Stem Cells International

Molecules and Biomaterial Technologies Affecting Stem Cell Differentiation

Lead Guest Editor: Lorenzo Lo Muzio
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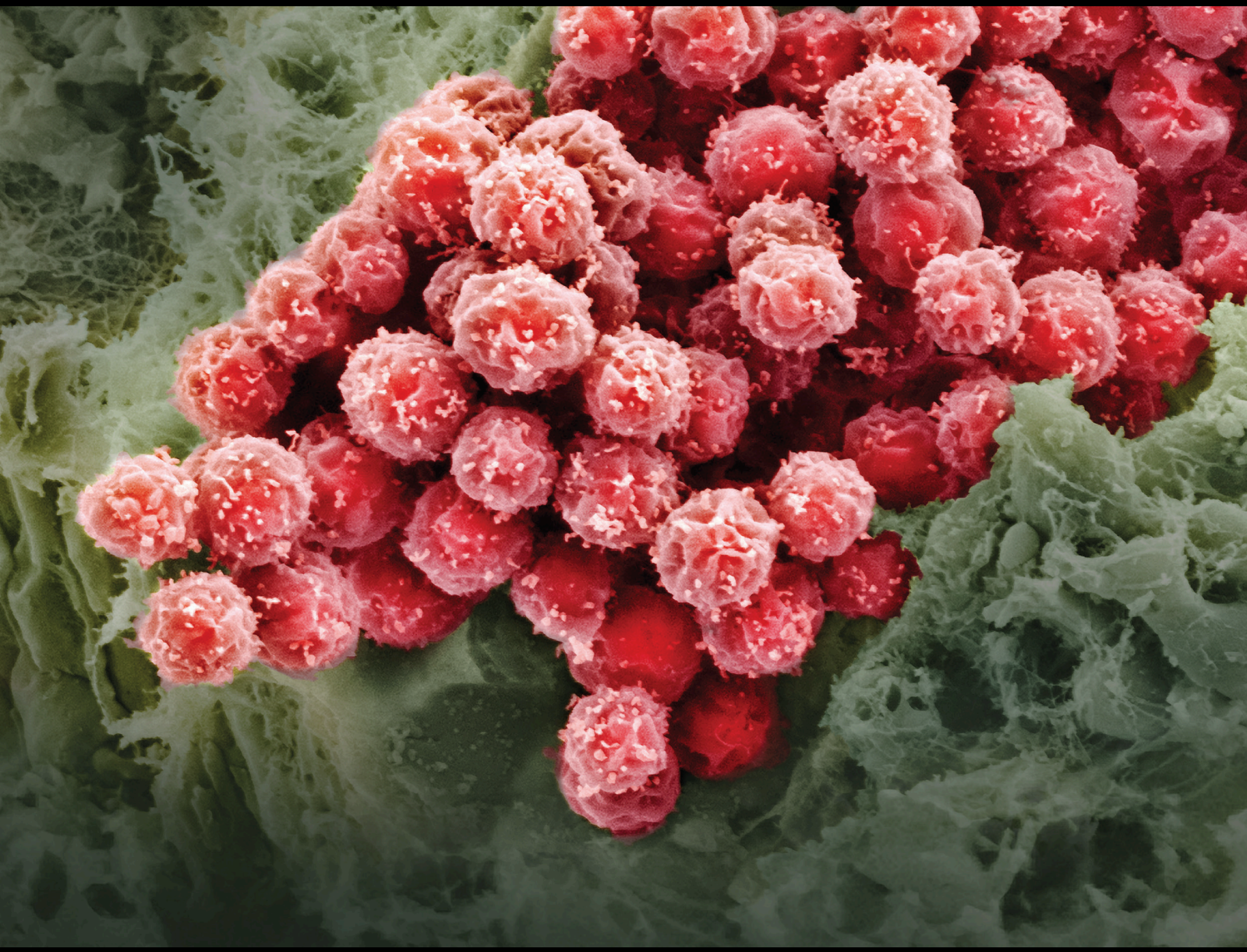

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Editorial

Molecules and Biomaterial Technologies Affecting Stem Cell Differentiation

Lorenzo Lo Muzio,[1] **Marco Mascitti**,[2] **Marcella La Noce**,[3] **Francesca Posa**,[1] **Yasusei Kudo**,[4] **and Nicola Cirillo**[5]

[1]*University of Foggia, Foggia, Italy*
[2]*Università Politecnica Delle Marche, Ancona, Italy*
[3]*Università Della Campania, Napoli, Italy*
[4]*Tokushima University, Tokushima, Japan*
[5]*University of Melbourne, Melbourne, Australia*

Correspondence should be addressed to Lorenzo Lo Muzio; lorenzo.lomuzio@unifg.it

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Mesenchymal Stem Cells (MSCs) are multipotent cells able to differentiate into specialized cells developing from mesoderm and to regenerate different tissues [1].

These adult stem cells were originally identified in the bone marrow, which is still considered the best cell source, but can also be isolated from several adult tissues such as adipose tissue, dental tissues, skin, brain, liver, and fetal tissues [2].

MSCs manifest peculiar stem cell properties of self-renewal and multipotency.

It has been extensively demonstrated that MSCs can be induced to differentiate *in vitro* into different cell types: not only mesodermal lineage cells, such as osteocytes, chondrocytes, and adipocytes, but also endothelial cells or hepatocytes [3]. Furthermore, MSC survival and differentiation towards a specific cell line can be influenced by molecular or physical factors.

Studies have convincingly demonstrated that MSCs are capable of repairing damaged tissues, particularly when appropriate microenvironmental conditions are present. This unique ability confers MSCs a tremendous potential for innovative therapeutic approaches, such as regenerative medicine, for the treatment of illness or disabilities [4].

This special issue highlights the most recent research progresses on factors, molecules, or stimuli derived from the extracellular microenvironment, which could affect MSCs' fate and commitment. Moreover, it includes a historical review about stem cell usage that also highlights their biological, religious, and ethical implications.

Bone regenerative medicine can exploit different strategies; among them, the most common approach consists in the use of MSCs grown on biocompatible scaffolds able to mimic their natural environment [5]. Often, these scaffolds are combined with factors that facilitate proliferation and osteogenic differentiation processes of MSCs [6, 7]. Nonetheless, there are several limitations associated with this type of methods [8]. A valid alternative is constituted by scaffolds coated with compounds similar to extracellular matrix components [9] and chemokines capable of inducing the recruitment, the adhesion, and the differentiation of MSCs to the damaged site [10]. In this line, stromal cell-derived factor-1α (SDF-1α) has been described as one of the key chemokines in promoting site-specific migration of healing MSCs [11].

Interestingly, L. Li et al. have demonstrated the ability of the microporous composites, developed combining OPF/BP

with SDF-1α, in promoting migration and osteogenic differentiation of rat bone marrow MSCs (BMSCs), which makes it a good material useful to increase bone regeneration.

MSC fate can be influenced not only by molecular factors but also by mechanical stimuli [12]. Several cellular mechanical loading models have been developed with the purpose of studying cell mechanoresponses.

In this regard, Y. Zhao et al. have authored a detailed article dedicated to the development of a novel device, called iStrain. It is an elastic membrane specifically designed to apply mechanical tension on human BMSCs in culture. The authors have demonstrated the capacity of this device in promoting BMSC differentiation toward the osteogenic and fibrogenic lineages.

The strategy based on the use of MSCs cultured on scaffolds is also extensively studied for its possible applications in cartilage tissue engineering [13]. Porous honeycomb-like sheets made of polylactic acid (PLA) are the most widely used scaffolds in this regard, although the optimal pore size has not yet been identified to ensure efficient cell adhesion and cartilage formation. M. Yagi et al., in their research, have proved the capacity of PLA honeycomb films in prompting cartilage formation starting from human synovial MSCs and identifying the 5 μm pores as the best dimension for *in vitro* cartilage formation.

Adipose tissue-derived MSCs (AT-MSCs) represent another attractive model of MSCs for cartilage tissue engineering. An innovative procedure for generating cartilage-like tissue is proposed by Q. T. Dang et al. The method presented is based on the use of AT-MSCs grown on a human dermal collagen matrix obtained from human skin, which could serve as a very reliable bioscaffold. This technical solution could also be used with other stem cells of mesenchymal origin.

These reports are really important in shaping our understanding of the mechanisms driving MSC differentiation *in vitro* with the purpose of identifying innovative therapeutic approaches for the regeneration of connective tissues as bone and cartilage.

In summary, these papers put together the most recent findings about molecules and materials suitable for MSC commitment and differentiation, very useful for clinicians and basic scientists.

Conflicts of Interest

The editors declare that they have no conflicts of interest regarding the publication of this Special Issue.

Lorenzo Lo Muzio
Marco Mascitti
Marcella La Noce
Francesca Posa
Yasusei Kudo
Nicola Cirillo

References

[1] A. M. Dimarino, A. I. Caplan, and T. L. Bonfield, "Mesenchymal stem cells in tissue repair," *Frontiers in Immunology*, vol. 4, p. 201, 2013.

[2] M. F. Pittenger, A. M. Mackay, S. C. Beck et al., "Multilineage potential of adult human mesenchymal stem cells," *Science*, vol. 284, no. 5411, pp. 143–147, 1999.

[3] L. Jackson, D. R. Jones, P. Scotting, and V. Sottile, "Adult mesenchymal stem cells: differentiation potential and therapeutic applications," *Journal of Postgraduate Medicine*, vol. 53, no. 2, pp. 121–127, 2007.

[4] M. F. Pittenger, D. E. Discher, B. M. Péault, D. G. Phinney, J. M. Hare, and A. I. Caplan, "Mesenchymal stem cell perspective: cell biology to clinical progress. Npj," *Regenerative Medicine*, vol. 4, no. 1, p. 22, 2019.

[5] K. M. Ferlin, M. E. Prendergast, M. L. Miller, D. S. Kaplan, and J. P. Fisher, "Influence of 3D printed porous architecture on mesenchymal stem cell enrichment and differentiation," *Acta Biomaterialia*, vol. 32, pp. 161–169, 2016.

[6] F. Posa, A. Di Benedetto, G. Ravagnan et al., "Bioengineering bone tissue with 3D printed scaffolds in the presence of oligostilbenes," *Materials*, vol. 13, no. 20, 2020.

[7] F. Rossi, M. Santoro, and G. Perale, "Polymeric scaffolds as stem cell carriers in bone repair," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 9, no. 10, pp. 1093–1119, 2015.

[8] Y. Qin, J. Guan, and C. Zhang, "Mesenchymal stem cells: mechanisms and role in bone regeneration," *Postgraduate Medical Journal*, vol. 90, no. 1069, pp. 643–647, 2014.

[9] A. Di Benedetto, F. Posa, M. Marazzi et al., "Osteogenic and chondrogenic potential of the supramolecular aggregate T-LysYal," *Frontiers in Endocrinology*, vol. 11, no. 285, 2020.

[10] S. Stamnitz and A. Klimczak, "Mesenchymal stem cells, bioactive factors, and scaffolds in bone repair: from research perspectives to clinical practice," *Cell*, vol. 10, no. 8, p. 1925, 2021.

[11] L. A. Marquez-Curtis and A. Janowska-Wieczorek, "Enhancing the migration ability of mesenchymal stromal cells by targeting the SDF-1/CXCR4 axis," *BioMed Research International*, vol. 2013, 561015 pages, 2013.

[12] A. J. Banes, M. Tsuzaki, J. Yamamoto et al., "Mechanoreception at the cellular level: the detection, interpretation, and diversity of responses to mechanical signals," *Biochemistry and cell biology = Biochimie et biologie cellulaire*, vol. 73, no. 7-8, pp. 349–365, 1995.

[13] A. R. Armiento, M. J. Stoddart, M. Alini, and D. Eglin, "Biomaterials for articular cartilage tissue engineering: learning from biology," *Acta Biomaterialia*, vol. 65, pp. 1–20, 2018.

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Research Article

SDF-1α/OPF/BP Composites Enhance the Migrating and Osteogenic Abilities of Mesenchymal Stem Cells

Linli Li,[1,2,3] **Xifeng Liu**,[1,2] **Bipin Gaihre**,[1,2] **Sungjo Park**,[4] **Yong Li**,[1,2] **Andre Terzic**,[4] **and Lichun Lu**[1,2]

[1]*Department of Physiology and Biomedical Engineering, Mayo Clinic, Rochester, MN 55905, USA*
[2]*Department of Orthopedic Surgery, Mayo Clinic, Rochester, MN 55905, USA*
[3]*Department of Orthopedics, Shanghai Fifth People's Hospital, Fudan University, Shanghai 200040, China*
[4]*Department of Cardiovascular Diseases and Center for Regenerative Medicine, Mayo Clinic, Rochester, MN 55905, USA*

Correspondence should be addressed to Lichun Lu; lu.lichun@mayo.edu

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In situ cell recruitment is a promising regenerative medicine strategy with the purpose of tissue regeneration without stem cell transplantation. This chemotaxis-based strategy is aimed at ensuring a restorative environment through the release of chemokines that promote site-specific migration of healing cell populations. Stromal cell-derived factor-1α (SDF-1α) is a critical chemokine that can regulate the migration of mesenchymal stem cells (MSCs). Accordingly, here, SDF-1α-loaded microporous oligo[poly(ethylene glycol) fumarate]/bis[2-(methacryloyloxy)ethyl] phosphate composites (SDF-1α/OPF/BP) were engineered and probed. SDF-1α/OPF/BP composites were loaded with escalating SDF-1α concentrations, namely, 0 ng/ml, 50 ng/ml, 100 ng/ml, and 200 ng/ml, and were cocultured with MSC. Scratching assay, Transwell assay, and three-dimensional migration model were utilized to assess the migration response of MSCs. Immunofluorescence staining of Runx2 and osteopontin (OPN), ELISA assay of osteocalcin (OCN) and alkaline phosphatase (ALP), and Alizarin Red S staining were conducted to assess the osteogenesis of MSCs. All SDF-1α/OPF/BP composites engendered a release of SDF-1α (>80%) during the first four days. SDF-1α released from the composites significantly promoted migration and osteogenic differentiation of MSCs documented by upregulated expression of osteogenic-related proteins, ALP, Runx2, OCN, and OPN. SDF-1α at 100 ng/ml was optimal for enhanced migration and osteogenic proficiency. Thus, designed SDF-1α/OPF/BP composites were competent in promoting the homing and osteogenesis of MSCs and thus offer a promising bioactive scaffold candidate for on-demand bone tissue regeneration.

1. Introduction

Biological repair of bone defects has been a major challenge for orthopedics [1, 2]. Autologous bone, allogenic bone, or osteoconductive biomaterials have been successfully used for the repair of bone defects in certain practices [3–5]. However, these methods have some limitations, such as donor site morbidity, limited sources, and immune rejection [3–5]. Tissue-engineered bone could be an effective substitute, with excellent bone regeneration potential and feasibility for commercialization [6, 7].

Conventional tissue engineering strategies depend on the transplantation of stem cells into biomaterials to recreate a functional tissue-equivalent [8, 9]. These methods also have several limitations, such as donor site morbidity, immune rejection, and transplanted cell apoptosis due to devascularization [3, 10]. Thus, an alternative method was designed to avoid these limitations, intending to induce the recruitment of stem cells to the scaffolds coated with chemokine at the bone defect sites. This method can recruit endogenous stem cells to the defect site to play reparative roles, maximizing the body's autoreparative ability [11]. Even though a lot of chemokines participate in this process, stromal cell-derived factor 1α (SDF-1α) has been recognized as one of the key factors [12].

SDF-1 is a 10 kDa chemokine that contains six spliced isoforms, of which SDF-1α is the main expressed isoform [12]. Previous studies have shown that SDF-1α exerts

pleiotropic effects in ischemic heart diseases: the gradient of SDF-1α could guide the recruitment of stem cells to the ischemic sites and also play a protective role through the activation of prosurvival signal transduction pathways [13, 14]. Its chemotactic and antiapoptotic properties indicate that this biochemical factor would be an ideal choice for cell homing studies [15].

Recent studies also showed the potential of SDF-1α in bone repair because it can stimulate bone marrow stromal stem cell (BMSC) migration and promote bone formation in bone defect models [16–18]. SDF-1α can also exert an antiapoptotic effect, protecting immature human BMSCs from apoptosis [1]. Wynn et al. showed that SDF-1α at the concentration of 30-50 ng/ml was optimal for the migration of human BMSCs [19]. The application of SDF-1α is limited by its short half-life and easy degradation by enzymes [13, 14]. Thus, the design of a suitable scaffold to both maintain the biological activity of SDF-1α and deliver it in a controlled manner remains a challenge.

Oligo[poly(ethylene glycol) fumarate] (OPF), a synthetic polymer, holds great potential as a drug delivery vehicle as it is biodegradable and can be developed into injectable formulations [20, 21]. Through the formation of a cross-linked hydrogel, OPF can act as a scaffolding matrix capable of promoting cell adhesion, proliferation, and differentiation, while also degrade in a predictable way [20, 21]. Moreover, incorporation of bis(2-(methacryloyloxy)ethyl) phosphate (BP) into OPF also showed improved mineralization and osteoblast precursor cell attachment, proliferation, and differentiation [20, 22]. Olthof et al. showed that functionalizing OPF hydrogels with 20% w/w BP could enhance the bone regeneration rate in both ectopic and orthotopic bone defect models, providing a useful approach to improve the biological and mechanical properties of polymer holding vast potential for bone regeneration [20].

In this study, microporous OPF/BP hydrogels were prepared by the salt leaching method. SDF-1α was loaded onto microporous OPF/BP hydrogel to obtain SDF-1α/OPF/BP composites, and the function of this delivery carrier on rat BMSC migration and osteogenic differentiation was investigated.

2. Materials and Methods

2.1. Preparation of OPF/BP Hydrogel. OPF was synthesized using poly (ethylene glycol) with an initial molecular weight (Mn) of 1 kDa according to the previously described method [23, 24].

OPF/BP hydrogel was made using the salt leaching method [20]. Firstly, OPF (0.5 g/ml), N-vinyl pyrrolidinone (NVP, 0.15 g/ml; Sigma Aldrich, St. Louis, MO), and Irgacure 2959 (I2959, 0.002 g/ml; Ciba Specialty Chemicals, Tarrytown, NY) were added and mixed in double-distilled water (ddH_2O). Then, 200 mg BP was added to make OPF/BP paste. The OPF/BP paste (50% w/w) was mixed with NaCl salt particles (50% w/w, 100-200 μm) to create the final paste for the composite. The resulting mixture was immediately transferred to 0.8 mm thick silicone rubber molds sandwiched between two glass plates. Then, the mixture was exposed to a UV light (UV-Handleuchte lamp A., Hartestein, Germany) to cross-link for 2 hours. After removal from the molds, hydrogels were punched into disc-shaped specimens using a cork borer (~5 mm in diameter) for further use. The composites were immersed in sterile ddH_2O to leach out the salt. Then, the microporous OPF/BP hydrogel was freeze-dried by lyophilization. Three samples were sent for scanning electron microscope (SEM) and atomic force microscopy (AFM) examination, respectively.

2.2. Characterization of OPF/BP Hydrogel

2.2.1. SEM. The morphology of OPF/BP hydrogels dried by lyophilization was observed using a scanning electron microscope (S-4700, Hitachi Instruments, Tokyo, Japan). For SEM imaging, the surface of the samples was sputter-coated with gold and palladium for 60 seconds and then detected at 3000 V accelerating voltage.

2.2.2. AFM. AFM was utilized to assess the nanoscale morphology of OPF/BP hydrogels using previously published protocols [25]. In brief, the OPF/BP hydrogel was placed onto the top of a fresh-surface mica disc (Ted Pella, Redding, CA). Nanoscale images (512×512 pixel resolution) were taken by a Nanoscope IV PicoFroce Multimode AFM (Bruker, Camarillo, CA), using the contact mode at room temperature. The surface roughness (Rq, root mean square) was calculated and averaged from five different areas on the AFM images.

2.3. Preparation of SDF-1α/OPF/BP Composites. The recombinant murine SDF-1α (PeproTech, Rocky Hill, NJ) was loaded onto the microporous OPF/BP by adsorption. Firstly, the SDF-1α was diluted in phosphate-buffered saline (PBS) (Gibco, Carlsbad, CA) in concentrations of 0 ng/ml, 250 ng/ml, 500 ng/ml, and 1000 ng/ml. Then, 50 μl SDF-1α solutions at different concentrations were pipetted evenly on one side of hydrogels. After 1 hour, other 50 μl SDF-1α solutions were pipetted evenly on the other side of the hydrogels. SDF-1α was loaded onto the OPF/BP hydrogels to generate four groups of composites with final SDF-1α concentrations of 0 ng/ml (control), 50 ng/ml (SDF50), 100 ng/ml (SDF100), or 200 ng/ml (SDF200), according to the culture system of 500 μl. Finally, the SDF-1α/OPF/BP composites were dried in the hood for 4 hours prior to further use.

2.4. In Vitro SDF-1α Release. Four samples of SDF-1α/OPF/BP composite in each group were placed in a 24-well plate (Corning, Corning, NY) and exposed to 1-, 2-, 3-, 4-, 5-, 14-, and 21-day culture in 500 μl complete medium (CM) (Dulbecco's modified Eagle's medium (DMEM) (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 100 U/ml penicillin-streptomycin (Gibco)). The medium was collected and subjected to ELISA assays of SDF-1α (RayBiotech, Peachtree Corners, GA), according to the manufacturer's protocol. The obtained values in the SDF50, SDF100, and SDF200 groups were subtracted by the values of the control group to preclude the preexisting SDF-1α. Finally, the concentrations of SDF-1α were calculated by the standard curve.

2.5. Two-Dimensional Migration Assay. Migration of rat BMSCs (Sprague-Dawley, Fisher Scientific, PA) was assessed by the scratching assay. Three replicates were done for each group. BMSCs were plated in a 24-well plate to create a confluent monolayer. After incubating the dishes properly for approximately 12 h in the incubator with 5% CO_2 and 95% relative humidity at 37°C, a linear wound midline was made across the bottom using a 200 μl sterile pipet tip. The dish was rinsed gently with phosphate-buffered saline (PBS) to remove any remaining cell debris. SDF-1α/OPF/BP composites were added into the corresponding well, and then, 500 μl DMEM was added into each well. Micrographs of the cells were taken using a digital Axiovert 25 Zeiss light microscope (Zeiss, Oberkochen, Germany) at time points 0 h and 48 h. The blank area without cells was quantified by ImageJ software (La Jolla, CA).

C-X-C chemokine receptor type 4 (CXCR4) expressions were assessed by immunofluorescence staining. Three replicates were done for each group. BMSCs were plated on the glass slides in a 24-well plate. SDF-1α/OPF/BP composites were added into the corresponding well, and then, 500 μl complete medium was added into each well. At 96 h, BMSCs were fixed with 4% paraformaldehyde (PFA) solution at room temperature for 1 hour and permeabilized by 0.2% Triton X-100 solution at room temperature for another hour. They were then immersed in 3% bovine serum albumin (BSA)/PBS solution at 37°C for 30 minutes to block nonspecific binding sites. The cells were then incubated with anti-CXCR-4 antibody (1 : 100 in PBS, Santa Cruz, sc-53534) at 4°C overnight. This was followed by the incubation with goat anti-rabbit IgG (Alexa Fluor 488, Abcam, ab150077) for 2 hours. Cell nuclei were finally labeled by 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO) at 37°C for 10 min. The stained cells were immediately imaged. The analysis of fluorescence integral density was done with ImageJ software.

The Transwell assay was performed using chambers with 8 μm pores purchased from Corning. Three replicates were done for each group. BMSCs were harvested and resuspended in 100 μl DMEM at a concentration of 1×10^6 cells and then seeded into the upper chambers of the 24-well plate. SDF-1α/OPF/BP composites were placed into lower chambers, and 400 μl DMEM was subsequently added into the lower chambers. Cells were incubated for 48 h in the incubator. Use a cotton-tipped applicator to carefully remove the remaining cells that have not migrated from the top of the membrane without damaging it. Then, the cells that migrated to the reverse side of the Transwell membrane were stained with calcein AM staining kit (Invitrogen, Carlsbad, CA). Micrographs of the cells were taken using a digital Axiovert 25 Zeiss fluorescence microscope at time points 24 h and 48 h. The ImageJ software was utilized to analyze the cell numbers at the reverse side of the Transwell membrane.

2.6. Three-Dimensional Migration Assay. Three replicates were done for each group. Rat BMSC spheroids were constructed using previously published protocols [26]. Rat BMSCs were trypsinized from the culture dishes and diluted in the complete medium at the concentration of 2×10^5/ml. Then, 50 μl cell solution was added to each well of Corning spheroid microplates to form cell spheroids with individual spheroids consisting of 10^4 cells. The microplate was centrifuged at 1500 rpm for 5 minutes and then maintained in the incubator. After 24 h, the spheroids were harvested, transferred to a 48-well plate, and suspended in 3 mg/ml collagen gel. After gel cross-linking, SDF-1α/OPF/BP composites and 500 μl DMEM were added into each well with spheroids (Figure 1(a)). Three replicates were done for each group. Micrographs were taken at time points 0 and 72 h, and calcein AM staining was performed at 72 h. The migration distance of BMSCs was calculated by the average distance from the core of spheroids.

2.7. Osteogenic Assay

2.7.1. Osteogenic Differentiation. Fifteen replicates were done for each group. SDF-1α/OPF/BP composites were placed into 24-well plates. Then, BMSCs were plated onto the SDF-1α/OPF/BP composites. On day 1, the complete medium was changed with osteogenic medium supplemented with 10 nM dexamethasone (DEX), 10 mM β-glycerophosphate sodium, and 50 mg/ml ascorbic acid (AA) (Sigma-Aldrich, St. Louis, MO). The medium was replaced every 3 days. On days 14 and 21, culture medium or cells were collected to detect their osteogenic abilities.

2.7.2. Immunofluorescence (IF) Staining. IF staining were constructed using previously published protocols [26]. On day 21, BMSCs under osteogenic differentiation were fixed with 4% paraformaldehyde (PFA) solution at room temperature for 1 hour and permeabilized by 0.2% Triton X-100 solution at room temperature for another hour. They were then immersed in 3% bovine serum albumin (BSA)/PBS solution at 37°C for 30 minutes to block nonspecific binding sites. The cells were then incubated with anti-RUNX2 antibody (1 : 100 in PBS, Abcam, ab23981) and anti-OPN antibody (1 : 100 in PBS, Abcam, ab8448) at 4°C overnight. This was followed by the incubation with goat anti-rabbit IgG (Alexa Fluor 488, Abcam, ab150077) for 2 hours. Cell nuclei were finally labeled by 4′,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO) at 37°C for 10 min. The stained cells were immediately imaged using LSM 780 Zeiss Confocal Microscopes (Zeiss, Oberkochen, Germany). The analysis of fluorescence stained area was done with ImageJ software.

2.7.3. Alkaline Phosphatase (ALP) Assay. On days 14 and 21, BMSCs were washed with PBS, trypsinized, and then resuspended in the complete medium. 50 μl cell suspension was used for the calculation of cell numbers with a hemacytometer. The left cell suspension was centrifuged at 1000 rpm and wash with PBS three times. Then, the BMSCs were lysed by 0.2% Triton X-100 solution by shaking for 30 minutes. The ALP concentration was measured using a QuantiChrome™ Alkaline Phosphatase Assay Kit (BioAssay Systems, Hayward, CA). The final ALP concentrations were normalized with the cell number.

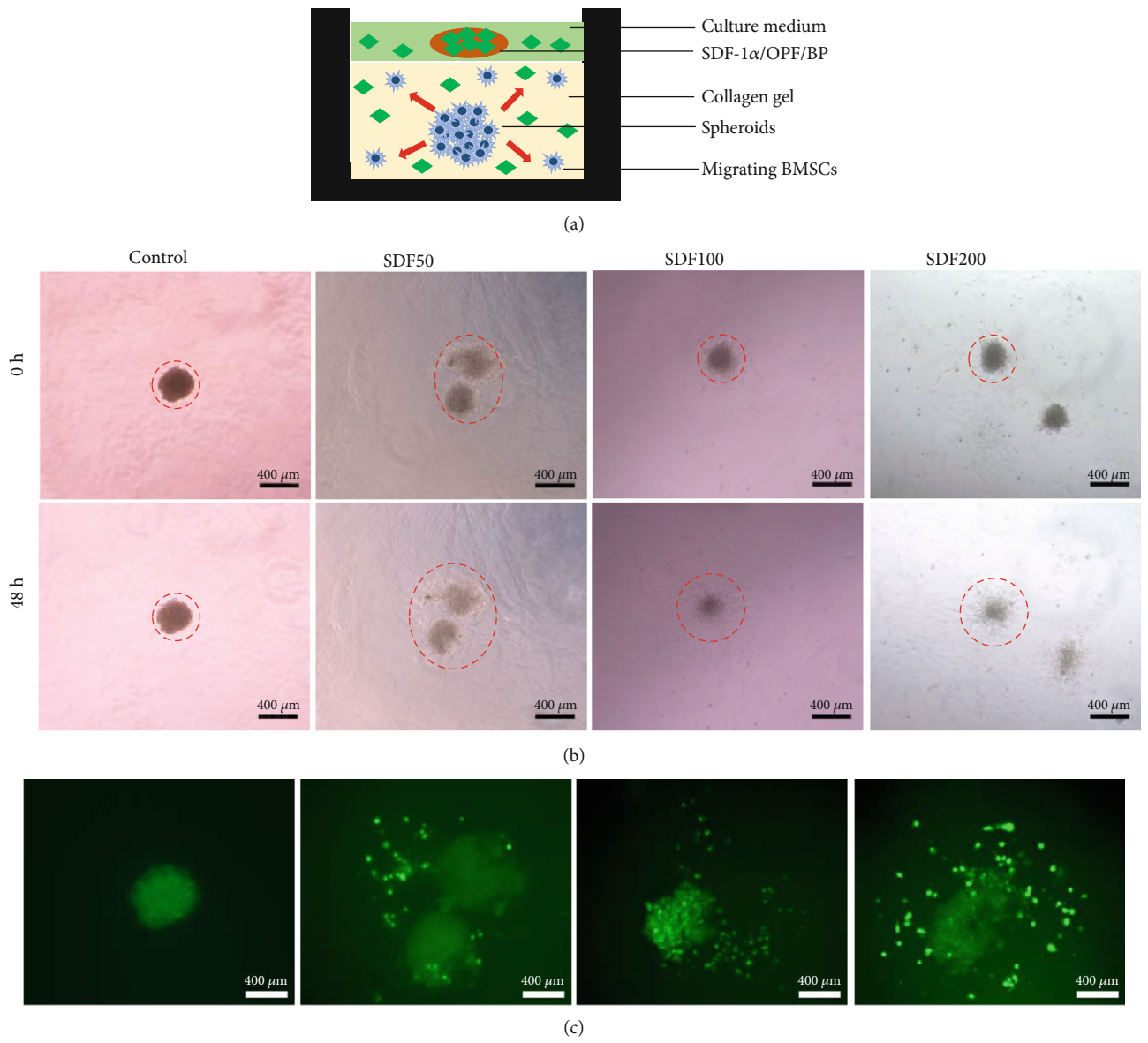

Figure 1: Three-dimensional migration assay of MSCs on the four groups of SDF-1α/OPF/BP composites. (a) Schematic illustration of the three-dimensional migration model. (b) 3D spheroid migration assay showed that the migrating distances of the SDF100 and SDF200 groups were higher than those of the SDF50 and control groups. (c) Calcein AM staining of 3D spheroids after 72 hours of culture with SDF-1α/OPF/BP composites. The red line indicates the distance of migrating cells.

2.7.4. Osteocalcin (OCN) Assay. On days 14 and 21, OCN concentration released into the culture medium was quantified utilizing the Rat Osteocalcin Enzyme Immunoassay Kit (Alfa Aesar, Haverhill, MA). Three replicates were done for each group. The final OCN concentrations were normalized with the cell number.

2.7.5. Alizarin Red S Staining. On day 21, BMSCs were fixed with 4% paraformaldehyde (PFA) solution for 1 hour. Then, the wells were washed in ddH_2O for 10 min before staining followed by the treatment with Alizarin Red S solution (Spectrum, Stamford, CT) for 30 minutes. After washing three times with ddH_2O, the wells were imaged. The analysis of the stained area was done with ImageJ software.

2.8. Statistical Analysis. GraphPad Prism 7 was used for statistical analysis. One-way analysis of variance (ANOVA) was used to determine the differences between the groups, and further, multiple comparisons were conducted using Tukey's honest significant difference test. A p value of <0.05 was considered to be significantly different.

3. Results

3.1. Characteristics of OPF/BP Microporous Hydrogel. SEM images of the OPF/BP hydrogels after salt leaching showed a highly porous interconnected network with micropores randomly distributed in the polymer matrix (Figures 2(b) and 2(c)). The average diameter of the micropores was

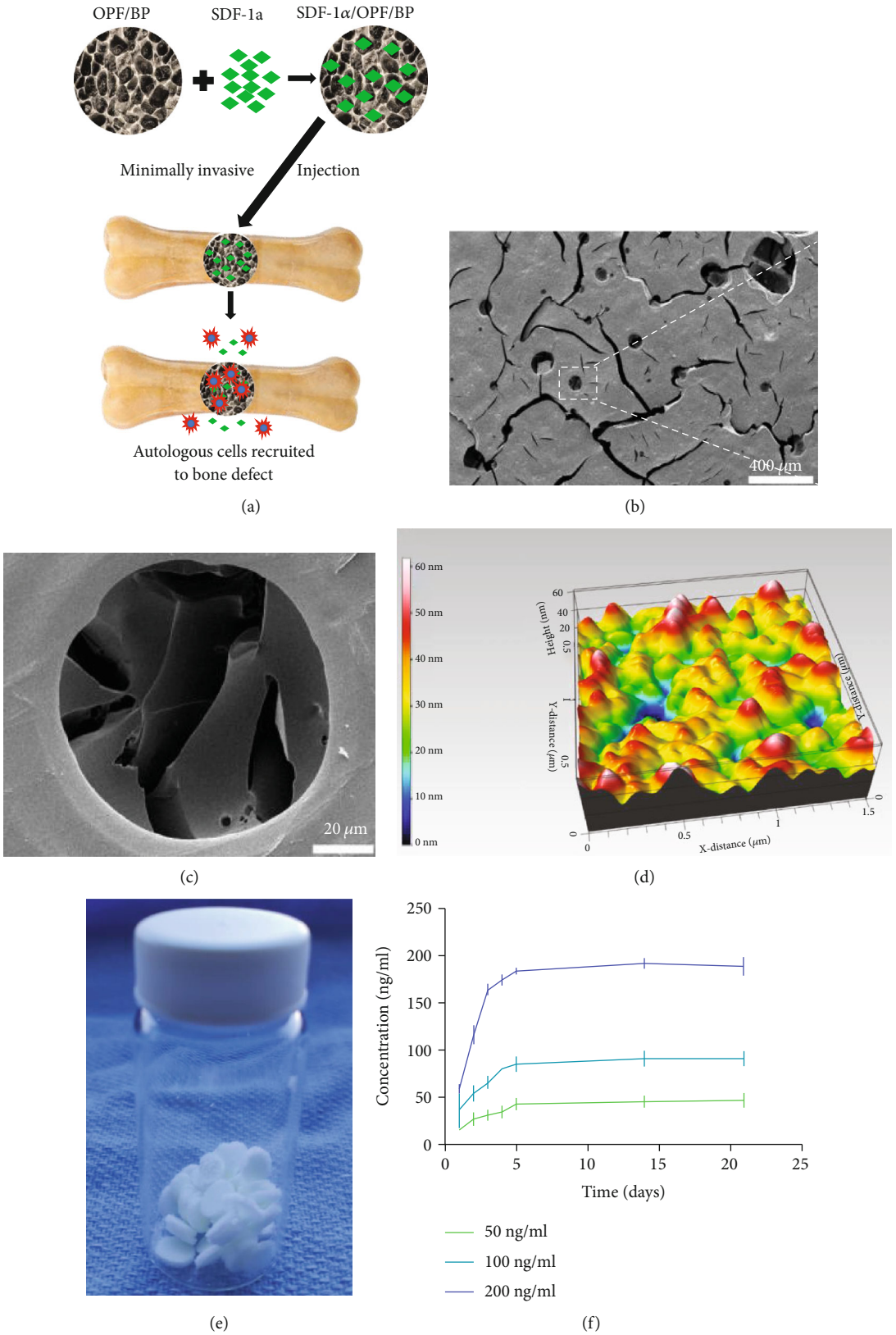

Figure 2: (a) Schematic illustration of the construction of SDF-1α/OPF/BP composites for the repair of the bone defect. (b, c) SEM images of OPF/BP hydrogel. (d) AFM image of OPF/BP hydrogel. (e) Photograph of OPF/BP hydrogel discs. (f) Release profiles of SDF-1α/OPF/BP composites showed the concentrations of SDF-1α in the culture medium. The release curve showed all groups reached 80% release percentage on the 4th day.

