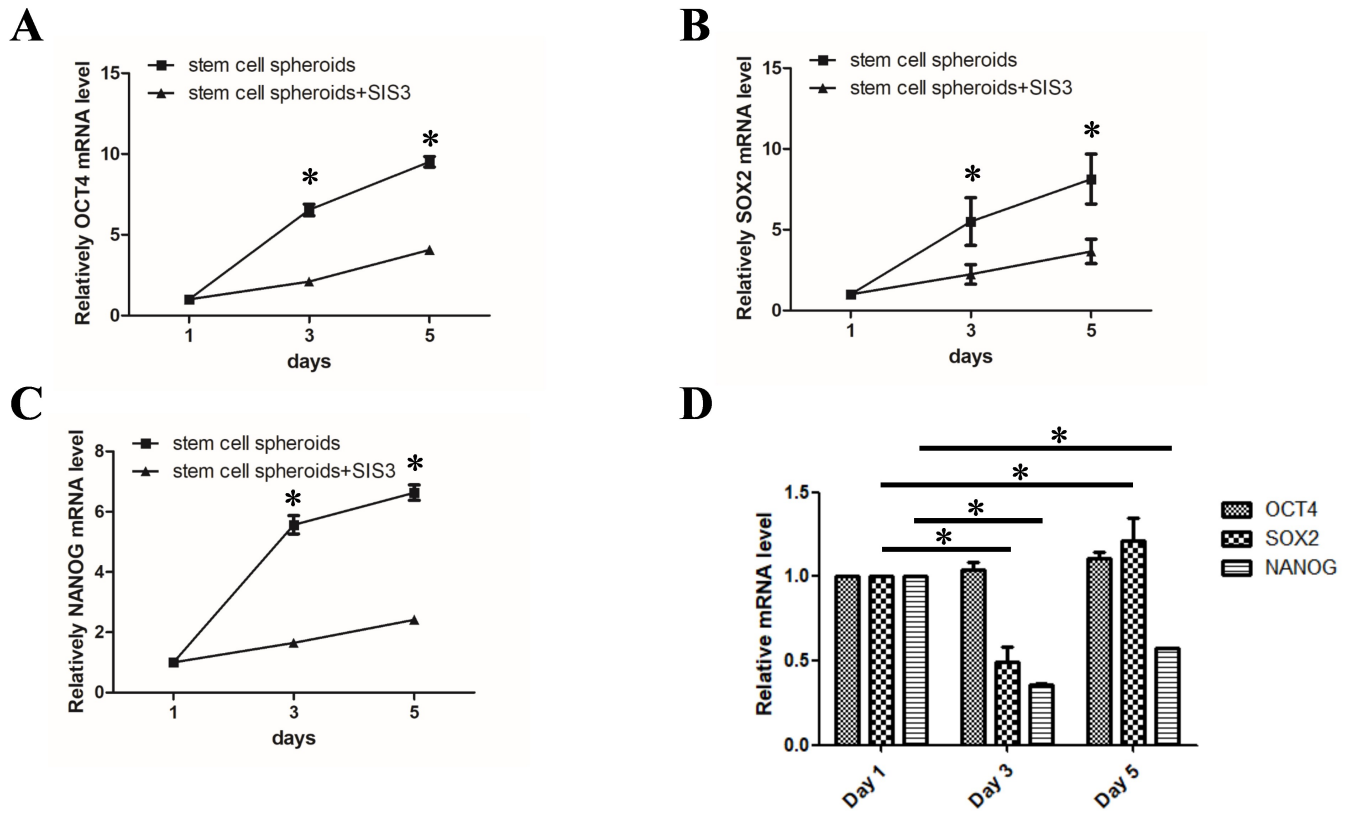

FIGURE 3. Relative mRNA expressions of pluripotency markers of OCT4, SOX2 and NANOG of SHED in spheroids and monolayers treated with or without SIS3. (A): The mRNA expression levels of OCT4 in SHED spheroids were significantly reduced when treated with SIS3 compared to the untreated group during the 5 days of 3D spheroid culture. (B): The mRNA expression levels of SOX2 in SHED spheroids were lower when treated with SIS3 compared to the untreated group during the 5 days of 3D spheroid culture. (C): The mRNA expression levels of NANOG in SHED spheroids were considerably reduced when treated with SIS3 compared to the untreated group during the 5 days of 3D spheroid culture. (D): The mRNA expression levels of OCT4, NANOG and SOX2 were measured on days 1, 3 and 5 of monolayer culture. (*$p < 0.05$). SIS3: specific inhibitor of Smad3. OCT4: octamer-binding transcription factor 4. SOX2: SRY-box transcription factor 2.

tension of cells, contributing to the preservation or enrichment of stemness in MSCs [71, 72].

