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Stem cell differentiation-related protein-loaded PLGA microspheres as a novel platform micro-typed scaffold for chondrogenesis

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Abstract

During cell differentiation for tissue regeneration, several factors, including growth factors and proteins, influence cascades in stem cells such as embryonic stem cells and mesenchymal stem cells (MSCs). In this study, transforming growth factor (TGF)-β3 and SOX9, which is an important protein in chondrocytes, were used to generate mature chondrocytes from human MSCs (hMSCs). For safe and effective delivery of bioactive molecules into hMSCs, biodegradable poly-(d,l-lactide-co-glycolide) (PLGA) microspheres (MSs) were coated with TGF-β3 and loaded with SOX9. Instead of SOX9 protein, release of the model protein FITC-bovine serum albumin (BSA) from PLGA MS was evaluated in vitro and in vivo by confocal laser microscopy and Kodak imaging. The bioactivities of TGF-β3 and SOX9 were evaluated by assessing α-helical formation using circular dichroism. PLGA MS loaded with FITC-BSA easily entered hMSCs without causing cytotoxicity. To confirm that internalization of PLGA MSs harboring TGF-β3 and SOX9 induced chondrogenesis of hMSCs, we performed several molecular analyses. By analysis, the specific marker gene expression levels in hMSCs adhered onto PLGA MSs coated with TGF-β3 and loaded with SOX9 were more than 3–5 times that of the control group both in vitro and in vivo. This result revealed that PLGA MS uptake and subsequent release of SOX9 induced chondrogenesis of hMSCs was enhanced by coating PLGA MSs with TGF-β3.

1. Introduction

The most important application of stem cells is their differentiation into desired cell types [1–3]. During organogenesis and wound healing, stem cell differentiation is extremely important. Many stem cells participate in wound healing [4–6]. Furthermore, transplanted stem cells do not readily survive after injection [7–9]. Therefore, in stem cell-based therapies, an adequate number of cells and the desired stem cell differentiation are necessary for successful tissue regeneration [10–12].

Recently, many researchers have focused on mesenchymal stem cells (MSCs), an important cell type located in every adult tissue. MSCs are multipotent and can differentiate into several tissues such as bone, cartilage, adipose tissue, and muscle [13–16]. Therefore, MSCs are an excellent choice for tissue regeneration [17–20]. Chondrogenesis involves several factors that regulate cascades involved in extra- and intra-cellular mechanisms mediated by specific transcription factors [21, 22]. Recent studies have attempted to deliver transcription factors or drugs that stimulate the differentiation of human MSCs (hMSCs) into specific cell types [23–25].

The transcription factor SOX9 is important for the first and last stages of cartilage formation [26, 27]. The SOX9 gene maintains collagen type II (COL II) expression in cartilage tissue, an important marker of mature chondrocytes. SOX9 protein and gene accumulation in cells excite the progress of prechondrogenic mesenchyme, and appear and disappear in cytosols and nucleus at high levels, which then formed mature chondrocytes where they regulate COL II and aggrecan expression as an enhancer [28, 29].

Transforming growth factor (TGF)-β3 stimulates the differentiation of stem cells into chondrocytes. This growth factor can physically interact with heparin, and increase its activity and stability for stem cell differentiation [30].
Powerful activators of cellular protein uptake are extremely difficult to generate because they need to be bioactive and stable in order to function efficiently. Carriers are required to deliver required factors into cells and block their denaturation until they reach the cytoplasm. Poly(ε-caprolactone-co-glycolide) (PCL-g) microspheres (MSs) are micro-type drug carriers that are degraded by hydrolysis in aqueous solutions and thereby can release the loaded drug simultaneously [31–33]. These materials have been loaded with various bioactive molecules such as peptides, genes, and proteins [34–36]. Loading PCL-g MSs with bioactive molecules is a potential means of delivering such molecules to desired cell types.

In a previous study, SOX9 pDNA coated PCL-g MSs, and also TGF-β3 coated PCL-g MSs, were tested for delivering micro-typed scaffolds of hMSCs. The chondrogenesis of hMSCs was highly observed in the cases of SOX9 pDNA and TGF-β3 coated PCL-g MSs [37]. However, the pDNA of SOX9 genes disappeared within 2 d due to the degradation of the PCL-g MSs.

Herein, we confirm that an improved micro-type scaffold carrying bioactive molecules is internalized by stem cells and stimulates their differentiation, as illustrated in scheme 1. To assess the induction of chondrogenic differentiation of hMSCs upon delivery of bioactive molecules using PCL-g MSs, the mRNA and protein levels of several chondrogenic differentiation markers were examined. Histological and immunofluorescence analyses showed that dual delivery of TGF-β3 and SOX9 stimulated the differentiation of hMSCs into chondrocytes.

### 2. Materials and methods

#### 2.1. Materials

PCL-g (average molecular weight, 33 000 Da; lactic acid:glycolic acid, 50:50; RG 503 H) was purchased from Boehringer Ingelheim (Ingelheim, Germany). Full-length human SOX9 protein (ab131911) was purchased from Abcam (Cambridge, UK). A Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Poly(vinyl alcohol) (PVA), dichloromethane (DCM), dimethyl sulfoxide (DMSO), and FITC-bovine serum albumin (FITC-BSA) (~70 kDa) were purchased from Sigma-Aldrich (St Louis, MO, USA).

