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Alireza Moshaverinia

Xingtian Xu

Chider Chen

Kentaro Akiyama

Malcolm L. Snead

See next page for additional authors

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Dental Mesenchymal Stem Cells Encapsulated in Alginate Hydrogel Co-Delivery Microencapsulation System for Cartilage Regeneration

Abstract

Dental-derived MSCs are promising candidates for cartilage regeneration, with high chondrogenic differentiation capacity. This property contributes to making dental MSCs an advantageous therapeutic option compared to current treatment modalities. The MSC delivery vehicle is the principal determinant for the success of MSC-mediated cartilage regeneration therapies. The objectives of this study were to: (1) develop a novel co-delivery system based on TGF- β 1 loaded RGD-coupled alginate microspheres encapsulating Periodontal Ligament Stem Cells (PDLSCs) or Gingival Mesenchymal Stem Cells (GMSCs); and (2) investigate dental MSC viability and chondrogenic differentiation in alginate microspheres. The results revealed the sustained release of TGF- β 1 from the alginate microspheres. After 4 weeks of chondrogenic differentiation *in vitro*, PDLSCs, GMSCs as well as human bone marrow mesenchymal stem cells (hBMMSC) (as positive control) revealed chondrogenic gene expression markers (Col II and Sox-9) *via* qPCR, as well as matrix positively stained by toluidine blue and safranin-O. In animal studies, ectopic cartilage tissue regeneration was observed inside and around the transplanted microspheres, confirmed by histochemical and immunofluorescent staining. Interestingly, PDLSCs showed more chondrogenesis than GMSCs and hBMMSCs (P

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Disciplines

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Author(s)

Alireza Moshaverinia, Xingtian Xu, Chider Chen, Kentaro Akiyama, Malcolm L. Snead, and Songtao Shi

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Dental mesenchymal stem cells encapsulated in alginate hydrogel co-delivery microencapsulation system for cartilage regeneration

Alireza Moshaverinia, DDS, MS, PhD^{*}, Xingtian Xu, DDS, Chider Chen, BSc, MSc, Kentaro Akiyama, DDS, PhD, Malcolm L Snead, DDS, PhD, and Songtao Shi, DDS, PhD

Center for Craniofacial Molecular Biology, Ostrow School of Dentistry, University of Southern California, Los Angeles, CA

Abstract

Dental-derived MSCs are promising candidates for cartilage regeneration, with high chondrogenic differentiation capacity. This property contributes to making dental MSCs an advantageous therapeutic option compared to current treatment modalities. The MSC delivery vehicle is the principal determinant for the success of MSC-mediated cartilage regeneration therapies. The objectives of this study were to: (1) develop a novel co-delivery system based on TGF- β 1 loaded RGD-coupled alginate microspheres encapsulating Periodontal Ligament Stem Cells (PDLSCs) or Gingival Mesenchymal Stem Cells (GMSCs); and (2) investigate dental MSC viability and chondrogenic differentiation in alginate microspheres. The results revealed the sustained release of TGF- β 1 from the alginate microspheres. After 4 weeks of chondrogenic differentiation *in vitro*, PDLSCs, GMSCs as well as human bone marrow mesenchymal stem cells (hBMMSC) (as positive control) revealed chondrogenic gene expression markers (Col II and Sox-9) *via* qPCR, as well as matrix positively stained by toluidine blue and safranin-O. In animal studies, ectopic cartilage tissue regeneration was observed inside and around the transplanted microspheres, confirmed by histochemical and immunofluorescent staining. Interestingly, PDLSCs showed more chondrogenesis than GMSCs and hBMMSCs ($P < 0.05$). Taken together, these results suggest that RGD-modified alginate microencapsulating dental MSCs make a promising candidate for cartilage regeneration. Our results highlight the vital role played by the microenvironment, as well as value of presenting inductive signals for viability and differentiation of MSCs.

Keywords

tissue engineering; cartilage regeneration; dental mesenchymal stem cells; alginate hydrogel; RGD tripeptide

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^{*}Corresponding Author: Alireza Moshaverinia, DDS, MS, PhD, Postdoctoral Research Fellow, Center for Craniofacial Molecular Biology (CCMB), Ostrow School of Dentistry, University of Southern California, 2250 Alcazar Street - CSA 103, Los Angeles, CA 90033, moshaver@usc.edu, Phone: (323) 442-3191, Fax: (323) 442-2981.