The 3D spheroid culture of SHED creates a cellular niche that more closely mimics the *in vivo* microenvironment [39]. The cellular niche of spheroids is presented in the ECM, facilitating cell-cell or cell-ECM interactions much more accessible than in monolayers [49]. This spheroid environment in the niche can help maintain stem cell quiescence, prevent differentiation and downregulate cell cycle progression genes [73]. Moreover, the spheroid culture of MSCs leads to a significant increase in ECM secretion, which is a heterogeneous network of proteins and polysaccharides that transmit physical and biological signals and can control stem cell fate [74, 75]. More importantly, besides determining specific lineage differentiation, ECM serves as a reservoir of growth factors with paracrine activity, playing an essential role in the beneficial effects of MSCs [39, 76, 77]. Paracrine secretion is one of the important mechanisms for the therapeutic application of MSCs in regenerative medicine and tissue engineering. Cytokines produced by MSCs in 3D spheroids exhibit multiple beneficial functions [78]. The 3D spheroid culture of MSCs promotes the paracrine secretion of therapeutic cytokines such as anti-inflammatory protein TSG-6 and angiogenic factors (VEGF and basic fibroblast growth factor (bFGF)), pro-inflammatory cytokines interleukin 1A (IL1A)/IL-1α, interleukin-1β (IL1B)/IL-1β, and interleukin 8 (IL8), anit-apoptotic factors (epidermal growth factor (EGF) and hepatocyte growth factor (HGF)), and the anti-oxidative factor IGF [12, 40]. As members of the transforming growth factors, the TGF-β signaling family controls the expression and activities of key transcription factors closely involved in the modulation of stem cell fate [79, 80]. Our study showed that 3D spheroid formation significantly increased the secretion and mRNA levels of TGF-β, as well as activated the TGF-β/Smad signaling pathway in SHED. The increased immunomodulatory activity of human MSCs in spheroids may be attributed to enhanced intercellular adhesions and cell contact-dependent signaling, which have also been associated with the heightened paracrine activity of MSC aggregates [81, 82].

TGF-β/Smad signals are known to play a crucial role in maintaining the pluripotent state of embryonic stem cells (ESCs) by regulating the expression of stem cell-specific markers [83]. In this study, we demonstrate that 3D spheroid formation induces upregulation of P-Smad3 expression in SHED and increases the expression levels of OCT4, NANOG and SOX2, primarily through the canonical