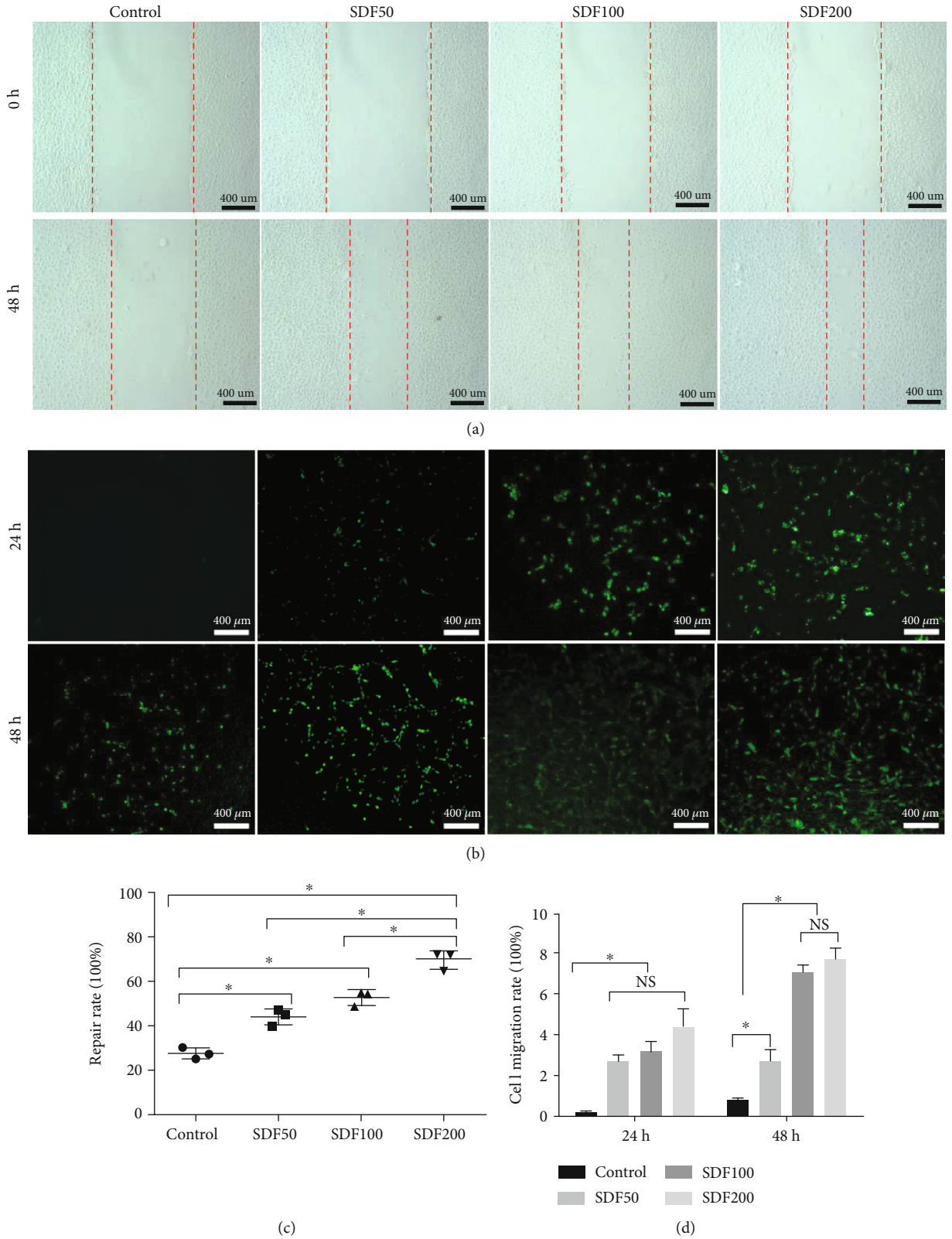

Figure 3: Two-dimensional migration assay of MSCs on the four groups of SDF-1α/OPF/BP composites. (a) Scratching assay of MSCs (light micrographs) and (b) Transwell assay of MSCs (fluorescent micrographs stained by calcein AM staining). (c) Scratching assay showed that the wound healing rates of the SDF100 and SDF200 groups were significantly higher ($^*p < 0.05$) than those of the SDF50 and control groups. (d) Transwell assay showed that the migrating rates of the three SDF groups were significantly higher ($^*p < 0.05$) than those of the control group on the first day, and the migrating rates of the SDF100 and SDF200 groups were significantly higher ($^*p < 0.05$) than those of the SDF50 and control groups on the second day. The red line indicates areas without migrating cells.

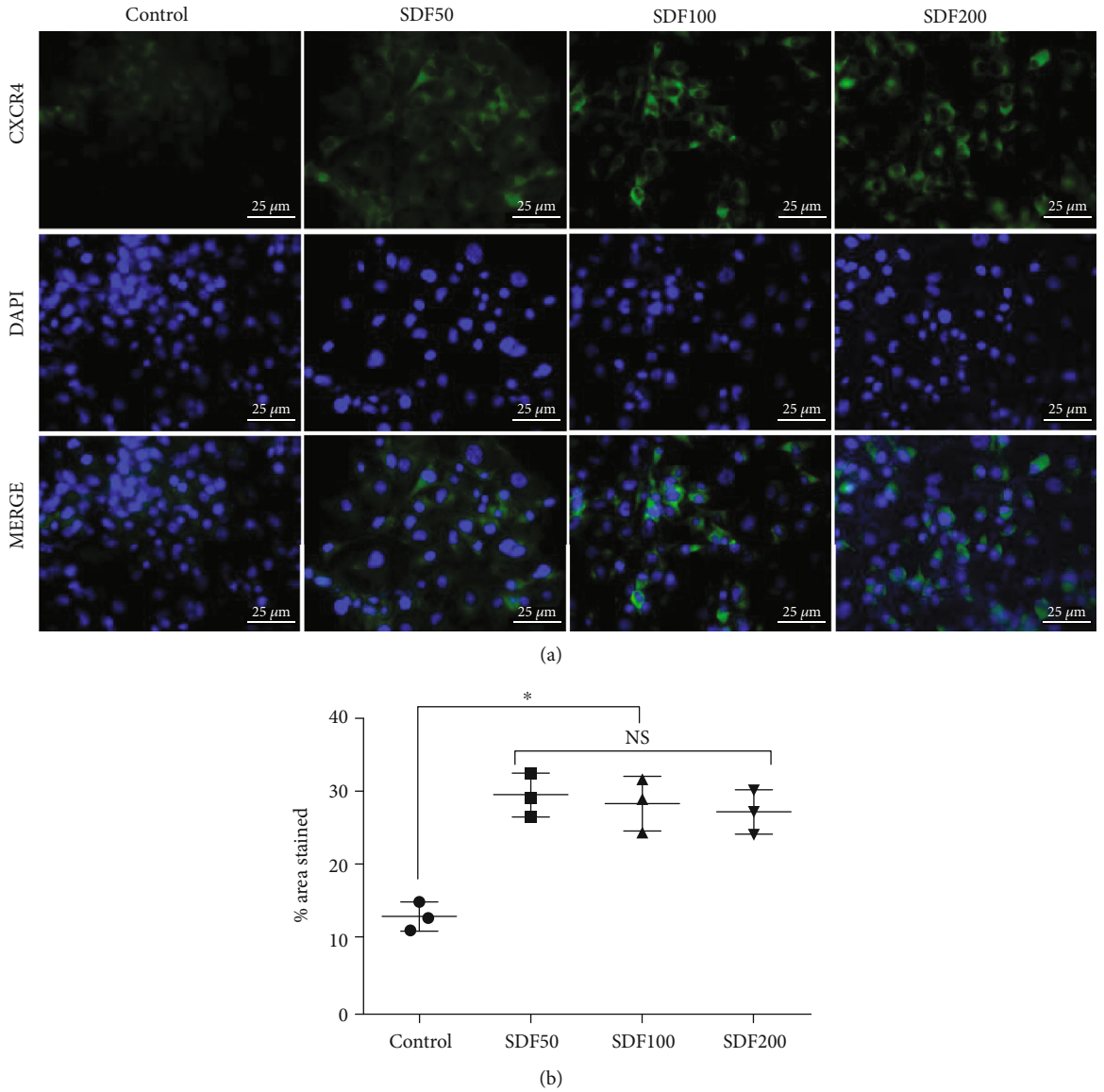

Figure 4: CXCR4 immunofluorescence staining of rat BMSCs. (a) CXCR4 immunofluorescence staining after 96 hours of coculture with SDF-1α/OPF/BP composites. (b) The percentage of CXCR4-positive areas of the SDF200, SDF100, and SDF50 groups was significantly higher ($^*p < 0.05$) than that of the control group.

about $118.3 \pm 26.4\,\mu$m. AFM scanning showed the average area roughness of OPF/BP hydrogels was 32.2 ± 29.8 nm (Figure 2(d)).

The release profiles of all SDF-1α/OPF/BP composites showed an initial burst release (50%) for the first two days. In the SDF200 group, the concentrations of SDF-1α released from the composites reached 163 ng/ml (81.5%) on the 3rd day, which leveled off to a more sustained release for the rest of the time course. In the SDF100 group, the concentrations of SDF-1α released from the composites reached 80 ng/ml (80%) on the 5th day and then leveled off to a more sustained release. Finally, in the SDF50 group, the concentrations of SDF-1α released from the composites reached 42 ng/ml (84%) on the 5th day. In all the groups, the release rate of SDF-1α reached about 90% on the 14th and 21st days (Figure 2(f)).

3.2. BMSC Chemotaxis to SDF-1α/OPF/BP Composites. The presence of SDF-1α enhanced the motility and migration of BMSCs in comparison to the control group. Cell migration was elucidated as the filling of the central gap area after making the scratch. During the wound healing assay, the average repair rate of the SDF200, SDF100, SDF50, and control group was 69.7%, 52.7%, 43.8%, and 27.3%, respectively. The cell migration rate of the SDF200 group was significantly higher ($p < 0.05$) than that of the other groups (Figures 3(a) and 3(c)).

Transwell assay showed that the average migration rate of the SDF200, SDF100, SDF50, and control group was 4.4%, 3.2%, 2.7%, and 0.2%, respectively, after 24 hours. After 48 hours, the corresponding average migration rate was 7.8%, 7.1%, 2.7%, and 0.8%. The migration rates of the SDF200 and SDF100 groups were significantly higher ($p < 0.05$) than

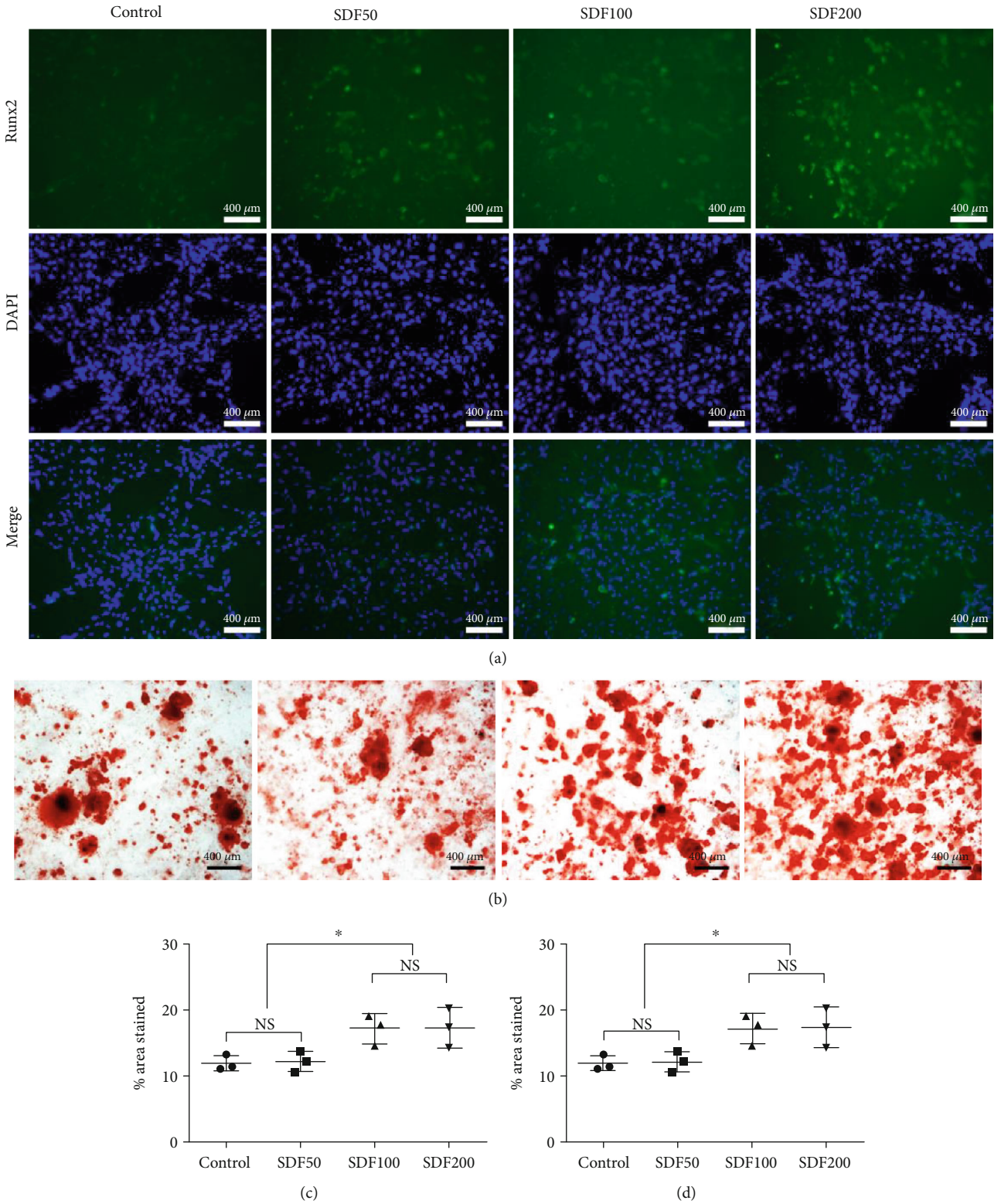

Figure 5: Osteogenic differentiation assays of MSCs after 21 days of culture on the four groups of SDF-1α/OPF/BP composites. (a) Runx2 immunofluorescence staining on the 21st day of osteogenic differentiation. (b) Alizarin Red S staining on the 21st day of osteogenic differentiation. (c) The percentage of Runx2-positive areas of the SDF200 and SDF100 groups was significantly higher ($^*p < 0.05$) than that of the SDF50 and control groups. (d) The percentage of Alizarin Red S stained area of the SDF200 and SDF100 groups was significantly higher ($^*p < 0.05$) than that of the SDF50 and control groups.

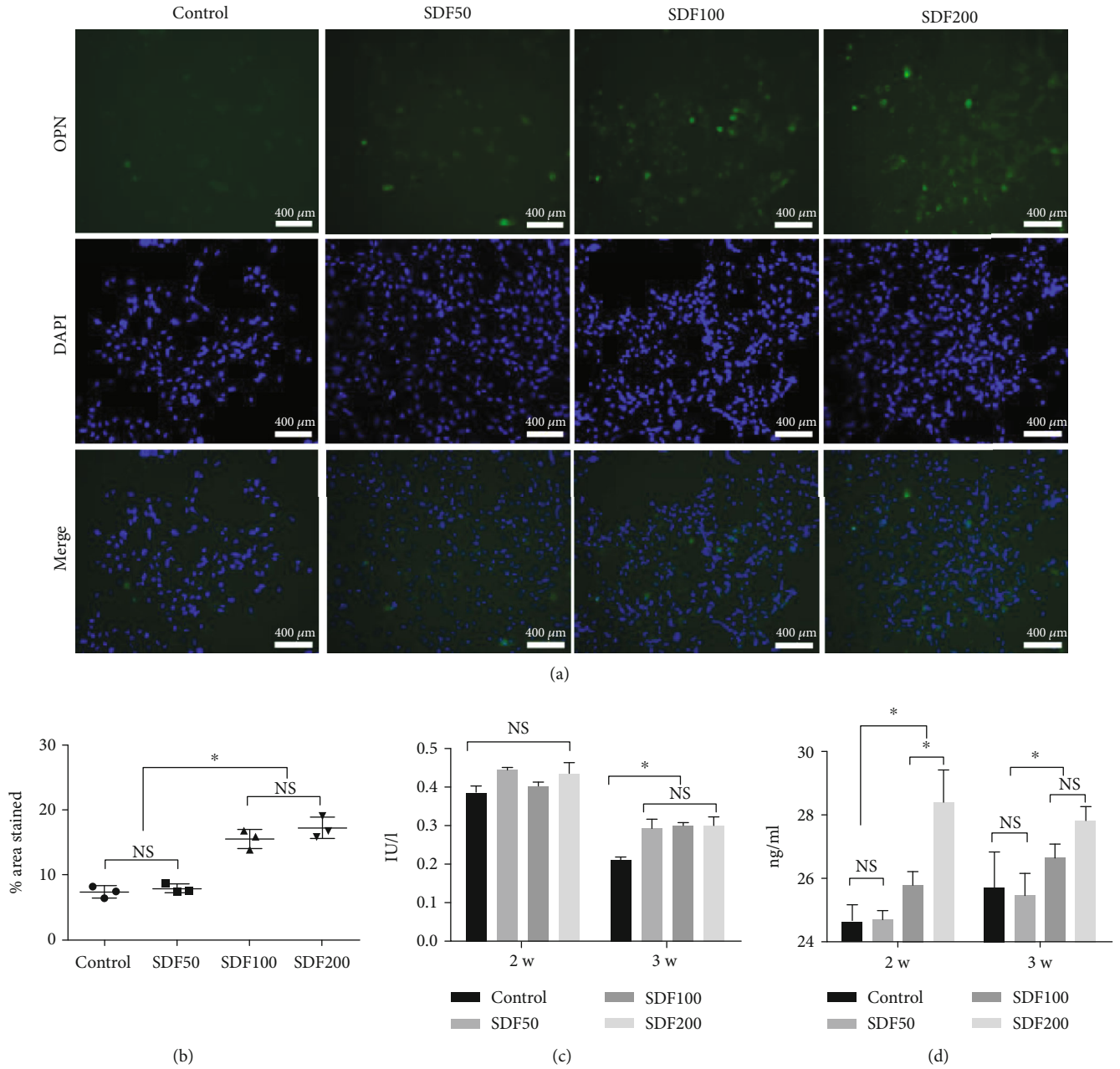

Figure 6: Osteogenic differentiation assays of MSCs after 14 and 21 days of culture on the four groups of SDF-1α/OPF/BP composites. (a) OPN immunofluorescence staining on the 21st day of osteogenic differentiation. (b) The percentage of OPN-positive areas of the SDF200 and SDF100 groups was significantly higher ($^*p < 0.05$) than that of the SDF50 and control groups. (c) ALP assay showed that ALP expression levels of the four groups were similar at day 14, while the expression levels in the control group were significantly lower ($^*p < 0.05$) than those of the other three groups on the 21st day. (d) OCN assay showed that the OCN expression levels in the SDF200 and SDF100 groups were significantly higher ($^*p < 0.05$) than those of the SDF50 and control groups on both days 14 and 21.

those of the SDF50 and control groups (Figures 3(b) and 3(d)).

The 3D migration assay showed that in the SDF200 and SDF100 groups, the border of spheroids extended more than 1.5 folds, while the SDF50 group extended less than 1.2 folds, and the control group almost stayed the same (Figures 1(b) and 1(c)), indicating the enhanced chemotaxis effect of SDF at higher concentrations.

SDF-1α enhanced the expression levels of CXCR-4 in BMSCs. The expression levels of CXCR-4 in the SDF50, SDF200, and SDF100 groups were significantly higher ($p < 0.05$) than those of the control group, but there was no statistical difference between SDF50, SDF200, and SDF100 groups ($p > 0.05$) (Figure 4).

3.3. BMSC Osteogenic Differentiation on SDF-1α/OPF/BP Composites. After 21 days of osteogenic differentiation, BMSCs in all the groups expressed Runx2 and OPN. The expression levels of Runx2 and OPN in the SDF200 and SDF100 groups were significantly higher ($p < 0.05$) than

those of the SDF50 and control groups, and there was no statistical difference between the SDF200 and SDF100 groups ($p > 0.05$) (Figures 5(a) and 5(c) and 6(a) and 6(b)). Alizarin Red S staining also confirmed that the SDF200 and SDF100 groups had higher mineral deposition than that of the SDF50 and control groups ($p < 0.05$), and there was no statistical difference between the SDF200 and SDF100 groups ($p > 0.05$) (Figures 5(b) and 5(d)).

On the 14th day, the expression levels of ALP showed no difference among the four groups, while its expression levels on the 21st day in the SDF200, SDF100, and SDF50 groups were higher than those of the control group with statistical significance ($p < 0.05$), and there was no statistical difference between the SDF50, SDF200, and SDF100 groups ($p > 0.05$) (Figure 6(c)). The expression levels of OCN in the SDF200 and SDF100 groups were also significantly higher ($p < 0.05$) than those of the SDF50 and control groups both on the 14th and 21st days. On the 14th day, the expression levels of OCN in the SDF200 group were also significantly higher than those of the SDF100 group, and on the 21st day, there was no statistical difference between the SDF200 and SDF100 groups ($p > 0.05$) (Figure 6(d)).

4. Discussion

In this study, we made SDF-1α/OPF/BP composites and investigated their potential on the migrating and osteogenic abilities of rat BMSCs. We found that SDF-1α released from the composites significantly promoted the homing and osteogenic abilities of BMSCs, and the optimum concentration of SDF-1α was 100 ng/ml.

Tissue engineering techniques dependent on stem cell transplantation are widely used in bone regeneration [26]. The drawbacks of the cell transplantation-based methods such as significant expense, time-wasting, potential infection, and low rates of cell survival (2-4%) limit their further clinical application [2, 10]. The cell guidance method, which motivates the migration and homing of stem cells, is an alternative approach. Thus, autologous MSC guidance is being considered as a feasible alternative treatment method as it does not require massive *in vitro* cell proliferation. Recently, a lot of chemokines or chemotactic drugs have been proved to possess the ability to recruit autologous cells to facilitate tissue regeneration [27, 28].

One promising factor in promoting cell migration is SDF-1α [29], a ligand of C-X-C chemokine receptor type 4 (CXCR4) expressed on the surface of many types of stem cells or progenitor cells [12]. SDF-1α plays a vital role in the recruitment of stem cells and also participates in the differentiation process of BMSCs, which is directly related to its ability to bind CXCR4 [12, 19]. However, there is a temporal mismatch between the peak expression of SDF-1α and the upregulation of CXCR4 [13]. Within an hour of injury, SDF-1α expression is rapidly upregulated, whereas CXCR4 peaks at 96 hours [30–32].

Previous studies have used OPF/BP microporous hydrogel as the carrier for BPM-2 (13 kDa), which has a similar molecular mass as SDF-1α (10 kDa), and showed that BMP-2 burst release (80% within 4 days) from OPF/BP composites produced more bone compared to other sustained release systems [22]. In this study, SDF-1α also showed a burst release from the OPF/BP hydrogel (80% within 4 days), which matched the upregulation of CXCR4 on stem cells *in vivo*. We also found that the SDF-1α could significantly enhance the expression of CXCR4 in rat BMSCs *in vitro*. This study showed that the burst release of SDF-1α could significantly improve the migration abilities of MSCs either in 2D migration assays or 3D spheroid assays. The migration abilities of MSCs were SDF-1α dose-dependent. The concentration of SDF-1α beyond 100 ng/ml, however, showed non-significant effects on the migration ability of MSCs. Previous studies also showed similar findings related to the optimum concentration of SDF-1α [14, 30].

OPF/BP can also provide a proper framework to achieve appropriate bone formation. The phosphates in the OPF-BP matrix contributed to the enhanced osteogenic activities such as attachment, proliferation of MSCs and osteoblasts, and tissue mineralization [20, 22]. Furthermore, SDF-1α is also known for its ability to promote the osteogenic differentiation of MSCs [17]. Thus, in this study, the composites developed combining OPF/BP with SDF-1α were able to induce increased mineral deposition and a significant increase in the expression levels of Runx2, OCN, OPN, and ALP—four vital osteogenic proteins representing both early and late stages of bone regeneration—to promote the osteogenic abilities of MSCs.

5. Conclusion

In conclusion, microporous SDF-1α/OP/BP composite promoted the migration and osteogenic differentiation of MSCs and thus represented a promising candidate material for bone tissue regeneration. The optimized concentration of SDF-1α for enhanced activities was determined to be 100 ng/ml in our study.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declared that they have no conflicts of interest to this work.

Acknowledgments

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References

[1] L. Mi, H. Liu, Y. Gao, H. Miao, and J. Ruan, "Injectable nanoparticles/hydrogels composite as sustained release system with stromal cell-derived factor-1α for calvarial bone regeneration," *International Journal of Biological Macromolecules*, vol. 101, pp. 341–347, 2017.

[2] S. J. Stephan, S. S. Tholpady, B. Gross et al., "Injectable tissue-engineered bone repair of a rat calvarial defect," *The Laryngoscope*, vol. 120, no. 5, pp. 895–901, 2010.

[3] W. Wang and K. Yeung, "Bone grafts and biomaterials substitutes for bone defect repair: a review," *Bioactive materials*, vol. 2, no. 4, pp. 224–247, 2017.

[4] V. Olivier, N. Faucheux, and P. Hardouin, "Biomaterial challenges and approaches to stem cell use in bone reconstructive surgery," *Drug Discovery Today*, vol. 9, no. 18, pp. 803–811, 2004.

[5] A. Kolk, J. Handschel, W. Drescher et al., "Current trends and future perspectives of bone substitute materials - from space holders to innovative biomaterials," *Journal of Cranio-Maxillo-Facial Surgery*, vol. 40, no. 8, pp. 706–718, 2012.

[6] W. S. Khan, F. Rayan, B. S. Dhinsa, and D. Marsh, "An Osteoconductive, Osteoinductive, and Osteogenic Tissue-Engineered Product for Trauma and Orthopaedic Surgery: How Far Are We?," *Stem Cells International*, pp. 1–7, 2012.

[7] X. Liu, A. L. Miller, S. Park et al., "Two-dimensional black phosphorus and graphene oxide nanosheets synergistically enhance cell proliferation and osteogenesis on 3D printed scaffolds," *ACS Applied Materials & Interfaces*, vol. 11, no. 26, pp. 23558–23572, 2019.

[8] L. Li, Y. He, X. Chen, and Y. Dong, "The role of continuous cerebrospinal fluid pulsation stress in the remodeling of artificial vertebral laminae: a comparison experiment," *Tissue Engineering. Part A*, vol. 25, no. 3-4, pp. 203–213, 2019.

[9] L. Li, X. Chen, Y. He, and Y. Dong, "Biological and mechanical factors promote the osteogenesis of rabbit artificial vertebral laminae: a comparison study," *Tissue Engineering. Part A*, vol. 24, no. 13-14, pp. 1082–1090, 2018.

[10] L. N. Niu, K. Jiao, Y. P. Qi et al., "Intrafibrillar silicification of collagen scaffolds for sustained release of stem cell homing chemokine in hard tissue regeneration," *The FASEB Journal*, vol. 26, no. 11, pp. 4517–4529, 2012.

[11] R. Subbiah and R. E. Guldberg, "Materials science and design principles of growth factor delivery systems in tissue engineering and regenerative medicine," *Advanced Healthcare Materials*, vol. 8, article e1801000, 2019.

[12] L. A. Marquez-Curtis and A. Janowska-Wieczorek, "Enhancing the migration ability of mesenchymal stromal cells by targeting the SDF-1/CXCR4 axis," *BioMed Research International*, vol. 2013, no. 561098, pp. 1–15, 2013.

[13] J. W. MacArthur Jr., B. P. Purcell, Y. Shudo et al., "Sustained release of engineered stromal cell-derived factor 1-α from injectable hydrogels effectively recruits endothelial progenitor cells and preserves ventricular function after myocardial infarction," *Circulation*, vol. 128, 11 Suppl 1, pp. S79–S86, 2013.

[14] B. P. Purcell, J. A. Elser, A. Mu, K. B. Margulies, and J. A. Burdick, "Synergistic effects of SDF-1α chemokine and hyaluronic acid release from degradable hydrogels on directing bone marrow derived cell homing to the myocardium," *Biomaterials*, vol. 33, no. 31, pp. 7849–7857, 2012.

[15] J. T. Schantz, H. Chim, and M. Whiteman, "Cell guidance in tissue engineering: SDF-1 mediates site-directed homing of mesenchymal stem cells within three-dimensional polycaprolactone scaffolds," *Tissue Engineering*, vol. 13, no. 11, pp. 2615–2624, 2007.

[16] Y. S. Liu, M. E. Ou, H. Liu et al., "The effect of simvastatin on chemotactic capability of SDF-1α and the promotion of bone regeneration," *Biomaterials*, vol. 35, no. 15, pp. 4489–4498, 2014.

[17] Q. Jin and W. V. Giannobile, "SDF-1 enhances wound healing of critical-sized calvarial defects beyond self-repair capacity," *PLoS One*, vol. 9, no. 5, article e97035, 2014.

[18] J. Ratanavaraporn, H. Furuya, H. Kohara, and Y. Tabata, "Synergistic effects of the dual release of stromal cell-derived factor-1 and bone morphogenetic protein-2 from hydrogels on bone regeneration," *Biomaterials*, vol. 32, no. 11, pp. 2797–2811, 2011.

[19] R. F. Wynn, C. A. Hart, C. Corradi-Perini et al., "A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow," *Blood*, vol. 104, no. 9, pp. 2643–2645, 2004.

[20] M. G. Olthof, M. A. Tryfonidou, X. Liu et al., "Phosphate functional groups improve oligo[(polyethylene glycol) fumarate] osteoconduction and BMP-2 osteoinductive efficacy," *Tissue Engineering. Part A*, vol. 24, no. 9-10, pp. 819–829, 2018.

[21] W. Wu, X. Liu, Z. Zhou, A. L. Miller, and L. Lu, "Three-dimensional porous poly(propylene fumarate)-co-poly(lactic-co-glycolic acid) scaffolds for tissue engineering," *Journal of Biomedical Materials Research. Part A*, vol. 106, no. 9, pp. 2507–2517, 2018.

[22] M. G. Olthof, D. H. Kempen, X. Liu et al., "Bone morphogenetic protein-2 release profile modulates bone formation in phosphorylated hydrogel," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 12, no. 6, pp. 1339–1351, 2018.

[23] M. Dadsetan, J. P. Szatkowski, M. J. Yaszemski, and L. Lu, "Characterization of photo-cross-linked oligo[poly(ethylene glycol) fumarate] hydrogels for cartilage tissue engineering," *Biomacromolecules*, vol. 8, no. 5, pp. 1702–1709, 2007.

[24] M. Dadsetan, T. E. Hefferan, J. P. Szatkowski et al., "Effect of hydrogel porosity on marrow stromal cell phenotypic expression," *Biomaterials*, vol. 29, no. 14, pp. 2193–2202, 2008.

[25] X. Liu, A. L. Miller, S. Park et al., "Functionalized carbon nanotube and graphene oxide embedded electrically conductive hydrogel synergistically stimulates nerve cell differentiation," *ACS Applied Materials & Interfaces*, vol. 9, pp. 14677–14690, 2017.

[26] L. Li, X. Liu, B. Gaihre, Y. Li, and L. Lu, "Mesenchymal stem cell spheroids incorporated with collagen and black phosphorus promote osteogenesis of biodegradable hydrogels," *Materials Science & Engineering. C, Materials for Biological Applications*, vol. 121, no. 111812, p. 111812, 2021.

[27] K. Andreas, M. Sittinger, and J. Ringe, "Toward _in situ_ tissue engineering: chemokine-guided stem cell recruitment," *Trends in Biotechnology*, vol. 32, no. 9, pp. 483–492, 2014.

[28] B. W. Vanden, "In situ tissue regeneration: chemoattractants for endogenous stem cell recruitment," *Tissue Engineering. Part B, Reviews*, vol. 20, no. 1, pp. 28–39, 2014.

[29] T. C. Lim, S. Rokkappanavar, W. S. Toh, L. S. Wang, M. Kurisawa, and M. Spector, "Chemotactic recruitment of adult neural progenitor cells into multifunctional hydrogels providing sustained SDF-1α release and compatible structural support," *The FASEB Journal*, vol. 27, no. 3, pp. 1023–1033, 2013.

[30] A. T. Askari, S. Unzek, Z. B. Popovic et al., "Effect of stromal-cell-derived factor 1 on stem-cell homing and tissue regeneration in ischaemic cardiomyopathy," *Lancet*, vol. 362, no. 9385, pp. 697–703, 2003.

[31] Y. L. Tang, W. Zhu, M. Cheng et al., "Hypoxic preconditioning enhances the benefit of cardiac progenitor cell therapy for treatment of myocardial infarction by inducing CXCR4 expression," *Circulation Research*, vol. 104, no. 10, pp. 1209–1216, 2009.

[32] M. S. Penn, "Importance of the SDF-1:CXCR4 axis in myocardial repair," *Circulation Research*, vol. 104, no. 10, pp. 1133–1135, 2009.

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Research Article

A Novel Tension Machine Promotes Bone Marrow Mesenchymal Stem Cell Osteoblastic and Fibroblastic Differentiation by Applying Cyclic Tension

Yi Zhao,[1] **Yiping Huang**,[1] **Lingfei Jia**,[2,3] **Ruoxi Wang**,[1] **Kuang Tan**,[1] **and Weiran Li**[1]

[1]*Department of Orthodontics, Peking University School and Hospital of Stomatology, Beijing 100081, China*
[2]*Department of Oral and Maxillofacial Surgery, Peking University School and Hospital of Stomatology, Beijing 100081, China*
[3]*Central Laboratory, Peking University School and Hospital of Stomatology, Beijing 100081, China*

Correspondence should be addressed to Weiran Li; weiranli@bjmu.edu.cn

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Bone marrow mesenchymal stem cells (BMSCs) are intraosseous stem cells, and the effects of tensile strain on BMSC differentiation mediate several bone-related treatments. To study the response of BMSCs under tension, we designed and developed a small cellular tension instrument, iStrain. When iStrain applied tension on BMSCs, these cells exhibited convergence in the alignment direction and lengthening of the cell processes and cell body. Real-time quantitative polymerase chain reaction (RT-qPCR) and western blotting demonstrated that iStrain-mediated cyclic tension promotes the differentiation of BMSCs toward osteogenesis and fibrogenesis. And the mRNA and protein expression of differentiation-related genes changes with the extension of tension time.

1. Introduction

Stem cells, which are defined as cells with the capacity to self-proliferate and differentiate into new functional cells in tissue [1], are important in maintaining tissue integrity and normal function. Bone marrow mesenchymal stem cells (BMSCs) are a specific type of stem cell in bone with active self-renewing and multidifferentiation abilities [2]. These cells have the potential to develop into osteocytes, chondrocytes, adipocytes, and cells of other embryonic lineages [2].

Traditionally, stem cell differentiation is achieved by adopting cocktails of various growth factors. However, the linkage between differentiation and mechanical stimuli has gradually been revealed as mechanical stimulation has shown regulatory effects on cell proliferation, differentiation, apoptosis, and migration [3–7]. The main types of mechanical stimulation *in vivo* include shear stress, compressive stress, and tensile stress [6]. Among these types, the effects of tensile stress on BMSCs are clinically important. BMSCs are the main functional cells in bone distraction, as they can differentiate into osteoblasts and initiate osteogenesis. In the process of distraction osteogenesis, continuous tensile stress plays an important role in promoting BMSC proliferation and differentiation [8]. The osteogenic differentiation of BMSCs is also involved in bone regenerative activity in rapid maxillary expansion during orthodontic treatment [9].

To study the mechanoresponses of cells, researchers have developed many cellular mechanical loading models. Yang et al. classified them as substrate deformation-based approaches, weight approaches, hydrostatic approaches, centrifugation approaches, fluid flow approaches, vibration approaches, and 3-D loading models [10]. Among these, the substrate deformation-based mechanical loading model is the most widely used cellular mechanical loading device, especially for tension. These devices usually use an elastic membrane as the deformation substrate on which the cells are cultured. With the stretch of the elastic membrane, the cells will undergo tension [10].

However, available tension devices currently have a large size and strictly rely on an external power supply, both of

which substantially constrict their applicable conditions and environment. To apply multiple strain loading modes in various circumstances, we designed a small stretching device named iStrain (Figures 1(a)–1(g)).

The objective of this study was to explore the response of BMSCs under different tension duration and the effects of tension on the osteoblastic and fibroblastic differentiation of BMSCs over time. To achieve this, we applied cyclic tensile strain to BMSCs using iStrain in vitro and observed the changes in cell morphology and internal structure, evaluated the survival and metabolic status of BMSCs by live/dead staining and Cell Counting Kit 8 (CCK-8) assays, and measured changes in mRNA and protein expression of differentiation-related genes by RT-qPCR and western blots.