#### 2.2. Preparation of protein-loaded PCL-g MSs

SOX9-loaded PCL-g MSs were prepared using an oil-in-water emulsion evaporation method, as previously reported [37]. Briefly, 4 g of PCL-g and 50 μg of SOX9 or FITC-
BSA were dissolved in 40 ml of DCM. The stock solution of SOX9 was dissolved in DMSO. Using a glass syringe and needle (20 G), the polymer solution was added drop-wise to 300 ml of aqueous solution containing 2% (w/v) PVA and then mixed with a magnetic stirrer at 600 rpm. After 2 h of continuous stirring, the MSs were centrifuged and washed twice. Empty MSs lacking SOX9 were prepared in the same way. Empty and SOX9-loaded MSs were labeled with Cy5.5-TGF-β3 following the manufacturer’s instructions. Cy5.5-NHS was added to the TGF-β3 solution, and the mixture was incubated for 30 min at 4 °C. During the reaction, the solution was briefly agitated every 10 min. Using Centricon (molecular weight cut-off, 10,000 Da), the labeled TGF-β3 solution was repeatedly washed with 2 ml of 0.15 M NaCl to eliminate ionic interactions between Cy5.5 and TGF-β3 until no Cy5.5 was detected in the filtrate by fluorescence spectrophotometry (675 nm). Finally, the MSs were collected by filtration, washed with distilled water, and freeze-dried. The sizes of MSs, as measured by scanning electron microscopy (SEM), ranged from 50 to 80 µm.

2.3. Characterization of PLGA MSs
The morphology of lyophilized MSs was observed by field-emission SEM (S-5000, Hitachi Ltd, Tokyo, Japan). Confocal microscopy (Zeiss LSM510 Meta, Göttingen, Germany) was conducted using a Zeiss 40×/1.20 NA water immersion objective lens. Fluorescence images were acquired using argon (wavelength, 488 nm) and helium–neon (wavelength, 543/633 nm) lasers.

2.4. Circular dichroism (CD) spectroscopy
CD spectroscopy was performed using a Jasco J-750 spectropolarimeter (Jasco Inc., Easton, MD, USA) at 4 °C. Samples were equilibrated at this temperature for 30 min prior to data collection. Equilibration was indicated by the absence of further changes in the CD signal when samples were incubated for longer amounts of time. All CD spectra measurements were recorded between 190 and 260 nm in a quartz cuvette with an optical path length of 1 mm. Data points were recorded with a 4.0 s response time and are expressed as molecular ellipticity [θ] in deg(θ) cm−2 dmol−1.

The optical activities of the various types of MS were determined via amino acid analysis to calculate the mean residue ellipticities.

2.5. In vitro studies of protein release from MSs
MSs were incubated in distilled water (DW) and maintained in a shaking incubator at 37 °C. Empty MSs were used as a control. The medium was removed,
Table 1. PCR primer sequences and product sizes.

| Gene     | Sequence                        |
|----------|---------------------------------|
| Aggrecan | (364bp) AGGTTGAACCTCCTGGCAGGT |
|          | (S) CTTGGAGGTCGTTGAGAAAGG      |
| COL II   | (336bp) CTGAAGGAGAGCTCTGCGC    |
|          | (AS) CCCCAGAAACACACAATCCCG     |
| COMP     | (250bp) AAAGCTGAGTGACGTCAC     |
|          | (AS) GGTAGCGGAGATGAGACCC       |
| SOX9     | (350bp) TTATGAGAATGACGAGCA     |
|          | (AS) CACACCATGAAGATGGTTTAT     |
| GAPDH    | (366bp) CGGCTGATGCTGCGTGGAG    |
|          | (AS) ATGATTTCTCGGAGAGCCCC      |

frozen, and replenished at 1, 3, 7, 10, 14, and 21 d. At appropriate intervals, samples were collected and centrifuged for 15 min at 13 000 rpm. Thereafter, 2 ml of supernatant was assayed for protein release and was replaced by 2 ml of fresh medium. During release experiments, the drug concentration in the DW was monitored by measuring the absorbance spectra over the wavelength range 220–270 nm. After equilibrium was reached, MSs were transferred to 3 ml of fresh DW and the process was repeated. In some cases, the process was also repeated once more. The characteristics of SOX9 (10 μg ml⁻¹) release from MSs (100 mg) in a dialysis membrane bag were evaluated, and then the PLGA MSs were moved to conical polypropylene tubes in 1 ml of α-minimal essential medium and placed on an orbital shaker. The empty PLGA MSs were used as the control. The medium was removed, frozen, and replenished at 1, 3, 7, 10, 14, and 21 d.

SDS-PAGE was performed to evaluate the release of BSA and TGF-β3 in denaturing conditions. Gels were washed twice with water, and protein bands were visualized by Coomassie Brilliant Blue G-250 staining. After 1 h, gels were destained using an aqueous solution of methanol (40% v/v) and acetic acid (10% v/v).

2.6. Nude mouse implantation and bioimaging

Animal experiments were approved by the Animal Care Committee of CHA University, Seoul, Korea. The back subcutis of Balb/c female mice (6 weeks old, n = 4 per group) was injected with cells and 50 mg of one of the following: PLGA MSs (control), TGF-β3-coated PLGA MSs, SOX9-loaded PLGA MSs, TGF-β3-coated SOX9-loaded PLGA MSs, FITC-BSA-loaded PLGA MSs, Cy5.5-TGF-β3-coated PLGA MSs, or FITC-BSA-loaded Cy5.5-TGF-β3-coated PLGA MSs. At 1, 2, and 3 weeks post-treatment, mice were sacrificed (n = 4 per time point) by an overdose injection of anesthetic, and skin, including the injection site (2 × 2 cm²), was carefully removed for biological examination. Skin flaps were photographed to record the tissue appearance around the treated site. In addition, in vivo animal experiments were conducted to evaluate the release of proteins from PLGA MSs. Briefly, 50 mg of PLGA MSs was subcutaneously injected into the flank of Balb/c nude mice (6 weeks old, female). At pre-determined time points, fluorescence images of injected nude mice were obtained using a 12-bit camera (Kodak Image Station 4000MM, New Haven, CT, USA) equipped with a special C-mount lens and a FITC and Cy5.5 bandpass emission filter (Omega Optical, Long Island, NY, USA).