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1. Introduction

Several treatment modalities have been introduced to regenerate or enhance the repair of articular cartilage, such as the grafting of autologous osteochondral tissue or the transplantation of autologous chondrocyte suspensions [1,2]. However, each of these strategies, the biological and mechanical properties of the formed tissue are inferior to those of native articular cartilage [3]. An advantageous alternative therapeutic option is regeneration of cartilage tissue using mesenchymal stem cells (MSCs). MSCs are multipotent cells that can differentiate into multiple lineages depending on the nature of the environmental signals which they receive. Specifically, MSCs undergo chondrogenesis and deposit a cartilage-specific matrix in pellet cultures and in a variety of biomaterials in the presence of appropriate growth factors. Most of the studies on chondrogenic differentiation have focused on applications using bone marrow MSCs (BMMSCs). However, it is well known that MSCs reside in a wide spectrum of post-natal tissue types including the orofacial tissues [4–6], while neural crest origin are attractive for craniofacial regenerative strategies as they might be more plastic to differentiate into craniofacial tissues [5–7]. Among the dental-derived MSCs, periodontal ligament stem cells (PDLSCs) and gingival mesenchymal stem cells (GMSCs) are of particular interest as they can be harvested easily, accessible through oral cavity and they can often be obtained as discarded biological samples in dental clinics [8,9]. Moreover, both *in vitro* and *in vivo* studies have confirmed the multilineage differentiation capabilities of these dental-derived MSCs [10,11].

However, an appropriate microenvironment and signaling molecules are required in order to effectively differentiate MSCs into chondrocytes [12]. It has been reported that growth factors such as: TGF- β 1, BMP-4, and FGF-2 are often required in the process of chondrogenesis [13]. Particularly, studies have reported that transforming growth factor-beta (TGF- β) plays an important role in chondrogenesis of MSCs [14] by stimulating chondrocyte proliferation while preventing cartilage hypertrophy [15]. In addition, it is well known that the cell delivery vehicle has an important role in the *in vivo* performance of MSCs and the success of the regenerative therapy. Therefore, we sought to design an appropriate microenvironment by engineering the physiochemical properties of the extracellular MSC microenvironment in order to tailor the niche characteristics and direct cell phenotype through differentiation [16,17]. Hydrogel biomaterials have been widely used for cartilage tissue engineering. Among the hydrogel biomaterials, alginates, which are natural hetero-polysaccharides isolated from brown sea algae, are of particular interest due to their unique properties including injectability and biodegradability [18,19]. Alginate can provide a 3D scaffold that facilitates the spatial distribution of MSCs, thus resulting in a structural organization that resembles the native *in vivo* microenvironments. Moreover, alginate microspheres have been used extensively for controlled delivery of growth factors (e.g. TGF- β) making them desirable biomaterials for chondrogenesis [20,21]. In the present study, we developed a novel co-delivery system that provides a 3D architecture of RGD-coupled alginate hydrogel loaded with a TGF- β 1 ligand based for microencapsulation of dental MSCs. This approach ensures optimized cartilage regeneration and provides a potential application for reconstruction of the temporomandibular joint disk and for applications in the appendicular skeleton.

2. Materials and methods

All the animal experiments in the current study were performed in accordance with the guidelines published by the Institutional Animal Care and Use Committee at the University of Southern California, and the American Association for Accreditation of Laboratory Animal Care.

2.1. Progenitor cell isolation and culture

Human PDLSCs and GMSCs were isolated and cultured according to previously published protocols by Seo *et al.* and Zhang *et al.* [9, 10]. The teeth and gingival tissues were obtained from healthy male patients (18–25 years old) undergoing third molar extractions according to IRB approval from the University of Southern California. Only subjects without any history of periodontal disease were included in this study.

For assay of colony forming units-fibroblastic (CFU-F), 0.1×10^6 cells were seeded in a culture dish and cultured for 14 days. Subsequently, the cells were stained with Toluidine Blue. A cell count of more than 50 in one colony was counted as a positive CFU-F. Passage 4 cells were used in the experiments and hBMSCs were used as the positive control group.

2.2. Flow cytometric analysis

Approximately 5×10^5 cells at passage four were incubated with specific PE- or FITC-conjugated mouse monoclonal antibodies for human CD34 and CD45 (as negative: markers for hematopoietic stem cells); and CD73, CD105, CD 146, and CD 166 (as positive: markers for mesenchymal stem cells) (BD Biosciences, San Jose, CA), or isotype-matched control IgGs (Southern Biotechnology Associates, Birmingham, Alabama) and subjected to flow cytometric analysis using a Beckman Coulter flow cytometer (Beckman Coulter, Brea, CA) and analyzed using FACScan software (BD Biosciences).

2.3. Biomaterial fabrication and cell encapsulation

Custom-made RGD-coupled alginate with high guluronic acid content (NovaMatrix FMC Biopolymer, Norway) was utilized in this study. Alginate was purified and partially oxidized (2%) to increase its degradability according to the methods in the literature [22, 23]. Subsequently, the alginate was mixed with TGF- β 1 (Abcam, Cambridge, MA) (50 μ g/mL) under rigorous stirring, concentrated, and freeze-dried under reduced pressure.