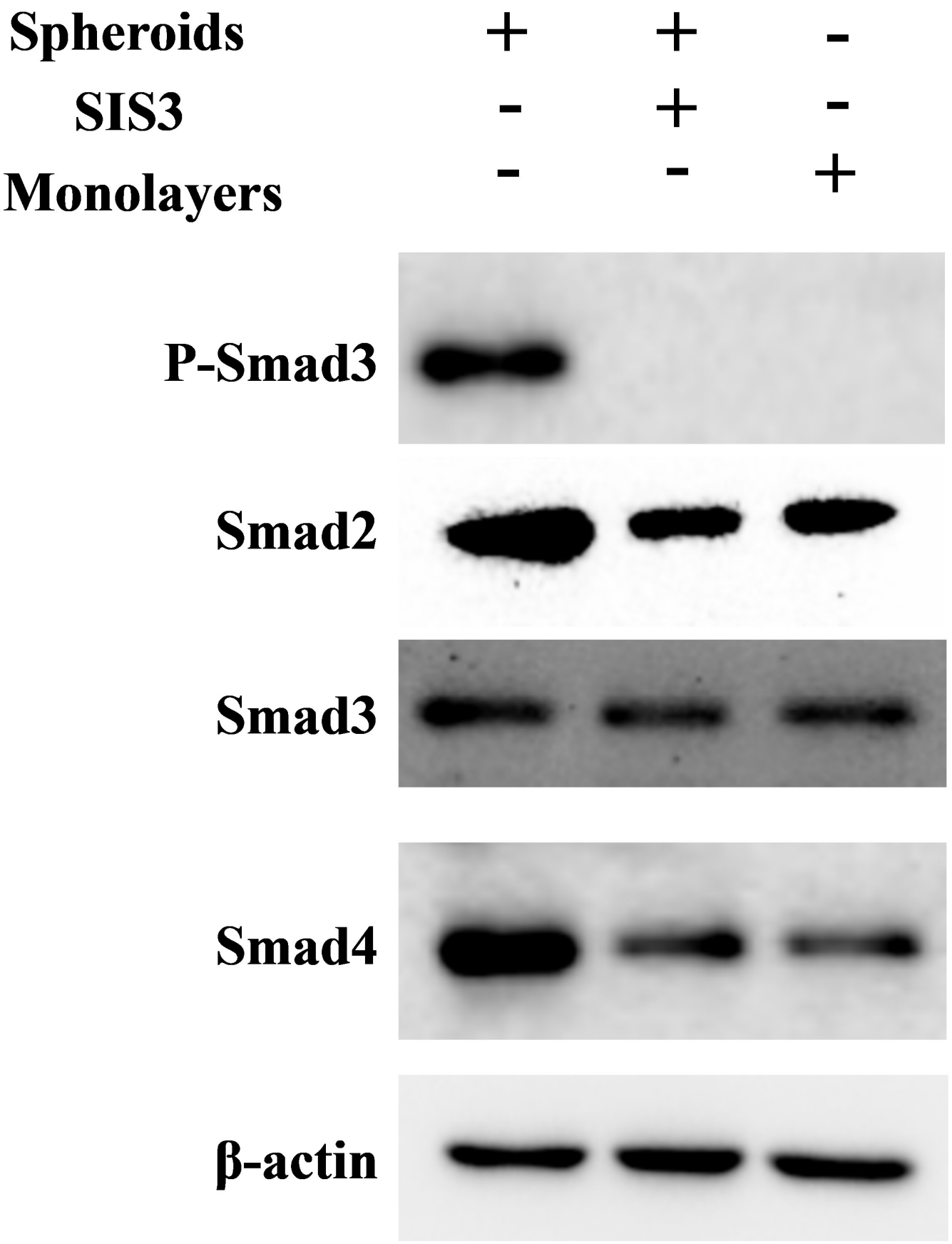

FIGURE 4. Western blot analysis of the expressions of Smads in monolayers and spheroid-cultured treated with or without SIS3 for 5 days. P-Smad3: phospho-Smad3. SIS3: specific inhibitor of Smad3.