2.7. Determination of the protein-loading efficiency

Briefly, 20 mg of lyed SOX9-loaded PLGA MSs was digested in 1 ml of 0.1 N NaOH containing 5% SDS and a protease inhibitor cocktail until the solution became transparent. A Micro BCA Protein Assay Kit (Pierce, Rockford, IL, USA) was used to determine the total amount of protein loaded in the PLGA MSs and the amount of protein released from MSs at different time points. The concentrations of empty (control), TGF-β3-coated, SOX9-loaded, and TGF-β3-coated SOX9-loaded PLGA MSs in the medium were evaluated by Western blot analysis and were quantitatively determined using the Micro BCA Protein Assay Kit. The percentage of total loaded protein that was released was calculated for every sample at various time points and is presented as a cumulative curve in figure 1(B). FITC-BSA was quantified using a Tecan Infinite M200 PRO spectrophotometer (Männedorf, Switzerland). Measurements were made at 25 °C using a 96-well plate. Samples were excited at 488 nm, and emission was monitored at 525 nm.

2.8. Cell culture

hMSCs were purchased from Lonza Walkersville Inc. (catalogue number, PT-2501; Walkersville, MD, USA). After thawing, hMSCs were cultured in MSCGM Basal Medium (catalogue number, PT-3001; Lonza Walkersville) in a humidified incubator containing 5% CO₂ at 37 °C. Cells were maintained at 37 °C in 5% CO₂ and 95% ambient air, and the culture medium was changed every 2 d. For chondrogenic culture, hMSCs (passage #5) and MSs were incubated in 5 ml polypropylene round-bottom tubes with gentle shaking. After incubation for 12 h, non-adhered cells were removed, and the tubes were incubated for cell growth. The culture medium was a serum-free medium containing 100 mg ml⁻¹ streptomycin and 100 IU ml⁻¹ penicillin.

2.9. RT-PCR and real-time-quantitative PCR (qPCR)

Isolated RNA samples were used to synthesize cDNA using a reverse transcriptase (SuperScript III, Invitrogen, Carlsbad, CA, USA) with oligo (dT) primers. Samples were amplified by PCR using platinum Taq Polymerase (Invitrogen) according to the manufacturer’s instructions and the following conditions: 30–35 cycles of 94 °C for 30 s, 55–61 °C for 45 s, and 72 °C for 45 s. All PCR primers and product sizes are listed in table 1. The level of GAPDH was used to control the input amount of RNA (determined once for each cDNA sample) and to normalize the levels of GAPDH expression.
all other genes measured in the same cDNA sample. The copy ratio of each analyzed cDNA was determined as the mean of three experiments. PCR products were separated by electrophoresis at 100 V on a 1.5% agarose gel in 0.5% Tris-acetate-EDTA buffer. Additionally, specific gene expression was measured via real-time PCR in an ExiCycler (Bioneer, Daejeon, Korea). In brief, 1 µl of each cDNA was amplified in a 20 µl assay containing 2.0 mM MgCl₂, 20 pM of each primer (table 1), and 1× Takara PCR Master Mix (Takara, Otsu, Japan). Samples were subjected to the following conditions in an ExiCycler: initial denaturation at 94 °C for 10 min, followed by 45 cycles of 94 °C for 40 s, 58 °C for 30 s, and 72 °C for 30 s. Relative quantification was calculated via the 2-ΔΔct (cycle-threshold) method. To confirm the amplification of specific transcripts, melting curve profiles were generated at the end of each PCR by cooling the sample to 40 °C and then heating it slowly to 95 °C while continuously measuring fluorescence.

2.10. Western blotting
Cells were lysed in radioimmunoprecipitation assay buffer (Pierce) supplemented with a complete protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Approximately 30–50 µg of protein was loaded onto 8–12% SDS polyacrylamide gels and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA, USA). Membranes were subsequently blocked in 2.5% skimmed milk prepared in Tris-buffered saline containing 0.01% Tween 20 and incubated with the following primary antibodies: anti-aggreca, anti-cartilage oligomeric matrix protein (COMP; Abcam), anti-COL II, anti-SOX9 (Chemicon, Temecula, CA, USA), and anti-β-actin (Sigma). The blots were visualized by chemiluminescence using Amersham ECL reagents (GE Healthcare, Little Chalfont, UK).

2.11. Histology and immunohistochemistry
hMSC-seeded PLGA MSs and mouse injection sites were processed for classical histology. Briefly, samples from each time point were embedded in an optimal cutting temperature compound (TISSUE-TEKs 4583, Sakura Finetek USA, Inc., CA), frozen, and cut into 10 mm sections at −20 °C. The nucleus and cytoplasm were stained with hematoxylin and eosin (H&E), respectively. In addition, cryosections (10 µm) of implanted scaffolds were stained with Alcian blue and Safranin-O for histological evaluation. Immunohistochemical analysis was conducted to identify COL II and SOX9 (Chemicon) by incubating the sections with specific primary antibodies (1:200) in a humidified environment. The sections were then rinsed in PBS, incubated with Alexa 488-conjugated goat anti-mouse and Alexa 555-conjugated donkey anti-rabbit immunoglobulin G (Molecular Probes), washed three times with PBS, and incubated with 4, 6-diamidino-2-phenylindole (1:1000; DAPI, Molecular Probes) for 5 min. Cells were visualized using a Zeiss LSM 510 META confocal microscope.