2. Materials and Methods

2.1. Cell Culture. Human BMSCs were purchased from ScienCell (San Diego, CA, USA). Cells were used for experiments between the 3rd and 7th passages and cultured in alpha-modified Eagle's medium with regular glucose (α-MEM; Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin solution (Gibco). Cells were incubated at 37°C in a humidified incubator containing 5% CO_2.

2.2. Tensile Strain Experiments. BMSCs were seeded at a density of 3.0×10^5 cells/cm^2 on collagen I-coated 6-well BioFlex plates (Flexcell International, Burlington, NC, USA) and then incubated in α-MEM until reaching 95% cell confluence. Subsequently, culture plates were subjected to cyclic tensile strain using iStrain with extension rates of 9%, 12%, and 15% at a frequency of 0.3 Hz for 12 h or subjected to cyclic tensile strain with a 12% extension rate at a frequency of 0.3 Hz for 6 h, 12 h, 24 h, 36 h, or 48 h. The BMSCs were then subjected to microscopic observation, immunohistochemistry staining, or RNA and protein extraction.

2.3. Live/Dead Staining. After exposure to cyclic tensile strain, BMSCs were stained with a live/dead staining kit (KGAF001, KeyGEN BioTECH Co Ltd., Nanjing, China). The culture medium was removed, and the BMSCs were washed three times with phosphate-buffered saline (PBS). Then, the BMSCs were stained in 2 ml of PBS supplemented with 0.4 μM calcein AM (stained live cells) and 1.6 μM propidium iodide (PI; stained dead cells) for 30 mins. The stained cells were observed using a fluorescence microscope (Nikon Eclipse 80i; Nikon Instech Co. Ltd., Kawasaki, Kanagawa, Japan). The live and dead cells exhibited green and red fluorescence, respectively.

2.4. CCK-8 Assays. CCK-8 assays were used to assess the proliferation of BMSCs treated with tensile strain. After exposure to cyclic tensile strain, BMSCs were seeded on 48-well plates. The medium was replaced every 2 days. 20 μl of CCK-8 solution was added to 200 μl of medium in one well of each group, and the cells were further cultured in a 5% CO_2 incubator for 4 h. Then, 100 μl of medium from each well was transferred to a new 96-well plate, and the absorbance at a wavelength of 450 nm was determined.

2.5. Cell Length Measurement. BMSCs were observed under a microscope, and cell length was measured. Six different positions of each well were selected, and six representative cells were taken at each position for measurement, and the results were obtained and analyzed.

2.6. Immunofluorescence Staining and Fluorescence Imaging. After application of tensile strain on BMSCs, BMSCs were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min. After the cells were washed with PBS, the BMSCs were subsequently permeabilized with 0.1% Triton X-100 in PBS for 10 min. Then, the cells were washed with PBS again and blocked with 5% goat serum (ZLI-9022, Zhongshan Golden Bridge Biotechnology, Beijing, China) for 1 h. Then, the BMSCs were incubated with CytoPainter Phalloidin-iFluor 488 Reagent (Abcam, Cambridge, MA, USA) diluted at 1 : 1000 for 1 h at room temperature. Finally, the BMSCs were stained with DAPI (Solarbio Science & Technology Co., Ltd., Beijing, China) for 5 min at room temperature and were observed and photographed using a confocal system for imaging (LSM 5 EXCITER, Carl Zeiss, Jena, Germany).

2.7. RT-qPCR. Total RNA was extracted from BMSCs following treatment using TRIzol reagent (Invitrogen, Gaithersburg, MD, USA). A PrimeScript RT Reagent Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China) was used to reverse transcribe cDNA from cellular RNA. RT-qPCR was performed using a Real-Time PCR Detection System (ViiA 7 Real-Time PCR System; Thermo Fisher Scientific, Wilmington, DE, USA) and FastStart Universal SYBR Green Master Mix (Roche Diagnostics GmbH, Mannheim, Germany) using the following parameters: 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 seconds at 95°C together with 1 min at 60°C. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was used as the internal normalization control. The sequences of mRNA primers used in this study are listed in Table 1.

2.8. Western Blot Analysis. BMSCs were washed with ice-cold PBS and solubilized with RIPA lysis buffer containing 1% protease inhibitor cocktail (Solarbio Science & Technology Co.). Protein concentrations were measured by a BCA Protein Assay Kit (Thermo Fisher Scientific), and concentrations were adjusted to be the same. After 4× loading buffer was added, samples were heat denatured, and a total of 40 μg of protein was used for western blot analysis. Prepared lysates containing equal amounts of protein were electrophoresed on a precast gel (Beyotime Institute of Biotechnology, Shanghai, China), and proteins were transferred to a polyvinylidene difluoride (PVDF) membrane. The transferred membranes were blocked with 5% BSA in TBST for 1 h at room temperature and were then incubated at 4°C overnight with anti-Runt-related transcription factor 2 (RUNX2) antibody; anti-osteopontin (OPN) antibody; anti-Collagen, type I, alpha 3 (COL-3) antibody; anti-Tenascin C (TN-C) antibody; anti-GAPDH antibody (Abcam); anti-Collagen, type I, alpha 1 (COL-1) antibody (Cell Signaling Technology, Danvers, MA, USA); or anti-Scleraxis (SCX) antibody (Santa Cruz Biotechnology, Dallas, TX, USA). After three washes with TBST, the membranes were

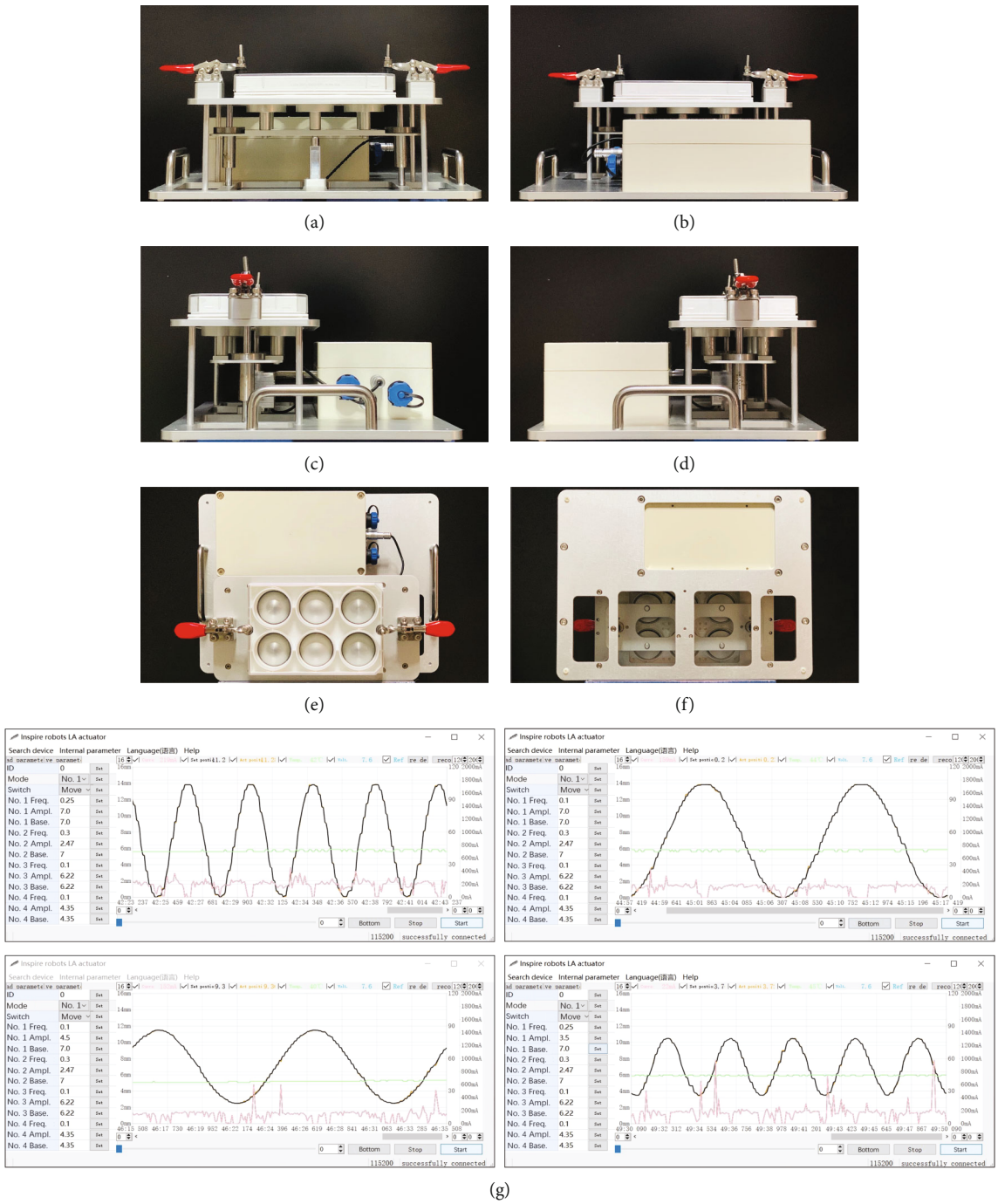


Figure 1: Images of the stretching device iStrain and its companion software. The front view (a), back view (b), side views (c, d), top view (e), and elevation view (f) of the stretching device iStrain. (g) Screenshots of the software showing different tension procedures.

incubated with anti-rabbit or anti-mouse secondary antibodies (ZB-2301 and ZB-2305, Zhongshan Golden Bridge Biotechnology, Beijing, China), which were diluted at 1 : 5000 at room temperature for 1 h. Chemiluminescence was produced using the Bio-Rad system, detected with a ChemiDoc MP Imaging System and analyzed using the ImageJ software.

2.9. Alizarin Red Staining. Alizarin red was used to detect calcium deposits formed by the BMSCs. After exposure to mechanical tension loading, BMSCs were reseeded in six-well plates. After 24 h of attachment, the medium was switched to α-MEM containing 10% fetal bovine serum, 1% penicillin-streptomycin solution, 10 mM β-glycerophosphate, 100 nM dexamethasone, 200 mM vitamin C, and 2 mM KH_2PO_4. After 14 days of culture, the BMSCs were fixed in 4% paraformaldehyde at room temperature for 15 min and subsequently stained with Alizarin red (1% solution).

2.10. Statistical Analysis. All data are presented as the mean and standard deviation from three independent experiments.

Table 1: mRNA primer sequences used in this study.

Gene	Forward sequence ($5' \longrightarrow 3'$)	Reverse sequence ($5' \longrightarrow 3'$)
GAPDH	GCATTGCCCTCAACGACCACT	CCATGAGGTCCACCACCCTGT
RUNX2	ACTACCAGCCACCGAGACCA	ACTGCTTGCAGCCTTAAATGACTCT
OCN	AGCAAAGGTGCAGCCTTTGT	GCGCCTGGGTCTCTTCACT
COL-1	TGGACGCCATCAAGGTCTACTGC	GGAGGTCTTGGTGGTTTTGTATTCG
OPN	CGAGGTGATAGTGTGGTTTATGG	GCACCATTCAACTCCTCGCT
SCX	CAGCCCAAACAGATCTGCACCTT	CTGTCTTTCTGTCGCGGTCCTT
COL-3	TGGTCTGCAAGGAATGCCTGGA	TCTTTCCCTGGGACACCATCAG
TN-C	GTCACCGTGTCAACCTGATG	GTTAACGCCCTGACTGTGGT
IL-6	AGACAGCCACTCACCTCTTCAG	TTCTGCCAGTGCCTCTTTGCTG
IL-1β	TGGCAACTGTTCCTG	GGAAGCAGCCCTTCATCTTT

GAPDH: glyceraldehyde 3-phosphate dehydrogenase; RUNX2: Runt-related transcription factor 2; OCN: osteocalcin; COL-1: collagen, type I, alpha 1; OPN: osteopontin; SCX: Scleraxis; COL-3: collagen, type I, alpha 3; TN-C: Tenascin C.

Differences among independent groups were analyzed by one-way analysis of variance (ANOVA) using IBM SPSS Statistics, version 26.0 (IBM Corp., Armonk, NY, USA). $P < 0.05$ was considered significant.

3. Device Design

The stretching device iStrain is a bioreactor invented to stimulate the tension state of cells from the muscle, lung, heart, blood vessel, skin, tendon, ligament, cartilage and bone *in vivo*. It is powered by a lithium battery and controlled by a computer program. The most obvious feature of iStrain is its compactness and battery power supply, which enable iStrain to adapt to a closed incubator with high humidity, suitable for cell culture to the maximum extent. Moreover, iStrain allows free programming to apply different force modes, including sine waves, triangular waves, and trapezoidal waves. The frequency and the specific duration of each action can also be adjusted on demand.

iStrain is composed of a single-chip microcomputer (inside the power bank; Figure 2(a), 6), a mechanical structure part and a power supply part. By using the visualized iStrain software on laptops, a personalized force mode can be easily set and switched. Then, through the USB port, the personalized force mode is transferred to the microcomputer inside the iStrain. After starting up, the microcomputer sends out instructions to the mechanical structure part. The mechanical structure part consists of the microlinear servo motor (MLSM; Figure 2(a), 11), the moving platform (Figure 2(a), 5) and the spherical stiffening elements (Figure 2(a), 4). When the instruction is acquired, the MLSM carries out linear motion according to the program set up in advance. With the cooperation of two linear bearings (Figure 2(a), 8), the MLSM drives the moving platform above it to move vertically. The spherical stiffening elements are fixed to the moving platform and are vertically below the elastic membranes of the cell culture plate. Thus, the vertical movement of the elements causes elastic deformation of the membranes and imposes regular stretching on the cells cultured on them (Figures 2(b) and 2(c)). The power supply part is composed of a lithium battery and a control switch. Both the single-chip microcomputer and the power supply part are packaged in one box (power bank, Figure 2(a), 6). The working time can reach 48 hours on a single charge.

For the maximum stretching effect of the equalized elastic membrane, the contact interface of the spherical stiffening element is designed as a spherical crown with low friction. The size of the element exactly fits the diameter of the cell culture well. When the element moves upwards, the elastic membrane will be deformed until the whole elastic membrane is pressed close to the spherical crown interface, making cells at all locations strained. A maximum of six spherical stiffening elements can be installed at the same time. By using different sizes of spherical stiffening elements, a 0-57% extension rate can be applied to the cells, and the precision of it can reach 0.01%.

With a size of 500 mm × 400 mm × 120 mm and a weight of 4900 g, the iStrain can be easily accommodated by regular incubators in a laboratory. All parts of the invention are moisture proof, and thus, the device can be used in a high-humidity environment and placed in a closed container as a whole without wiring.

4. Results

4.1. Cell Viability of BMSCs under Cyclic Tension. By using iStrain, we applied cyclic tensile strain to BMSCs at a frequency of 0.3 Hz for 6 h, 12 h, 24 h, 36 h, or 48 h with a 12% extension rate and investigated the damage of tensile strain to the cell viability of BMSCs utilizing live/dead staining and CCK-8 assays. Live/dead staining revealed that the number of dead cells increased with the extension of time from 0 h (negative control) to 48 h, and there was a remarkable increase between 12 h and 24 h, indicating significantly increased cell damage between 12 h and 24 h (Figure 3(a)). According to the CCK-8 assay results, there was no significant difference in the first three days between the different time groups. On day 5, there was a slight increase in the 6 h, 12 h, and 24 h time groups and a slight decrease in the 36 h and 48 h time groups compared with the control group (Figure 3(b)). Based on this, we hypothesized that a short period of tension would not have a significant negative effect

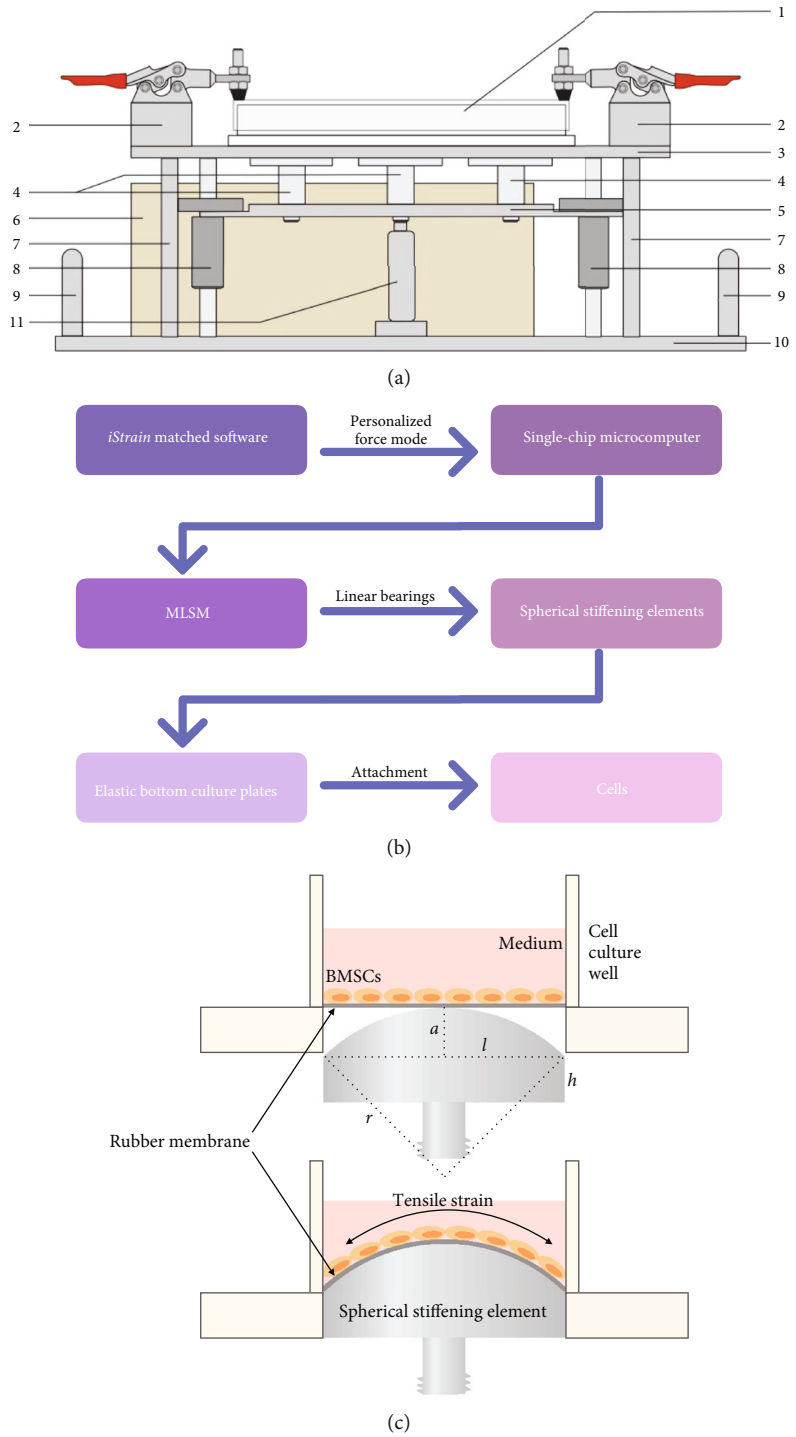
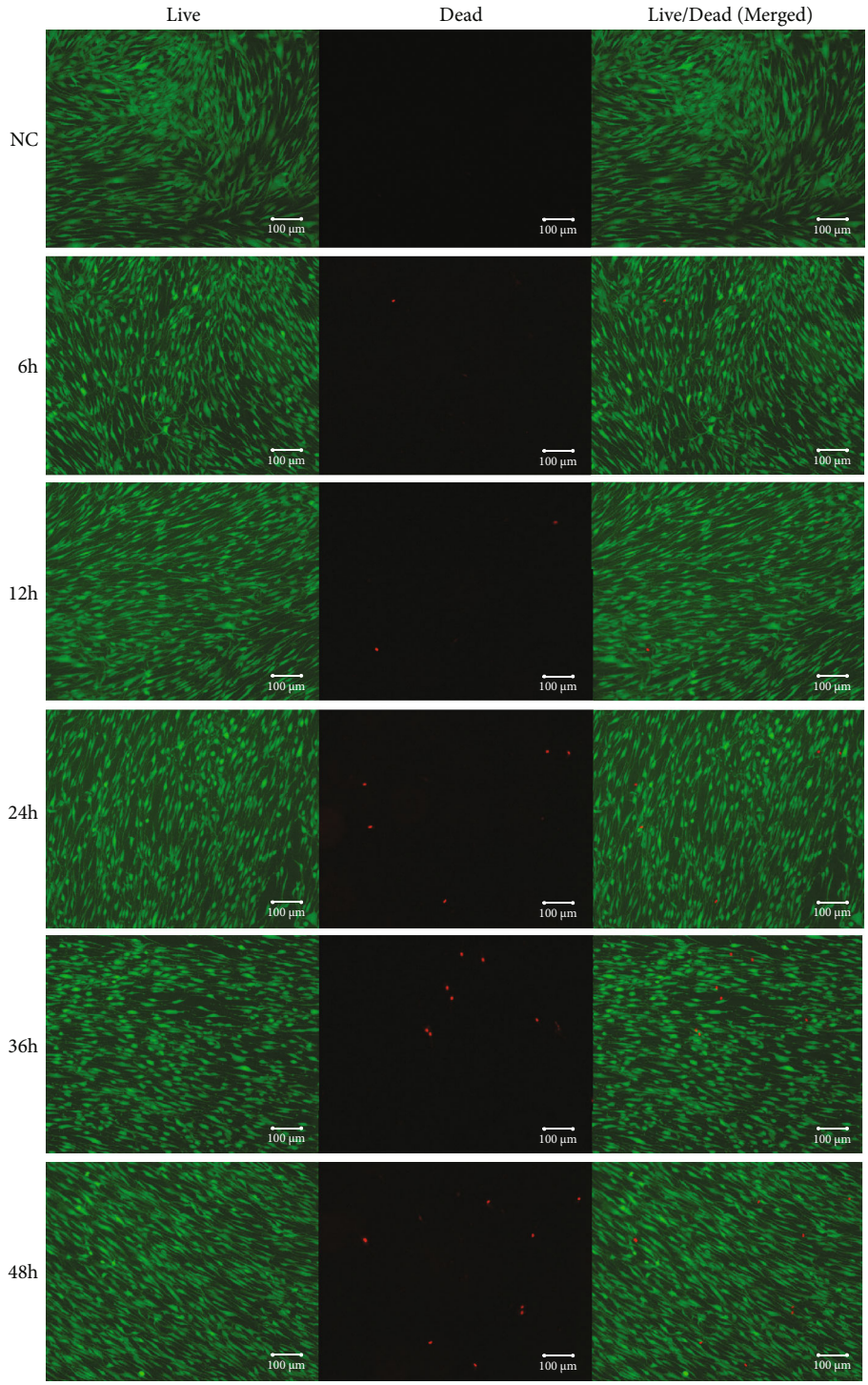


FIGURE 2: The structure and working principle of iStrain. (a) The structure of iStrain. 1: 6-well BioFlex plate; 2: culture plate fixtures; 3: culture plate support platform; 4: spherical stiffening elements; 5: movable platform; 6: power bank; 7: vertical support column; 8: linear bearings; 9: handles; 10: pedestal; 11: MLSM. (b) The workflow diagram of iStrain. A personalized force pattern is formed on iStrain companion software, and then, the force pattern is transferred to the microcomputer. After start-up, the microcomputer sends commands to the MLSM, which moves linearly according to the preset program. With the cooperation of two linear bearings, the MLSM drives the movable platform together with the spherical stiffening elements above it to move vertically, thus applying force to the elastic bottom of the culture plate and stretching the cells cultured on it. (c) The structure of the spherical stiffening element depends on five variables: h, l, ε, r, and a. h is the distance from the rubber membrane to the lowest bottom of the culture plate. l is the radius of the rubber membrane. For the BioFlex plates, $h = 5$ mm and $l = 16.5$ mm. ε refers to the extension rate, which is determined for each independent experiment. r refers to the radius of the spherical part, and a refers to its height. r and a are calculated as follows: $\varepsilon\% = [(r \bullet \arcsin(l/r))/l - 1] \times 100\%$; $a = r - \sqrt{(r^2 - l^2)}$.



(a)

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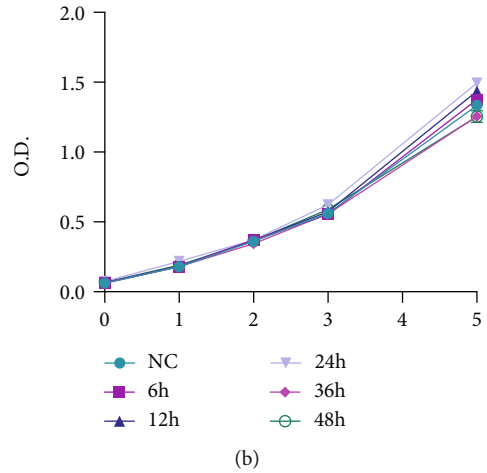


(b)

Figure 3: Cell viability of BMSCs under cyclic tension. (a) Live/dead staining of the negative control group and cyclic tension group at 6 h, 12 h, 24 h, 36 h, and 48 h. Live cells are stained with green fluorescence. Dead cells are stained with red fluorescence. (b) CCK-8 assay results show the effect of tensile strain lasting different times on cell proliferation of BMSCs. Different icons represent the negative control group and different time groups of 6 h, 12 h, 24 h, 36 h, and 48 h.

on cell viability and proliferation, but extending the tension time to 24 h would affect cell viability, and continuing to extend the tension time to 36 h would affect cell viability and cell proliferation.

4.2. Cyclic Tension from iStrain Influences BMSC Morphology and Actin Filament Arrangement. By using iStrain, we applied cyclic tensile strain to BMSCs at a frequency of 0.3 Hz for 6 h, 12 h, 24 h, 36 h, and 48 h with a 12% extension rate. Under the microscope, the unstimulated control BMSCs were randomly arranged, whereas with the extension of the application time, the BMSCs exposed to the cyclic tensile strain were gradually arranged unidirectionally, perpendicular to the direction of the tensile force, accompanied by elongation of the cell processes and cell body. When the tension time exceeded 24 h, the intercellular space was gradually increased, and the cells were sparser (Figure 4(a)). By measuring the cell length under the microscope, we found that cell length gradually increased with extension of tension time and reached a peak at 12 h, followed by a decrease in cell length from 24 h to 48 h. Nevertheless, the cell lengths of the 24 h, 36 h, and 48 h groups were still longer than those of the negative control group (Figure 4(b)). These morphological and alignment changes were also confirmed by the arrangement of actin filaments. Immunohistochemistry staining demonstrated that actin filaments were arranged parallel in BMSCs treated with cyclic tensile strain but randomly oriented in the control cells (Figure 4(c)). The elongation of the cell processes and cell bodies was also observed with immunohistochemical staining of actin filaments.

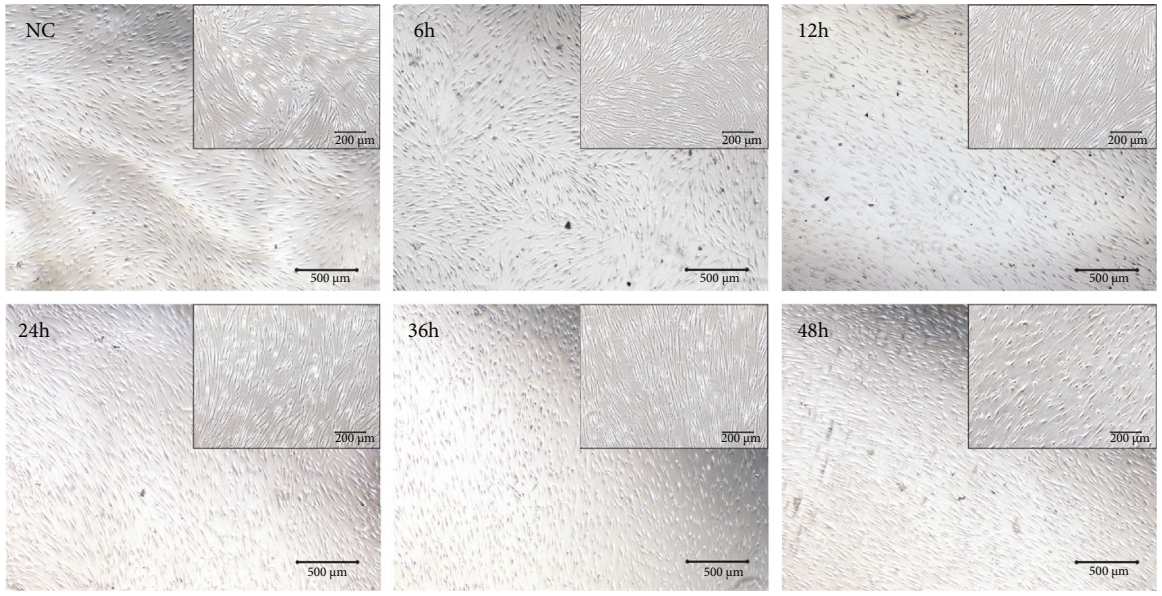
4.3. Cyclic Tension from iStrain Influences the Expression of Osteoblastic Differentiation-Related Genes in BMSCs. Figures 5(a) and 5(b) show the changes in the mRNA expression of osteoblastic differentiation-related genes in BMSCs under different force values of cyclic tension for 12 h. The expression of RUNX2 and osteocalcin (OCN) was increased in the tension groups and peaked when the extension rate was 12%. Hence, we chose an elongation of 12% for the subsequent tension experiments.

Figures 5(c)–5(e) show the time course of mRNA expression of osteoblastic differentiation-related genes in BMSCs in response to cyclic tension force. Compared with that of the control group, the mRNA expression of RUNX2, COL-1, and OPN mRNA, detected by qPCR, gradually peaked at 12 h and 24 h with mechanical tension loading and then decreased thereafter.

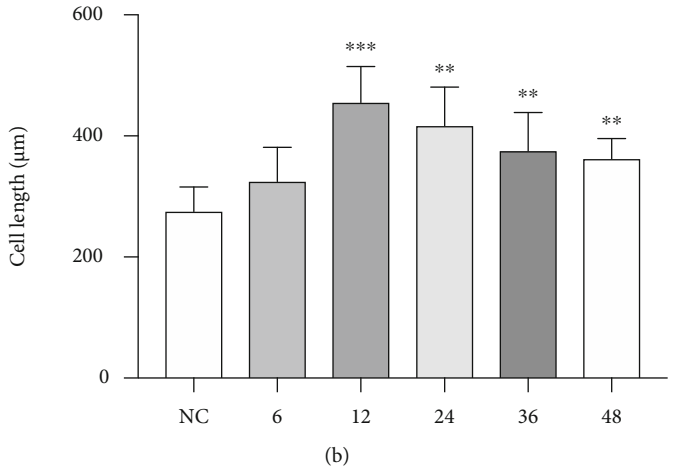
4.4. Cyclic Tension from iStrain Influences the Expression of Fibroblastic Differentiation-Related Genes in BMSCs. Figures 5(f)–5(h) show the time course of mRNA expression of fibroblastic differentiation-related genes in BMSCs in response to cyclic tension force. Compared with that of the control group, the mRNA expression of SCX, COL-3, and TN-C mRNA gradually peaked at 12 h, which was followed by a gradual decrease.

4.5. Cyclic Tension from iStrain Influences the Expression of Inflammatory Genes in BMSCs. Figures 5(i) and 5(j) show the time course of changes in the mRNA expression of inflammation-related genes in BMSCs in response to cyclic tension forces. IL-6 and IL-1β mRNA expression, compared to controls, was upregulated in a time-dependent manner in the mechanically stimulated groups.

4.6. Cyclic Tension from iStrain Influences the Protein Expression of Osteoblastic and Fibroblastic Differentiation-Related Genes in BMSCs. Figures 5(k)–5(q) demonstrate the changes in the expression of osteogenic-related proteins and fibroblastic differentiation-related proteins in BMSCs in response to cyclic tension force. Figure 5(k) shows the representative western blot results of RUNX2, COL-1, OPN, SCX, COL-3, TN-C, and GAPDH. Compared with that of the control group, the protein expression of RUNX2, COL-1, and OPN gradually peaked at 12 h and 24 h of mechanical tension loading and then decreased thereafter (Figures 5(l)–5(n)). The



(a)



(b)

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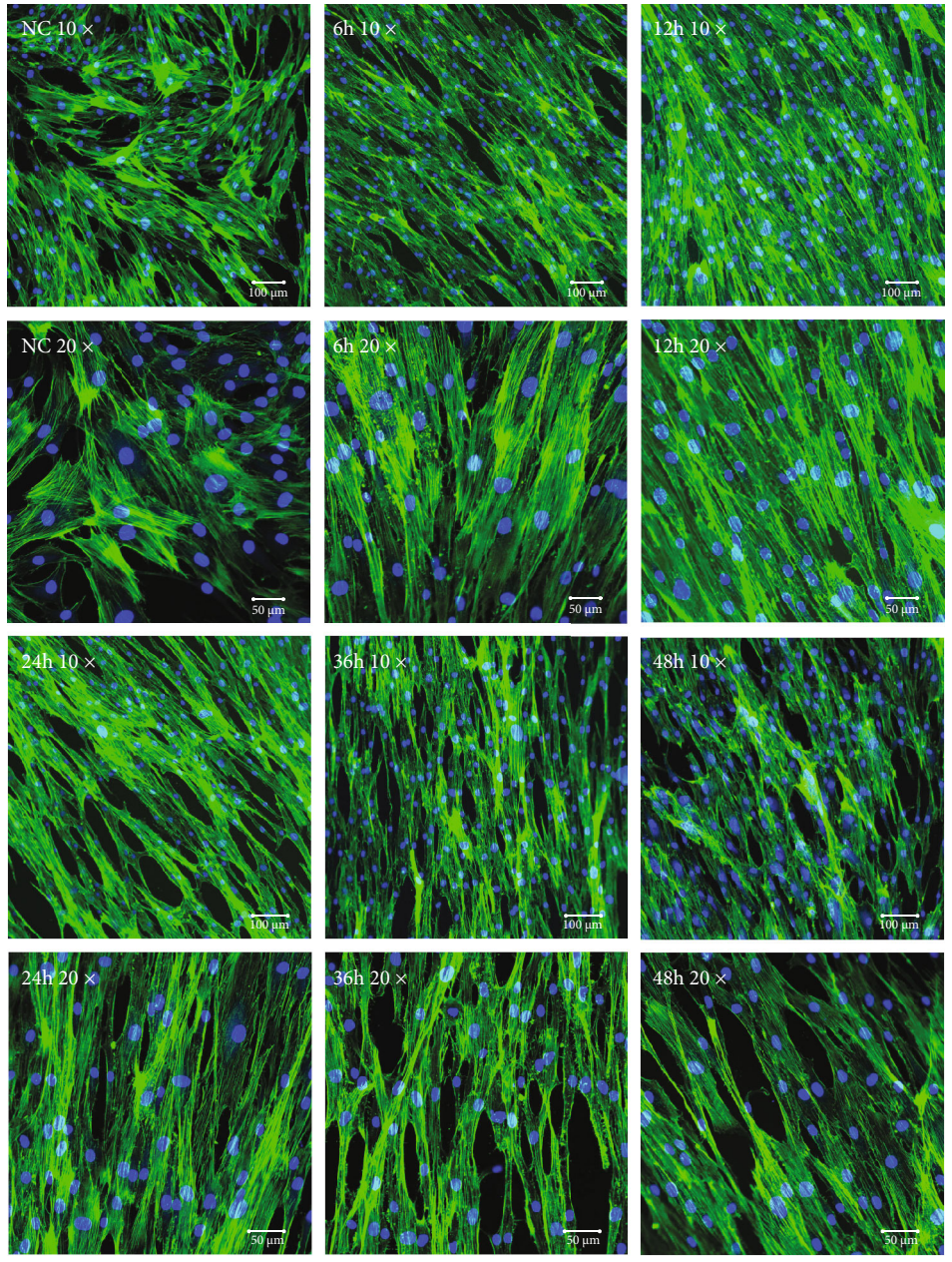


(c)

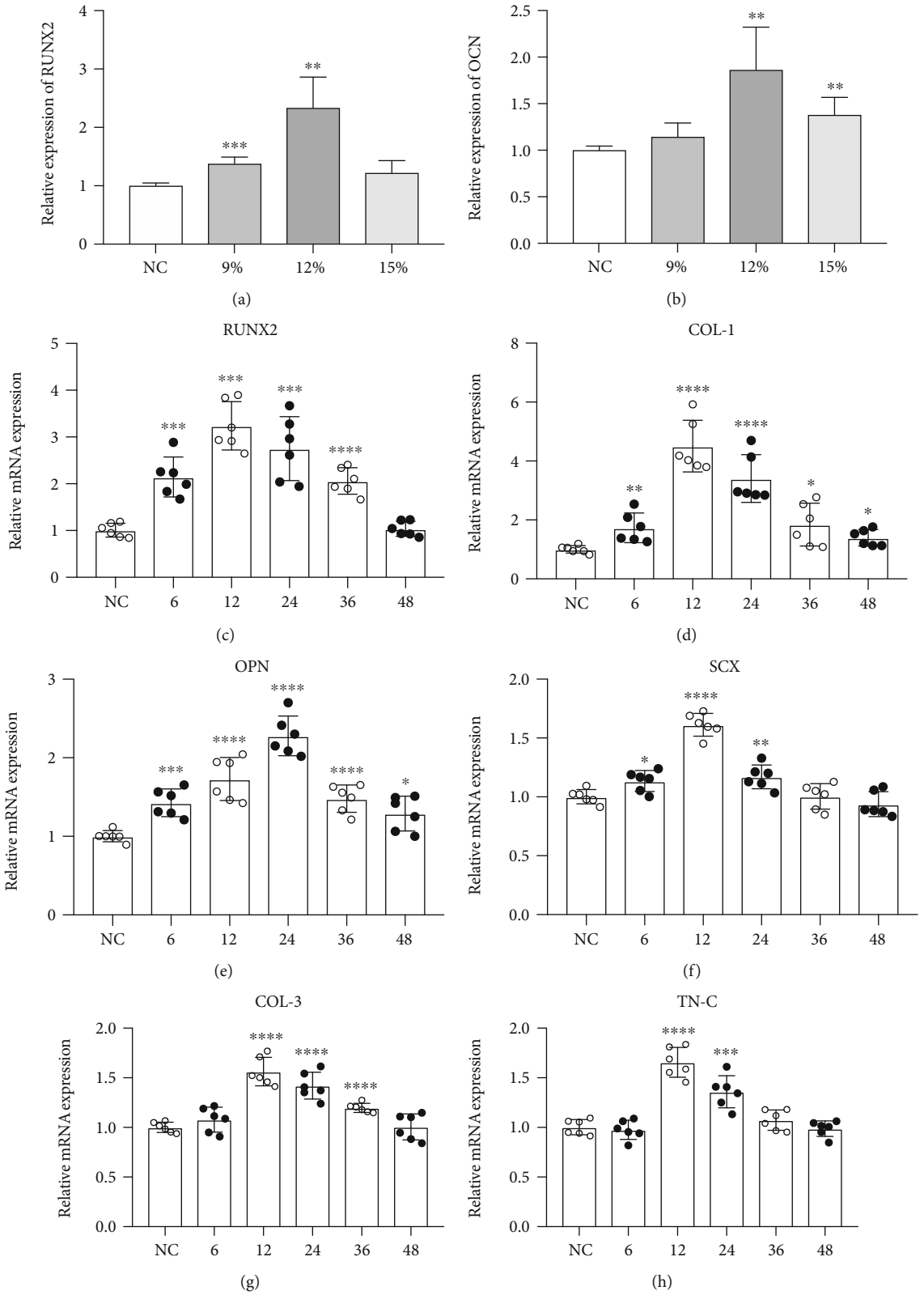
Figure 4: Cyclic tension influences BMSC arrangement and morphology. (a) Morphology and arrangement of BMSCs observed under the 4x and 10x microscopes after 6 h, 12 h, 24 h, 36 h, and 48 h of cyclic tension compared with those of the control group (without loading). Scale bars: 500 μm/200 μm. (b) Cell length of BMSCs measured under the microscope after 6 h, 12 h, 24 h, 36 h, and 48 h of cyclic tension compared with that of the control group. $^{**}P < 0.01$ and $^{***}P < 0.001$. (c) The green fluorescence indicates the F-actin. The blue fluorescence indicates the cell nucleus. Representative photographs of the control and cyclic tension groups at 6 h, 12 h, 24 h, 36 h, and 48 h under 10x and 20x microscopy are shown. Scale bars: 100 μm/50 μm.

protein expression of SCX, COL-3, and TN-C also gradually peaked at 12 h and 24 h of mechanical tension loading and decreased thereafter (Figures 5(o)–5(q)).