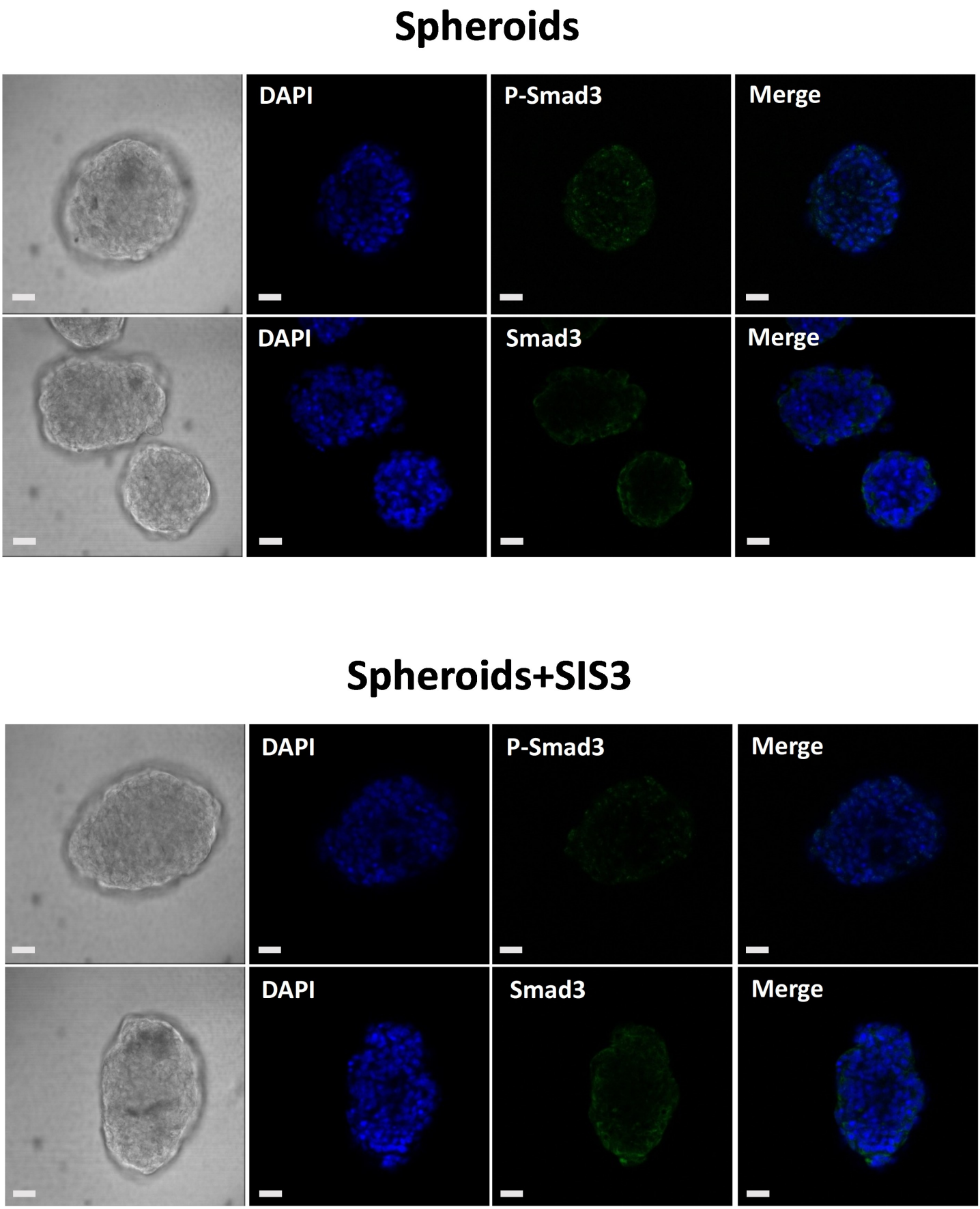

FIGURE 5. Represent images of p-Smad3 nuclear translocation and localization of Smad3, assayed by immunofluorescence confocal microscopy in spheroids. Nuclei, DAPI (blue) and Smads (green). Scale bar: 20 μm.

Smad3 signaling pathway. Although the precise mechanism underlying the activation of TGF-β/Smad signaling by these interactions is unclear, the results suggest that TGF-β/Smad signaling involves a priming process that enhances the stemness properties of SHED in spheroids. TGF-β-mediated phosphorylation of Smad3 on spheroids is essential for TGF-β/Smad signal transduction. Previous studies have reported that Smads can bind to the promoter of pluripotent genes and regulate their expression [52]. However, it is uncertain whether the binding of Smads to target genes occurs through the formation of complexes with other transcription factors, and the functional significance of any competition between SMAD2/3 and Smad1/5/8 for the cofactor Smad4 remains unclear.

5. Conclusions

In summary, this study investigated the regulation of stemness in SHED cultured in 3D spheroids. The results revealed that SHED spheroids activated the TGF-β/Smad signaling pathway and enhanced the stemness properties of SHED. The higher levels of TGF-β produced by SHED spheroids may contribute to cellular transduction and the multiple beneficial functions of MSCs, thereby indicating that MSC spheroids might have promising therapeutic applications in regenerative medicine and tissue engineering.

AVAILABILITY OF DATA AND MATERIALS

Not applicable.

AUTHOR CONTRIBUTIONS

JX and HWL—designed the research study. WBW and XMS—performed the research. HYK, SQQ, XHZ, QHZ, XHY and XTZ—analyzed the data. JX and JJ—wrote the manuscript. All authors read and approved the final manuscript.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

SHEDs were isolated from patients after obtaining informed consent from their parents at the department of pediatric dentistry of Shenzhen Longgang E.N.T. Hospital (Shenzhen, China). The study was approved by the Ethics Committee of Shenzhen Longgang E.N.T. Hospital (Reference number of approval of the Ethics Committee: ZSSOM No. 2021-0130).

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

[1] Guo H, Zhao W, Liu A, Wu M, Shuai Y, Li B, *et al*. SHED promote angiogenesis in stem cell-mediated dental pulp regeneration. Biochemical and Biophysical Research Communications. 2020; 529: 1158–1164.

[2] Liang C, Liao L, Tian W. Stem cell-based dental pulp regeneration: insights from signaling pathways. Stem Cell Reviews and Reports. 2021; 17: 1251–1263.

[3] Wu M, Liu X, Li Z, Huang X, Guo H, Guo X, *et al.* SHED aggregate exosomes shuttled miR-26a promote angiogenesis in pulp regeneration *via* TGF-β/SMAD2/3 signalling. Cell Proliferation. 2021; 54: e13074.

[4] Xu X, Liang C, Gao X, Huang H, Xing X, Tang Q, *et al*. Adipose tissue–derived microvascular fragments as vascularization units for dental pulp regeneration. Journal of Endodontics. 2021; 47: 1092–1100.

[5] Zhu X, Liu J, Yu Z, Chen CA, Aksel H, Azim AA, *et al*. A miniature swine model for stem cell-based de novo regeneration of dental pulp and dentin-like tissue. Tissue Engineering Part C: Methods. 2018; 24: 108–120.

[6] Iohara K, Utsunomiya S, Kohara S, Nakashima M. Allogeneic transplantation of mobilized dental pulp stem cells with the mismatched dog leukocyte antigen type is safe and efficacious for total pulp regeneration. Stem Cell Research & Therapy. 2018; 9: 116.

[7] Hilkens P, Bronckaers A, Ratajczak J, Gervois P, Wolfs E, Lambrichts I. The angiogenic potential of DPSCs and SCAPs in an *in vivo* model of dental pulp regeneration. Stem Cells International. 2017; 2017: 2582080.

[8] Oubenyahya H. Stem cells from dental pulp of human exfoliated teeth: current understanding and future challenges in dental tissue engineering. Stem Cells International. 2021; 24: 9–20.