2.12. Statistical analysis
A two-tailed student t-test was used to test the statistical significance of the differences between the experimental groups. A paired t-test was used to compare the results obtained for the MSs both loaded and coated with proteins. A p-value of <0.05 was considered statistically significant.

3. Results and discussion

3.1. Confirmation of protein-loaded TGF-β3-coated PLGA MSs in vitro
To confirm that PLGA MS were loaded with protein and coated with TGF-β3, we first evaluated the morphologies of PLGA MSs by SEM and detected the fluorescence of TGF-β3 and proteins by confocal laser microscopy (figures 1(A) and (B)). The mean diameter of PLGA MSs coated with TGF-β3 or loaded with SOX9 was 50–80 µm. When around 25 µg of SOX9 protein was added to 1 g of PLGA MSs (data not shown), ~18 µg of SOX9 protein was loaded into the MSs, indicating a high loading efficiency (around 72%). The loading capacity was detected using a model protein (FITC-BSA) (figure 1(B)). Green labeling, representing FITC-BSA, was clearly identified in the core of the PLGA MSs by confocal laser microscopy (figure 1(B(b))). We also confirmed the coating of PLGA MSs with Cy5.5-TGF-β3, Red labeling, representing Cy5.5-TGF-β3, covered PLGA MSs (figure 1(B(c))). In the case of FITC-BSA-loaded Cy5.5-TGF-β3-coated PLGA MSs, yellow and orange labeling was clearly observed at the core and surface, respectively. Therefore, PLGA MS were loaded with FITC-BSA and coated with Cy5.5-TGF-β3 (figure 1(B(d))). These results indicate that PLGA MSs could be loaded with SOX9 and coated with TGF-β3. Well-characterized micro-type vehicles and scaffolds are good candidates for stem cell differentiation when applied to wound sites. From the above results, we suggest that this micro-typed scaffold is a good choice for chondrogenic differentiation because TGF-β3 will stimulate adhered stem cells, and SOX9 will be released from PLGA MSs and internalized, without being denaturated.

During the release of proteins and growth factors from PLGA MSs, the proteins had to keep their unique character. For the detection of their nature, Coomasie blue staining, detection for degradation, and CD, represented by the bioactivity detected by α-helical formation, were employed for releasing proteins (figures 1(C) and (D)). These results showed that the BSA proteins (model proteins) loaded into PLGA MSs maintained their stability for three weeks (figure 1(C(b))). In addition, the TGF-β3-coated growth factors on PLGA MSs showed a similar pattern of stability for three weeks (figure 1(C(a))). Not only the stability but also the bioactivity, the loaded-proteins and coated-growth factors
were tested using CD, which revealed the second structure of proteins (α-helical formation) (figure 1(D)). The second structure of proteins was clearly revealed in the 222 nm wavelength after three weeks, even for a small amount of BSA and TGF-β3 (figures 1(D(a) and (b))). This phenomenon indicated that the released proteins and growth factors are coincident with the degradation of PLGA MSs and have potential capacity for displaying their unique functions when they were delivered into cells. From the above results, the protein loaded and growth factor coated PLGA MSs will maintain their stability and bioactivity for a long period, which is enough to differentiate the stem cells into matured chondrocytes.

We tested the degradation of hollow MSs incubated in aqueous solution for three weeks by performing SEM. The shape of the various types of hollow MSs changed from spherical to deformed, indicating that hollow MSs were degraded (figure 2(A)). This is extremely important because protein release is dependent on MS degradation. Proteins must be maintained in hollow MSs to stimulate adhered hMSCs.

The release of FITC-BSA and Cy5.5-TGF-β3 from hollow MS was determined by ELISA analyses (figure 2(B)). Large amounts of FITC-BSA and Cy5.5-TGF-β3 were released from hollow MSs in 1 d, and this release plateaued after 21 d. Therefore, FITC-BSA and Cy5.5-TGF-β3 were released over a long period, which could stimulate adhered hMSCs to differentiate into mature chondrocytes.

3.2. Evaluation of protein-loaded PLGA MSs in vivo

PLGA MSs carrying TGF-β3 and/or proteins were injected into nude mice and imaged using xenozene (figures 3(A)–(D)). The various types of scaffold were viewed by green and blue (pseudo-color) labeling, which represented FITC and Cy5.5, respectively. After PLGA MS injection, the intensity of green and blue labeling steadily decreased over three weeks, suggesting that FITC-BSA and Cy5.5-TGF-β3 were released from implanted PLGA MSs. Surprisingly, BSA and TGF-β3 showed almost the same release pattern when they were delivered together in comparison to when they were delivered separately.

The luminescence of released FITC- and Cy5.5-labeled factors was monitored using confocal laser microscopic views (figures 3(E)–(H)). The fluorescence intensities of FITC, Cy5.5, and FITC/Cy5.5 were maintained for a long amount of time, decreasing to about 20% after three weeks (data not shown). This is consistent with the release patterns obtained in vitro. Similar to the Kodak imaging results, the levels of FITC- and Cy5.5-labeled proteins delivered by PLGA MSs steadily decreased over three weeks. FITC-BSA and Cy5.5-TGF-β3 exhibited similar patterns when they were delivered together in comparison to when they were delivered separately.