4.7. Cyclic Tension from iStrain Influences the Mineralization of BMSCs. Figure 6 shows the Alizarin red staining result of BMSCs in the different time groups. Alizarin red staining revealed a time-dependent increase in the color intensity, which suggests the mineralization ability increasing in BMSCs with the extension of tension time.

5. Discussion

In this research, to study the effects of tensile strain stimuli on BMSCs, we developed a small-scale program-controlled mechanical tension device, iStrain. By using this device, we



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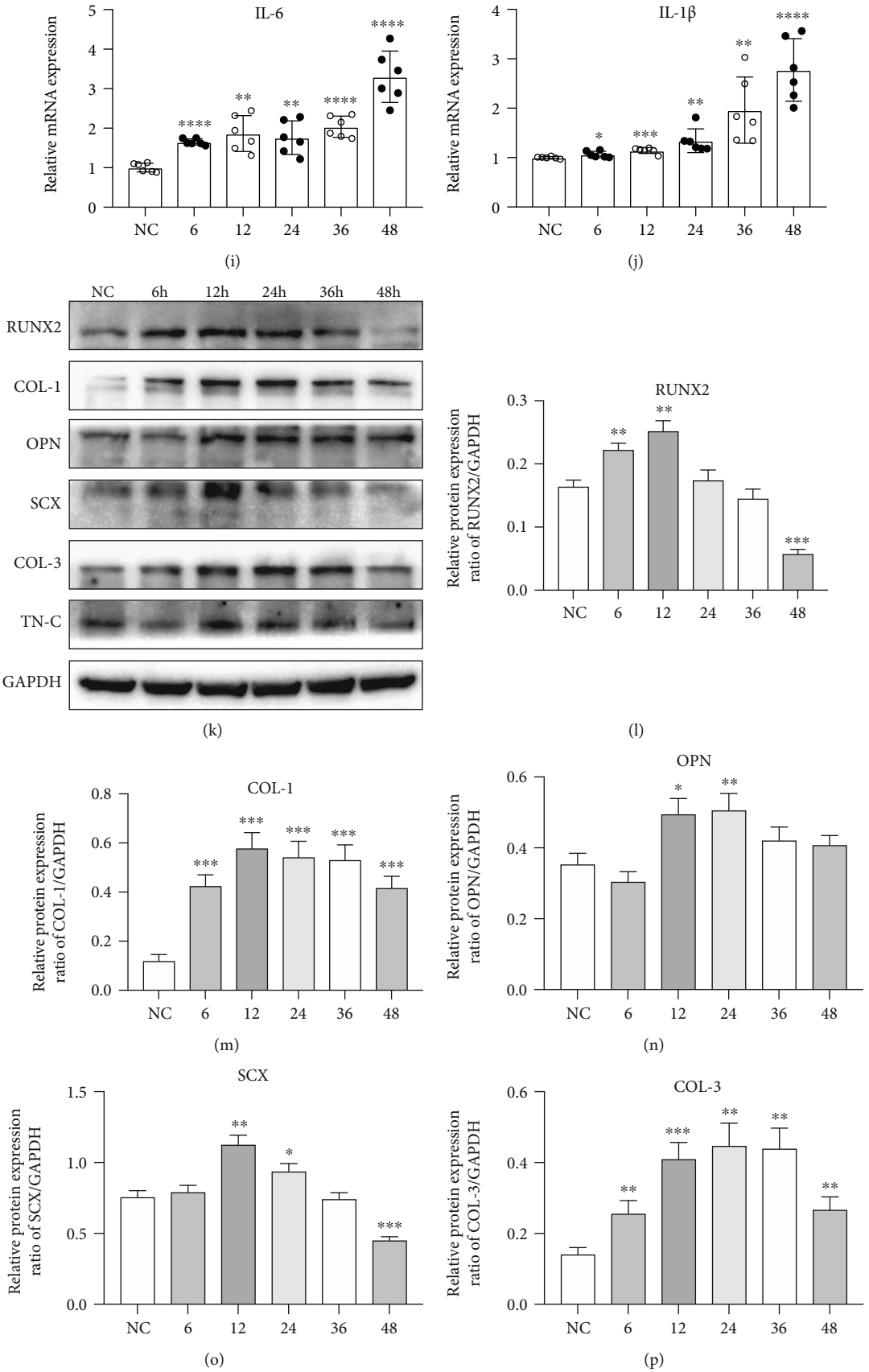

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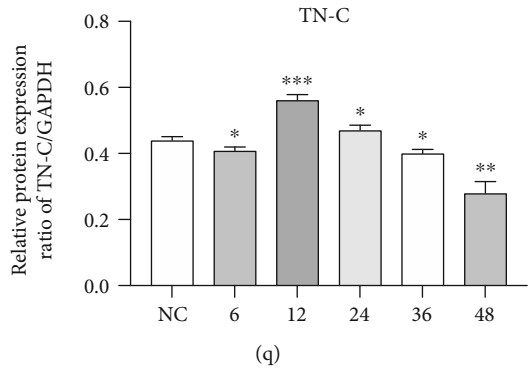

Figure 5: Cyclic tension promotes BMSC osteoblastic and fibroblastic differentiation. The mRNA expression levels of RUNX2 (a) and OCN (b) in response to cyclic tension force under different tension force values (including the control or with extension rates of 9%, 12%, and 15%) for 12 h. The mRNA expression levels of RUNX2 (c), COL-1 (d), OPN (e), SCX (f), COL-3 (g), TN-C (h), IL-6 (i), and IL-1β (j) in response to cyclic tension force with a 12% extension rate for the control or 6 h, 12 h, 24 h, 36 h, or 48 h of loading. Gene expression was calibrated using the GAPDH housekeeping gene. Data represent the mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs. the control. (h) Representative chemiluminescent images of western blot analysis of RUNX2, COL-1, OPN, SCX, COL-3, TN-C, and GAPDH in BMSCs. (l-q) Western blot analysis of RUNX2 (l), COL-1 (m), OPN (n), SCX (o), COL-3 (p), and TN-C (q). Gray value was calibrated using GAPDH. Data represent the mean ± SD; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ vs. the control.

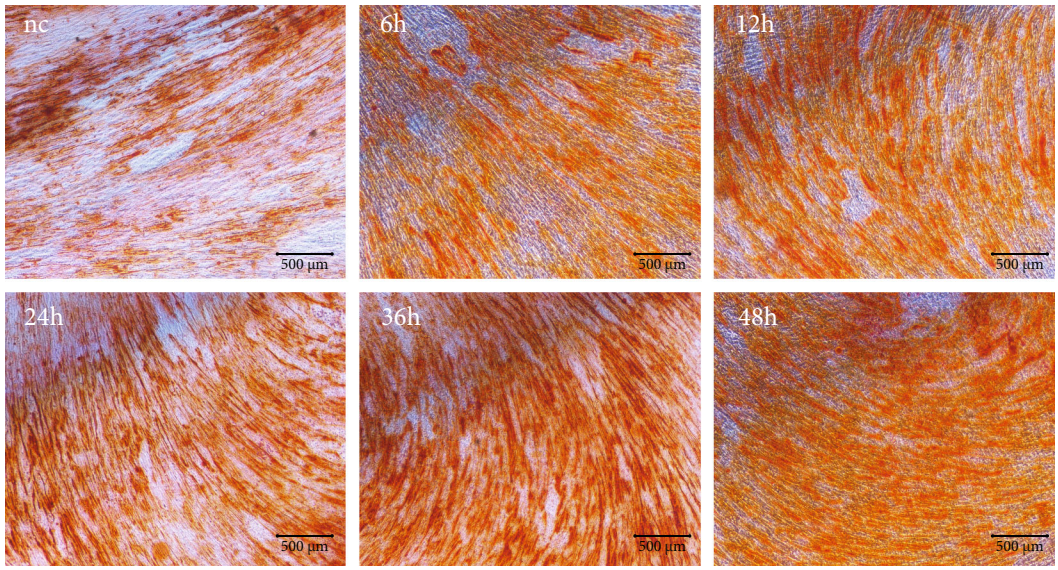

Figure 6: Cyclic tension promotes BMSC mineralization. The Alizarin red staining result of BMSCs in the control group and mechanically stimulated groups of 6 h, 12 h, 24 h, 36 h, and 48 h. Scale bars: 500 μm.

first investigated the suitable extension rate for BMSCs. According to similar preliminary experimental research methods, the elongation rate of the BMSC tension assay was mostly set to vary from 10% to 15% [11, 12]. Considering that the force application method of iStrain may be different from that used by other researchers, we first set the extension rate to three values: 9%, 12%, and 15%. We examined the mRNA expression of RUNX2 and OCN in each group by RT-qPCR and found that the expression of RUNX2 and OCN increased in the tensile groups, and the peak appeared in the group with a 12% extension rate. Therefore, we chose the 12% extension rate for subsequent experiments, including microscope observation, immunohistochemistry staining, RNA and protein extraction, and Alizarin red staining. Then, we utilized RT-qPCR and western blotting to analyze the mRNA and protein expression of osteogenic differentiation- and fibroblastic differentiation-related genes. Cyclic tensile strain stimuli will promote the osteogenic and fibroblastic differentiation of BMSCs, as shown by the increased expression of RUNX2, COL-1, OPN, SCX, COL-3, and TN-C. In the mechanical tension-stimulated groups, the mRNA and protein expression of most genes peaked when the tension duration was between 12 h and 24 h rather than longer time point. There are two possible factors to explain this. First, cell viability and proliferation will be harmed by long-term tensile strain, as we noted before. Second, with the

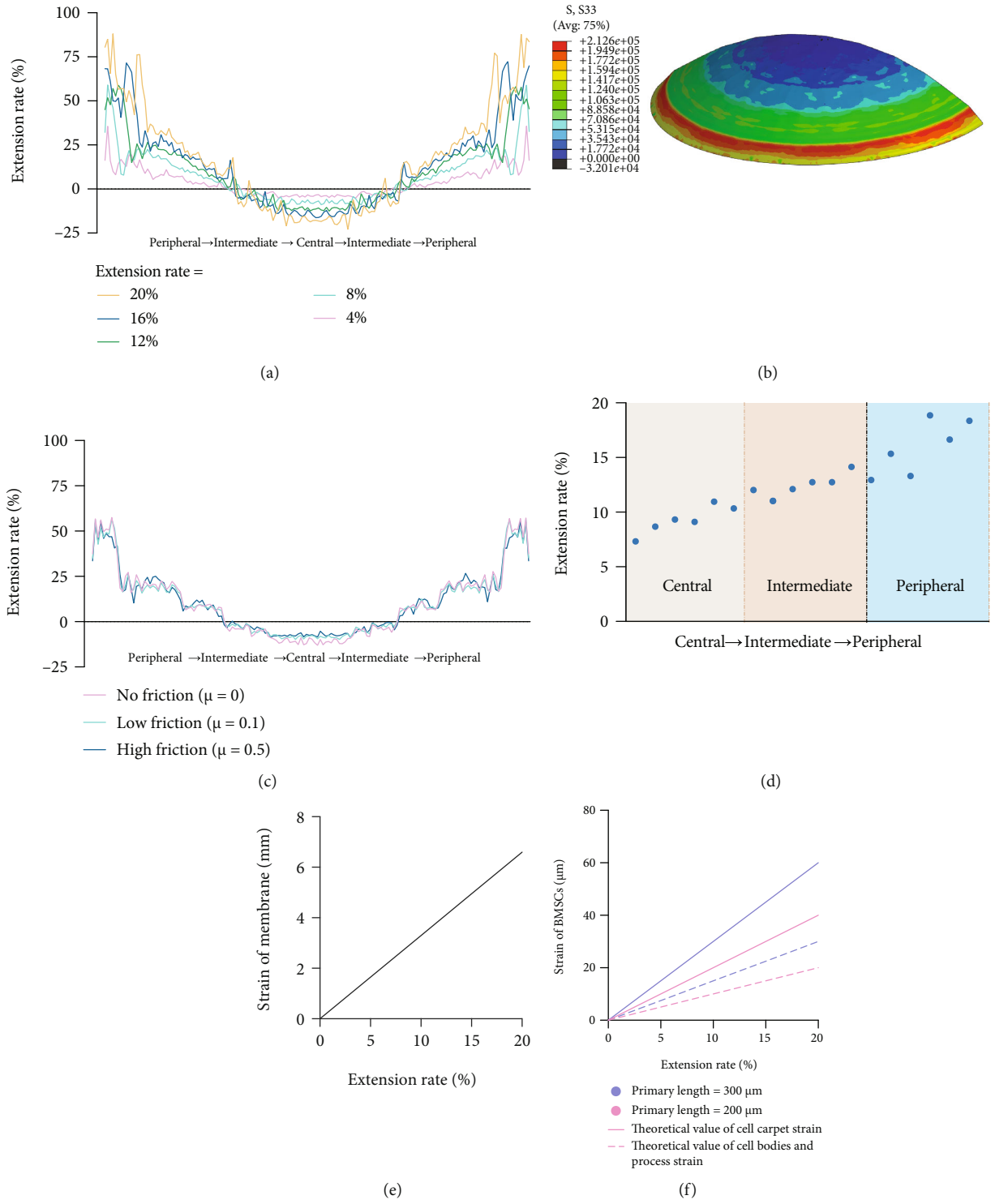

Figure 7: Strain of the membrane and BMSCs. (a) The strain on the membrane with different extension rates based on the three-dimensional finite element analysis. No friction between the membrane and the spherical stiffening elements supposed. The strain is determined as the ratio of the distances between two points under the stretched and unstretched conditions. (b) The three-dimensional rendering model of the distribution of tension force. (c) The three-dimensional finite element analysis of strain on the membrane under different frictions with 12% extension rate. (d) The distribution of strain from the central area to the peripheral area of the membrane. (e) The strain of the membrane equals extension rate times the primary length of the elastic membrane ($2l = 33$ mm). (f) The theoretical value of cell carpet strain is equal to the primary length times extension rate. The theoretical value of cell bodies and process strain is equal to half of the primary length times extension rate.

extension of time, the inflammatory reactions in BMSCs may be intensified, which will be harmful for osteoblastic and fibroblastic differentiation of BMSCs. The harmful effect of long-time cyclic tension on cell viability and proliferation can also explain the reason of the phenomena of increased intercellular space and sparse BMSC arrangement when the tension time exceeds 24 h, as less cell renewal and more cell death under long-time cyclic tension. The reason of why there are slight differences of mRNA and protein expression peak between different genes is correlated with different timing of the appearance of relevant markers. For example, compared with OPN, RUNX2 is a relative early marker of the osteogenic process. The mRNA and protein expression of RUNX2 peaked at 12 h, suggesting that early osteogenic differentiation activity is more active when the tension time is closed to 12 h, while the mRNA and protein expression of OPN peaked at 24 h, suggesting that late osteogenic differentiation activity is more active when the tension time is closed to 24 h.

When using iStrain for force application, BMSCs seeded on the elastic membrane will be strained together with the membrane. According to the manufacturer's protocols, the material of the bottom of BioFlex® culture plates is a type of flexible silicone elastomer, which is linear elastic isotropic. When we used iStrain to stretch the membrane, the deformation of the membrane is always in the range of elastic deformation. Plus, the surface of the spherical stiffening elements is designed as frictionless and stainless to minimize the friction between the elements and the membrane.

We also analyzed the strain of the membrane using three-dimensional finite element analysis by ANSYS® Software, version 19.0 (ANSYS Inc., Canonsburg, PA, USA). In the situation where friction was negligible, we compared the strain of the membrane with different extension rates. The results showed that the strain was closed to the theoretical extension rate in the intermediate area while smaller in the central area and larger in the peripheral area. The difference of the strain between different locations was smaller when the extension rate was relatively small and became larger when the extension rate increased (Figure 7(a)). The distribution of tension force was represented by different colors in the three-dimensional rendering model based on the three-dimensional finite element analysis, demonstrating an increasing trend from the central to the peripheral area (Figure 7(b)). And then, we compared the strain of the membrane under different frictions with 12% extension rate. The result showed that the strain has no significant changes between different friction groups (Figure 7(c)). Moreover, we also examined the strain of the membrane at different locations with 12% theoretical extension rate using flour particles as Wang et al. applied [13]. The extension rate was closed to 12% in the intermediate area while smaller in the central area and larger in the peripheral area, but the difference between them was considered to be acceptable (Figure 7(d)).

To sum up, the cells in the center of the membrane were deformed less than the ones next to the edges, once they were deformed spherically. The curve of the extension rate rose slowly from the center to the peripheral area and then ascended steeply in the most peripheral area. So, in our opinion, cell deformation in most part of the membrane could be considered uniform, except for the most peripheral area. And the experimental value of the extension rate was close to the theoretical one. However, the degree of the deviation from uniform stretching increased with the extension rate. Once the extension rate exceeded 16%, the nonuniformity of strain was thought to become unacceptable to be neglected.

The strain of the elastic membrane is equal to the primary length of the membrane times the extension rate, which can be written as the strain of the elastic membrane $= 2l \times$ extension rate (l is the radius of the membrane, which equals half of the primary length of the membrane) (Figure 7(e)). According to the research by Bieler et al., cell carpets show full strain transfer, while cell processes and cell bodies show about 50% strain transfer [14]. Therefore, the theoretical value of cell carpet strain is equal to the primary length times extension rate. The theoretical value of cell bodies and processes strain is equal to half of the primary length times extension rate (Figure 7(f)).

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

All authors have no conflicts of interest.

Authors' Contributions

Yi Zhao and Yiping Huang contributed to this article equally. Yi Zhao contributed to cell experiment, device design, collection of data and analysis, statistical analysis, and writing of the manuscript. Lingfei Jia contributed to the data collection and statistical analysis. Ruoxi Wang and Kuang Tan contributed to cell experiment. Weiran Li and Yiping Huang contributed to the design of this study as well as the revision of the manuscript. All authors have read and approved the final article.

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References

[1] R. Schofield, "The stem cell system," *Biomedicine & Pharmacotherapy= Biomedecine & Pharmacotherapie*, vol. 37, no. 8, pp. 375–380, 1983.

[2] B. Zhao, J. Wang, C. Ren et al., "Mechanical stretching promotes the differentiation of BMSC into pelvic floor ligament fibroblasts," *Oncotarget*, vol. 5, 2018.

[3] A. J. Banes, M. Tsuzaki, J. Yamamoto et al., "Mechanoreception at the cellular level: the detection, interpretation, and diversity of responses to mechanical signals," *Biochemistry and Cell Biology*, vol. 73, no. 7-8, pp. 349–365, 1995.

[4] A. G. Petrov and P. N. Usherwood, "Mechanosensitivity of cell membranes. Ion channels, lipid matrix and cytoskeleton," *European Biophysics Journal*, vol. 23, no. 1, pp. 1–19, 1994.

[5] C. A. Lambert, B. Nusgens, and C. M. Lapière, "Mechano-sensing and mechano-reaction of soft connective tissue cells," *Advances in Space Research*, vol. 21, no. 8-9, pp. 1081–1091, 1998.

[6] S. Wada, H. Kanzaki, T. Narimiya, and Y. Nakamura, "Novel device for application of continuous mechanical tensile strain to mammalian cells," *Biology Open*, vol. 6, no. 4, pp. 518–524, 2017.

[7] G. C. Reilly and A. J. Engler, "Intrinsic extracellular matrix properties regulate stem cell differentiation," *Journal of Biomechanics*, vol. 43, no. 1, pp. 55–62, 2010.

[8] P. Hu, X. Zhu, C. Zhao, J. Hu, E. Luo, and B. Ye, "Fak silencing impairs osteogenic differentiation of bone mesenchymal stem cells induced by uniaxial mechanical stretch," *Journal of Dental Sciences*, vol. 14, no. 3, pp. 225–233, 2019.

[9] J. Li, X. Wang, F. Yang et al., "Matrine enhances osteogenic differentiation of bone marrow-derived mesenchymal stem cells and promotes bone regeneration in rapid maxillary expansion," *Archives of Oral Biology*, vol. 118, p. 104862, 2020.

[10] L. Yang, Y. Yang, S. Wang, Y. Li, and Z. Zhao, "_In vitro_ mechanical loading models for periodontal ligament cells: from two- dimensional to three-dimensional models," *Archives of Oral Biology*, vol. 60, no. 3, pp. 416–424, 2015.

[11] B. Zhang, Q. Luo, Z. Chen et al., "Cyclic mechanical stretching promotes migration but inhibits invasion of rat bone marrow stromal cells," *Stem Cell Research*, vol. 14, no. 2, pp. 155–164, 2015.

[12] H. Yu, W. Yu, Y. Liu, X. Yuan, R. Yuan, and Q. Guo, "Expression of HIF-1α in cycling stretch-induced osteogenic differentiation of bone mesenchymal stem cells," *Molecular Medicine Reports*, vol. 20, pp. 4489–4498, 2019.

[13] J. Wang, B. Fan, Y. Wei, X. Suo, and Y. Ding, "A simple multi-well stretching device to induce inflammatory responses of vascular endothelial cells," *Lab on a Chip*, vol. 16, no. 2, pp. 360–367, 2016.

[14] F. H. Bieler, C. E. Ott, M. S. Thompson et al., "Biaxial cell stimulation: a mechanical validation," *Journal of Biomechanics*, vol. 42, no. 11, pp. 1692–1696, 2009.

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Research Article

Optimal Pore Size of Honeycomb Polylactic Acid Films for In Vitro Cartilage Formation by Synovial Mesenchymal Stem Cells

Misaki Yagi,[1] Mitsuru Mizuno,[1] Ryota Fujisawa,[1] Hisako Katano,[1] Kentaro Endo,[1] Nobutake Ozeki,[1] Yuriko Sakamaki,[2] Hideyuki Koga,[3] and Ichiro Sekiya[1]

[1]*Center for Stem Cell and Regenerative Medicine, Tokyo Medical and Dental University, 1-5-45, Bunkyo-ku, Yushima, Tokyo, Japan*
[2]*Research Core, Tokyo Medical and Dental University, Tokyo, Japan*
[3]*Department of Joint Surgery and Sports Medicine, Tokyo Medical and Dental University, Tokyo, Japan*

Correspondence should be addressed to Ichiro Sekiya; sekiya.arm@tmd.ac.jp

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Background. Tissue engineering of cartilage requires the selection of an appropriate artificial scaffold. Polylactic acid (PLA) honeycomb films are expected to be highly biodegradable and cell adhesive due to their high porosity. The purpose of this study was to determine the optimal pore size of honeycomb PLA films for in vitro cartilage formation using synovial mesenchymal stem cells (MSCs). *Methods*. Suspensions of human synovial MSCs were plated on PLA films with different pore sizes (no pores, or with 5 μm or 20 μm pores) and then observed by scanning electron microscopy. The numbers of cells remaining in the film and passing through the film were quantified. One day after plating, the medium was switched to chondrogenic induction medium, and the films were time-lapse imaged and observed histologically. *Results*. The 5 μm pore film showed MSCs with pseudopodia that extended between several pores, while the 20 μm pore film showed MSC bodies submerged into the pores. The number of adhered MSCs was significantly lower for the film without pores, while the number of MSCs that passed through the film was significantly higher for the 20 μm pore film. MSCs that were induced to form cartilage peeled off as a sheet from the poreless film after one day. MSCs formed thicker cartilage at two weeks when growing on the 5 μm pore films than on the 20 μm pore films. *Conclusions*. Honeycomb PLA films with 5 μm pores were suitable for in vitro cartilage formation by synovial MSCs.

1. Background

Tissue engineering of cartilage requires the appropriate selection of cells [1]. Several cell candidates are currently available, including chondrocytes, induced pluripotent stem (iPS) cells, and mesenchymal stem cells (MSCs). The use of chondrocytes is invasive, as cell collection requires that normal cartilage be sacrificed [2]; however, iPS cells require more time and effort than other cell types for cartilage differentiation [3]. MSCs are therefore more useful, as their cell sources are easy to harvest, the cells proliferate well, and they can be induced to differentiate into cartilage. Synovial MSCs are particularly attractive as a cell source for tissue engineering of cartilage because of their high chondrogenic differentiation potential [4, 5].

The appropriate selection of artificial material is also important for cartilage engineering [6] in addition to the selection of synovial MSCs. Several artificial scaffold materials are already in clinical use [7]. In the field of orthopedics, one of the most popular scaffold materials is polylactic acid (PLA) because of its biodegradability; however, cells can have difficulty adhering to it. For this reason, PLA is not yet in common use as a scaffold for cells used clinically for cartilage regeneration. Efforts made to overcome this adhesion problem have included spinning of PLA nanofibers and arranging PLA fibers in lattice patterns [8, 9], but the types of PLA scaffolds that are most suitable for tissue formation have not yet been identified.

PLA can be prepared in the form of honeycomb-like sheets, and these are expected to show highly biodegradability and improved cell adhesion due to their high porosity [10]. Porous films can be formed from water droplet templates by the breath figure method, and this method has attracted considerable interest because of its simplicity and

wide applicability to a variety of materials. Recently, honeycomb films have been prepared by the breath figure technique [11, 12]; however, the best pore size for efficient cell adhesion and cartilage formation has not been established. The purpose of the present study was to determine the optimal pore size of honeycomb PLA films for in vitro cartilage formation by synovial MSCs.

2. Methods

2.1. Preparation of Synovial MSCs. All methods were carried out in accordance with relevant guidelines and regulations. All procedures performed in the study involving human participants were in accordance with the Declaration of Helsinki [13]. This study was approved by the Medical Research Ethics Committee of Tokyo Medical and Dental University (M2017-142), and informed consent was obtained from all study subjects.

Human synovium was harvested from the knees of patients with osteoarthritis who underwent total knee arthroplasty operations, and cell culture was performed according to the method established in our previous reports [4, 14, 15]. Briefly, the synovium was minced and digested at 37°C for 3 h in a solution of 3 mg/mL collagenase (Sigma Aldrich, MO, USA), and the digested cells were filtered through a 70 μm cell strainer (Greiner Bio-one GmbH, Kremsmuenster, Austria). The obtained nucleated cells were cultured in 150 cm^2 culture dishes (Nalge Nunc International, Thermo Fisher Scientific, MA, USA) in 18 mL alpha minimum essential medium (αMEM, Thermo Fisher Scientific) containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific). The cells were treated with 0.25% trypsin-EDTA (Thermo Fisher Scientific) at 37°C for 5 min, harvested, and cryopreserved as passage 0. For cell culture, the frozen cells were slowly thawed, plated, and incubated for 4 days as passage 1. These passage 1 cells were then replated at 50 cells/cm^2, cultured for 14 days, and the resulting passage 2 cells were used for analyses.

2.2. Differentiation Assays. The differentiation potential of the MSCs was evaluated as in previous studies [15–17]. Chondrogenesis was examined by suspending 1.25×10^5 synovial MSCs in 0.5 mL chondrogenic induction medium consisting of Dulbecco's modified Eagle's medium (DMEM, Thermo Fisher Scientific) containing 10 ng/mL transforming growth factor-β3 (TGF-β3, Miltenyi Biotec, Bergisch Gladbach, Germany), 500 ng/mL bone morphogenetic protein 2 (BMP-2, Medtronic, MN, USA), 40 μg/mL proline (Sigma-Aldrich), 100 nM dexamethasone (Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), 100 μg/mL pyruvate (Sigma-Aldrich), 50 μg/mL ascorbate-2-phosphate (Fujifilm Wako Pure Chemical Corporation), and 50 mg/mL 1% ITS Premix (BD: Becton, Dickinson and Company, NZ, USA). The cells were pelleted by centrifugation at 500 × g for 10 min and then cultured for 21 days. After centrifugation, the pellets were sectioned and stained with toluidine blue (Fujifilm Wako Pure Chemical Corporation) for morphological analysis. Adipogenesis was determined by suspending 100 synovial MSCs in a 60 cm^2 dish and culturing in culture medium for 14 days to produce cell colonies. The adherent cells were cultured for a further 21 days in adipogenic induction medium consisting of α-MEM supplemented with 100 nM dexamethasone, 0.5 mM isobutylmethylxanthine (Sigma-Aldrich), and 50 mM indomethacin (Fujifilm Wako Pure Chemical Corporation). Adipocytes were stained with oil red O (Muto Pure Chemicals, Tokyo, Japan).

Calcification was studied by plating 100 synovial MSCs in a 60 cm^2 dish and culturing for 14 days in culture medium to allow formation of cell colonies. The adherent cells were further cultured in a calcification induction medium consisting of α-MEM supplemented with 50 μg/mL ascorbic acid 2-phosphate, 10 nM dexamethasone, and 10 mM β-glycerophosphate (Sigma-Aldrich). After 21 days, calcification was assessed by alizarin red staining (Merck Millipore, MA, USA).

2.3. PLA Honeycomb Films. Circular PLA films with a diameter of 6 mm and thickness of 5 μm were prepared as three types: a PLA film without any pores (0 μm), one with 5 μm pores, and one with 20 μm pores. Before cell plating, the films were immersed in 70% ethanol, washed with phosphate-buffered saline (PBS, Thermo Fisher Scientific) for hydrophilization, and incubated with FBS overnight at 4°C.

2.4. Scanning Electron Microscopy (SEM). Synovial MSCs (1×10^4 cells) were suspended in 500 μL αMEM with 10% FBS and plated on PLA films in a 24-well plate. After 2 h, the films were fixed in 2.5% glutaraldehyde (TAAB Laboratories and Equipment Ltd., Berks, England) for 2 h and washed overnight in 0.1 M PBS at 4°C. Each film specimen was then postfixed with 1% osmium tetroxide (TAAB Laboratories and Equipment Ltd.) for 2 h at 4°C and dehydrated in graded ethanol solutions (Fujifilm Wako Pure Chemical Corporation). After exchanging with 3-methyl butyl acetate (Fujifilm Wako Pure Chemical Corporation) and critical point drying, the specimen was coated with platinum and the surface was observed by SEM (S-4500, Hitachi Ltd., Tokyo, Japan) [18].

2.5. Surface Markers. Synovial MSCs (5×10^5 cells) at passage 3 in 500 μL αMEM containing 10% FBS were dropped onto films in the wells of a 24-well plate. A 1 mL volume of αMEM containing 10% FBS was added, and the cells were incubated for 24 h at 37°C and 5% CO_2. Adherent cells were then detached by treating with 0.25% trypsin-EDTA for 5 min. The cells were suspended in Hank's balanced salt solution (HBSS, Thermo Fisher Scientific) at a density of 5×10^5 cells/mL and treated for 30 minutes on ice with antibodies for CD14, CD29 (integrin β1), CD31, CD44, CD45, CD49c (integrin α3), CD49f (integrin α6), CD51/CD61 (integrin $\alpha v\beta$3), CD61, CD73, CD106, and CD146 (all from BD). The data were analyzed by FACS Verse (BD) and FlowJo software (Tree Star Inc., OR, USA). Cells positively stained with Ghost Dye Violet 510 (Tonbo Biosciences, CA, USA) were removed as dead cells. Isotype controls were prepared as negative controls (BD). The expressions of CD29, CD49c, CD49f, and CD51/CD61 by cells seeded on plastic dishes (just before seeding on the film) were examined in the same manner.

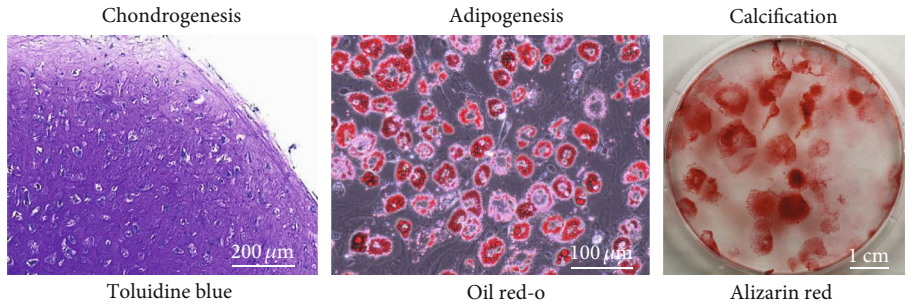

Figure 1: Multipotency of synovial MSCs. Synovial MSCs were cultured in chondrogenic, adipogenic, and calcification induction media. A section of the cartilage pellet was stained with toluidine blue, and the culture dishes for adipogenesis and calcification were stained with oil red O and alizarin red, respectively.

2.6. Quantitative Evaluation of Adherent Cells. Synovial MSCs were stained with DiI (Thermo Fisher Scientific) and 3×10^3 synovial MSCs suspended in 500 μL αMEM with 10% FBS were plated on the PLA films in a 24-well plate. After 24 h, the films and plates were fixed with 10% neutral formalin (Fujifilm Wako Pure Chemical Corporation). The MSCs remaining on the PLA films and the MSCs passing through the film to the bottom of the plates were observed, and the numbers of the cells were counted from the images obtained with a microscope analysis system (BZ-X810, KEYENCE, Osaka, Japan). Partial images of 4×4 area of film and a 10×10 area of bottom were taken at 4x magnification of the objective lens and automatically combined using software (BZ-X analyzer, KEYENCE). The regions with a fluorescence intensity above a fixed value within the film or on the bottom were defined as cells, and the number of regions was counted using imaging software (BZ-X analyzer). The numbers of nonadherent cells were calculated from the numbers of adherent cells on film and bottoms.