[9] Mattei V, Delle Monache S. Delle monache dental pulp stem cells (DPSCs) and tissue regeneration: mechanisms mediated by direct, paracrine, or autocrine effects. Biomedicines. 2023; 11: 386.

[10] Anoop M, Datta I. Stem cells derived from human exfoliated deciduous teeth (SHED) in neuronal disorders: a review. Current Stem Cell Research & Therapy. 2021; 16: 535–550.

[11] Fujii Y, Hatori A, Chikazu D, Ogasawara T. Application of dental pulp stem cells for bone and neural tissue regeneration in oral and maxillofacial region. Stem Cells International. 2023; 2023: 2026572.

[12] Bartosh TJ, Ylöstalo JH, Mohammadipoor A, Bazhanov N, Coble K, Claypool K, *et al*. Aggregation of human mesenchymal stromal cells (MSCs) into 3D spheroids enhances their antiinflammatory properties. Proceedings of the National Academy of Sciences of the United States of America. 2010; 107: 13724–13729.

[13] Sakai VT, Zhang Z, Dong Z, Neiva KG, Machado MAAM, Shi S, *et al*. SHED Differentiate into functional odontoblasts and endothelium. Journal of Dental Research. 2010; 89: 791–796.

[14] Mitrano TI, Grob MS, Carrión F, Nova-Lamperti E, Luz PA, Fierro FS, *et al*. Culture and characterization of mesenchymal stem cells from human gingival tissue. Journal of Periodontology. 2010; 81: 917–925.

[15] Costa MHG, Serra J, McDevitt TC, Cabral JMS, da Silva CL, Ferreira FC. Dimethyloxalylglycine, a small molecule, synergistically increases the homing and angiogenic properties of human mesenchymal stromal cells when cultured as 3D spheroids. Biotechnology Journal. 2021; 16: e2000389.

[16] Shi S, Bartold PM, Miura M, Seo BM, Robey PG, Gronthos S. The

efficacy of mesenchymal stem cells to regenerate and repair dental structures. Orthodontics and Craniofacial Research. 2005; 8: 191–199.

[17] Cordeiro MM, Dong Z, Kaneko T, Zhang Z, Miyazawa M, Shi S, *et al*. Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth. Journal of Endodontics. 2008; 34: 962–969.

[18] Rosa V, Zhang Z, Grande RH, Nör JE. Dental pulp tissue engineering in full-length human root canals. Journal of Dental Research. 2013; 92: 970–975.

[19] Sugiaman VK, Djuanda R, Pranata N, Naliani S, Demolsky WL, Jeffrey. Tissue Engineering with stem cell from human exfoliated deciduous teeth (SHED) and collagen matrix, regulated by growth factor in regenerating the dental pulp. Polymers. 2022; 14: 3712.

[20] Shi X, Mao J, Liu Y. Pulp stem cells derived from human permanent and deciduous teeth: Biological characteristics and therapeutic applications. Stem Cells Translational Medicine. 2020; 9: 445–464.

[21] Wang SK, Komatsu Y, Mishina Y. Potential contribution of neural crest cells to dental enamel formation. Biochemical and Biophysical Research Communications. 2011; 415: 114–119.

[22] Leathers TA, Rogers CD. Time to go: neural crest cell epithelial-to-mesenchymal transition. Development. 2022; 149: dev200712.

[23] Fan H, Li Y, Yuan F, Lu L, Liu J, Feng W, *et al*. Up-regulation of microRNA-34a mediates ethanol-induced impairment of neural crest cell migration *in vitro* and in zebrafish embryos through modulating epithelial-mesenchymal transition by targeting Snail1. Toxicology Letters. 2022; 358: 17–26.

[24] Riekstina U, Cakstina I, Parfejevs V, Hoogduijn M, Jankovskis G, Muiznieks I, *et al*. Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. Stem Cell Reviews and Reports. 2009; 5: 378–386.

[25] Luk ST, Ng KY, Zhou L, Tong M, Wong TL, Yu H, *et al*. Deficiency in embryonic stem cell marker reduced expression 1 activates mitogen-activated protein kinase kinase 6-dependent p38 mitogen-activated protein kinase signaling to drive hepatocarcinogenesis. Hepatology. 2020; 72: 183–197.

[26] Hung TH, Huang Y, Yeh CT, Yeh CN, Yu J, Lin CC, *et al*. High expression of embryonic stem cell marker SSEA3 confers poor prognosis and promotes epithelial mesenchymal transition in hepatocellular carcinoma. To be published in Biomedical Journal. 2023. [Preprint].

[27] Kim M, Kim YH, Tae G. Human mesenchymal stem cell culture on heparin-based hydrogels and the modulation of interactions by gel elasticity and heparin amount. Acta Biomaterialia. 2013; 9: 7833–7844.