It was necessary to evaluate any cytotoxic effects on hMSCs of protein-loaded PLGA MSs, which are rapidly degraded in aqueous environments and can...
cause local acidic conditions. First, we investigated the morphologies of PLGA MSs carrying bioactive molecules and cells simultaneously (figure S1(A) (stacks.iop.org/BMM/11/055003/mmedia)). Upon loading and coating with proteins, the size of PLGA MSs was maintained due to extracellular matrix (ECM) production by embedded hMSCs (figures S1(A(b)–(d))). This confirmed that the embedded hMSCs proliferated and maintained their functions.

Next, we measured the viability of hMSCs adhered to various types of PLGA MSs. The viability of hMSCs adhered to PLGA MSs harboring SOX9 and/or TGF-β3 was similar to that of hMSCs adhered to control PLGA MSs (figure S1(B)). Therefore, the viability of hMSCs was not affected by PLGA MSs carrying SOX9 and/or TGF-β3.

hMSCs were cultured in round-bottom tubes and imaged at various time points (figure S1(C)). PLGA MSs loaded with SOX9 and/or coated with TGF-β3 did not have any cytotoxic effect on hMSCs over three weeks (figures S1(C(m) and (n))). After three weeks, only green labeling, representing live cells, was detected in hMSCs adhered to TGF-β3-coated SOX9-loaded PLGA MS, whereas red labeling, representing dead cells, was detected in hMSCs adhered to TGF-β3-coated PLGA MSs. A low level of red labeling was detected in hMSCs adhered to SOX9-loaded PLGA MS after 3 d and three weeks (figures S1(C(e) and (f))).

3.3. Investigation of SOX9 encapsulation by PLGA MSs

A schematic view of SOX9 encapsulated PLGA MSs for protein production related to chondrocytes is shown in figure 4(A). The Micro BCA assay was used to detect the release of SOX9 from PLGA MSs (figure 4(B)). SOX9 was released from PLGA MSs coated with or without TGF-β3 (figure 4(B)). Therefore, SOX9 was encapsulated by PLGA MSs and released as these MSs were degraded.

We examined SOX9 expression in hMSCs adhered to various types of PLGA MS (figure 4(C)). SOX9 protein expression was much higher in hMSCs adhered to TGF-β3-coated PLGA MS than in hMSCs adhered to SOX9-loaded PLGA MS after 3 d and three weeks (figures S1(C(e) and (f))).
β3-coated PLGA MSs, and SOX9 protein expression was greatly increased in these hMSCs.

3.4. Induction of chondrogenic differentiation of hMSCs in vitro by SOX9-loaded TGF-β3-coated PLGA MSs

We have confirmed the effect of SOX9-loaded TGF-β3-coated PLGA MSs on the chondrogenic differentiation of adhered hMSCs. During cartilage generation, specific factors, such as aggrecan, COMP, COL II, and SOX9, in stem cells and chondrocytes are important. These factors are markers of chondrocytes. We have investigated the chondrogenesis of hMSCs adhered to SOX9-loaded TGF-β3-coated PLGA MSs. RT-PCR and qPCR confirmed that the mRNA levels of these factors were high in hMSCs adhered to SOX9-loaded TGF-β3-coated PLGA MSs (figures 5(A) and (B)). The delivery of SOX9 or TGF-β3 alone into hMSCs slightly increased the aforementioned mRNA levels; however, such hMSCs did not express all the mature chondrocyte markers. Upon delivery of SOX9 alone, hMSCs expressed SOX9 and COMP genes (figure 5(A)). By contrast, delivery of TGF-β3 influenced gene expression of COL II and COMP. However, all the aforementioned chondrogenic markers were expressed when both SOX9 and TGF-β3 were delivered (figure 5(A)). Therefore, delivery of neither SOX9 nor TGF-β3 alone was sufficient for chondrogenesis, and dual delivery of these factors is a unique means of inducing chondrogenesis of hMSCs. The levels of aggrecan, COMP, COL II, and SOX9 were confirmed by real-time qPCR. Similar findings were obtained in this analysis; expression of these factors was highest upon dual delivery of SOX9 and TGF-β3 (figure 5(B)).

During cartilage regeneration, the levels of specific ECM components need to increase. Therefore, we confirmed the expression of cartilage-related ECM components in cultures of differentiated hMSCs adhered to PLGA MSs (figure 5(C)). GAGs, markers of cartilage tissue, were detected in cultures of hMSCs adhered to PLGA MSs loaded with SOX9 and/or coated with TGF-β3. The level of GAGs was highest in cultures of hMSCs adhered to SOX9-loaded TGF-β3-coated PLGA MSs. Normalized GAG production was two-fold higher in hMSCs adhered to SOX9-loaded TGF-β3-coated PLGA MSs than in hMSCs adhered to TGF-β3-coated PLGA MSs. GAG production was slightly higher in hMSCs adhered to SOX9-loaded TGF-β3-coated PLGA MSs than in hMSCs adhered to SOX9-coated PLGA MSs, and the former is a suitable candidate for delivering bioactive molecules and cells simultaneously (figure 5(C)).