2.7. In Vitro Cartilage Formation of MSCs Plated on PLA Films. A 500 μL volume of αMEM with 10% FBS containing 5×10^5 synovial MSCs was dropped onto films in the wells of a 24-well plate. A further 1 mL αMEM with 10% FBS was added, and the cells were incubated for 24 h. The medium was then switched to chondrogenic induction medium (this was considered time 0). The medium was exchanged every 1–3days.

2.8. Time-Lapse Images. Synovial MSCs seeded on PLA films were observed with a microscope (EVOS XL Core Cell Imaging System, Thermo Fisher Scientific) at time 0, 1, 3, and 5 days. The film and cell sheet areas at each time point were defined manually using ImageJ software (National Institutes of Health, MD, USA), and the area ratio was calculated.

2.9. Histology. Synovial MSCs seeded on PLA films were washed with PBS, fixed in 4% paraformaldehyde (PFA, Fujifilm Wako Pure Chemical Corporation) at 4°C for 1 h, and embedded in 2% agarose gel (Fujifilm Wako Pure Chemical Corporation). The framework of the PLA film was then removed, and the cylindrical agarose gel holding the PLA film was embedded in paraffin, sliced, stained with safranin-o/fast green (Fujifilm Wako Pure Chemical Corporation), and observed with a microscope (BZ-X810).

The cartilage thickness was measured by drawing a single line along the long axis of the cartilage, determining the midpoint of both ends of the cartilage on that line, and then drawing seven perpendicular lines 500 μm on both sides of that midpoint. The midpoints of both ends of the cartilage were determined on each vertical line, and the minimum width through these points was determined. Finally, an average thickness of the cartilage and the coefficient of variance for cartilage thickness at 7 points were calculated using ImageJ.

2.10. Statistical Analysis. The Shapiro-Wilk test was used to confirm the normality of the data ($P > 0.05$). The analysis between the two groups were calculated by a paired Student's t test. Cell counts and time courses were statistically analyzed by two-way analysis of variance (ANOVA) with Tukey's multiple comparisons test using GraphPad Prism 8 software (GraphPad Software, CA, USA). All statistical analysis methods are described in the figure legends. Two-tailed P values of <0.05 were considered statistically significant.

3. Results

3.1. SEM Images of Honeycomb PLA Films after Plating MSCs. Synovial MSCs showed multipotency for chondrogenesis, adipogenesis, and calcification (Figure 1). At 2 h after plating onto the film without pores (0 μm), MSCs showed extended pseudopodia and attachment to the film (Figure 2(a)). MSCs on the 5 μm pore film also showed pseudopodia that extended and adhered to several pores. MSCs on the 20 μm pore film showed submergence of the cell body into the pore and pseudopodia extending around the pore.

3.2. Surface Markers of Synovial MSCs Plated onto Honeycomb Films. One day after seeding onto films with pore sizes of 0, 5, and 20 μm, the MSCs in each film expressed 100% of the positive MSC markers CD44, CD73, and CD90, and less than 5% of the negative MSC markers CD14, CD45, CD106, and CD146 (Figure 2(b)). The expression of the four different integrins in MSCs did not differ among the films, but the expression of integrin α6 was

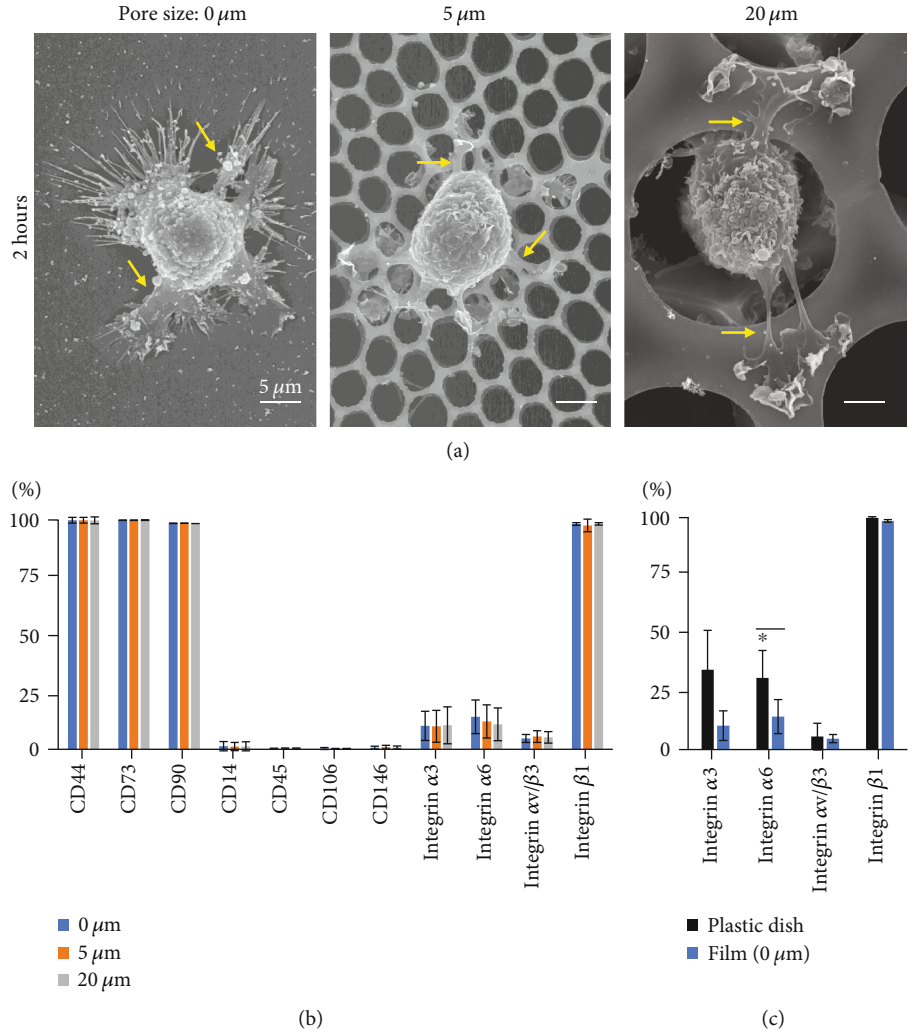

Figure 2: SEM images and surface markers of synovial MSCs plated onto honeycomb films. (a) SEM images of honeycomb PLA films after plating synovial MSCs. Synovial MSCs (1.0×10^4 in 500 μL medium) were plated on PLA films without pores (0 μm) or with 5 μm and 20 μm pores and observed from above after 2 h. Representative pseudopodia are indicated with yellow arrows. (b) Surface markers of synovial MSCs one day after seeding onto the film. Means and standard deviations are shown ($n = 3$). (c) Surface markers of synovial MSCs before and one day after seeding onto 0 μm film. $^*P < 0.05$. P values were determined by paired Student's t test.

significantly lower in MSCs on films than in MSCs cultured in plastic dishes (Figure 2(c)).

3.3. MSC Numbers Remaining in and Passing through Films. One day after seeding the MSCs (Figure 3(a)), fewer cells were observed in the film without pores than in the films with pores, and more cells were observed in the bottom of the dish containing the film with 20 μm pores than dishes containing the other film types (Figure 3(b)). The number of cells on the film with no pores was 770 ± 100 cells, which was significantly lower than the cell number of 1110 ± 60 for the 5 μm pore film or 1100 ± 120 cells for the 20 μm pore film (Figure 3(c)). The number of cells on the bottom was essentially 0 cells for both the film with no pores and the 5 μm pore film, which was significantly lower than 170 ± 110 cells noted for the 20 μm pore film. The number of cells not adhered to the film or the bottom of the dish was 2200 ± 100 cells for the film with no pores and was significantly higher than the 1900 ± 60 cells obtained with the 5 μm pore film. The value was also significantly higher than 1700 ± 130 cells obtained with the 20 μm pore film.

3.4. The Early Phase of Cartilage Formation. During in vitro cartilage formation by MSCs, the cell sheet formed by the plated MSCs peeled off the film without pores at one day, assumed a round shape at three days, and was maintained as a cartilage mass at five days (Figure 4(a)). By contrast, the cell sheets formed by MSCs plated on the 5 and 20 μm pore films remained sheet-like and did not peel off the film. Quantitative evaluation showed that the area of the sheet of MSCs plated on the film without pores significantly decreased to $20 \pm 10\%$ at 1 day, while the area of the sheet of MSCs plated on the film with 5 and 20 μm pores was maintained at 100% for 5 days (Figure 4(b)).

3.5. Effect of Pore Size of the PLA Film on Cartilage Formation. Synovial MSCs were plated on the three types

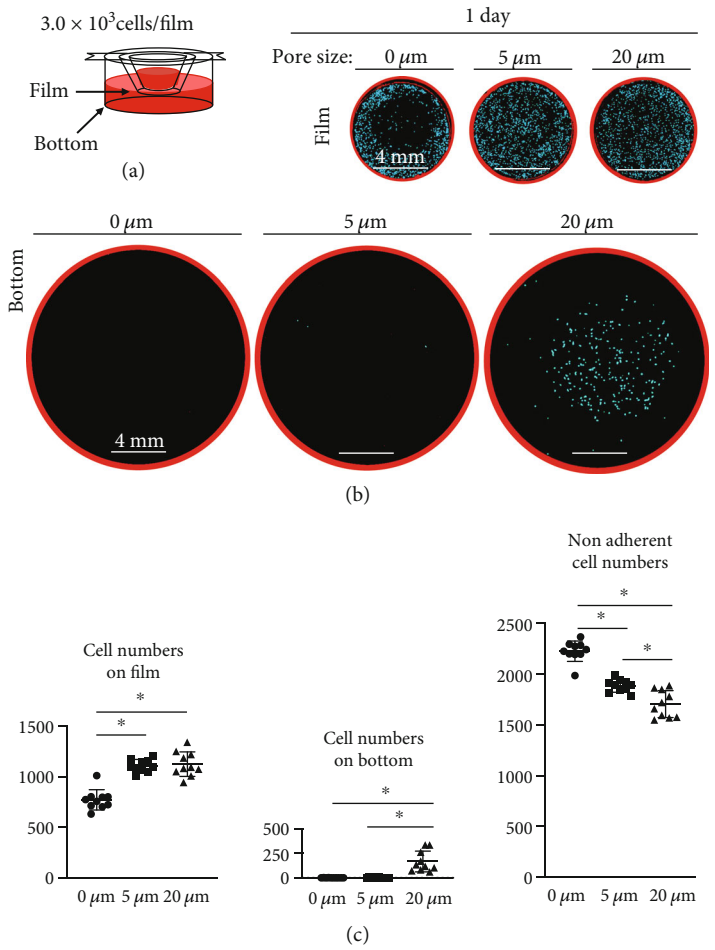

Figure 3: Analysis of numbers of synovial MSCs remaining in and passing through PLA films. (a) Experimental setting. (b) Images of MSCs remaining on PLA films and MSCs passing to the bottoms of the plates. DiI-stained cells were observed from above and image processed and are shown in blue. (c) Quantitative evaluation of cell numbers on the films, on the bottoms of the dishes, and nonadherent cells. Means and standard deviations are shown ($n = 10$). $^*P < 0.05$. P values were determined by one-way ANOVA with Tukey's multiple comparisons test.

of PLA films, cultured in the chondrogenic induction medium for two weeks, and then observed histologically (Figure 5(a)). MSCs plated on all three PLA films produced extracellular matrix, as confirmed by red staining with safranin O and they formed cartilage. The MSCs plated on the films without pores were spherical in shape, whereas the MSCs plated on the films with pores were sheet-like. The thickness of the sheet-like cartilage formed by MSCs plated on 5 μm pore film (Figure 5(b)) was 630 ± 130 μm and was significantly thicker than the 460 ± 100 μm thick sheet-like cartilage formed by plating MSCs on the 20 μm pore film (Figure 5(c)). The coefficient of variance for thickness of sheet-like cartilage measured at seven different points was 0.05 ± 0.01 for MSCs seeded on 5 μm pore film and was significantly lower than the 0.15 ± 0.11 thickness measured for MSCs seeded on the 20 μm pore film. This suggests the formation of a more even cartilage by MSCs seeded on 5 μm pore film than on 20 μm pore film.

3.6. Cartilage Formation of MSCs Plated on 5 μm Pore PLA Films. Synovial MSCs plated on 5 μm pore PLA films and cultured in the chondrogenic induction medium for three weeks showed multilayered cells above the film and a small number of cells below the film (Figure 6). A few layers of cells were observed above and below the film and a slight cartilage matrix was produced at one week, and the thickness of the cartilage increased at two weeks and further increased to 300–500 μm at 3 weeks. The thickness ratio of the cartilage above and below the film was approximately 3 : 1.

4. Discussion

Synovial MSCs seeded on PLA films without pores exhibited extended pseudopodia. Some reports have indicated that the pseudopodia perform the function of cell adhesion [18, 19]; however, in the present study, the number of adherent cells was on the film without pores was only about one third of the number on the PLA films with pores. This finding does not mean that the pseudopodia of synovial MSCs have no ability to adhere to films without pores; rather, it means that the pseudopodia of synovial MSCs are not sufficiently adhesive to withstand removal of the film from the dish, fixation of the cells with formalin, and microscopy observation. In

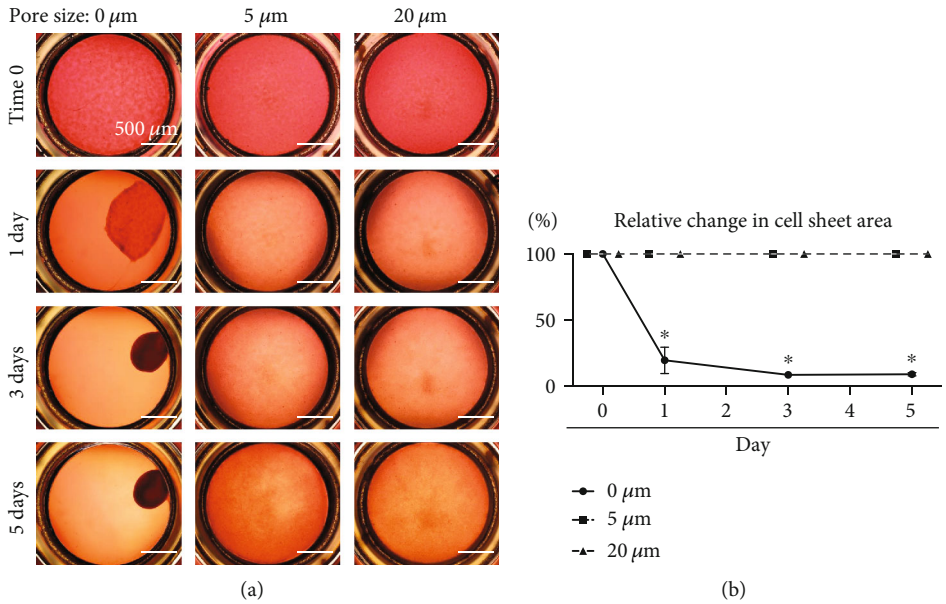

Figure 4: Early phase of cartilage formation by synovial MSCs on PLA films. (a) Time lapse pictures. MSCs (5.0×10^5 in 500 μL medium) were plated, cultured in chondrogenic induction medium, and then continuously observed. (b) Relative change in cell sheet area. Means and standard deviations are shown ($n = 3$). $^*P < 0.05$. P values were determined by the two-way ANOVA with Tukey's multiple comparisons test.

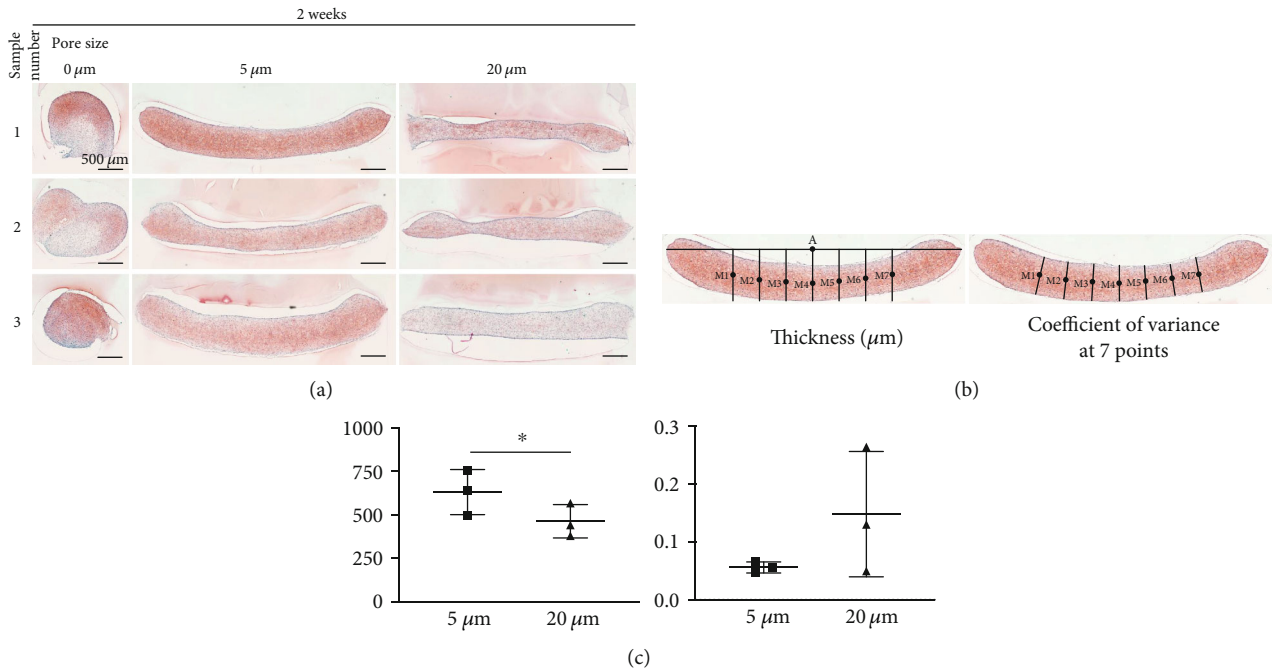

Figure 5: Histological images and thickness of cartilage derived from synovial MSCs plated on PLA films. (a) Histological images. MSCs (5.0×10^5 in 500 μL medium) were plated, cultured in chondrogenic induction medium for 14 days, and stained with safranin O. The results of three different samples are shown. (b) Method for measuring the cartilage thickness. A single line was drawn along the long axis of the cartilage, the midpoint of both ends of the cartilage was determined on this line (point A), and seven perpendicular lines were drawn every 500 μm on both sides from "point A." The midpoints of both ends of the cartilage were determined on each vertical line (M1-M7), and the minimum width through these points was determined. (c) Average thickness of the cartilage and the coefficient of variance for cartilage thickness at 7 points. Means and standard deviations are shown ($n = 3$). $^*P < 0.05$. P values were determined by a paired Student's t test.

other words, pores of a suitable size are useful to ensure efficient functioning of the pseudopodia.

The expression of surface markers by the synovial MSCs that adhered to the film did not differ with the presence or size of the pores, but the expression of integrin $\alpha 6$ was reduced compared to cells cultured on plastic dishes. The PLA film we used is a thin sheet of less than 10 μm thickness and has softer physical properties than a plastic dish. The expression level of integrin $\alpha 6$ might be altered when MSCs adhere to soft PLA materials, since the expression levels of other integrins depend on mechanosensing of the rigidity of the materials by the adhering cells [20–24].

Chondrogenic induction was observed in MSCs plated on the PLA films without pores, but the cell sheet peeled off from the films after one day and formed a spherical cartilage mass after two weeks. The ascorbate-2-phosphate contained in the chondrogenic induction medium promotes the production of extracellular matrix and the aggregation of MSCs [25]. The observation of peeling of the cell sheets from the films during the process of cartilage formation indicates that the strength of aggregation of the MSCs is greater than the strength of adhesion of the MSCs to the PLA films without pores.

MSCs plated on 5 μm pore films formed the thickest cartilage among the three types of films used in this study. This is because the MSCs did not fall through the pores after plating; instead, they firmly adhered to the pores via their pseudopodia. This firm adhesion prevented the cells from peeling off after chondrogenic differentiation. The chondrogenic differentiation of MSCs using scaffolds is affected by the size of the scaffold pores [26, 27]. In the current study, the film with the 5 μm pore size was the best of the three tested films for cartilage formation by synovial MSCs under the conditions used here. MSCs seeded on 20 μm pore films formed thinner and more uneven cartilage compared to MSCs seeded on 5 μm pore films. The number of cells that adhered to the film one day after seeding was the same, but the number of cells that passed through the film and fell to the bottom was higher for the 20 μm pore films.

Although the MSCs extended their pseudopodia and were caught in the pores, some of the cells passed through the 20 μm pores, probably because the MSC body is less than 20 μm in diameter [28] and therefore smaller than the pores. One possibility why the thickness and smoothness of the cartilage formed at 2 weeks differed between the films with 5 μm and 20 μm pores, even though the number of cells remaining in the film at 1 day was the same, might be that cells fell through the 20 μm pores from 1 day to 2 weeks after seeding, especially in the early period before the cartilage matrix was fully formed.

The pore size in honeycomb films can be adjusted in a range from several tens of nanometers to tens of micrometers by controlling the template water droplets by the breath figure method. The honeycomb films have the added advantage of forming uniformly sized pores when compared to conventional porous scaffolds with size-distributed pores [11]. The pore sizes reported for honeycomb films have been found suitable for proliferation and function of endothelial cells [29], myocytes [30], and hepatocytes [31]. Kawano et al. compared PLA honeycomb films with 1.6 μm, 3.2 μm, and 4.7 μm pores and reported that the number of adherent bone marrow MSCs increased as the pore size increased [32]. This finding concurred with our results and suggests that a suitable pore size for MSC adhesion to a PLA honeycomb film is approximately 5 μm.

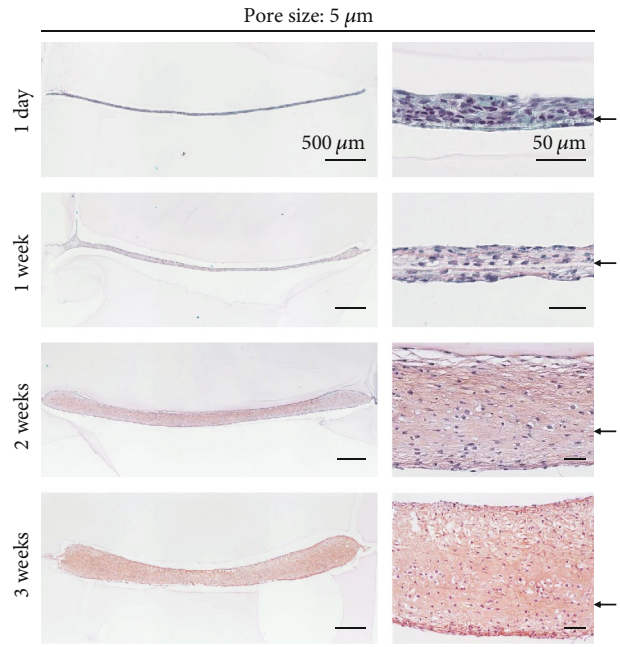

Figure 6: Temporal alteration in histological images of in vitro cartilage formation of synovial MSCs plated on PLA films with 5 μm pores. MSCs (5.0×10^5 in 500 μL medium) were plated, cultured in chondrogenic induction medium for three weeks, and stained with safranin O. The arrow indicates the PLA film.

One limitation of our study is that we did not conduct in vivo investigations of the effectiveness of implanting honeycomb PLA films plated with MSCs into cartilage defects. Therefore, we cannot say at this time whether cartilage defects will be repaired when transplanted with undifferentiated MSCs adhering to 5 μm pore honeycomb PLA films or whether the MSCs will require further ex vivo differentiation into cartilage sheets. We also do not know whether the PLA film will be absorbed after implantation, or when this will occur. Another point that requires clarification is whether a PLA film will be recognized as a foreign substance in the joint and cause inflammation.

We compared in vitro cartilage sheet formation by synovial MSCs using honeycomb PLA films with 0, 5, and 20 μm pores. MSCs on the 5 μm pore film showed pseudopodia that extended out to several pores. MSCs on the 20 μm pore film showed cell bodies submerged in the pores. MSCs plated on 5 μm pore films formed the thickest and most even cartilage layer among the three types of films. Honeycomb PLA films with 5 μm pores were therefore considered suitable for in vitro cartilage formation by synovial MSCs.

Abbreviations

PLA: Polylactic acid
MSCs: Mesenchymal stem cells.

Data Availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethical Approval

All methods were carried out in accordance with relevant guidelines and regulations. All procedures performed in the study involving human participants were in accordance with the Declaration of Helsinki. This study was approved by the Medical Research Ethics Committee of Tokyo Medical and Dental University (M2017-142).

Consent

Informed consent was obtained from all study subjects.

Conflicts of Interest

The authors declare that they have no competing interests.

Authors' Contributions

MY performed the experiments, data collection, and rough draft writing; MM performed the experiments, data collection, and draft writing; RF performed the experiments and data collection; HKa, KE, NO, and HKo are responsible for the project administration; YS is responsible for the scanning electron microscopy; IS is responsible for the conceptualization, supervision, and final writing. All authors reviewed and approved the manuscript.

Acknowledgments

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References

[1] J. C. Bernhard and G. Vunjak-Novakovic, "Should we use cells, biomaterials, or tissue engineering for cartilage regeneration?," *Stem Cell Research & Therapy*, vol. 7, no. 1, p. 56, 2016.

[2] P. Niemeyer, D. Albrecht, S. Andereya et al., "Autologous chondrocyte implantation (ACI) for cartilage defects of the knee: A guideline by the working group "Clinical Tissue Regeneration" of the German Society of Orthopaedics and Trauma (DGOU)," *The Knee*, vol. 23, no. 3, pp. 426–435, 2016.

[3] A. Yamashita, M. Morioka, Y. Yahara et al., "Generation of scaffoldless hyaline cartilaginous tissue from human iPSCs," *Stem Cell Reports*, vol. 4, no. 3, pp. 404–418, 2015.

[4] Y. Sakaguchi, I. Sekiya, K. Yagishita, and T. Muneta, "Comparison of human stem cells derived from various mesenchymal tissues: superiority of synovium as a cell source," *Arthritis & Rheumatism*, vol. 52, no. 8, pp. 2521–2529, 2005.

[5] C. De Bari and A. J. Roelofs, "Stem cell-based therapeutic strategies for cartilage defects and osteoarthritis," *Current Opinion in Pharmacology*, vol. 40, pp. 74–80, 2018.

[6] A. R. Armiento, M. J. Stoddart, M. Alini, and D. Eglin, "Biomaterials for articular cartilage tissue engineering: learning from biology," *Acta Biomaterialia*, vol. 65, pp. 1–20, 2018.

[7] D. da Silva, M. Kaduri, M. Poley et al., "Biocompatibility, biodegradation and excretion of polylactic acid (PLA) in medical implants and theranostic systems," *Chemical Engineering Journal*, vol. 340, pp. 9–14, 2018.

[8] A. Grémare, V. Guduric, R. Bareille et al., "Characterization of printed PLA scaffolds for bone tissue engineering," *Journal of Biomedical Materials Research Part A*, vol. 106, no. 4, pp. 887–894, 2018.

[9] K. Kim, M. Yu, X. Zong et al., "Control of degradation rate and hydrophilicity in electrospun non-woven poly(d,l-lactide) nanofiber scaffolds for biomedical applications," *Biomaterials*, vol. 24, no. 27, pp. 4977–4985, 2003.

[10] Y. Fukuhira, E. Kitazono, T. Hayashi et al., "Biodegradable honeycomb-patterned film composed of poly(lactic acid) and dioleoylphosphatidylethanolamine," *Biomaterials*, vol. 27, no. 9, pp. 1797–1802, 2006.

[11] H. Yabu, "Fabrication of honeycomb films by the breath figure technique and their applications," *Science and Technology of Advanced Materials*, vol. 19, no. 1, pp. 802–822, 2018.

[12] H. Yamazaki, K. Ito, H. Yabu, and M. Shimomura, "Formation and control of line defects caused by tectonics of water droplet arrays during self-organized honeycomb-patterned polymer film formation," *Soft Matter*, vol. 10, no. 16, p. 2741, 2014.

[13] W H Organization, "World Medical Association Declaration of Helsinki," *Jama*, vol. 310, no. 20, 2013.

[14] M. Mizuno, H. Katano, Y. Shimozaki et al., "Time-lapse image analysis for whole colony growth curves and daily distribution of the cell number per colony during the expansion of mesenchymal stem cells," *Scientific Reports*, vol. 9, no. 1, p. 16835, 2019.

[15] M. Mizuno, H. Katano, Y. Mabuchi et al., "Specific markers and properties of synovial mesenchymal stem cells in the surface, stromal, and perivascular regions," *Stem Cell Research & Therapy*, vol. 9, no. 1, p. 123, 2018.

[16] R. Fujisawa, M. Mizuno, H. Katano et al., "Cryopreservation in 95% serum with 5% DMSO maintains colony formation and chondrogenic abilities in human synovial mesenchymal stem cells," *BMC Musculoskeletal Disorders*, vol. 20, no. 1, p. 316, 2019.

[17] Y. Kohno, M. Mizuno, N. Ozeki et al., "Comparison of mesenchymal stem cells obtained by suspended culture of synovium from patients with rheumatoid arthritis and osteoarthritis," *BMC Musculoskeletal Disorders*, vol. 19, no. 1, p. 78, 2018.

[18] S. Suzuki, M. Mizuno, Y. Sakamaki et al., "Morphological changes in synovial mesenchymal stem cells during their adhesion to the meniscus," *Laboratory Investigation*, vol. 100, no. 7, pp. 916–927, 2020.

[19] J. C. Adams, "Cell-matrix contact structures," *Cellular and Molecular Life Sciences*, vol. 58, no. 3, pp. 371–392, 2001.

[20] A. Elosegui-Artola, E. Bazellières, M. D. Allen et al., "Rigidity sensing and adaptation through regulation of integrin types," *Nature Materials*, vol. 13, no. 6, pp. 631–637, 2014.

[21] P. H. Krebsbach and L. G. Villa-Diaz, "The role of integrin $\alpha 6$ (CD49f) in stem cells: more than a conserved biomarker,"

Stem Cells and Development, vol. 26, no. 15, pp. 1090–1099, 2017.

[22] H. Lv, L. Li, M. Sun et al., "Mechanism of regulation of stem cell differentiation by matrix stiffness," *Stem Cell Research & Therapy*, vol. 6, no. 1, 2015.

[23] N. Strohmeyer, M. Bharadwaj, M. Costell, R. Fässler, and D. J. Müller, "Fibronectin-bound $\alpha 5\beta 1$ integrins sense load and signal to reinforce adhesion in less than a second," *Nature Materials*, vol. 16, no. 12, pp. 1262–1270, 2017.

[24] A. van Wijnen, R. Olivares-Navarrete, E. M. Lee et al., "Substrate stiffness controls osteoblastic and chondrocytic differentiation of mesenchymal stem cells without exogenous stimuli," *Plos One*, vol. 12, no. 1, 2017.

[25] W. Ando, K. Tateishi, D. Katakai et al., "In vitro generation of a scaffold-free tissue-engineered construct (TEC) derived from human synovial mesenchymal stem cells: biological and mechanical properties and further chondrogenic potential," *Tissue Engineering Part A*, vol. 14, no. 12, pp. 2041–2049, 2008.

[26] A. Matsiko, J. P. Gleeson, and F. J. O'Brien, "Scaffold mean pore size influences mesenchymal stem cell chondrogenic differentiation and matrix deposition," *Tissue Engineering Part A*, vol. 21, article 3-4, pp. 486–497, 2015.

[27] C. M. Murphy, M. G. Haugh, and F. J. O'Brien, "The effect of mean pore size on cell attachment, proliferation and migration in collagen-glycosaminoglycan scaffolds for bone tissue engineering," *Biomaterials*, vol. 31, no. 3, pp. 461–466, 2010.

[28] M. Mizuno, H. Katano, K. Otabe et al., "Complete human serum maintains viability and chondrogenic potential of human synovial stem cells: suitable conditions for transplantation," *Stem Cell Research & Therapy*, vol. 8, no. 1, p. 144, 2017.

[29] S. Yamamoto, M. Tanaka, H. Sunami et al., "Effect of honeycomb-patterned surface topography on the adhesion and signal transduction of porcine aortic endothelial cells," *Langmuir*, vol. 23, no. 15, pp. 8114–8120, 2007.

[30] K. Arai, M. Tanaka, S. Yamamoto, and M. Shimomura, "Effect of pore size of honeycomb films on the morphology, adhesion and cytoskeletal organization of cardiac myocytes," *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 313-314, pp. 530–535, 2008.

[31] M. Tanaka, K. Nishikawa, H. Okubo et al., "Control of hepatocyte adhesion and function on self-organized honeycomb- patterned polymer film," *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, vol. 284-285, pp. 464–469, 2006.

[32] T. Kawano, M. Sato, H. Yabu, and M. Shimomura, "Honeycomb-shaped surface topography induces differentiation of human mesenchymal stem cells (hMSCs): uniform porous polymer scaffolds prepared by the breath figure technique," *Biomater. Sci.*, vol. 2, no. 1, pp. 52–56, 2014.

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Review Article

Stem Cells: A Historical Review about Biological, Religious, and Ethical Issues

Ioannis Alexandros Charitos,[1,2] **Andrea Ballini**,[3,4] **Stefania Cantore**,[5] **Mariarosaria Boccellino**,[4] **Marina Di Domenico**,[4] **Elisa Borsani**,[6] **Riccardo Nocini**,[7] **Michele Di Cosola**,[8] **Luigi Santacroce**,[5] **and Lucrezia Bottalico**[1]

[1]*Interdepartmental Research Center for Pre-Latin, Latin and Oriental Rights and Culture Studies (CEDICLO), University of Bari, Bari, Italy*
[2]*Department of Emergency and Urgency, National Poisoning Centre, Riuniti University Hospital of Foggia, Foggia, Italy*
[3]*Department of Biosciences, Biotechnologies and Biopharmaceutics, Campus Universitario "Ernesto Quagliariello", University of Bari "Aldo Moro", Bari, Italy*
[4]*Department of Precision Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy*
[5]*Department of Interdisciplinary Medicine, University of Bari, School of Medicine, Bari, Italy*
[6]*Division of Anatomy and Physiopathology, Department of Clinical and Experimental Sciences, University of Brescia, Brescia, Italy*
[7]*Section of Ear Nose and Throat (ENT), Department of Surgical Sciences, Dentistry, Gynecology and Pediatric, University of Verona, Verona, Italy*
[8]*Department of Clinical and Experimental Medicine, University of Foggia, Foggia, Italy*

Correspondence should be addressed to Andrea Ballini; andrea.ballini@me.com
and Stefania Cantore; stefaniacantore@pec.omceo.bari.it

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Stem cells can be used to replace damaged cells or regenerate organs and have broadened our knowledge of the development and progression of certain diseases. Despite significant advances in understanding stem cell biology, several problems limit their use. These problems are related not only to the growth of tumors in animal models and their rejection in transplant cases but also to ethical and social issues about the use of embryonic cells. The ethical-scientific debate on this type of cells has taken on great interest both for their application in regenerative medicine and for the potential possibilities in the field of cell and gene therapy. Different points of view often have the expression of a perception that depends on scientific goals or opportunities or on religious traditions and beliefs. Therefore, as the questions and doubts about when life begins, so do the answers for the use of these cells as therapy or otherwise. So, in addition to the origin of stem cells, there are currently some social bioethical (such as political and legislative issues) and religious dilemmas. The purpose of the study is aimed at being a narrative on the history of stem cells and the evolution of their use to date, as well as to clarify the bioethical position of the various religions today in comparison with the social ones regarding the research and use of embryonic and adult ones. Hence, their biological hypostasis regarding the concepts of "conception" and "fertilization" and their development and therapeutic use compared to those of the main theological doctrines.