[28] Diomede F, Rajan TS, Gatta V, D'Aurora M, Merciaro I, Marchisio M, *et al*. Stemness maintenance properties in human oral stem cells after long-term passage. Stem Cells International. 2017; 2017: 5651287.

[29] Zhang S, Liu P, Chen L, Wang Y, Wang Z, Zhang B. The effects of spheroid formation of adipose-derived stem cells in a microgravity bioreactor on stemness properties and therapeutic potential. Biomaterials. 2015; 41: 15–25.

[30] Bloom AB, Zaman MH. Influence of the microenvironment on cell fate determination and migration. Physiological Genomics. 2014; 46: 309–314.

[31] Chen S, Ikemoto T, Tokunaga T, Okikawa S, Miyazaki K, Yamada S, *et al*. Newly generated 3D Schwann-like cell spheroids from human adipose-derived stem cells using a modified protocol. Cell Transplantation. 2022; 31: 9636897221093312.

[32] Baraniak PR, Cooke MT, Saeed R, Kinney MA, Fridley KM, McDevitt TC. Stiffening of human mesenchymal stem cell spheroid microenvironments induced by incorporation of gelatin microparticles. Journal of the Mechanical Behavior of Biomedical Materials. 2012; 11: 63–71.

[33] Yamada Y, Okano T, Orita K, Makino T, Shima F, Nakamura H. 3D-cultured small size adipose-derived stem cell spheroids promote bone regeneration in the critical-sized bone defect rat model. Biochemical and Biophysical Research Communications. 2022; 603: 57–62.

[34] McBeath R, Pirone DM, Nelson CM, Bhadriraju K, Chen CS. Cell Shape, cytoskeletal tension, and RhoA regulate stem cell lineage commitment. Developmental Cell. 2004; 6: 483–495.

[35] Cesarz Z, Tamama K. Spheroid culture of mesenchymal stem cells. Stem Cells International. 2016; 2016: 9176357.

[36] Vidyasekar P, Shyamsunder P, Sahoo SK, Verma RS. Scaffold-free and scaffold-assisted 3D culture enhances differentiation of bone marrow stromal cells. In Vitro Cellular & Developmental Biology—Animal. 2016; 52: 204–217.

[37] Di Stefano AB, Grisafi F, Perez-Alea M, Castiglia M, Di Simone M, Meraviglia S, *et al*. Cell quality evaluation with gene expression analysis of spheroids (3D) and adherent (2D) adipose stem cells. Gene. 2021; 768: 145269.

[38] Yamada KM, Cukierman E. Modeling tissue morphogenesis and cancer in 3D. Cell. 2007; 130: 601–610.

[39] Kim HJ, Sung IY, Cho YC, Kang MS, Rho GJ, Byun JH, *et al*. Three-dimensional spheroid formation of cryopreserved human dental follicle-derived stem cells enhances pluripotency and osteogenic induction properties. Tissue Engineering and Regenerative Medicine. 2019; 16: 513–523.

[40] Potapova IA, Gaudette GR, Brink PR, Robinson RB, Rosen MR, Cohen IS, *et al*. Mesenchymal stem cells support migration, extracellular matrix invasion, proliferation, and survival of endothelial cells *in vitro*. Stem Cells. 2007; 25: 1761–1768.

[41] Frith JE, Thomson B, Genever PG. Dynamic three-dimensional culture methods enhance mesenchymal stem cell properties and increase therapeutic potential. Tissue Engineering Part C: Methods. 2010; 16: 735–749.

[42] Kim SJ, Kim EM, Yamamoto M, Park H, Shin H. Engineering multi-cellular spheroids for tissue engineering and regenerative medicine. To be published in Advanced Healthcare Materials. 2020. [Preprint].

[43] Chen LC, Wang HW, Huang CC. Modulation of inherent niches in 3D multicellular MSC spheroids reconfigures metabolism and enhances therapeutic potential. Cells. 2021; 10: 2747.

[44] Choi J, Choi W, Joo Y, Chung H, Kim D, Oh SJ, *et al.* FGF2-primed 3D spheroids producing IL-8 promote therapeutic angiogenesis in murine hindlimb ischemia. NPJ Regenerative Medicine. 2021; 6: 48.

[45] Hosseini V, Maroufi NF, Saghati S, Asadi N, Darabi M, Ahmad SNS, *et al*. Current progress in hepatic tissue regeneration by tissue engineering. Journal of Translational Medicine. 2019; 17: 383.

[46] Macková H, Hlídková H, Kaberova Z, Proks V, Kučka J, Patsula V, *et al*. Thiolated poly (2-hydroxyethyl methacrylate) hydrogels as a degradable biocompatible scaffold for tissue engineering. Materials Science and Engineering: C. 2021; 131: 112500.