We further confirmed the chondrogenic differentiation of hMSCs adhered to PLGA MSs by performing histological analyses of cartilage-specific ECM components (figure 5(D)). The cellular distribution was first observed to assess the proliferation of adhered hMSCs (figures 5(D(a)–(d))). By H&E staining, many cells were observed in cultures con-
Figure 5. Evaluation of in vitro chondrogenic differentiation of hMSCs adhered to PLGA MSs loaded with SOX9 and/or coated with TGF-β3. (A) RT-PCR analysis of aggrecan COMP, SOX9, and COL II, which are markers of mature chondrocytes. The total RNA was prepared from hMSCs grown in culture tubes containing MSs for 21 d. GAPDH was used as an internal control. (B) Real-time qPCR analysis of chondrogenic gene expression levels. Aggrecan, COMP, SOX9, and COL II gene expression was quantified using samples cultured for 21 d. Data represent the means ± SD (n = 3). *p < 0.05, **p < 0.005. (C) GAG/DNA assay of hMSCs to quantify chondrogenesis. (D) Histological analysis of hMSCs adhered to PLGA MSs loaded with SOX9 and/or coated with TGF-β3. (a)–(d) H&E staining, (e)–(h) Alcian blue staining, and (i)–(l) Safranin-O staining. Bar, 50 µm.

Figure 6. Western blot and histological analyses of in vitro chondrogenic differentiation of hMSCs adhered to PLGA MSs loaded with SOX9 and/or coated with TGF-β3. (A) Western blot analysis of hMSCs adhered to various types of PLGA MS. (B) Immunohistological analysis of hMSCs adhered to various types of PLGA MS. (a)–(d) DAPI staining, (e)–(h) COL II labeling, (i)–(l) SOX9 labeling, and (m)–(p) merged images. Bar, 50 µm.
containing SOX9-loaded TGF-β3-coated PLGA MSs, while few cells were observed in cultures containing PLGA MSs loaded with SOX9 or coated with TGF-β3 alone (figure 5(D(d))). Therefore, SOX9-loaded TGF-β3-coated scaffolds support cell proliferation and growth. Polysaccharides and proteoglycans, types of GAGs, were detected by Alcian blue and Safranin-O staining, respectively. In cultures containing SOX9-loaded TGF-β3-coated PLGA MSs, blue staining (figure 5(D(h))), representing polysaccharides, and orange staining (figure 5(D(l))), representing proteoglycans, was observed. Therefore, SOX9-loaded TGF-β3-coated PLGA MSs promoted chondrogenic differentiation of hMSCs.

Finally, the effect of SOX9-loaded TGF-β3-coated PLGA MSs on the chondrogenesis of hMSCs was examined by western blot and immunohistological analyses (figures 6(A) and (B)). A western blot analysis was performed to evaluate the expression of proteins related to mature chondrocytes following treatment of hMSCs with various types of PLGA MS. SOX9, COMP, COL II, and aggrecan were clearly detected in hMSCs adhered to SOX9-loaded TGF-β3-coated PLGA MSs (figure 6(A)). Although these proteins were also expressed in hMSCs adhered to PLGA MSs loaded with SOX9 or coated with TGF-β3 alone (figure 6(A)), their expression levels were not as high. We confirmed that SOX9-loaded TGF-β3-coated PLGA MSs had the potential to induce chondrogenic differentiation of hMSCs (figure 6(B)). As expected, hMSCs treated with SOX9-loaded TGF-β3-coated PLGA MSs expressed COL II and SOX9 proteins, which were represented by vivid green and red labeling, respectively (figures 6(B(g) and (k))).

3.5. Evaluation of the effect of SOX9-loaded TGF-β3-coated PLGA MSs on hMSCs in vivo

We investigated the effect of injection of PLGA MSs and hMSCs on cartilage regeneration in nude mice. hMSCs and PLGA MSs loaded with SOX9 and/or coated with TGF-β3 were implanted into the backs of mice. Three weeks later, the injection sites were excised, and the expression of mature chondrocyte markers was investigated similar to the in vitro analyses. mRNA expression of aggrecan, COMP, COL II, and SOX9 was assessed by RT-PCR and real-time qPCR (figures 7(A) and (B)). Expression of COL II, SOX9, COMP, and aggrecan was highest in hMSCs adhered to SOX9-loaded TGF-β3-coated PLGA MSs (figure 7(A)), and aggrecan expression was eight-fold higher in these cells than in the control cells (figure 7(B)).
To confirm chondrogenesis, it is necessary to determine the GAG content because this is an important ECM component of cartilage. Therefore, we tested GAG production using implanted hMSCs adhered to various types of PLGA MS. Upon dual delivery of SOX9 and TGF-β3, hMSCs exhibited chondrogenic differentiation, as assessed by GAG production (figure 7(C)). This indicates that hMSCs survived in the implanted environment by secreting ECM components to assist their growth and proliferation. Ultimately, hMSCs improved their microenvironment, resulting in chondrogenic differentiation.

We confirmed the expression of polysaccharides and proteoglycans using hMSCs delivered together with various types of PLGA MS. First, cellular distribution, which reflects cell survival, growth, and proliferation, was assessed (figures 7(D(a)–(d))). Implanted hMSCs were clearly observed when delivered together with SOX9-loaded TGF-β3-coated PLGA MSs (figure 7(D(d))). The implanted cells increased in number and covered the scaffold. However, when delivered together with PLGA MSs lacking SOX9 and TGF-β3, there were few hMSCs (figure 7(D(a))). The intensities of Alcian blue staining (figure 7(D(h))), representing polysaccharides, and Safranin-O staining (figure 7(D(I))), representing proteoglycans, were high upon injection of SOX9-loaded TGF-β3-coated PLGA MSs. This indicates that the microenvironment supported the chondrogenesis of implanted hMSCs.