1. Introduction

Stem cells are undifferentiated cells that have not yet developed structures or proteins and are characteristic of a specific type of cell or tissue. Thus, they contribute to the creation of all mature cells in the human body and are the basis of every cell, tissue, and organ. A stem cell is able to guarantee both self-renewal and differentiation. The way to do this is through its asymmetrical division. However, cell division can be both symmetrical and asymmetrical. The symmetrical

division produces identical cells (cell cloning). Asymmetric division is typical of stem cells and therefore creates cells destined to differentiate (different from the cell of origin) together with cells identical to those of origin (i.e., still stem cells). In this way, self-renewal is guaranteed, and at the same time, a progeny of differentiated cells is created which in turn will be transferred to the affected tissues or organs [1, 2]. Thus, the stem cells must also be able to differentiate into the various types of cells and to regenerate functional tissues in vivo. Stem cells are a population of undifferentiated cells characterized by (I) their ability to proliferate widely (ability to self-renew) and to differentiate into different types of cells and tissues (potential for differentiation) and (II) usually arise from a single cell (clonal property). There are five types of stem cells according to their origin: (a) embryonic stem cells (ESCs) are isolated from the internal cell mass of the blastocyst. They consist of populations of pluripotent cells that can produce the primitive ectoderm during embryogenesis. A particular group of stem cells is those of the dental-derived stem cells (d-DSCs) which can differentiate *in vitro* into layer-like tissues of mesoderm, endoderm, and ectoderm such as those of adipocytes and neural cells [2–6]. (b) Amniotic epithelial cells (AECs) arise from the amniotic membrane of the human placenta. They express multivalent ESC markers (such as oct-4, Nanog, and alkaline phosphatase), do not express telomerase, and do not form teratomas *in vivo* after transplantation. They can differ *in vitro* from both endoderm (such as pancreatic endocrine cells and hepatocytes), mesoderm (such as cardiomyocytes), and from the ectoderm (such as in nerve cells) [4, 5]. (c) Fetal stem cells (FSCs) are mostly isolated (from embryonic cadaver tissue organs or are from tissue-specific embryonic stem cells), up to the 12^{th} week of pregnancy. They can therefore be transplanted without rejection reactions and have the therapeutic advantage over ESCs of not causing teratomas *in vivo* [7–10]. (d) The umbilical cord epithelium (UCE) is derived from the epithelial amniotic membrane and is a source of pluripotent stem cells. They can differentiate into various functional progenitor cells (such as hematopoietic, dendritic, neural, hepatocytes, pancreatic cells, and endothelial cells), and (e) adult somatic stem cells are produced during ontogenesis [11–13]. They are found in specialized areas in almost all mammalian organs and tissues such as bone marrow, heart, kidneys, brain, skin, eyes, gastrointestinal tract, liver, pancreas, lungs, breasts, prostate, testicles, and ovaries. Moreover, four types of multipotent adult stem cells have been described: (1) isolated from long-term cultures of nonphenotypic adherent cells from human or mouse/rat mesenchymal tissue such as bone marrow and muscle tissue, (2) unrestricted ones isolated from umbilical cord blood (UCSCs) cultures, (3) inducible (MIAMI) isolated from marrow, and (4) multipotent from human bone marrow (h-BMSC). However, there is a simpler distinction of stem cells which is the one that divides them according to their origin and their developmental plasticity that is in embryonic and somatic. However, there are multiplicative somatic stem cells (Table 1) [11, 13, 14].

Stem cells are used in the research field of medicine for the treatment of various diseases such as hematological (e.g., bone marrow transplantation), ophthalmology (e.g., age-related macular degeneration), endocrinological (diabetes), neurological, genetically modified fat cells, and drug development (Figure 1).

To date, three stem cell-based therapies have been considered: (a) already differentiated cell transplantation with specific types of cells that derive from embryonic or somatic stem cells and include patient's own stem cells and grow and differentiate under special processing conditions in the laboratory (e.g., insulin-producing cells for the treatment of diabetes), (b) stimulation of endogenous stem cells in an individual (e.g., by administering growth factors) with the possibility of inducing or increasing the processes of self-repair, and (c) direct administration of stem cells to the patient, so that a colonization process begins in the desired part of the body and thus the differentiation continues into the desired cell type (Figure 2) [15–18].

However, depending on the choice of technique and the type of stem cells, we will also have certain limitations (Table 2). Hematopoietic stem cell transplantation from bone marrow, peripheral blood, or cord blood is used to treat hematological malignancies or congenital immunodeficiencies (such as β-thalassemia, Fanconi anemia, sickle cell anemia, acute and chronic leukemia, F leukemia, non-Hodgkin's lymphoma, Hodgkin's disease, and others). Stem cell transplantation belonging to the same patient (autologous) was also used to preserve bone marrow in patients who had received a high dose of chemotherapy. The use of h-BMSCs as a therapy to modulate immune responses is known. In fact, it has been shown that these cells can express histamine H1, H2, and H4 receptors and histamine itself then stimulating the production of interleukin 6 (IL-6) [18–20]. Finally, several studies mention the role of stem cells in the relationship with periodontitis antibodies and particularly in CVD (cardiovascular disease) biomarkers in the early stages. It was noted that some patients with periodontitis had serum and salivary increase in *Aggregatibacter actinomycetemcomitans* high IgG titers and high hs-CRP (high-sensitivity C-reactive protein) with a negative effect. The burden of periodontal bacteria can lead to an increase in plasma and salivary levels of suPAR (soluble urokinase-type plasminogen activator receptor) and hs-CRP [21–23]. In fact, suPAR proved to be a valuable prognostic biomarker for the correlation between periodontitis and related cardiovascular diseases [21]. It has recently been noted that during a periodontal disease that is associated with endothelial dysfunction, there is a relevant relationship between the disease and the EPCs (circulating endothelial progenitor cells) [24]. EPCs are a subtype of bone marrow-derived stem cells, and they have hematopoietic and endothelial stem cells and thus participate in the integrity of the vessels. Indeed, EPC has been suggested as an indicator of endothelial dysfunction and the cumulative risk of cardiovascular disorders. EPC identification occurs through the search for markers CD34, CD133, and the kinase insertion domain receptor (KDR). Furthermore, EPCs increase is correlated with the presence of positive to serum biomarkers [21–24].

Autologous transplantation is also used for the treatment of difficult autoimmune diseases but also as a means of gene

Table 1: The different sources of stem cells with different plasticity and differentiation.

Stem cell plasticity				
Totipotent/omnipotent	Pluripotent	Multipotent	Oligopotent	Unipotent
They are cells with the most undifferentiated cell form during embryonic development (e.g., the fertilized oocyte (zygote)) up to the stage of the first blastomeres (i.e., three to four days after fertilization). Are single cells that can give rise to a new organism with adequate maternal support. So, it can give rise to all extra-embryonic tissues, tissues of the body, and of the germ line.	They can differentiate into cell types from the ectoderm, endoderm, and mesoderm, which then produce all cell types for all tissues and organs. The best-known pluripotent stem cells are embryonic (isolated from the internal cell mass (ICM) of the blastocyst).	They can differentiate into discrete cell types (e.g., several types of blood cell-like lymphocytes, monocytes, neutrophils, bone cells or other nonblood cell type, and others. The best now is the mesenchymal cells (MSCs) in the bone marrow, adipose tissue, ∗Wharton's jelly in umbilical cord blood, dental tissues, and peripheral blood.	These stem cells can self-renew and differentiate into two or more cells that belong to a specific type of tissue (e.g., hematopoietic stem cells, the bronchioalveolar stem cells, or BASCs).	They can self-renew and differentiate into a single specific cell type forming a single cell line (e.g., the muscle stem cells).

∗Inside the Wharton's jelly (substantia gelatinea funiculi umbilicalis) in umbilical cord, there is a cell population that has stem characteristics and is made up of the mesenchymal cells of the layer (MSCs). They exhibit cell adhesion characteristics while phenotypically expressing a specific set of surface antigens (including CD73, CD90, and CD105 stem cells).

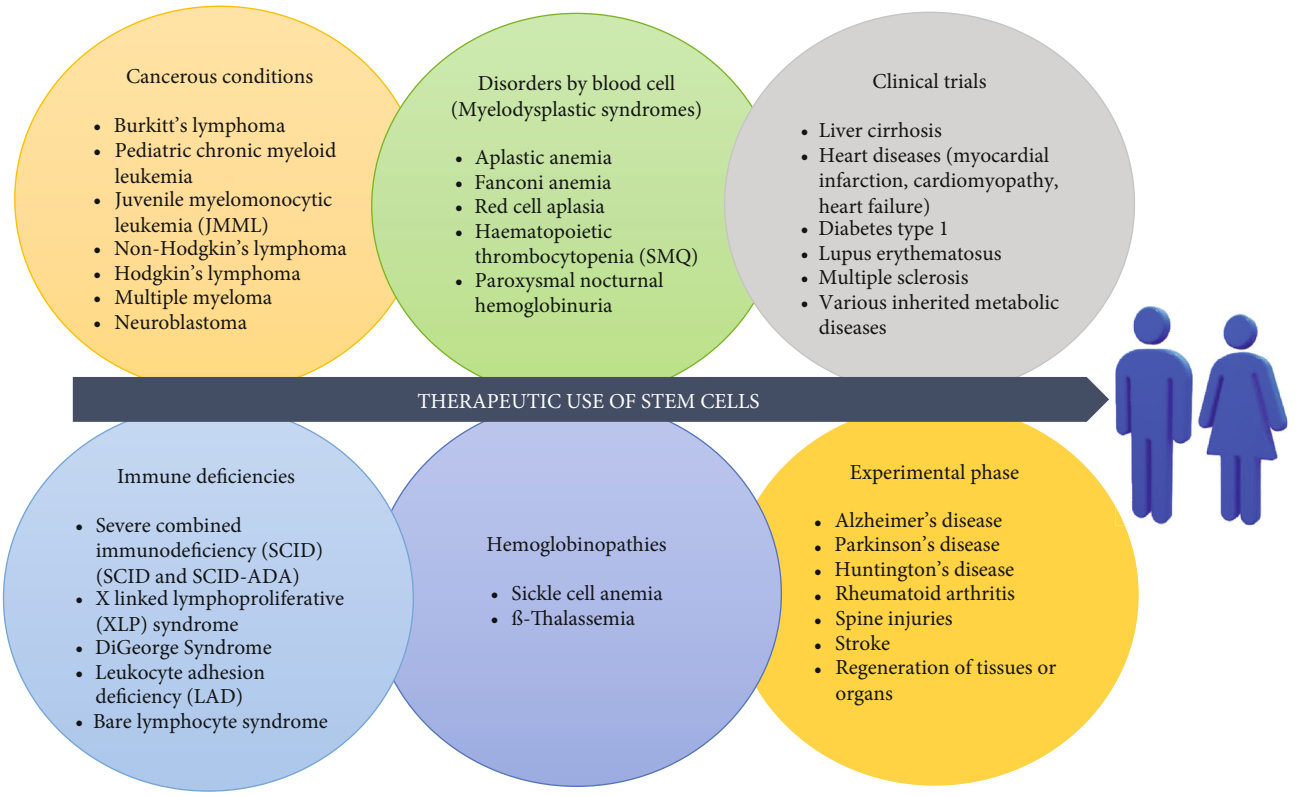

Figure 1: Summary of the diseases treated or even therapies in clinical trials or experimentation with stem cells.

therapy which is still in the experimental stage. Additionally, stem cell transplantation is used as a treatment for some types of cancer (such as breast and neuroblastoma). As for the treatment of diabetes, perhaps the production of functional pancreatic cells from stem cells ready for transplantation could be a new therapeutic approach [25, 26].

Scientific interest has mostly turned to embryonic stem cells as in the case of certain neurological diseases (e.g., stroke, Alzheimer's disease, and Parkinson's disease). On this prospect, the use of neural stem cells (NSCs) as regenerative therapies, the combination with the cannabinoids for medical use is very promising. It has been noted that they act as

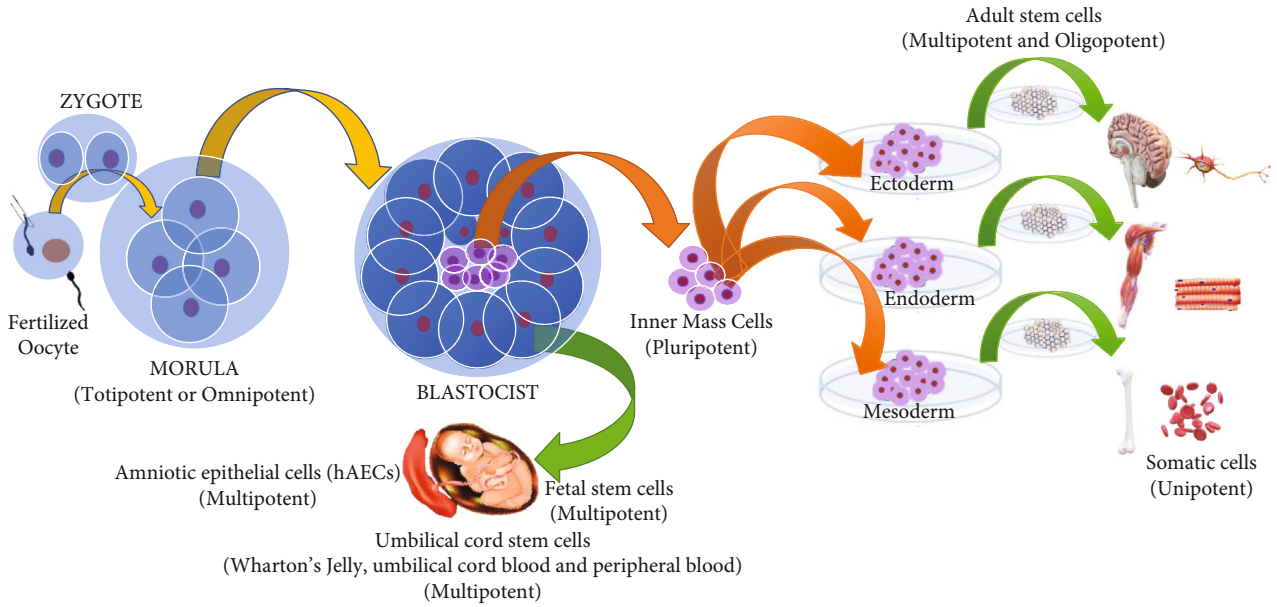

Figure 2: The origin, the collection process, and the plasticity of stem cells: after fertilization of the natural oocyte or in vitro the pluripotent embryonic stem cells are created as internal cell mass inside a blastocyst. The stem cells show that their range of potential is wide and that cells of one tissue can, under experimental conditions and with appropriate culture cell, reprogram to mature cells in another, different tissue from which they come from. So, they can function as multiples. This is called plasticity and is the basis of cell therapy. Fetal stem cells are found within the organs of the fetus. To this source belongs the embryonic corpse tissue (which can be obtained after a spontaneous abortion due to illness, etc.) and afterwards with the appropriate culture, reprogrammed and functioning such as multipotent cells.

Table 2: Main differences and limitations for use of stem cells.

Limits of stem cells		
Embryonic stem cells		Adult stem cells
In vitro fertilization	Nuclear transport	
Limited number of cell lines for federally funded research needs. Risks for the creation of teratomas (tumors) from the transplantation of undifferentiated stem cells.	It has not yet been achieved in humans. Risks for the creation of teratomas (tumors) from the transplantation of undifferentiated stem cells.	Production of a limited number of cell types. They are not found in all tissues. Difficulties in identification, isolation, preservation, and cultivation in the laboratory.

potent regulators of NSCs, thus, also having the potential to modulate various neurogenic characteristics. Using embryonic stem cells, certain types of nerve cells can be produced ex vivo, which, if transplanted into the body, could repair focal organic damage (such as experimentally, even in spinal injuries). Furthermore, using the differentiation power of stem cells, congenital defects such as *Krabbe disease*, cheiloschisis, osteogenesis imperfecta, and others can be cured [25–29]. In the dental field, dental mesenchymal cell (MSCs) research can be applied for the replacement of dental and oral tissues against dental caries, periodontitis, etc., as well as the use of platelet-rich plasma (PRP) and platelet-rich fibrin (PRP) extracts for the dental implantology process [13, 30–34].

Finally, for treatment in patients with inflammatory bowel disease (IBD), they develop microbiota dysbiosis with immune dysregulation. Furthermore, it was noted that mesenchymal stem cells (MSCs) can produce similar effects, and targeted investigations on the interactions between these cells and gut microbiota could lead to a new therapeutic approach. The use of probiotics and/or fecal transplantation can be an effective adjuvant therapeutic strategy [35–39].

2. Stem Cells in the History

2.1. The Early Years. The two zoologists Theodor Heinrich Boveri (1862-1915) and Valentin Häcker (1864-1927) used the term stem cell to describe cells committed to give rise to the germline. Boveri was also a comparative anatomist who during his cytology and genetic studies discovered that some cells could regenerate with subsequent functional differentiation. On this basis, he thinks that even cancer cells start from a cell with chromosome scrambled leading to continuous and uncontrollably dividing cell [40]. He thus wanted to explain the theory of embryonic nature tumor etiology and pathogenesis as previously interpreted by Julius Cohnheim as did Max Askanazy (1865-1940), Felix Marchand (1846-1928), and Robert Bonnet (1851-1921) with the blastomere theory of teratoma-like tumors. However, still today, some oncogenetic theories for some tumors still concern the cells of this

type of cells [41–49]. The histologist Franz Ernst Christian Neumann who was carrying out his studies on bone marrow and Alexander Alexandrowitsch Maximow (1874-1928) claim that there is a common cell precursor that differs to give all the mature blood cells. On this Maximow, he developed the concept of polyblasts. It will be later that these cells with the aim of regenerating and differentiating themselves were called stem cells by Ernst Haeckel (1834-1919). He is starting from the Mendelian concept thinking that could not always explain heredity and phenotypic traits and so he puts into action the new concept of research that he will call phenogenetics. In fact, he argued that in multicellular beings, there is a first line of cells that call *Stammzellen (Stamm*: phylum + *zellen*: cells) which leads to phylogeny that is to evolution through evolution proliferation and differentiation capacity. Then, he introduced the foundations for the theory of hematopoiesis [50].

In the early 60s, Ernest Armstrong McCulloch (1926-2011), a biophysicist, and James Edgar Till, (born 1931) a cell biologist, were the pioneers for the study of stem cells with the quantitative clonal method. After they had introduced the cells into the bone marrow of the previously irradiated laboratory mice, they observed nodules at the level of the spleen. In fact, these cell colonies each came from a single progenitor cell [51]. Later together with the molecular biologist Lou Siminovitch (born in 1920), they realize that cells could functionally self-renew by creating these colonies [52]. Thanks to the oncologist and immunologist Georges Mathé (1922-2010) in 1958, we have the first successful allogeneic bone marrow transplant with people who are not relatives, and in 1963, he treated a patient with leukemia for the first time thanks to the bone marrow transplant. Previously, together with Marcel Legrain and René Kuss, he performed the first kidney grafts among unrelated donors with success [53].

2.2. Towards the Evolution of the 21st Century Era. In 1981, the two biologists Sir Martin John Evans (born in 1941) with Matthew Kaufman (1942-2013) cultivated mouse embryonic stem cells for the first time in the laboratory, and subsequently in 2007, together with Mario Capecchi and Oliver Smithies, they won the Nobel Prize in physiology and medicine. In 1988, the first umbilical cord blood stem cell transplant took place in a child with Fanconi's anemia. Since then, more than 6,000 transplants have been performed in which the transplant is entrusted to a relative or another stem cell recipient, provided there is compatibility (heterologous transplant) [54].

This is because they used embryonic stem cells to induce specific gene modifications in mice by isolating the embryonic stem cell from the embryo and implanting it in adult female mice. This will open new horizons for research and therapy. In 1992, the first public and private stem cell bank was established in the United States. In 1995, umbilical cord stem cells were introduced of equal value in therapeutic applications with bone marrow stem cells [54].

In 1998, the biologist James Alexander Thomson through his research led him to the discovery of human embryonic stem cells (published under the title Embryonic Stem Cell Lines derived from Human Blastocysts), and in 2007, he discovered the method of human-induced pluripotent stem cells (iPS), i.e., by converting skin cells into cells that closely resemble human embryonic stem cells (published article under the title Induced Pluripotent Stem Cell Lines Derived from Human Somatic Cells) [55, 56].

In 2001, the first study on umbilical cord stem cell transplantation in adults was published, and in 2004, Gesine Koegler and colleagues found that in the umbilical cord blood, in addition to hematopoietic stem cells, there are pluripotent stem cells [57].

Finally, in 2012, Shinya Yamanaka and John Gurdon were awarded the Nobel Prize for Physiology or Medicine for the discovery that mature cells can be converted to stem cells; hence, they can be manipulated to become pluripotent [58].

3. Ethical Issues in the Use of Stem Cells

3.1. Epistemology and Ethical Social Approach. Stem cell research then is linked to moral issues that are raised around the world. On the one hand, the development of adequate and innovative therapies for the treatment of serious diseases makes their research indispensable, but on the other, moral questions emerge from the use of embryos and therefore the limits and conditions of this research. In addition, there is the question of the limitations of intellectual property on the use of research materials and how to access medical and therapeutic technologies. It is important to understand the future use of gametes, embryos, or somatic cells offered by donors together with the protection of privacy (including that of donors). Hence, using them raises questions relating to personal data [59]. At the same time, there is a debate on the safety and efficacy of treatments in progress, as well as on the understanding, by patients, of the origin of the materials used in the treatments. For stem cells isolated from the umbilical cord, there are two main related issues: (a) the appropriate time that donor consent should be obtained for the use of the resulting medical data and (b) the related issues concerning maintenance and the cold storage in specific banks. Concerning bone marrow mesenchymal stem cells, questions arise regarding pain and risks to the donor during the cell isolation process [60].

Then, questions are raised relating to complete information consent of the participants on the whole experimental process. The importance of some institutional issues concerns the careful consideration of the intellectual property right that governs stem cell research related to medical treatments and ethics. In fact, for this reason, the guidelines have been created over time by the International Society for Stem Cell Research (ISSCR) [17, 61]. Thus, it is important to review the objectives of the information and consent process on cell and tissue donors and to collect data thereafter. Finally, the collection of other somatic cells (such as adult skin cells) raises some distinct problems such as limited discomfort and the patient's physical risk during the isolation process. In fact, the first source of stem cell production is the excess fertilized eggs left over from *in vitro* fertilization, and that after the removal of the pluripotent stem cells, the

fertilized eggs are destroyed [62]. This is where two moral issues arise: (a) that it is intolerable to annihilate ("kill") fertilized eggs that can potentially develop into a human being; (b) morally, it is unacceptable to use these "potential" human beings to an end. However, according to medical and biological science, the fertilized egg of the first fourteen days does not yet contain a personalized life, as it begins to form after fourteen days of fertilization [63]. Therefore, there is no existence of a "human person" that can be attributed to the fetus in the first few days. It is not subject to duties and rights to be able to recognize a situation like that attributed as in the human being. Thus, it is not about "killing" by taking stem cells.

Furthermore, there can be no concern when there are abortion laws that allow the destruction of the fetus until the first twelve weeks of pregnancy since the freedom of the woman is valued by the legislator as a superior legal good and by the fetus of the first three months. Thus, a possible source of stem cells is cadaveric embryonic tissue after spontaneous or voluntary abortion. But for this to happen, it is necessary to obtain the consent of the pregnant woman if this would not lead to an insult to the woman's personality [64]. Therefore, you must be adequately informed before giving your consent for both the use of the fetal tissue and the source of research funding [59]. However, the creation of human embryos for research purposes only is prohibited.

The distinction between pluripotent and omnipotent stem cells is seen as ethically crucial in medicine's science. After the complete development of the pluripotent stem cells, there is the possible evolution of them towards complete and equal organisms such as the embryo of origin [65, 66]. Thus, it is likely that a reproductive technique may be developed in the future which will create people with similar external characteristics. This is morally at fault because the creation of human beings would weigh on the free development of the personality and on their autonomy of life. This cannot happen in a society that respects the value of human life. Some bioethics committee proposes that it would be more ethically appropriate to strictly prohibit the acquisition of stem cells for the purpose of culturing omnipotent. Therefore, to obtain stem cells from fertilized eggs, it is necessary to obtain the written consent of the gamete donors, as these fertilized eggs are elements of their personality. This consent must specify that the fetus will not be used for third party reproductive purposes and that in case of refusal it will not have negative consequences on future medical care needs. However, research on fetal tissue is indicated in case of interruption of medical problems as it can help to understand the causes of the anomalies with prenatal or preimplantation tests when it comes to couples who have resorted to *in vitro* fertilization. Thus, thanks to the bioethical dilemmas regarding the extraction of stem cells from the human embryonic blastocyst, an attempt was made to extract the stem cells three days after fertilization in the division phase of 6-8 cells after biopsy and without altering the development of the embryo which is then implanted in the uterus [67].

As regards adult stem cells, this source does not present legal but bioethical problems. This is because it does not endanger the person from whom they are removed, and for this reason, scientists should focus on finding these same stem cells. The condition to be considered is the donor's consent with the guarantees already provided in detail in the convention on human rights and biomedicine. As in previous stem cell sources, the financial counterpart agreement is being strictly prohibited. It would also be advisable to keep the donor anonymous as in transplants. Another source of stem cells would be that of cloning. Cloning as a process is the replacement of the egg nucleus with the nucleus of a mature somatic cell, which contains all the genetic information necessary to create a human organism.

Nevertheless, reproductive cloning is explicitly prohibited [68]. Therefore, without allowing for an evolution of this organism but only taking the stem cells in the first days of its evolution, we can define therapeutic cloning; therefore, the reception of stem cells by organisms created by intentional cloning and for the sole purpose of research is prohibited. Therapeutic cloning should be exempted from the general prohibition in Article 18 of the Council of Europe Convention on Human Rights and Biomedicine [69]. The storage and management of stem cells are important. However, another aspect of the stem cell issue then is the function of the banks that store and prepare these cells for use. Precisely because the use of stem cells as tools for disease treatment is limited, the existence of such banks has no real value.

However, if these banks exist and operate, it is imperative that those interested in their services are protected and not misinformed. Interested parties should be informed about medical data relating to stem cell research and about the law on the protection of personal data [67]. Finally, the new pandemic disease from the novel SARS-CoV-2 coronavirus and the planned procedures that perform allogeneic and autologous hematopoietic cell transplantation (HCT) have been disrupted. The European Society for Blood and Marrow Transplantation (EBMT) has drawn up guidelines for the more comfortable management issues of transplant candidates for both recipients and donors [70–72].

3.2. Theological Bioethical Approach Dilemmas on the Use of Stem Cells. According to the Christian's doctrine only Human, such as a psychosomatic unit, from the moment of conception, can become free thanks to his/her own will by the grace of God, in communion, that is, with divine energy, thus, removing his body from tenderness and from decomposition. From the moment of its conception, the fetus, as a "potential person," is a structured psychosomatic entity. The main approach to the question of the beginning of life is made by the Christian thought, with spiritual criteria refers to a human being who was created as an image of the creator God and exists as an independent being from the first moment of his conception. After these considerations, we understand that the Christian point of view is different from medical epistemology. This is because, only at the beginning of a pregnancy is the concept of "potential human being" introduced, and at birth, one can speak of a human face, since from that moment, the newborn is entitled to special legal protection. On the other hand, "fertilized eggs" or *in vitro* embryos, which are placed in a nutrient medium, will not grow with certainty. In fact, some may not even reach the

blastocyst stage or may not be of sufficient quality to be transferred to the female uterus. Instead, the valid and grown ones must be cryopreserved after the fifth day in case they are redundant because otherwise, they will stop growing and therefore existing. Continuing this previous thought, we can understand that this "image" is sometimes attributed to the autonomy of man and sometimes to the soul together with the body [73–75].

The Roman Catholic Church, represented by the Bishop or Pope of Rome, is against the use of embryonic stem cells from embryos created exclusively for therapy and research (this appears at the Pontifical Academy for Life in August 2000), which concludes with the use of only stem cells by adults for this purpose. Indeed, there is the suggestion of the use of adult stem cells and that this type of cell is more suitable for the advancement of research and therapeutic applications [73, 75, 76]. The position of the Roman Catholic Church, from 1869 onwards, is that the moment of conception is also the beginning of the entity of the human person. However, the Roman Catholic Church, before 1869, which by decision of Pope Pius IX, adopted now of conception, as the beginning of the life of a complete man, according to Thomas Aquinas. He claimed that the fetus acquires a soul after 40 days of fertilization. So, during its development, it is not considered human until it acquires a soul, and it can happen with a continuing pregnancy. At the beginning, the embryo lives like a plant because it is not an independent essence. After the transformation from the state of a vegetable being into an animal and that only in the final phase of its development is the rational soul created (which is given only by God) transforming it into the human essence. Thus, the Catholic ethical doctrine does not allow the use of embryonic stem cells from embryos created exclusively for this purpose, by means of assisted reproduction procedures or for the purpose of reproductive cloning [77, 78].

The point of view of the Protestant doctrines on the stem cell issue presents several points of view. Mainly, the theological and ethical point of view begins with the doctrine that recognizes human beings as entities that will have a relationship first with God and then with others and with itself. Therefore, the human fetus is considered a developing human being that, if not born, will not have these relationships. Thus, he opposes the use of early embryonic stem cells, as well as the question of reproductive cloning, because all this is considered against human dignity [73, 74, 78]. Finally, the Lutheran theologian and professor of systematic theology at Pacific Lutheran Theological Seminary (USA), Theodore Frank Peters, have very moderate ideas. He criticizes the Catholic doctrine for having combined the concept of the divine union of the soul with the body (that has become from the animal in Human rational essence) with the uniqueness of the new genome until it is deposited on the walls of the uterus between the 12th and the 14th day later fertilization. He also makes considerations about the excess and abandoned embryos that are placed for cryopreservation in the refrigerators of assisted reproduction clinics; in fact, since they are destined to be destroyed, he thinks whether they should be attributed to research. Their use for research purposes can lead to important therapeutic breakthroughs [79, 80].

In the Orthodox church (former imperial Christian Roman church) doctrine, represented by the Ecumenical Patriarch and Archbishop of New Rome or Constantinople (today Istanbul), the research on the use of embryonic stem cells is based on the position on the moral status of embryos. According to this theological doctrine, the psyche ($\psi\upsilon\chi\acute{\eta}$) is an immaterial divine gift that builds the essence of a personality at the origin of its life. Through being it completely differentiates itself from the inanimate and unreasonable creation, summarizing within it (as being with intellect) the creation both material and spiritual (divine) that causes the life. The body is initially presented in the form of a single cell, the fertilized egg, resulting from the fusion of the nuclei of two germ cells, a male and a female, which through this karyogamy phenomenon unite both the living energies of the souls of the carriers giving new vital energy, i.e., the psyche. So, it already preexists from the beginning immediately after this merger. As the body develops, the psyche also reveals its energies [81].

Therefore, without psyche, there can be not existing life, because in biblical theology it is considered as the "breath of life." For every scientific process, the Orthodox Church carefully follows the reasoning and methods that can go to the detriment of the concepts of the Human "*prosopon*" ("face": as the distinct divine individual energy) and the Human *hypostasis* (the individual unique existence) to which they confer a unique and supreme value in the rational beings thus the humans [82].

Hence, the fetus has the status of a perfect human being, to be subsequently perfected by divine energy. The Orthodox Church considers that God during the creation of the human being gave him all the necessary conditions to develop his abilities to exercise for good. This means that man has acquired a specific divine quality and every scientific achievement is a consequence of this. However, the Church recognizes the therapies that come from somatic stem cells, but also expresses her theological objections when derived from embryonic cells [80]. These concepts determine what is morally acceptable or not in the Orthodox Church as a fundamental bioethical principle. This theological and bioethical axis has remained unchanged over time in Orthodox theology compared to other Western Churches. However, the theological bioethical theory of the Orthodox Church refuses to search for stem cells or part of a variety of embryonic stem cells but has a positive effect on migratory cell search of adult stem cells. Finally, it allows the research and use of umbilical cord stem cells, because they come from an adult organism at the time of delivery and their collection does not present an ethical or moral theological dilemma [77–80].

According to the Jewish religion, the fetus according to the Old Testament and in the Talmud after the forty days of its development is considered in part a human being, which has respect and protection. Research on embryonic stem cells is morally acceptable since the embryo before 40 days is "as if it were water" and not an entity with a real soul.

For the Islamic religion, referring to the Koran, the fetus acquires psyche and image at the end of its fourth month of pregnancy. He has since been considered a person who enjoys rights. Hence, stem cell research may be acceptable if

Table 3: Main bioethical concerns of stem cells.

Bioethical reserves of stem cell resources	
Embryonic stem cells	Adult stem cells
In vitro fertilization/somatic cell nuclear transfer (SCNT)∗ Consent of donors and subsequent destruction of blastocysts (social/religious questions) ∗The administration of oocytes requires the consent of the donor and suspicions for their possible use in reproductive cloning.	Nonsignificant bioethical reservations on their use, which mostly concern informed consent and noncommercialization

stem cell research is acceptable because the fetus cannot be considered a complete human being [65, 75, 78, 79].

In Buddhism, it is particularly important that the use of embryonic stem cells can be used if one intends to do research aimed at healing and improving the lives of patients, but research done for financial gain is not acceptable. In Hinduism, however, the destruction of embryos is morally not permissible. However, has no official position on stem cell research. The issue in Buddhism is that it does not accept any cause of pain in all living beings but considering that before 14 days the fetus does not feel pain, we can say that research on embryonic stem cells can be performed (Table 3) [65, 75, 78, 83].

4. Conclusions

The use of stem cells in the treatment of diseases is one of the major research achievements of recent years. Isolating fetal and adult stem cells is challenging. Umbilical cord blood stem cells are less of a challenge to isolate as they are obtained from placental blood at birth after the umbilical cord is cut. In recent years, adult stem cells can be reprogrammed back to their pluripotent state. This is an important evolution in this scientific field. The use of embryonic stem cells is controversial because it requires the destruction of an embryo, and adult stem cells are preferred for the treatment of diseases. When the stem cells come from the recipient himself, there is no risk of rejection. The potential of stem cells is varied as the stem cells of one tissue can, under appropriate conditions, mature into cells of another different tissue and function as omnipotent and not simply as multipotent. This phenomenon is called plasticity and is the basis of cell therapies. Regenerative medicine is the one that could benefit most from the use of pluripotent stem cells. Activating pluripotent stem cells to produce specialized cell types will enable a renewable source of cells and tissues to be used as transplants and alleviate many diseases and disabilities. Thousands of patients around the world have already benefited from stem cell treatment that is still evolving today for many important pathologies such as neurological ones. Thus, the use of stem cells contributes to (a) basic research on human development, (b) the safe and more specialized development of drugs, and (c) the treatment of repair or replacement of damaged tissues and organs of the body in the context of the regenerative medicine field. The embryonic stem cell or the embryo *in vitro* could be used for scientific purposes only because the fate of its survival simply depends solely on its implantation in the uterus. On the other hand, there is the opinion that even the embryo *in vitro* has a potential that is its ability to evolve into a human being and, therefore, considers every stem cell precious for the same reason. For science and in particular, for biologists and doctors, the fetus is a set of cells without a "substantial" human being and that "therapeutic" cloning differs from "reproductive" cloning because it aims at curing diseases and therefore must be morally acceptable.

In summary, the process of obtaining human embryonic stem cells from embryos *in vitro* finds the opposite side of the Christian Churches, because the embryo becomes a person from the first moment of conception. But here, we can notice in an interdisciplinary way, that is, between theology and science a different interpretation of terms that leads to a concurrent error. It is obvious that the perceptions and objections that exist today in the main Christian doctrines that are both in the Catholic and Evangelical and Orthodox Churches seem to be based on a misunderstanding of what is it, a conception whether made natural or with medical assistance. Therefore, one cannot speak of an early fetal existence because it does not exist.

The *in vitro* fertilized egg, although it is in a nutrient medium and can grow under suitable conditions (not, obviously in all cases) to the blastocyst stage, does not constitute an "embryo". To do this, it is necessary to transfer it to the uterus and to grow after successful "implantation." So, there is conception (both in the case of *in vitro* fertilization and through the natural process) only if the fertilized egg is successfully implanted and the pregnancy begins. Otherwise, there is no conception. Thus, there exists an error interpretation between the term conception and fertilization, and the use of the term "embryo" as the fertilized egg (or the embryo *in vitro*), which cannot yet be considered as a real fetus but only if transferred to the uterus for its subsequent development. Hence, the definition of the term conception should not be equated with the term fertilization. Therefore, it cannot be considered that an early fetal existence exists, even the egg fertilized *in vitro* up to the blastocyst stage, does not constitute an "embryo". Because, as we have already mentioned, its transfer, successful implantation, and thus its growth in the uterus are mandatory. Finally, it is of fundamental importance to start a constructive debate on the issue that allows the comparison and ethical supervision of embryonic stem cells. Due to the recent embryonic stem cell research, there is still no clear and specific regulation for each case. The 2016 ISSCR guidelines offer a reason for comparison and therefore for updating given the latest knowledge and experiments.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

Concept and design were done by I.A.C., A.B., and L.B.; acquisition, analysis, and interpretation of data were done by I.A.C. and L.B.; drafting of the manuscript was done by I.A.C., L.S., and A.B.; critical revision of the manuscript for important intellectual content was done by A.B., M.D.C., M.D.D., S.C., M.B., R.N., E.B., and L.B.; administrative, technical, or material support were done by L.S., M.B., and S.C.; supervision was done by A.B. and L.S. All authors have read and agreed to the published version of the manuscript. Ioannis Alexandros Charitos and Andrea Ballini contributed equally as co-first authors. Michele Di Cosola, Luigi Santacroce, and Lucrezia Bottalico contributed equally as co-last authors.