[47] Chang PC, Lin ZJ, Luo HT, Tu CC, Tai WC, Chang CH, *et al*. Degradable RGD-functionalized 3D-printed scaffold promotes osteogenesis. Journal of Dental Research. 2021; 100: 1109–1117.

[48] Williams DF. On the mechanisms of biocompatibility. Biomaterials. 2008; 29: 2941–2953.

[49] Cheng NC, Wang S, Young TH. The influence of spheroid formation of human adipose-derived stem cells on chitosan films on stemness and differentiation capabilities. Biomaterials. 2012; 33: 1748–1758.

[50] Kasprzak C, Brown JR, Feller K, Scott PJ, Meenakshisundaram V, Williams C, *et al*. Vat photopolymerization of reinforced Styrene-Butadiene elastomers: a degradable scaffold approach. ACS Applied Materials & Interfaces. 2022; 14: 18965–18973.

[51] Li SN, Wu JF. TGF-β/SMAD signaling regulation of mesenchymal stem cells in adipocyte commitment. Stem Cell Research & Therapy. 2020; 11: 41.

[52] Xu RH, Sampsell-Barron TL, Gu F, Root S, Peck RM, Pan G, *et al*. NANOG is a direct target of TGF-β/activin-mediated SMAD signaling in human ESCs. Cell Stem Cell. 2008; 3: 196–206.

[53] Vallier L, Mendjan S, Brown S, Chng Z, Teo A, Smithers LE, *et al*. Activin/Nodal signalling maintains pluripotency by controlling Nanog expression. Development. 2009; 136: 1339–1349.

[54] Guo L, Zhou Y, Wang S, Wu Y. Epigenetic changes of mesenchymal stem cells in three-dimensional (3D) spheroids. Journal of Cellular and Molecular Medicine. 2014; 18: 2009–2019.

[55] Han X, Tang S, Wang L, Xu X, Yan R, Yan S, *et al.* Multicellular spheroids formation on hydrogel enhances osteogenic/odontogenic differentiation of dental pulp stem cells under magnetic nanoparticles induction. International Journal of Nanomedicine. 2021; 16: 5101–5115.

[56] Stuart MP, Matsui RAM, Santos MFS, Côrtes I, Azevedo MS, Silva KR, *et al*. Successful low-cost scaffold-free cartilage tissue engineering using human cartilage progenitor cell spheroids formed by micromolded nonadhesive hydrogel. Stem Cells International. 2017; 2017: 7053465.

[57] Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, *et al*. SHED: stem cells from human exfoliated deciduous teeth. Proceedings of the National Academy of Sciences of the United States of America. 2003; 100: 5807–5812.

[58] Elsafadi M, Shinwari T, Al-Malki S, Manikandan M, Mahmood A, Aldahmash A, *et al.* Convergence of TGF-β and BMP signaling in regulating human bone marrow stromal cell differentiation. Scientific Reports. 2019; 9: 4977.

[59] Kim D, Lee AE, Xu Q, Zhang Q, Le AD. Gingiva-derived mesenchymal stem cells: potential application in tissue engineering and regenerative medicine—a comprehensive review. Frontiers in Immunology. 2021; 12: 667221.

[60] Zhou H, He Y, Xiong W, Jing S, Duan X, Huang Z, *et al*. MSC based gene delivery methods and strategies improve the therapeutic efficacy of neurological diseases. Bioactive Materials. 2022; 23: 409–437.

[61] Prasajak P, Rattananinsruang P, Chotinantakul K, Dechsukhum C, Leeanansaksiri W. Embryonic stem cells conditioned medium enhances Wharton's jelly-derived mesenchymal stem cells expansion under hypoxic condition. Cytotechnology. 2015; 67: 493–505.

[62] Park E, Patel AN. Changes in the expression pattern of mesenchymal and pluripotent markers in human adipose-derived stem cells. Cell Biology International. 2010; 34: 979–984.

[63] Chen K, Li X, Li N, Dong H, Zhang Y, Yoshizawa M, *et al*. Spontaneously formed spheroids from mouse compact bone-derived cells retain highly potent stem cells with enhanced differentiation capability. Stem Cells International. 2019; 2019: 8469012.

[64] Yamamoto M, Kawashima N, Takashino N, Koizumi Y, Takimoto K, Suzuki N, *et al*. Three-dimensional spheroid culture promotes odonto/osteoblastic differentiation of dental pulp cells. Archives of Oral Biology. 2014; 59: 310–317.

[65] Drela K, Sarnowska A, Siedlecka P, Szablowska-Gadomska I, Wielgos M, Jurga M, *et al*. Low oxygen atmosphere facilitates proliferation and maintains undifferentiated state of umbilical cord mesenchymal stem cells in an hypoxia inducible factor-dependent manner. Cytotherapy. 2014; 16: 881–892.