The expression of mature chondrocyte-related markers, indicating chondrogenic differentiation of implanted hMSCs induced by co-delivery of SOX9 and TGF-β3, was validated by a western blot analysis (figure 8(A)). Similar to the in vitro culture system, levels of COL II, SOX9, COMP, and aggrecan were high when hMSCs were injected together with SOX9-loaded TGF-β3-coated PLGA MSs. The protein levels of these chondrocyte-related markers were similar to their mRNA levels in hMSCs adhered to SOX9-loaded TGF-β3-coated PLGA MSs. Therefore, these mRNAs were translated into proteins, causing chondrogenesis.

The expression of proteins such as SOX9 and COL II in hMSCs injected together with PLGA MSs was detected by immunohistological analysis (figure 8(B)). SOX9 and COL II, represented by red and green labeling, respectively, were released from hMSCs and localized around PLGA MSs. SOX9 and COL II localizations differed according to the type of PLGA MS injected (figures 8(B(p)–(t))). No labeling of SOX9 or COL II was observed in the control group. However, both red and green labeling was detected around PLGA MSs loaded with SOX9 or coated with TGF-β3 (figures 8(B(n) and (s))). SOX9 and COL II co-localized around PLGA MSs that harbored both SOX9 and TGF-β3 (figure 8(B(s))), but did not co-localize around PLGA MSs that harbored only SOX9 or TGF-β3 (figures 8(B(q) and (r))). Therefore, endogenous SOX9 and released exogenous SOX9 proteins internalized in hMSCs were co-localized around the PLGA MS scaffolds (figure 8(B(s))). Also, SOX9 and COL II proteins produced from hMSCs cultured in chondrogenic media in vitro was different from the hMSCs that were cultured in PLGA MSs loaded with SOX9 and coated with TGF-β3 in vivo (figure 8(B(t))). This means that the hMSCs were sufficient to differentiate in the condition of PLGA MSs loaded with SOX9 or coated with TGF-β3.
4. Conclusions

In this study, PLGA MSs loaded with SOX9 and coated with TGF-β3 were generated to deliver bioactive molecules and hMSCs. In these PLGA MSs, which did not have cytotoxic effects, SOX9 and TGF-β3 were bioactive and stable. Surprisingly, these factors could induce chondrogenic differentiation of hMSCs adhered to PLGA MSs. Several analyses indicated that PLGA MSs are useful for delivering these factors into hMSCs and thereby promoting chondrogenesis.