References

[1] W. Zakrzewski, M. Dobrzyński, M. Szymonowicz, and Z. Rybak, "Stem cells: past, present, and future," *Stem Cell Research & Therapy*, vol. 10, no. 1, p. 68, 2019.

[2] T. Boroviak and J. Nichols, "The birth of embryonic pluripotency," *Philosophical Transactions of the Royal Society B: Biological Sciences*, vol. 369, no. 1657, p. 20130541, 2014.

[3] T. Vazin and W. J. Freed, "Human embryonic stem cells: derivation, culture, and differentiation: a review," *Restorative Neurology and Neuroscience*, vol. 28, no. 4, pp. 589–603, 2010.

[4] A. Ballini, S. Cantore, S. Scacco et al., "A comparative study on different stemness gene expression between dental pulp stem cells vs. dental bud stem cells," *European Review for Medical and Pharmacological Sciences*, vol. 23, no. 4, pp. 1626–1633, 2019.

[5] S. Cantore, V. Crincoli, A. Boccaccio et al., "Recent advances in endocrine, metabolic and immune disorders: mesenchymal stem cells (MSCs) and engineered scaffolds," *Endocrine, Metabolic & Immune Disorders-Drug Targets*, vol. 18, no. 5, pp. 466–469, 2018.

[6] J. W. T. Kusena, M. Shariatzadeh, R. J. Thomas, and S. L. Wilson, "Understanding cell culture dynamics: a tool for defining protocol parameters for improved processes and efficient manufacturing using human embryonic stem cells," *Bioengineered*, vol. 12, no. 1, pp. 979–996, 2021.

[7] C. Qiu, Z. Ge, W. Cui, L. Yu, and J. Li, "Human amniotic epithelial stem cells: a promising seed cell for clinical applications," *International Journal of Molecular Sciences*, vol. 21, no. 20, p. 7730, 2020.

[8] S. H. Ghamari, M. Abbasi-Kangevari, T. Tayebi, S. Bahrami, and H. Niknejad, "The bottlenecks in translating placenta-derived amniotic epithelial and mesenchymal stromal cells into the clinic: current discrepancies in marker reports," *Frontiers in Bioengineering and Biotechnology*, vol. 8, p. 180, 2020.

[9] P. V. Guillot, K. O'Donoghue, H. Kurata, and N. M. Fisk, "Fetal stem cells: betwixt and between," *Seminars in Reproductive Medicine*, vol. 24, no. 5, pp. 340–347, 2006.

[10] H. Abdulrazzak, D. Moschidou, G. Jones, and P. V. Guillot, "Biological characteristics of stem cells from foetal, cord blood and extraembryonic tissues," *Journal of the Royal Society Interface*, vol. 7, 6, pp. S689–S706, 2010.

[11] M. L. Weiss and D. L. Troyer, "Stem cells in the umbilical cord," *Stem Cell Reviews*, vol. 2, no. 2, pp. 155–162, 2006.

[12] G. Liu, B. T. David, M. Trawczynski, and R. G. Fessler, "Advances in pluripotent stem cells: history, mechanisms, technologies, and applications," *Stem Cell Reviews and Reports*, vol. 16, no. 1, pp. 3–32, 2020.

[13] F. Posa, G. Colaianni, M. Di Cosola et al., "The myokine irisin promotes osteogenic differentiation of dental bud-derived MSCs," *Biology*, vol. 10, no. 4, p. 295, 2021.

[14] M. Di Domenico, A. Feola, P. Ambrosio et al., "Antioxidant Effect of Beer Polyphenols and Their Bioavailability in Dental-Derived Stem Cells (D-dSCs) and Human Intestinal Epithelial Lines (Caco-2) Cells," *Stem Cells International*, vol. 2020, 13 pages, 2020.

[15] A. Ballini, A. Boccaccio, R. Saini, P. Van Pham, and M. Tatullo, "Dental-derived stem cells and their secretome and interactions with bioscaffolds/biomaterials in regenerative medicine: from the in vitro research to translational applications," *Stem Cells International*, vol. 2017, 3 pages, 2017.

[16] M. Reisman and K. T. Adams, "Stem cell therapy: a look at current research, regulations, and remaining hurdles," *Pharmacy and Therapeutics*, vol. 39, no. 12, pp. 846–857, 2014.

[17] I. Hyun, M. Munsie, M. F. Pera, N. C. Rivron, and J. Rossant, "Toward guidelines for research on human embryo models formed from stem cells," *Stem Cell Reports*, vol. 14, no. 2, pp. 169–174, 2020.

[18] N. Granot and R. Storb, "History of hematopoietic cell transplantation: challenges and progress," *Haematologica*, vol. 105, no. 12, pp. 2716–2729, 2020.

[19] R. C. Zhang, W. Q. Du, J. Y. Zhang et al., "Mesenchymal stem cell treatment for peripheral nerve injury: a narrative review," *Neural Regeneration Research*, vol. 16, no. 11, pp. 2170–2176, 2021.

[20] K. Nemeth, T. Wilson, B. Rada et al., "Characterization and function of histamine receptors in human bone marrow stromal cells," *Stem Cells*, vol. 30, no. 2, pp. 222–231, 2012.

[21] G. Isola, A. Polizzi, A. Alibrandi, R. C. Williams, and R. Leonardi, "Independent impact of periodontitis and cardiovascular disease on elevated soluble urokinase-type plasminogen activator receptor (suPAR) levels," *Journal of Periodontology*, 2020.

[22] F. Inchingolo, F. S. Martelli, C. Gargiulo Isacco et al., "Chronic periodontitis and immunity, towards the implementation of a personalized medicine: a translational research on gene single nucleotide polymorphisms (SNPs) linked to chronic oral dysbiosis in 96 Caucasian patients," *Biomedicine*, vol. 8, no. 5, p. 115, 2020.

[23] G. Isola, A. Polizzi, R. Patini, S. Ferlito, A. Alibrandi, and G. Palazzo, "Association among serum and salivary A. actinomycetemcomitans specific immunoglobulin antibodies and periodontitis," *BMC Oral Health*, vol. 20, no. 1, p. 283, 2020.

[24] R. T. Mendes and D. Fernandes, "Endothelial dysfunction and periodontitis: the role of inflammatory serum biomarkers," *Dent Hypotheses*, vol. 7, no. 1, pp. 4–11, 2016.

[25] A. Ul Hassan, G. Hassan, and Z. Rasool, "Role of stem cells in treatment of neurological disorder," *International Journal of Health Sciences*, vol. 3, no. 2, pp. 227–233, 2009.

[26] J. S. Lunn, S. A. Sakowski, J. Hur, and E. L. Feldman, "Stem cell technology for neurodegenerative diseases," *Annals of Neurology*, vol. 70, no. 3, pp. 353–361, 2011.

[27] R. S. Rodrigues, D. M. Lourenço, S. L. Paulo et al., "Cannabinoid actions on neural stem cells: implications for pathophysiology," *Molecules*, vol. 24, no. 7, p. 1350, 2019.

[28] I. A. Charitos, R. Gagliano-Candela, L. Santacroce, and L. Bottalico, "The Cannabis spread throughout the continents and its therapeutic use in history," *Endocrine, Metabolic & Immune Disorders Drug Targets*, vol. 21, no. 3, pp. 407–417, 2021.

[29] W. Y. Li, Q. B. Zhu, L. Y. Jin, Y. Yang, X. Y. Xu, and X. Y. Hu, "Exosomes derived from human induced pluripotent stem cell-derived neural progenitor cells protect neuronal function under ischemic conditions," *Neural Regeneration Research*, vol. 16, no. 10, pp. 2064–2070, 2021.

[30] S. Acharya, A. I. Lumley, and Y. Devaux, "Non-coding RNAs and stem cells: the dream team for neural regeneration in Parkinson's disease?," *Neural Regeneration Research*, vol. 16, no. 10, pp. 2017-2018, 2021.

[31] Q. T. Dang, T. D. Huynh, F. Inchingolo et al., "Human chondrocytes from human adipose tissue-derived Mesenchymal stem cells seeded on a dermal-derived collagen matrix sheet: our preliminary results for a ready to go biotechnological cartilage graft in clinical practice," *Stem Cells International*, vol. 2021, 12 pages, 2021.

[32] A. Di Benedetto, F. Posa, C. Carbone et al., "NURR1 downregulation favors osteoblastic differentiation of MSCs," *Stem Cells International*, vol. 2017, Article ID 7617048, 10 pages, 2017.

[33] F. Mastrangelo, S. Scacco, A. Ballini et al., "A pilot study of human mesenchymal stem cells from visceral and subcutaneous fat tissue and their differentiation to osteogenic phenotype," *European Review for Medical and Pharmacological Sciences*, vol. 23, no. 7, pp. 2924–2934, 2019.

[34] D. M. Dohan Ehrenfest, M. Del Corso, F. Inchingolo, and J. B. Charrier, "Selecting a relevant in vitro cell model for testing and comparing the effects of a Choukroun's platelet-rich fibrin (PRF) membrane and a platelet-rich plasma (PRP) gel: tricks and traps," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, vol. 110, no. 4, pp. 409–411, 2010.

[35] A. Di Benedetto, F. Posa, S. De Maria et al., "Polydatin, natural precursor of resveratrol, promotes osteogenic differentiation of mesenchymal stem cells," *International Journal of Medical Sciences*, vol. 15, no. 9, pp. 944–952, 2018, eCollection 2018.

[36] D. K. W. Ocansey, L. Wang, J. Wang et al., "Mesenchymal stem cell-gut microbiota interaction in the repair of inflammatory bowel disease: an enhanced therapeutic effect," *Clinical and Translational Medicine*, vol. 8, no. 1, p. 31, 2019.

[37] A. Ballini, G. Dipalma, C. G. Isacco et al., "Oral microbiota and immune system crosstalk: a translational research," *Biology (Basel)*, vol. 9, no. 6, p. 131, 2020.

[38] L. Santacroce, I. A. Charitos, and L. Bottalico, "A successful history: Probiotics and their potential as antimicrobials," *Expert Review of Anti-infective Therapy*, vol. 17, no. 8, pp. 635–645, 2019.

[39] N. Han, L. Jia, L. Guo et al., "Balanced oral pathogenic bacteria and probiotics promoted wound healing via maintaining mesenchymal stem cell homeostasis," *Stem Cell Research & Therapy*, vol. 11, no. 1, 2020.

[40] S. Hansford and D. G. Huntsman, "Boveri at 100: Theodor Boveri and genetic predisposition to cancer," *The Journal of Pathology*, vol. 234, no. 2, pp. 142–145, 2014, https://mattiolihealth.com/wp-content/uploads/2020/11/Med-Histor-2-2020.pdf.

[41] A. H. Maehle, "Ambiguous cells: the emergence of the stem cell concept in the nineteenth and twentieth centuries," *Notes and Records of the Royal Society*, vol. 65, no. 4, pp. 359–378, 2011.

[42] E. Grundmann, "Die Vorstellungen von Julius Cohnheim zur Geschwulstentstehung und Metastasierung im Blickwinkel neuer Forschungsergebnisse," *Zentralblatt f ü r allgemeine Pathologie und pathologische Anatomie*, vol. 130, no. 4, pp. 323–331, 1985.

[43] S. Cantore, A. Ballini, D. De Vito et al., "Characterization of human apical papilla-derived stem cells," *Journal of Biological Regulators and Homeostatic Agents*, vol. 31, no. 4, pp. 901–910, 2017.

[44] D. Vermesan, H. Vermesan, S. I. Dragulescu et al., "Secondary pathologic fractures in osteosarcoma: prognosis and evolution," *European Review for Medical and Pharmacological Sciences*, vol. 13, no. 2, pp. 71–76, 2009.

[45] F. Inchingolo, L. Santacroce, A. Ballini et al., "Oral cancer: a historical review," *International Journal of Environmental Research and Public Health*, vol. 17, no. 9, p. 3168, 2020.

[46] G. Baniebrahimi, F. Mir, and R. Khanmohammadi, "Cancer stem cells and oral cancer: insights into molecular mechanisms and therapeutic approaches," *Cancer Cell International*, vol. 20, no. 1, p. 113, 2020.

[47] E. Pellegrino and M. G. Gutierrez, "Human stem cell-based models for studying host-pathogen interactions," *Cellular Microbiology*, vol. 1, article e13335, 2021.

[48] S. Topi, L. Santacroce, L. Bottalico et al., "Gastric cancer in history: a perspective interdisciplinary study," *Cancers (Basel)*, vol. 12, no. 2, p. 264, 2020.

[49] S. Takaishi, T. Okumura, and T. C. Wang, "Gastric cancer stem cells," *Journal of Clinical Oncology*, vol. 26, no. 17, pp. 2876–2882, 2008.

[50] M. Ramalho-Santos and H. Willenbring, "On the origin of the term "stem cell"," *Cell Stem Cell*, vol. 1, no. 1, pp. 35–38, 2007.

[51] A. Ballini, A. Di Benedetto, D. De Vito et al., "Stemness genes expression in naive vs. osteodifferentiated human dental-derived stem cells," *European Review for Medical and Pharmacological Sciences*, vol. 23, no. 7, pp. 2916–2923, 2019.

[52] M. Girlovanu, S. Susman, O. Soritau et al., "Stem cells - biological update and cell therapy progress," *Medicine and Pharmacy Reports*, vol. 88, no. 3, pp. 265–271, 2015.

[53] G. Brunetti, A. Di Benedetto, F. Posa et al., "High expression of TRAIL by osteoblastic differentiated dental pulp stem cells affects myeloma cell viability," *Oncology Reports*, vol. 39, no. 4, pp. 2031–2039, 2018.

[54] The Nobel Prizer, "Gene modification in mice," 2007, https://www.nobelprize.org/prizes/medicine/2007/advanced-information/.

[55] J. A. Thomson, J. Itskovitz-Eldor, S. S. Shapiro et al., "Embryonic stem cell lines derived from human blastocysts," *Science*, vol. 282, no. 5391, pp. 1145–1147, 1998.

[56] M. Boccellino, D. Di Stasio, G. Dipalma et al., "Steroids and growth factors in oral squamous cell carcinoma: useful source

of dental-derived stem cells to develop a steroidogenic model in new clinical strategies," *European Review for Medical and Pharmacological Sciences*, vol. 23, no. 20, pp. 8730–8740, 2019.

[57] K. K. Ballen, B. R. Logan, M. J. Laughlin et al., "Effect of cord blood processing on transplantation outcomes after single myeloablative umbilical cord blood transplantation," *Biology of Blood and Marrow Transplantation*, vol. 21, no. 4, pp. 688–695, 2015.

[58] A. Colman, "Profile of John Gurdon and Shinya Yamanaka, 2012 Nobel laureates in medicine or physiology," *Proceedings of the National Academy of Sciences*, vol. 110, no. 15, pp. 5740-5741, 2013.

[59] J. B. McCormick and H. A. Huso, "Stem cells and ethics: current issues," *Journal of Cardiovascular Translational Research*, vol. 3, no. 2, pp. 122–127, 2010.

[60] "International Society for Stem Cell Research (ISSCR), guidelines for stem cell research and clinical translation," 2016, https://www.isscr.org/policy/guidelines-for-stem-cell-research-and-clinical-translation.

[61] J. Kurtzberg, A. D. Lyerly, and J. Sugarman, "Untying the Gordian knot: policies, practices, and ethical issues related to banking of umbilical cord blood," *Journal of Clinical Investigation*, vol. 115, no. 10, pp. 2592–2597, 2005.

[62] H. P. Schreiber, "Ethische und biopolitische Probleme der Stammzellforschung," *Therapeutische Umschau*, vol. 63, no. 11, pp. 711–715, 2006.

[63] H. T. Greely, "Moving human embryonic stem cells from legislature to lab: remaining legal and ethical questions," *PLoS Med*, vol. 3, no. 5, p. e143, 2006.

[64] J. Sugarman, "Human stem cell ethics: beyond the embryo," *Cell Stem Cell*, vol. 2, no. 6, pp. 529–533, 2008.

[65] L. Østnor, *Stem Cells, Human Embryos and Ethics*, Publisher Springer Netherlands, 2008.

[66] C. T. Scott, "Stem cells: new frontiers of ethics, law, and policy," *Neurosurgical Focus*, vol. 24, no. 3-4, article E24, 2008.

[67] M. S. Rao and C. I. Civin, "Translational research: toward better characterization of human embryonic stem cell lines," *Stem Cells*, vol. 23, no. 10, pp. 1453–1459, 2005.

[68] T. Caulfield, K. Kamenova, U. Ogbogu et al., "Research ethics and stem cells: is it time to re-think current approaches to oversight?," *EMBO Reports*, vol. 16, no. 1, pp. 2–6, 2015.

[69] V. Volarevic, B. S. Markovic, M. Gazdic et al., "Ethical and safety issues of stem cell-based therapy," *International Journal of Medical Sciences*, vol. 15, no. 1, pp. 36–45, 2018.

[70] C. Sun, J. Yue, N. He, Y. Liu, X. Zhang, and Y. Zhang, "Fundamental principles of stem cell banking," *Advances in Experimental Medicine and Biology*, vol. 951, pp. 31–45, 2016.

[71] A. D. Inchingolo, A. M. Inchingolo, I. R. Bordea et al., "SARS-CoV-2 disease adjuvant therapies and supplements breakthrough for the infection prevention," *Microorganisms*, vol. 9, no. 3, p. 525, 2021.

[72] I. A. Charitos, A. Ballini, L. Bottalico et al., "Special features of SARS-CoV-2 in daily practice," *World Journal of Clinical Cases*, vol. 8, no. 18, pp. 3920–3933, 2020.

[73] M. Cherry, *Religious Perspectives on Bioethics-Routledge Annals of Bioethics*, EditoreTaylor & Francis, 2013.

[74] L. Walters, "Human embryonic stem cell research: an intercultural perspective," *Kennedy Institute of Ethics Journal*, vol. 14, no. 1, pp. 3–38, 2004.

[75] "Religious Groups' Official Positions on Stem Cell Research," https://www.pewforum.org/2008/07/17/religious-groups-official-positions-on-stem-cell-research/.

[76] "Declaration on the production and the scientific and therapeutic use of human embryonic stem cells," https://www.vatican.va/roman_curia/pontificalacademies/actlife/documents/rc_pa_acdlife_doc_20000824_cellule-staminali_en.html.

[77] L. Ostnor, "Stem cells from human embryos for research the theological discussion within Christianity," in *Stem Cells, Human Embryos and Ethics*, pp. 209-210, Springer, Dordrecht, 2008.

[78] G. Heine, "Theological arguments in the human stem cell debate: a critical evaluation," in *Stem Cells, Human Embryos and Ethics Interdisciplinary Perspectives*, L. Ostnor, Ed., pp. 228-229, Springer, Dordrecht, 2007.

[79] P. Ted, *The stem cell debate*, Fortress press, Minneapolis, 2007.

[80] N. Allum, A. Allansdottir, G. Gaskell et al., "Religion and the public ethics of stem-cell research: attitudes in Europe, Canada and the United States," *PLoS One*, vol. 12, no. 4, article e0176274, 2017.

[81] G. Makarios, *Stem Cell Research and Use, Theological and Bioethical Approach*, GRIGORI Publisher, Athens, 2014.

[82] H. Engelhardt Jr., "The discourses of orthodox Christian medical ethics," in *The Cambridge World History of Medical Ethics*, R. Baker and L. McCullough, Eds., pp. 211–217, Cambridge University Press, Cambridge, 2008.

[83] A. Sagan and P. Singer, "The moral status of stem cells," *Metaphilosophy*, vol. 38, no. 2-3, pp. 264–284, 2007.

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Research Article

Human Chondrocytes from Human Adipose Tissue-Derived Mesenchymal Stem Cells Seeded on a Dermal-Derived Collagen Matrix Sheet: Our Preliminary Results for a Ready to Go Biotechnological Cartilage Graft in Clinical Practice

Quan Tran Dang,[1] Thao Duy Huynh,[2] Francesco Inchingolo,[3] Gianna Dipalma,[3] Alessio Danilo Inchingolo,[3] Stefania Cantore,[3] Gregorio Paduanelli,[3] Kieu Cao Diem Nguyen,[3] Andrea Ballini,[4,5] Ciro Gargiulo Isacco,[3] and Cong Toai Tran[2,6]

[1]*University of Medicine and Pharmacy, Ho Chi Minh City, Vietnam*
[2]*Pham Ngoc Thach University of Medicine, Ho Chi Minh City, Vietnam*
[3]*Department of Interdisciplinary Medicine, University of Bari "Aldo Moro", Bari, Italy*
[4]*Department of Biosciences, Biotechnologies and Biopharmaceutics, Campus Universitario "Ernesto Quagliariello", University of Bari "Aldo Moro", Bari, Italy*
[5]*Department of Precision Medicine, University of Campania "Luigi Vanvitelli", Naples, Italy*
[6]*School of Medicine, Viet Nam National University, Ho Chi Minh City, Vietnam*

Correspondence should be addressed to Andrea Ballini; andrea.ballini@me.com

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Background. The articular cartilage is unique in that it contains only a single type of cell and shows poor ability for spontaneous healing. Cartilage tissue engineering which uses mesenchymal stem cells (MSCs) and adipose tissue-derived mesenchymal stem cells (AT-MSCs) is considered an attractive treatment for cartilage lesions and osteoarthritis. The establishment of cartilage regenerative medicine is an important clinical issue, but the search for cell sources able to restore cartilage integrity proves to be challenging. The aim of this study was to create cartilage grafts from the combination of AT-MSCs and collagen substrates. *Methods*. Mesenchymal stem cells were obtained from human donors' adipose tissue, and collagen scaffold, obtained from human skin and cleaned from blood vessels, adipose tissues, and debris, which only preserve dermis and epidermis, were seeded and cultured on collagen substrates and differentiated to chondrocytes. The obtained chondrocyte extracellular matrix of cartilage was then evaluated for the expression of chondrocyte-/cartilage-specific markers, the Cartilage Oligomeric Matrix Protein (COMP), collagen X, alpha-1 polypeptide (COL10A1), and the Collagen II, Human Tagged ORF Clone (COL2A1) by using the reverse transcription polymerase chain reaction (RT-PCR). *Results*. Our findings have shown that the dermal collagen may exert important effects on the quality of in vitro expanded chondrocytes, leading in this way that the influence of collagen skin matrix helps to produce highly active and functional chondrocytes for long-term cartilage tissue regeneration. *Conclusion*. This research opens up the possibility of generating cartilage grafts with the precise purpose of improving the existing limitation in current clinical procedures.

1. Introduction

Cartilage is one of the most important structural tissues and together with bone is subjected to deterioration under the effect of multiple conditions such as aging, metabolic disorders, hormonal deficit, and traumatic injuries [1]. Compared to bone tissue, cartilage has poor or no capability of self-repairing due to a complete avascularity and lack of nerve

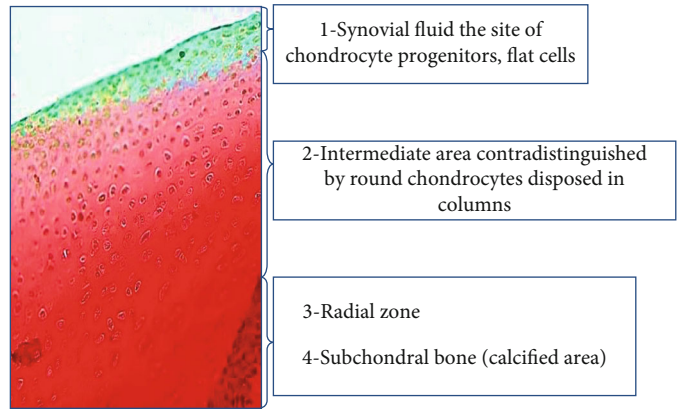


Figure 1: Structure of human cartilage. The cartilage tissue consists of 4 different substrates: 1, the superficial area, a mélange of synovial fluid, chondrocytes, stem cells, and flat cells; 2, an intermediate zone; 3, the radial zone; and 4, the subchondral calcified area close to the bone.

and lymphatic support [1]. For this reason, therapeutic regenerative approaches have encountered strong limitations and poor outcomes [2]. The chondrocytes are cells of mesenchymal origin and form the 5% of the entire cartilage tissue though they work as main homeostasis factors producing and regulating the remaining 95% of cartilage compound made of extracellular matrix (ECM), of which collagen type 2 and proteoglycans (PGs) are the main components [3, 4].

Cartilage tissue histologically consists of four substrates, the most superficial one in relation with the synovial fluid and chondrocyte progenitors, the intermediate area contradistinguished by round chondrocytes followed by the radial zone, and a final calcified area close to subchondral bone (Figure 1) [4, 5]. Any trauma leading to cartilage degeneration, arthritis, affects 26% of adult population of industrialized countries and constitutes one of the highest costs sustained by the national health system [3].

One of the great challenges in modern surgery and reconstructive medicine is to achieve an acceptable long-lasting result of joint biomechanical bone interface functionality and regeneration due to complexity of the inner structure mainly composed by cartilage and its extracellular matrix [5–7]. Despite the use of mesenchymal stem cells (MSCs), their secretome and interactions with bioscaffolds/biomaterials demonstrated to be a useful tool in regenerative medicine, showing significant differentiation capacity in endochondral bone formation; the chondrocyte hypertrophic end stage is almost inevitable [8, 9].

Currently, few approaches have been used such as surgical techniques like the use of autologous/allogeneic or cell regenerative approaches with the infiltration of autologous/allogeneic MSCs and chondrocytes [7, 10–13]. Limitations to these techniques are seen on medium long-term postsurgery/transplantation due to chondrocyte limited self-regenerative capacity and low number of transplanted cells during the implantation procedure; additionally, transplanted chondrocytes tend to lose their capacity of producing new ECM over the time, an event which contributes to generate either inflammation or infection with a consequent loss and/or rejection of both transplanted tissue and cells [14].

The aim of this study was to present an innovative procedure for the generation of a cartilage-like tissue by using a regenerative approach with adipose tissue-derived MSCs (AT-MSCs) seeded on human dermal collagen matrix obtained from human skin.

2. Materials and Methods

2.1. MSCs from Adipose Tissue Isolating Procedure. All procedures were performed under informed consent, in accordance with human study protocols approved by the Declaration of Helsinki for the reuse of human biospecimens in scientific research and the Vietnamese National Health Institute Committee of Biosolution (Research n93IRB-VN01013; grant number B2019-44-01). Fat tissues were originally collected from consented donors and tested for different panels of infectious agents such as HIV, HBV, HCV, and VDRL. Abdominal fat was harvested endoscopically in a lipoaspirate form and transferred to a laboratory facility for further process [15]. Fat was collected in proper sterile containers with DMEM/F12 (Gibco, Grand Island NY, USA), FBS 10%, (Gibco, Grand Island NY, USA), and gentamicin 50 μg/ml (Gibco, Grand Island NY, USA). Tissue was then transferred into dishes and cleaned with a PBS-streptomycin-penicillin solution, foreign tissues were removed, and fat tissues were finely chopped and transferred into a dispase-collagenase enzymatic solution (3 : 1 ratio) and incubated for 90 minutes at 37°C. The sample was then centrifuged (Universal 32, Zentrifugen, Germany) for 5 minutes at a speed of 3,000 rpm; the bottom sediment was collected and filtered by a cell strainer with diameter of 70 μM (BD Falcon™). The obtained material was recentrifuged at a speed of 3,000 rpm for 5 minutes, and the sediment was collected and incubated into T-25 cm^2 flask (Nunc, Wiesbaden, Germany) with DMEM/F12 (Gibco, Grand Island NY, USA) and FBS 10% (Gibco, Grand Island NY, USA) and incubated at 37°C with 5% CO^2. The medium was replaced every 2 days. A tiny fraction of cells was collected and counted by Trypan Blue (Merck KGaA, Darmstadt, Germany). After five days, a colony with the typical spindle-like shape started forming, and at 80–90% of confluence, cells were harvested by enzymatic digestion by using a combination of Trypsin-EDTA (Gibco, Grand Island NY, USA) at 37°C for 5 minutes; suspended cells were collected and washed by PBS and centrifuged for

5 min at 3,000 rpm; the sediment of attached pellet at the bottom was removed by using serum-free media (Gibco, Grand Island NY, USA) and cultured in T-25 cm^2 flask with 5 ml of DMEM/F12 with 10% FBS. The average amount of adherent cells was $0.9\,(\pm 0.37) \times 10^6 - 10^7$ cells per cm^2. Cell viability at the time of passage was nearly 100%. The MSCs were able to proliferate up to 3rd generation. The experiment was conducted by proceeding with same methodology with several fat tissue samples. Cells were tested by flow cytometry for MSC-specific cluster of differentiation (CD) markers to confirm their mesenchymal phenotype. The cells resulted negative for hematopoietic cell markers including CD14, CD45, and HLA-DR and were positive for exclusive markers such as CD73, CD90, and CD105.

2.2. Differentiation of AT-MSCs to Chondrocytes In Vitro. The MSCs at 80 to 90% of confluence were collected and transferred in an appropriate container and induced to chondrogenic phenotype by using a chondrogenic differentiation kit (StemPro™ Chondrogenesis Differentiation Kit, Thermo Fisher, Foster City, CA, USA), following the indications of manufacturer's instructions. MSCs were collected and washed to eliminated basal medium culture and were eventually cultured with the chondrogenic medium seeded on derm collagen scaffold and incubated at 37°C for a period between 2 and 3 weeks; the medium was changed every 3 days. Chondrocyte-like cells were then stained by Alcian Blue, Safranin-O, and hematoxylin and eosin (H-E) stain [16]. All stained samples were observed at a Nikon Eclipse 1000 optical microscope with different magnifications (Nikon Corporation, Tokyo, Japan).

2.3. Alcian Blue Staining Procedure. Alcian Blue (Merck KGaA, Darmstadt, Germany) is a dye used to assess the presence of chondrocytes; the stain reveals the sulphated proteoglycan in cartilage tissue [16]. Cell samples were deparaffinised with xylene substitute, followed by 3 changes of 5 minutes per change. Then, each sample was hydrated in 100% ethanol solution followed by 2 changes of 2 minutes each. The 3rd step was the hydration of samples in 95% ethanol followed by 2 changes of 2 minutes each and again a hydration in 70% ethanol for 2 minutes with a subsequent hydration in 50% ethanol for 2 minutes. At this step, samples were rinsed in di-H_2O for 5 minutes and incubated in 3% acetic acid for 3 minutes and subsequently stained with 1% Alcian Blue solution with a pH 2.5 for 30-60 minutes washed with tap water for 2 minutes and rinsed in di-H_2O. The samples were cleared in xylene substitute with 2 changes of 2 minutes each.

2.4. Safranin-O Staining Procedure. Safranin-O (Merck KGaA, Darmstadt, Germany) allowed to confirm the presence of active chondrocytes by detecting the presence of cartilaginous proteoglycans, mucin, and mast cell granules on a formalin-fixed, paraffin-embedded cartilage tissue-like sample [16]. The final stain confirmed the presence of cartilage-like tissue and mucin in orange/red color, cell nuclei were stained in black, and the background appeared in bluish-green stain. Cell samples were deparaffinised, placed on a slide rinsed with distilled water, stained for 10 minutes with Weigert's iron hematoxylin, and washed with running tap water for 10 minutes. The cells were then stained with fast green solution for 5 minutes, rinsed with acetic acid solution, and stained in 0.1% solution of Safranin-O for 5 minutes. The samples were dehydrated and cleared with ethyl, alcohol (95%), ethyl alcohol (100%), and xylene for 2 minutes each, and resinous medium was used to fix.

2.5. The H-E Staining. The H-E staining technique is one of the principal tissue stains used in histology. The hematoxylin stains cell nuclei blue, and eosin stains the extracellular matrix and cytoplasm pink, with other structures taking on different shades, hues, and combinations of these colors. H-E stain was performed using a diagnostic kit (Celnovte Biotech Zhengzhou City, Henan Province, China). Moreover, in this study, the H-E Saigon (H-SG) staining method was presented (under patenting). This type of unique stain is similar to the more conventional H-E staining methodology, and the procedure however is highly sensitive for type 2 collagen and connective tissues. The samples were incubated in Bouin's solution to bind the dye to the tissue and let overnight at room temperature. Then, the samples were briefly rinsed under water thoroughly and with Weigert's iron hematoxylin and phosphotungstic acid (PTAH) for 10-15 minutes then rinsed under water for 5 minutes. The successive step uses Orange-G to stain the sample for 5-10 minutes, followed by rinsing under water for 2-3 minutes. Then, it is possible to proceed with one step blue/green (blue methylene/fast green) for 10-15 minutes and straight to fuchsin acid 1%, for 1 minute. Finally, the sample was rinsed under running water, dehydrated, cleared, and mounted in a diagnostic slide (all reagents were purchased from Merck KGaA, Darmstadt, Germany).

2.6. RT-PCR Procedure. The chondrocyte phenotype obtained from AT-MSCs was evaluated for the expression of chondrocyte-/cartilage-specific markers, the Cartilage Oligomeric Matrix Protein (COMP), collagen X, alpha-1 polypeptide (COL10A1), and the Collagen II, Human Tagged ORF Clone (COL2A1) by using the reverse transcription polymerase chain reaction (RT-PCR) following the manufacturer's instructions. RNA was isolated by using TRIzol (Gibco, Grand Island NY, USA) following the manufacturer's protocol. The extracted RNA was dissolved in diethylpyrocarbonate-treated water and successively transcribed to cDNA by using RT-PCR. cDNA has been amplified by using Applied Biosystems ABI GeneAmp PCR (Thermo Fisher, Foster City, CA, USA) system at 94°C for 40 s, 56°C for 50 s, and 72°C for 60 s for 35 cycles after an initial denaturation at 94°C for 5 min [15, 17, 18], using the primers reported in Table 1 and actin as internal control.

2.7. Collagen Matrix Scaffold from Skin Samples. A collagen matrix scaffold was obtained from human skin samples donated by consent patients from the Traumatic Unit of the Ho Chi Minh City Orthopaedics Traumatic Hospital. The samples were collected with previous official permission of three independent institutions: (I) Orthopaedic Traumatic

Table 1: RT-PCR primers used to identify, respectively, COMP, COL10A1, and COL2A1 genes.