[66] Gustafsson MV, Zheng X, Pereira T, Gradin K, Jin S, Lundkvist J, *et al*. Hypoxia requires notch signaling to maintain the undifferentiated cell state. Developmental Cell. 2005; 9: 617–628.

[67] Dong L, Wang Y, Zheng T, Pu Y, Ma Y, Qi X, *et al*. Hypoxic hUCMSC-derived extracellular vesicles attenuate allergic airway inflammation and airway remodeling in chronic asthma mice. Stem Cell Research & Therapy. 2021; 12: 4.

[68] Gorgun C, Ceresa D, Lesage R, Villa F, Reverberi D, Balbi C, *et al*. Dissecting the effects of preconditioning with inflammatory cytokines and hypoxia on the angiogenic potential of mesenchymal stromal cell (MSC)-derived soluble proteins and extracellular vesicles (EVs). Biomaterials. 2021; 269: 120633.

[69] Guo L, Zhao RCH, Wu Y. The role of microRNAs in self-renewal and differentiation of mesenchymal stem cells. Experimental Hematology. 2011; 39: 608–616.

[70] Cheung TH, Quach NL, Charville GW, Liu L, Park L, Edalati A, *et al*. Maintenance of muscle stem-cell quiescence by microRNA-489. Nature. 2012; 482: 524–528.

[71] Zhang D, Kilian KA. The effect of mesenchymal stem cell shape on the maintenance of multipotency. Biomaterials. 2013; 34: 3962–3969.

[72] Jozkowiak M, Hutchings G, Jankowski M, Kulcenty K, Mozdziak P, Kempisty B, *et al.* The stemness of human ovarian granulosa cells and the role of resveratrol in the differentiation of MSCs—a review based on cellular and molecular knowledge. Cells. 2020; 9: 1418.

[73] Moritani Y, Usui M, Sano K, Nakazawa K, Hanatani T, Nakatomi M, *et al*. Spheroid culture enhances osteogenic potential of periodontal ligament mesenchymal stem cells. Journal of Periodontal Research. 2018; 53: 870–882.

[74] Gonzalez-Fernandez T, Tenorio AJ, Saiz AM Jr, Leach JK. Engineered cell-secreted extracellular matrix modulates cell spheroid mechanosensing and amplifies their response to inductive cues for the formation of mineralized tissues. Advanced Healthcare Materials. 2022; 11: e2102337.

[75] Raik S, Sharma P, Kumar S, Rattan V, Das A, Kumar N, *et al*. Three-dimensional spheroid culture of dental pulp-derived stromal cells enhance their biological and regenerative properties for potential therapeutic applications. The International Journal of Biochemistry & Cell Biology. 2023; 160: 106422.

[76] Liu F, Qiu H, Xue M, Zhang S, Zhang X, Xu J, *et al.* MSC-secreted TGF-β regulates lipopolysaccharide-stimulated macrophage M2-like polarization *via* the Akt/FoxO1 pathway. Stem Cell Research & Therapy. 2019; 10: 345.

[77] Ashraf R, Sofi HS, Sheikh FA. Experimental protocol of MSC differentiation into neural lineage for nerve tissue regeneration using polymeric scaffolds. Methods in Molecular Biology. 2020; 2125: 109–117.

[78] Xu Y, Shi T, Xu A, Zhang L. 3D spheroid culture enhances survival and therapeutic capacities of MSCs injected into ischemic kidney. Journal of Cellular and Molecular Medicine. 2016; 20: 1203–1213.

[79] Grafe I, Alexander S, Peterson JR, Snider TN, Levi B, Lee B, *et al.* TGF-β family signaling in mesenchymal differentiation. Cold Spring Harbor Perspectives in Biology. 2018; 10: a022202.

[80] de la Grange P, Jolly A, Courageux C, Ben Brahim C, Leroy P. Genes coding for transcription factors involved in stem cell maintenance are repressed by TGF-β and downstream of Slug/Snail2 in COPD bronchial epithelial progenitors. Molecular Biology Reports. 2021; 48: 6729–6738.

[81] Zimmermann JA, Mcdevitt TC. Pre-conditioning mesenchymal stromal cell spheroids for immunomodulatory paracrine factor secretion. Cytotherapy. 2014; 16: 331–345.

[82] Mochizuki Y, Kogawa R, Takegami R, Nakamura K, Wakabayashi A. Co-microencapsulation of islets and MSC CellSaics, mosaic-like aggregates of MSCs and recombinant peptide pieces, and therapeutic effects of their subcutaneous transplantation on diabetes. Biomedicines. 2020; 8: 318.

[83] Itoh F, Watabe T, Miyazono K. Roles of TGF-β family signals in the fate determination of pluripotent stem cells. Seminars in Cell & Developmental Biology. 2014; 32: 98–106.

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