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Notes

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References

[1] Mariano E D, Teixeira M J, Marie S K and Lepski G 2015 Adult stem cells in neural repair: current options, limitations and perspectives World J. Stem Cells 7 477–82
[2] Zhou Y, Snead M L and Tamerler C 2015 Bio-inspired hard-to-soft interface for implant integration to bone Nanomedicine 11 431–4
[3] Nabekura T and Lanier L L 2014 Antigen-specific expansion and differentiation of natural killer cells by alloantigen stimulation J. Exp. Med. 211 2455–65
[4] Kaestner K H 2015 An epigenomic road map for endoderm development Cell Stem Cell 16 343–4
[5] Kuwahara A, Ozone C, Nakano T, Saito K, Eiraku M and Sasai Y 2015 Generation of a ciliary margin-like stem cell niche from human retinal tissue J. Exp. Med. 224 2373–8
[6] Poglayen G and Vrtovec B 2015 Stem cell therapy for chronic heart failure Curr. Opin. Cardiol. 30 301–10
[7] Hu X et al 2015 Dextran-coated fluorapatite crystals doped with Yb(3+)/Ho(3+) for labeling and tracking chondrogenic differentiation of bone marrow mesenchymal stem cells in vitro and in vivo Biomaterials 52 441–51
[8] Cook C A, Hahn K C, Morrisette-Mcalmon J B and Grayson W L 2015 Oxygen delivery from hyperbarically loaded microtanks extend cell viability in anoxic environments Biomaterials 52 376–84
[9] Wezel F, Pearson J and Southgate J 2014 Plasticity of in vitro-generated urethelial cells for functional tissue formation Tissue Eng. A 20 1358–68
[10] Skedelik A S, Jaiswal P K and Khan W S 2012 Clinical applications of mesenchymal stem cells in the treatment of fracture non-union and bone defects Curr. Stem Cell Res. Ther. 7 127–33
[11] Xu X, Wang W, Kratz K, Fang I, Li Z, Kurtz A, Ma N and Lendlein A 2014 Controlling major cellular processes of human mesenchymal stem cells using microwell structures Adv. Healthcare Mater. 3 1991–2003
[12] Kang H, Shih Y R and Varghese S 2015 Biomimeralized matrices dominate soluble cues to direct osteogenic differentiation of human mesenchymal stem cells through adenosine signaling Biomaterials 16 1030–61
[13] Sangamier A P, Chandra S, Bhadur D and Khanna A 2015 Effect of HSA coated iron oxide labeling on human umbilical cord derived mesenchymal stem cells Nanotechnology 26 125103
[14] Ghorbani F M, Kafshaf B, Shokrollahi P, Seyedjafarif E and Ardestehrlajimi A 2015 PCL/chitosan/Zn-doped nHA electrosprun nanocomposite scaffold promotes adipose derived stem cells adhesion and proliferation Carbohydr. Polym. 118 135–42
[15] Luo Y, Shen H, Fang Y, Cao Y, Huang J, Zhang M, Dai J, Shi X and Zhang Z 2015 Enhanced proliferation and osteogenic differentiation of mesenchymal stem cells on graphene oxide-incorporated electrosprun poly(lactic-co-glycolic acid) nanofibrous mats ACS Appl. Mater. Interfaces 7 6331–9
[16] Kim M K, Seo B F, Kim K J, Lee S J, Ryu Y H and Rhie J W 2015 Secretory factors of human chorion-derived stem cells enhance activation of human fibroblasts Cytotherapy 17 301–9
[17] Gu W, Song J, Li X M, Wang D, Guo X J and Xu W G 2015 Mesenchymal stem cells alleviate airway inflammation and emphysema in COPD through down-regulation of cyclooxygenase-2 via p38 and ERK MAPK pathways Sci. Rep. 5 8733
[18] Van Pham P and Phan N K 2015 Production of good manufacturing practice-grade human umbilical cord blood-derived mesenchymal stem cells for therapeutic use Methods Mol. Biol. 1283 73–85
[19] Roos–Burgo B, Sanchez–Guio F, Del Canizo C and De Las Rivas J 2014 Transcriptomic portrait of human mesenchymal stromal/stem cells isolated from bone marrow and placenta BMC Genomics 15 910
[20] Hino K, Saito A, Kido M, Kanemoto S, Asada R, Takai T, Cui M, Cui X and Imazumi K 2014 Master regulator for chondrogenesis, SOX9, regulates transcriptional activation of the endoplasmic reticulum stress transducer BEEH7/CREB3L2 in chondrocytes J. Biol. Chem. 289 13610–20
[21] Oztan H, Okada M, Yamashita A, Nakagawa K, Yoshikawa H and Tsumaki N 2013 Direct induction of chondrogenic cells from human dermal fibroblast culture by defined factors PLoS ONE 8 e77365
[22] Jeon S Y, Park J S, Yang H N, Lim H J, Yi S W, Park H and Park K H 2014 Co-delivery of Cbfa-1-targeting siRNA and SOX9 protein using PLGA nanoparticles to induce chondrogenesis of human mesenchymal stem cells Biomaterials 35 8236–48
[23] Chang J C, Su H L and Hau S H 2008 The use of peptide-delivery to protect human adipose-derived adult stem cells from damage caused by the internalization of quantum dots Biomaterials 29 925–36
[24] Hsieh S C, Wang F F, Hung S C, Chen Y J and Wang Y J 2006 The internalized CdSe/ZnS quantum dots impair the chondrogenesis of bone marrow mesenchymal stem cells J. Biomed. Mater. Res. B 79 95–101
[25] Kim I H, Park J S, Yang H N, Woo D G, Jeon S Y, Do H J, Lim H Y and Park K H 2011 The use of biodegradable PLGA nanoparticles to mediate SOX9 gene delivery in human mesenchymal stem cells (hMSCs) and Induce chondrogenesis Biomaterials 32 6268–78
[26] Park J S, Yang H N, Woo D G, Jeon S Y and Park K H 2013 Multilineage differentiation of human-derived dermal fibroblasts transfected with genes coated on PLGA nanoparticles plus growth factors Biomaterials 34 8582–97
[27] Lauing K L, Cortes M, Domowicz M S, Henry J G, Baria A T and Schwartz N B 2014 Aggrecan is required for growth plate cartilage architecture and differentiation Dev. Biol. 396 224–36
[28] Zhang K, Guo J, Ge Z and Zhang J 2014 Nanosecond pulsed electric fields (nsPEFs) regulate phenotypes of chondrocytes through Wnt-β-catenin signaling pathway Sci. Rep. 4 5836
[29] Lei J, McLane L T, Curtis J E and Temenoff J S 2014 Characterization of a multilayer heparin coating for biomolecule presentation to human mesenchymal stem cell spheroids Biomater. Sci. 2 666–73
[31] Xia J, Wang Z, Huang D, Yan Y, Li Y and Guan J 2015 Asymmetric biodegradable microdevices for cell-borne drug delivery ACS Appl. Mater. Interfaces 7 6293–9
[32] Lee C H, Hsieh M J, Chang S H, Lin Y H, Liu S I, Lin T Y, Hung K C, Pang J H and Juang J H 2014 Enhancement of diabetic wound repair using biodegradable nanofibrous metformin-eluting membranes: in vitro and in vivo ACS Appl. Mater. Interfaces 6 3979–86
[33] Wang H, Cheng M, Hu J, Wang C, Xu S and Han C C 2013 Preparation and optimization of silver nanoparticles embedded electrospun membrane for implant associated infections prevention ACS Appl. Mater. Interfaces 5 11014–21
[34] Xiong N, Dong X Y, Zheng J, Liu F F and Sun Y 2015 Design of LVFFARK and LVFFARK-functionalized nanoparticles for inhibiting amyloid β-protein fibrillation and cytotoxicity ACS Appl. Mater. Interfaces 7 5650–62
[35] Su S, Tian Y, Li Y, Ding Y, Ji T, Wu M, Wu Y and Nie G 2015 ‘Triple-punch’ strategy for triple negative breast cancer therapy with minimized drug dosage and improved antitumor efficacy ACS Nano 9 1367–78
[36] Zhou P, Cheng X, Xia Y, Wang P, Zou K, Xu S and Du J 2014 Organic/inorganic composite membranes based on poly(L-lactic-co-glycolic acid) and mesoporous silica for effective bone tissue engineering ACS Appl. Mater. Interfaces 6 20895–903
[37] Park J S, Yang H N, Woo D G, Jeon S Y and Park K H 2012 SOX9 gene plus heparinized TGF-β3 coated dexamethasone loaded PLGA microspheres for inducement of chondrogenesis of hMSCs Biomaterials 33 7151–63