NM_000095 (*GenBank*) COMP (Cartilage Oligomeric Matrix Protein)	*NM_016685.2* (*GenBank*) *Mus musculus cartilage oligomeric matrix protein (COMP)*	*NM_000493* (*GenBank*) COL10A1 (collagen X, alpha-1 polypeptide)	*NM_001844* (*GenBank*) COL2A1 (Collagen II, Human Tagged ORF Clone)
Forward GCTCTGTGGCATACAGGAGA	Forward TGGGTGATGCCTGTGATAGT	Forward CGCTGAACGATACCAAATGCCC	Forward CCTGGCAAAGATGGTGAGACA
Reverse CATAGAATCGCACCCTGATG	Reverse CGTCATTGTCATCATCGTCA	Reverse TGGACCAGGAGTACCTTGCTCT	Reverse CCTGGTTTTCCACCTTCACCTG

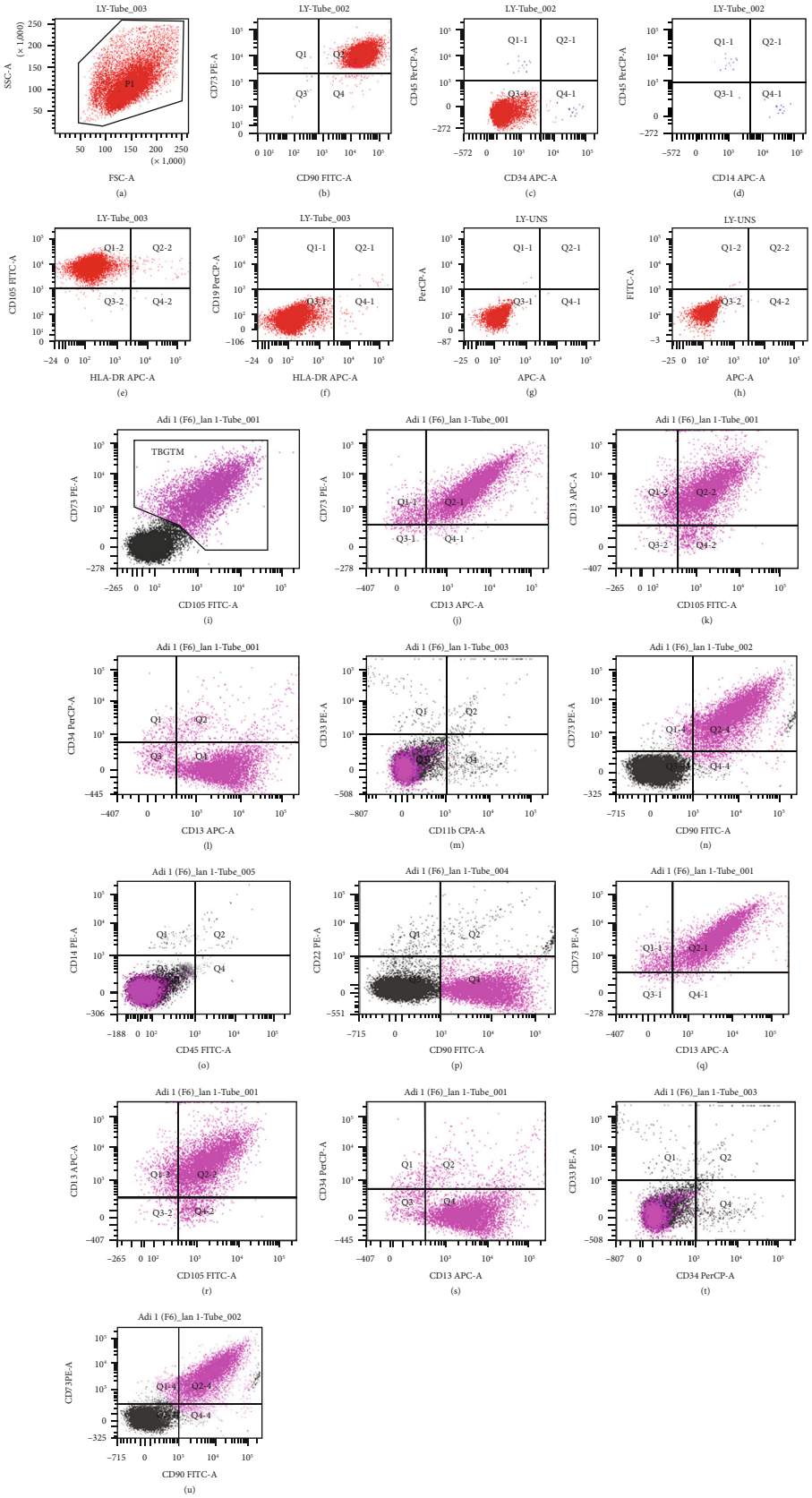
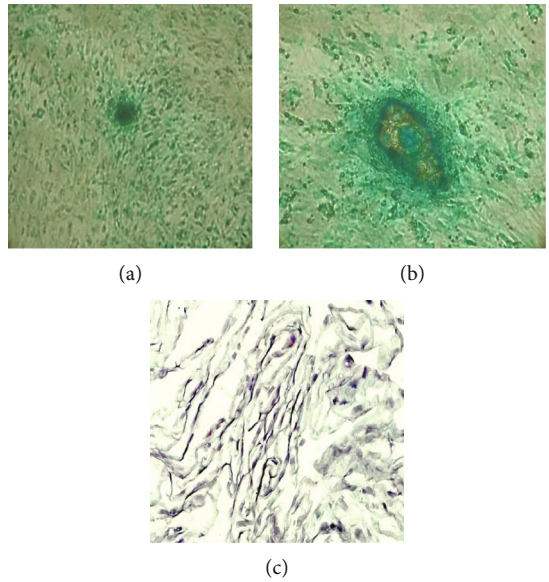


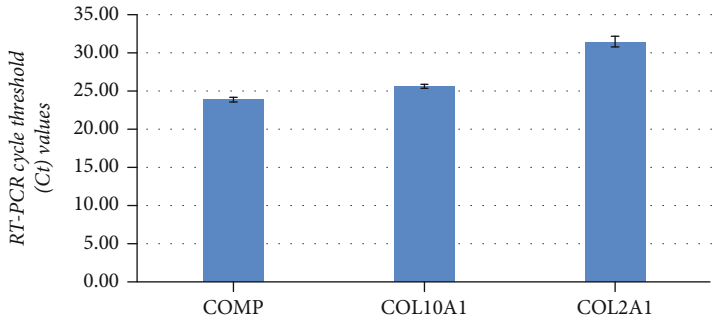
Figure 2: Flow cytometry results confirmed the presence of MSC markers (a–u) $CD13^{+}CD73^{+}CD90^{+}CD105^{+}$ and SSC^{low} and a negative expression for CD 11b, CD14, CD19, CD34, CD45, and HLA-DR.



(a) (b)

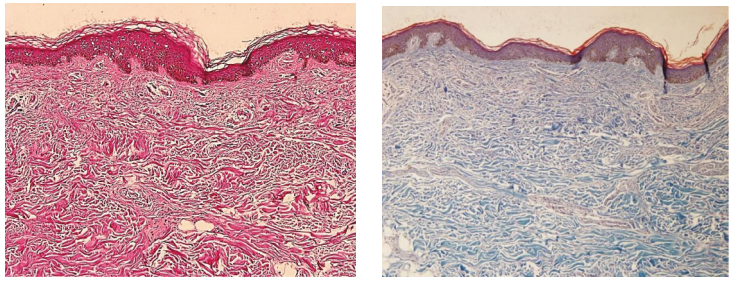
(c)

Figure 3: AT-MSC chondrocyte-like cells at 14 days: (a) Alcian Blue magnification ×10; (b) Alcian Blue magnification ×40; (c) H-E stain of chondrocytes magnification ×20.



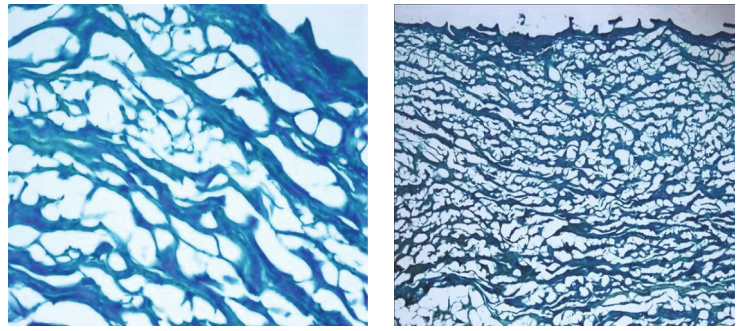
	COMP	COL10A1	COL2A1
AVERAGE	23.98	25.77	31.60
Standard deviation	0.32	0.25	0.71
MT (melting temperature)	87°C	86°C	84.8°C

Figure 4: RT-PCR cycle threshold shows a positive expression for cartilage extracellular matrix genes: (a) the Cartilage Oligomeric Matrix Protein-COMP; (b) collagen type 1-COL10A1; (c) the collagen type 2-COL2A1.



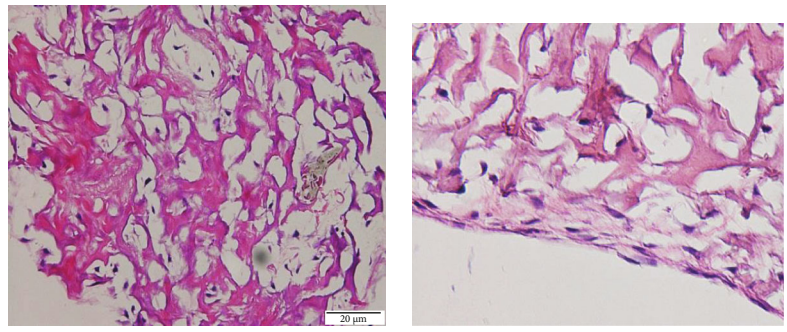
(a) (b)

Figure 5: Human skin before the process of creating the skin-derm scaffold at magnification 10x: (a) H-E stain; (b) H-SG stain.



(a) (b)

Figure 6: Human-derived derm-skin after removal of the epidermis, fat tissues, blood vessels, and debris part: (a) H-SG stain, magnification 60x; (b) H-SG stain magnification 10x.



(a) (b)

Figure 7: Human-derived derm-skin cartilage with attached mature chondrocytes at 21 days: H-E stain, magnification 20x (a) and magnification 40x (b).

Hospital Ethic Committee; (II) Ho Chi Minh City Health Department Ethic Committee; and (III) Ministry of Health of Socialist Republic of Vietnam (MOH).

Skin sample of the size of 3 cm × 3 cm was collected in appropriate containers with sterile buffer PBS solution with gentamycin and brought to the laboratory facility. The samples were processed in a sterile environment and cleaned from blood vessels, adipose tissues, and debris preserving only the dermis and epidermis. Cleared samples were successively treated with NaCl 1 M, EDTA, hypotonic solution, SDS solution, and PBS.

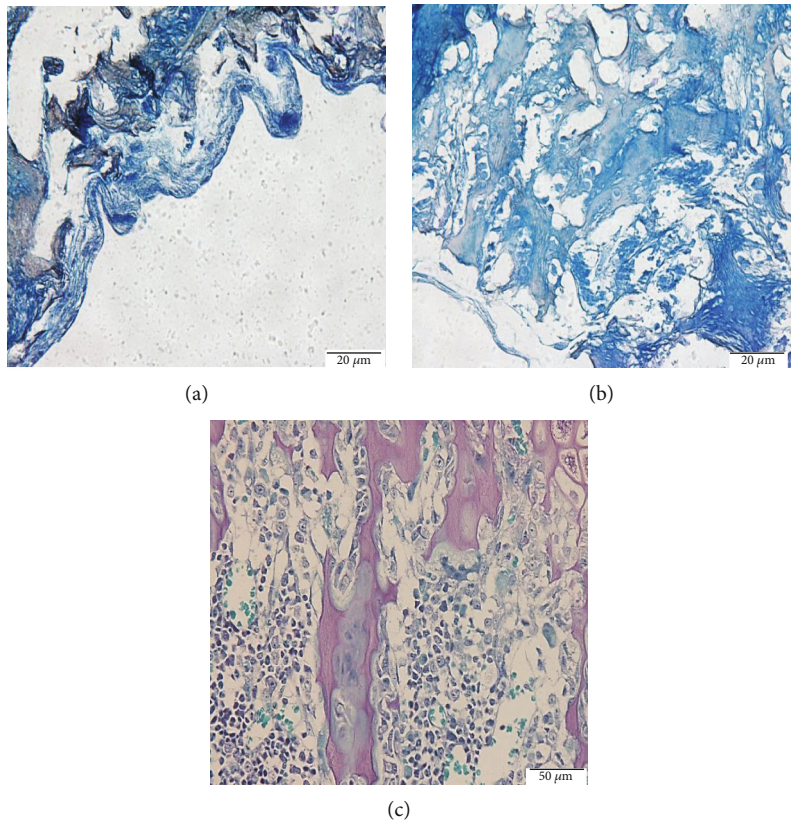
After each step with the above solution, the samples were collected and centrifuged at room temperature at 200/rpm for 5 minutes for 3 times; the samples were then collected and put in cold temperature to dry up. This step involved 3 subphases in a refrigerator at 4°C for 30 minutes, at -20°C for 1 hour and, eventually moved at -80°C for 24 hours. The samples were successively stored for 48 hours at 0.4 millibar pressure. Samples were packed and sterilized with gamma rays at a dose of 25 kGy and stored at 4°C.

Collagen matrix was evaluated before and after by two specific methods, the gel permeation chromatography (GPC) and the high-performance liquid chromatography (HPLC) to quantify collagen content in collagen dermal scaffold before and after treatment to monitor changes in collagen content in the steps of the treatment process. Scaffold samples with and without chondrocytes were then photographed at an APREO scanning electron microscope (SEM) (Thermo Fisher, Foster City, CA, USA).

In addition, a test of toxicity was performed to assess a grade of negative effect on cells by the collagen scaffold (ISO: 10993-5), at 24 hours, 48 hours, and 1 week to show that all samples were eventually free of toxins or any other dangerous alteration, and histology outcomes confirmed the good growth and features of implanted cells.

2.8. Flow Cytometry Analysis and Procedure. The AT-MSCs, once reaching the enough confluence (80-100%), were enzymatically harvested by Trypsin-EDTA (0.25%-0.02%). The cell suspension was centrifuged at 3000 rpm for 5 minutes. Pellets were then washed two times by PBS and analysed by flow cytometry for CD panel expression: CD45, CD34, CD11b, CD13, CD19, CD14, CD105, CD73, and CD90. The analysis was performed 3 times, (modality 2-laser, 6-color) by BD FACSCanto™ (Amersham Biosciences Corp, Piscataway, NJ, USA), (2-laser, 6-color).

2.9. The Construction of Cartilage-Like Membrane by Using Human AT-MSC Chondrocytes. At the 3rd passage, the AT-MSCs were induced to chondrocytes by using the StemPro™ Chondrogenesis Differentiation Kit. Chondrocytes at 90% confluence were enzymatically harvested,



(a) (b)

(c)

Figure 8: Cartilage-like tissue stained by Alcian Blue: magnification 20x (a), 40x (b), and chondrocytes embedded in cartilage matrix Alcian Blue at day 21 (magnification 50x) (c).

10^6–10^7 cells/cm^2, and seeded on the prepared skin collagen scaffold by using the centrifugation force of 1000 rpm for 1 minute and repeated 5 times. Cells and scaffold were successively stained by H-E and Safranin-O and analysed by SEM to assess a 3D presence and distribution of cells on the scaffold.

3. Results

3.1. Isolation Culture, Differentiation of AT-MSCs, and Their CD Marker Expression. After being isolated and collected from adipose tissues, cells were successively immerged and cultured into a basal DMEM plus 10% FBS. The CD panel expression is as follows: CD13, CD105, CD73, and CD90.

Flow cytometry results showed positive expression for $CD13^+CD73^+CD90^+CD105^+$and SSC^{low} and negative expression for markers CD34, CD11b, CD14, CD19, CD45, and HLA-DR (APC-A (allophycocyanin); fluorescein isothiocyanate (FITC); forward scattered area (FSC-a); cytotoxic cryopreservant (Per-CPA)) (Figure 2).

3.2. AT-MSC-Derived Chondrocytes In Vitro Stains and RT-PCR Outcomes. At week 2, the AT-MSCs were collected and induced to chondrocytes by using the StemPro™ Chondrogenesis Differentiation Kit. The chondrocytes were successively stained by H-E and Alcian Blue. After 14 days, the result was a typical cluster of spindle-fibroblast-like cells which were largely present (Figure 3).

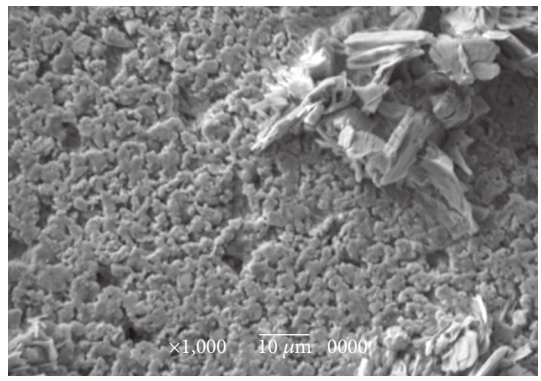


Figure 9: Derm-skin scaffold with collagen sheet, without cells, at SEM magnification ×1,000 (bar: 10 μm). The scaffold proved to have adequate intercompartmental space to allot and home chondrocytes to build up a final cartilage-like matrix.

The RT-PCR analysis procedure was used to assess the expression of typical cartilage extracellular matrix genes like the COMP, COL10A1, and COL2A1, showing reverse transcription. Genes have been detected with a cycle threshold (Ct) value of less than 38 cycles of amplification (Figure 4). Is well known that cycle threshold (Ct) values inversely correlate to the amount of target nucleic acid in the sample. According to universal standard protocols, a positive result

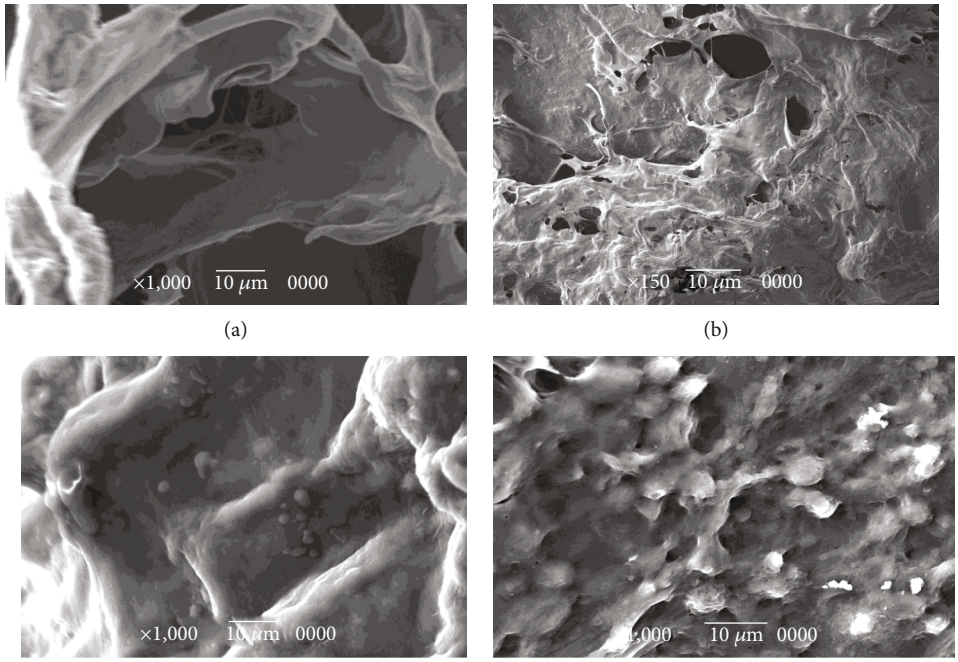


Figure 10: (a, b) Derm-skin scaffold with collagen sheet, without chondrocytes at SEM magnification ×1,000 (a) and magnification ×150 (b). (c, d) The SEM shows the presence of well-integrated chondrocytes at 15-21 days, magnification ×1,000. The chondrocytes were able to construct and modify the scaffold environment.

indicates that investigated markers were detected with a Ct value of less than 38 cycles of amplification.

3.3. Results of Collagen Scaffold Obtained from Human Derm-Skin. The derm-skin collagen matrix scaffolds, obtained from human skin samples and then deeply cleaned and enzymatically treated to obtain a pure matrix without cells or residuals such as blood, fat deposits, or debris, followed a final sterilization with gamma irradiation. The untreated skin samples were stained by H-E and H-SG (Figures 5–8), and the obtained scaffolds were analysed using SEM. The derm/skin scaffolds showed to have enough intercompartmental space to allocate and home chondrocytes to build up a final cartilage-like matrix (Figures 9 and 10).

4. Discussion

In this translational study, we chose to use human dermal substrate as vector to transduce human AT-MSC-derived chondrocytes since the human skin/dermis remains one of the most efficient sites of type II collagen matrix, significant to accommodate chondrocytes in generating very close biosimilar cartilage-like tissue [19, 20]. After a long-term follow-up (11 years) on 61 patients after autologous chondrocyte transplantation graft procedures, it was observed that all of the specimens stained positive for COMP and aggrecan. Hyaline-like cartilage stained positive for type II collagen in the predominant part of the tissue (>50%). The fibrous area stained positive for type I collagen stiffness measurements in hyaline cartilage which were twice those in fibrous cartilage samples [21]. Basically, the repair mechanism was seen in lesions that were fibrous in appearance, and patients received native type II collagen (with or without drug support like acetaminophen) which showed good to excellent long-term outcomes [22, 23].

Therefore, we anticipate the use of skin/derm as a high functional biovector for delivery of substitute cartilage-like substrate. Such approach would potentially allow surgeons to treat articular cartilage lesions with transduced MSCs in a comfortable level of security and in a relatively short healing period.

Furthermore, this strategy may allow us to avoid the current adversities coming from the two step-operative methodology of current cartilage regenerative approach that involves the choice of compatible biomaterial, the generation of *in vitro* chondrocytes from MSCs, and their insertion into the damaged articulation. The *in vitro*-derived MSC chondrocytes located within fibrous injured tissue are primarily spindle-shaped fibroblast-like cells capable of synthesizing type I collagen that is not fully compatible with the *in vivo* normal articular chondrocyte/cartilage tissue conformation. The outcomes of this technique showed the formation of fibrocartilaginous or bone-like tissues both histologically and mechanically different from the surrounding endogenous cartilage tissue [21–24]. These types of side effects are also common even with the use of the latest generation of biomaterial solutions such as fibrin glue, acellular matrix, collagen gels, and alginate up to hyaluronic acid-derived oligostilbenoids, polylactic acid, and polyglycolic acid [25–32].

Technically, the use of autologous chondrocytes is not always a practice free of several issues. Indeed, they must be expanded *in vitro* before being implanted during a second

surgery that is not always an easy practicability; on the other hand, the use of autologous cartilage is strictly reserved to the short availability of autologous tissue.

Thus, the limitations are seen either in terms of biointegration or purely as surgical procedure. For instance, fibrin glue inserted might be gradually replaced by fibrous tissue in a period of less than 21 days and it could function as the main barrier against chondrocyte migration which constrains the correct healing process [20, 33]. The typology of chondrocytes obtained are collagen type I producer and a two weeks' analysis and confirmed a sensitive low and less amounts of both cartilage-specific chondrocyte DNA and proteoglycans compared to those seeded with type II collagen sponges [34, 35]. With regard to hyaluronic compounds, though representing a valid support in cartilage regenerative therapy, these products still remain too fluid to be used as functional scaffolds to accommodate cells. In addition, this procedure does not solve the problem of collagen type II matrix, essential for a full recovery of functional cartilage tissue. Moreover, the structural architecture of the scaffold is equally important. Besides, it should be mentioned that quite limited other factors may compromise a correct growth and development of chondrocytes toward their use in cartilage repair and, as studies have investigated, basal medium used to growth cells *in vitro* may also eventually negatively affect this process [36].

In addition, vitamin D appears to play an important role in bone and cartilage metabolism since its receptors are widely found in human articular chondrocytes. Thus, effects of variation of vitamin D and its supplementation in diet may directly impact cartilage and bone biology [37–40].

Some studies suggested the direct use of seeded chondrocytes on specific semisolid elastic biomaterials that exhibited the capacity of these cells to produce cartilage *in vivo*, due to intrinsic inductive mechanism of the defected cartilage that may act as a "bioactive chamber with natural settings" [40–46].

Therefore, those findings, in line with our results, showed that it is actually possible to carry on with one-step graft and implantation surgery procedure using a very ductile biocompatible material like the autologous skin/derm. This option may improve the quality of the regenerated articular cartilage by supporting cell adhesion, cell growth, and gene expression, specific for cartilage regeneration. Our findings showed that the dermal collagen may exert important effects on the quality of *in vitro* expanded chondrocytes, leading in this way that the influence of collagen skin matrix helps to produce highly active and functional chondrocytes for long-term cartilage tissue regeneration. The presented data showed that skin/derm can function as a very reliable bioscaffold for AT-MSC-derived chondrocytes and we can also speculate that the obtained scaffold fit into an articular cartilage defect (Figures 5–8). Chondrocytes seeded onto derm/-skin scaffold acquired the typical columnar conformation and shape of those found on human cartilage (Figures 9 and 10). This solution avoids also collateral effects and rejection associated with allograft transplants. Thus, the idea was to combine a very ductile technical solution with autologous MSC transplantation that not necessarily should come from adipose tissue but from the bone marrow, dental compartment, synovial fluid, and/or peripheral blood.

5. Conclusion

In summary, there is a huge promise to advance current cartilage therapies toward achieving a consistently successful approach for addressing cartilage afflictions. The results of the current *in vitro* study showed a biocartilage graft solution, which demonstrated chondrocyte formation. These findings confirm that the cells on the skin/derm scaffold were able to produce cartilage tissue gradually throughout collagen type I and II matrix as well as on the surface of the substrate. Therefore, these results may be considered a valid and solid first step toward the generation of a complete and full-scale compatible portion of cartilage-like tissue containing both chondrocyte and essential substrates such as collagen type II and solid cartilage to support and replace injured tissues. Further *in vivo* research and investigation are necessary to establish whether this approach would be pertinent in the context of articular joint cartilage repair. Translational cooperation between research groups and clinicians is key to the safe and successful progression of tissue engineering for cartilage regeneration therapy, with the ultimate goal of providing all patients who have cartilage-related diseases with the opportunity to repair and conserve their joint rather than replacing it.

Data Availability

The experimental data used to support the findings of this study are included within the article.

Ethical Approval

The entire study was conducted according to Vietnamese Ministry of Health guidelines. All procedures were performed in accordance with the declaration of Helsinki for the reuse of human biospecimens in scientific research and the human study protocols approved by the Vietnamese National Health Institute Committee of Biosolution Research n93IRB-VN01013.

Consent

The human subjects involved in this research received an informed consent.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors' Contributions

All the authors were responsible for conceptualization, formal analysis, validation, and investigation. Ciro Gargiulo Isacco and Andrea Ballini were responsible for writing, review, and editing. All authors have read and agreed to the published version of the manuscript. Quan T. Dang, Thao D. Huynh, and Francesco Inchingolo contributed equally as co-first authors. Cong Toai Tran, Ciro Gargiulo Isacco, and

Andrea Ballini designed and performed the experiments, analysed the data, supervised the research, and contributed equally as co-last authors.

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References

[1] M. J. Lammi, J. Piltti, J. Prittinen, and C. Qu, "Challenges in fabrication of tissue-engineered cartilage with correct cellular colonization and extracellular matrix assembly," *International Journal of Molecular Sciences*, vol. 19, no. 9, p. 2700, 2018.

[2] M. Lo Monaco, G. Merckx, J. Ratajczak et al., "Stem cells for cartilage repair: preclinical studies and insights in translational animal models and outcome measures," *Stem Cells International*, vol. 2018, 22 pages, 2018.

[3] T. I. Malinin, W. Mnaymneh, H. K. Lo, and D. K. Hinkle, "Cryopreservation of articular cartilage. Ultrastructural observations and long-term results of experimental distal femoral transplantation," *Clinical Orthopaedics and Related Research*, vol. 303, pp. 18–32, 1994.

[4] P. E. Harrison, I. K. Ashton, W. E. Johnson, S. L. Turner, J. B. Richardson, and B. A. Ashton, "The in vitro growth of human chondrocytes," *Cell and Tissue Banking*, vol. 1, no. 4, pp. 255–260, 2000.

[5] M. Pei and F. He, "Extracellular matrix deposited by synovium-derived stem cells delays replicative senescent chondrocyte dedifferentiation and enhances redifferentiation," *Journal of Cellular Physiology*, vol. 227, no. 5, pp. 2163–2174, 2012.

[6] A. Boccaccio, A. E. Uva, M. Fiorentino, G. Monno, A. Ballini, and A. Desiate, "Optimal load for bone tissue scaffolds with an assigned geometry," *International Journal of Medical Sciences*, vol. 15, no. 1, pp. 16–22, 2018.

[7] E. V. Medvedeva, E. A. Grebenik, S. N. Gornostaeva et al., "Repair of damaged articular cartilage: current approaches and future directions," *International Journal of Molecular Sciences*, vol. 19, no. 8, p. 2366, 2018.

[8] A. Ballini, S. Cantore, S. Scacco, D. Coletti, and M. Tatullo, "Mesenchymal stem cells as promoters, enhancers, and playmakers of the translational regenerative medicine 2018," *Stem Cells International*, vol. 2018, 2 pages, 2018.

[9] A. Ballini, A. Boccaccio, R. Saini, P. Van Pham, and M. Tatullo, "Dental-derived stem cells and their secretome and interactions with bioscaffolds/biomaterials in regenerative medicine: from the in vitro research to translational applications," *Stem Cells International*, vol. 2017, 3 pages, 2017.

[10] S. K. Williams, D. Amiel, S. T. Ball et al., "Prolonged storage effects on the articular cartilage of fresh human osteochondral allografts," *The Journal of Bone and Joint Surgery*, vol. 85, no. 11, pp. 2111–2120, 2003.

[11] Y. D. Levy, S. Görtz, P. A. Pulido, M. C. JC, and W. D. Bugbee, "Do fresh osteochondral allografts successfully treat femoral condyle lesions?," *Clinical Orthopaedics and Related Research*, vol. 471, no. 1, pp. 231–237, 2013.

[12] M. B. Pabbruwe, E. Esfandiari, W. Kafienah, J. F. Tarlton, and A. P. Hollander, "Induction of cartilage integration by a chondrocyte/collagen-scaffold implant," *Biomaterials*, vol. 30, no. 26, pp. 4277–4286, 2009.

[13] S. Cantore, V. Crincoli, A. Boccaccio et al., "Recent advances in endocrine, metabolic and immune disorders: mesenchymal stem cells (MSCs) and engineered scaffolds," *Endocrine, Metabolic & Immune Disorders Drug Targets*, vol. 18, no. 5, pp. 466–469, 2018.

[14] Y. He, A. S. Siebuhr, N. U. Brandt-Hansen et al., "Type X collagen levels are elevated in serum from human osteoarthritis patients and associated with biomarkers of cartilage degradation and inflammation," *BMC Musculoskeletal Disorders*, vol. 15, no. 1, p. 309, 2014.

[15] F. Mastrangelo, S. Scacco, A. Ballini et al., "A pilot study of human mesenchymal stem cells from visceral and subcutaneous fat tissue and their differentiation to osteogenic phenotype," *European Review for Medical and Pharmacological Sciences*, vol. 23, no. 7, pp. 2924–2934, 2019.

[16] N. Schmitz, S. Laverty, V. B. Kraus, and T. Aigner, "Basic methods in histopathology of joint tissues," *Osteoarthritis and Cartilage*, vol. 18, pp. S113–S116, 2010.

[17] T. Debnath, U. Shalini, L. K. Kona et al., "Development of 3D alginate encapsulation for better chondrogenic differentiation potential than the 2D pellet system," *Journal of Stem Cell Research & Therapy*, vol. 5, no. 4, p. 276, 2015.

[18] L. Zhang, J. Hu, and K. A. Athanasiou, "The role of tissue engineering in articular cartilage repair and regeneration," *Critical Reviews in Biomedical Engineering*, vol. 37, no. 1-2, pp. 1–57, 2009.

[19] A. Klinder, S. Kussauer, B. Hiemer, A. Wree, R. Bader, and A. Jonitz-Heincke, "Influence of conditioned media on the re-differentiation capacity of human chondrocytes in 3D spheroid cultures," *Journal of Clinical Medicine*, vol. 9, no. 9, p. 2798, 2020.

[20] D. Deponti, A. Di Giancamillo, F. Gervaso et al., "Collagen scaffold for cartilage tissue engineering: the benefit of fibrin glue and the proper culture time in an infant cartilage model," *Tissue Engineering. Part A*, vol. 20, no. 5-6, pp. 1113–1126, 2014.

[21] L. Peterson, M. Brittberg, I. Kiviranta, E. L. Akerlund, and A. Lindahl, "Autologous chondrocyte Transplantation," *The American Journal of Sports Medicine*, vol. 30, no. 1, pp. 2–12, 2017.

[22] F. Bakilan, O. Armagan, M. Ozgen, F. Tascioglu, O. Bolluk, and O. Alatas, "Effects of native type II collagen treatment on knee osteoarthritis: a randomized controlled trial," *The Eurasian Journal of Medicine*, vol. 48, no. 2, pp. 95–101, 2016.

[23] P. Kannu, J. F. Bateman, S. Randle et al., "Premature arthritis is a distinct type II collagen phenotype," *Arthritis and Rheumatism*, vol. 62, no. 5, pp. 1421–1430, 2010.

[24] M. Schnabel, S. Marlovits, G. Eckhoff et al., "Dedifferentiation-associated changes in morphology and gene expression in primary human articular chondrocytes in cell culture," *Osteoarthritis and Cartilage*, vol. 10, no. 1, pp. 62–70, 2002.

[25] G. Brunetti, M. F. Faienza, L. Piacente et al., "High dickkopf-1 levels in sera and leukocytes from children with 21-hydroxylase deficiency on chronic glucocorticoid treatment," *American Journal of Physiology. Endocrinology and Metabolism*, vol. 304, no. 5, pp. E546–E554, 2013.

[26] M. Marrelli, A. Pujia, F. Palmieri et al., "Innovative approach for the in vitro research on biomedical scaffolds designed

and customized with CAD-CAM technology," *International Journal of Immunopathology and Pharmacology*, vol. 29, no. 4, pp. 778–783, 2016.

[27] B. Perniconi, D. Coletti, P. Aulino et al., "Muscle acellular scaffold as a biomaterial: effects on C2C12 cell differentiation and interaction with the murine host environment," *Frontiers in Physiology*, vol. 5, p. 354, 2014.

[28] M. Tatullo, F. Genovese, E. Aiello et al., "Phosphorene is the new graphene in biomedical applications," *Materials*, vol. 12, no. 14, p. 2301, 2019.

[29] F. Posa, A. Di Benedetto, G. Ravagnan et al., "Bioengineering bone tissue with 3D printed scaffolds in the presence of oligostilbenes," *Materials*, vol. 13, no. 20, p. 4471, 2020.

[30] A. Di Benedetto, F. Posa, S. De Maria et al., "Polydatin, natural precursor of resveratrol, promotes osteogenic differentiation of mesenchymal stem cells," *International Journal of Medical Sciences*, vol. 15, no. 9, pp. 944–952, 2018.

[31] M. Tatullo, M. Marrelli, G. Falisi et al., "Mechanical influence of tissue culture plates and extracellular matrix on mesenchymal stem cell behavior: a topical review," *International Journal of Immunopathology and Pharmacology*, vol. 29, no. 1, pp. 3–8, 2015.

[32] A. Di Benedetto, F. Posa, M. Marazzi et al., "Osteogenic and chondrogenic potential of the supramolecular aggregate T-LysYal®," *Frontiers in Endocrinology*, vol. 11, p. 285, 2020.

[33] D. Alkaya, C. Gurcan, P. Kilic, A. Yilmazer, and G. Gurman, "Where is human-based cellular pharmaceutical R&D taking us in cartilage regeneration?," *3 Biotech*, vol. 10, no. 4, p. 161, 2020.

[34] R. L. Davies and N. J. Kuiper, "Regenerative medicine: a review of the evolution of autologous chondrocyte implantation (ACI) therapy," *Bioengineering*, vol. 6, no. 1, p. 22, 2019.

[35] S. Nehrer, H. A. Breinan, A. Ramappa et al., "Canine chondrocytes seeded in type I and type II collagen implants investigated in vitro," *Journal of Biomedical Materials Research*, vol. 38, no. 2, pp. 95–104, 1997.

[36] Z. Sheikh, S. Najeeb, Z. Khurshid, V. Verma, H. Rashid, and M. Glogauer, "Biodegradable materials for bone repair and tissue engineering applications," *Materials*, vol. 8, no. 9, pp. 5744–5794, 2015.

[37] F. Posa, A. Di Benedetto, E. A. Cavalcanti-Adam et al., "Vitamin D Promotes MSC Osteogenic Differentiation Stimulating Cell Adhesion and $\alpha V\beta 3$ Expression," *Stem Cells International*, vol. 2018, 9 pages, 2018.

[38] C. Pascual-Garrido, M. E. Angeline, R. Ma et al., "Low levels of vitamin D have a deleterious effect on the articular cartilage in a rat model," *HSS Journal*, vol. 12, no. 2, pp. 150–157, 2016.

[39] F. Posa, A. Di Benedetto, G. Colaianni et al., "Vitamin D effects on osteoblastic differentiation of mesenchymal stem cells from dental tissues," *Stem Cells International*, vol. 2016, 9 pages, 2016.

[40] M. A. Szychlinska, R. Imbesi, P. Castrogiovanni et al., "Assessment of vitamin D supplementation on articular cartilage morphology in a young healthy sedentary rat model," *Nutrients*, vol. 11, no. 6, p. 1260, 2019.

[41] A. Di Benedetto, F. Posa, C. Carbone et al., "NURR1 downregulation favors osteoblastic differentiation of MSCs," *Stem Cells International*, vol. 2017, 10 pages, 2017.

[42] W. Shui, L. Yin, J. Luo et al., "Characterization of chondrocyte scaffold carriers for cell-based gene therapy in articular cartilage repair," *Journal of Biomedical Materials Research. Part A*, vol. 101, no. 12, pp. 3542–3550, 2013.

[43] H. Chiang, C. J. Liao, Y. H. Wang et al., "Comparison of articular cartilage repair by autologous chondrocytes with and without in vitro cultivation," *Tissue Engineering Part C: Methods*, vol. 16, no. 2, pp. 291–300, 2010.

[44] G. Jacob, K. Shimomura, and N. Nakamura, "Osteochondral injury, management and tissue engineering approaches," *Frontiers in Cell and Developmental Biology*, vol. 8, 2020.

[45] Y. Wu, B. Ayan, K. K. Moncal et al., "Hybrid bioprinting of zonally stratified human articular cartilage using scaffold-free tissue strands as building blocks," *Advanced Healthcare Materials*, vol. 9, no. 22, p. 2001657, 2020.

[46] Y. Chen, X. Ouyang, Y. Wu, S. Guo, Y. Xie, and G. Wang, "Co-culture and mechanical stimulation on mesenchymal stem cells and chondrocytes for cartilage tissue engineering," *Current Stem Cell Research & Therapy*, vol. 15, no. 1, pp. 54–60, 2020.