PDLSC, GMSC, as well as hBMSC (as a positive control) were encapsulated in TGF- β 1 ligand (50 μ g/mL) loaded alginate at a density of 2×10^6 cells/mL of alginate solution [24]. Microbead formation was accomplished using a microfluidic device with a two-channel fluid jacket microencapsulator equipped with a micropipette. Alginate (200 μ Lh $^{-1}$) and soybean oil (10 mLh $^{-1}$) (Sigma) were injected into the device using syringe pumps. Alginate droplets were sheared off by the soybean oil, flowed out of the device, and were added dropwise into a petri dish containing 100 mM CaCl $_2$ solution to form microspheres. The formed constructs were incubated at 37°C for 45 min to complete cross-linking and then washed three times in non-supplemented DMEM. Alginate hydrogel without cells was used as the negative control in this study.

2.4. *In vitro* release study

TGF- β 1 (50 μ g/mL) loaded alginate microspheres were incubated in 500 mL of high-glucose DMEM supplemented with 100 U/mL penicillin and 100 μ g/mL streptomycin in 48-well plates on a rotational shaker at 37°C for one week. At each selected time interval, the medium was collected and analyzed for released TGF- β 1 using an anti-human recombinant TGF- β 1 ELISA kit (Abcam). At the end of release study, the residual entrapped TGF- β 1 was extracted by dissolving the scaffolds in 10% sodium citrate solution in distilled water and the percentage of cumulative released TGF- β 1 was measured.

2.5. Cell survival assay

Survival of the encapsulated MSCs was measured as described previously [22, 23] using calcein AM to stain live cells and ethidium bromide homodimer-1 to stain dead cells

(Invitrogen, Carlsbad, CA). The percentage of live cells was measured using ImageJ software. Additionally, an MTT assay (Invitrogen) was utilized to further evaluate the metabolic activity of encapsulated MSCs according to methods previously published [25].

2.6. *In vitro* chondrogenic assay

Encapsulated PDLSCs and GMSCs as well as hBMMSCs (2×10^6 in 1 mL of alginate) were cultured with a chondrogenic medium containing DMEM with 15% FBS, 2 mM L-glutamine, 1% ITS (BD Bioscience), 100 nM Dex, 100 μ M ascorbic acid, 2 mM sodium pyruvate (R&D Systems, Minneapolis, MN), 100 U/mL penicillin, and 100 μ g/mL streptomycin.

Four weeks after the induction, the samples were fixed with 4% PFA, and paraffin sections were made. Chondrogenic differentiation, as indicated by matrix production, was determined by staining with 0.1% safranin-O (Sigma-Aldrich, St. Louis, MO) and 0.1% toluidine blue (Sigma-Aldrich) solution. Because the polyanions of alginate stained intensely by Safranin-O and toluidine blue resulted in a strong background, the sections were first washed with Ca^{2+} -free PBS to remove the cross-linker Ca^{2+} and dissociate the alginate prior to staining.

Sections were immunolabeled using primary Sox 9 and Collagen II antibodies (Abcam) at 4°C overnight, detected using Alexa fluor conjugated secondary antibody (1:200 dilution; Invitrogen), and counterstained with DAPI. The chondrogenic assays were determined from 3 independent samples for each experimental group. Five areas were randomly selected from each sample, then the positive area in the field was calculated with NIH Image-J software (NIH, Bethesda, MD) and shown as a percentage of the area over total field area.

2.7. RNA isolation, reverse transcription and real time PCR

PDLSC, GMSC, as well as hBMMSC were encapsulated in TGF- β 1 ligand (50 μ g/mL) loaded alginate in presence or absence of 100 ng/ml human recombinant BMP-4 or 50 μ g/mL FGF2 (both from Invitrogen) as previously described. After 2 weeks of culturing in chondrogenic media, extraction of RNA from the encapsulated cells was performed by collecting 10 alginate hydrogels and dissolving them for 15–20 min by gentle stirring in a sterile depolymerization buffer consisting of 50 mM sodium citrate dehydrate and 80 mM sodium chloride (Sigma Aldrich, St Louis, MO). Following dissolution, the decapsulated cells were centrifuged at 9400 g for 10 min and the pellet was washed with PBS and centrifuged again at 9400 g for 3 min. Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's recommendations. Single-stranded cDNA synthesis was performed with 100 ng total RNA using a Superscript III cDNA synthesis kit (Invitrogen). Data were analyzed by the $2^{-\text{Ct}}$ method, with normalization to the Ct of the housekeeping gene GAPDH. Primer and probe sequences are described in Table 1.

2.8. Dental MSC-mediated tissue regeneration *in vivo*

PDLSCs, GMSCs, and hBMMSCs were encapsulated in TGF- β 1 loaded alginate systems and transplanted subcutaneously (approximately 4×10^6 cells) into the dorsal surface of 5-month-old Beige nude XID III (NU/NU) mice (Harlan, Livermore, CA; N=5 for each group). All animals were treated according to the Guidelines and Regulations for the Use and Care of Animals at University of Southern California. After 8-weeks the mice were sacrificed, the constructs and the surrounding tissue were surgically removed and analyzed using histological, histochemical and immunofluorescence staining.

2.9. Histological and histochemical assessment

For histological examination, harvested specimens were fixed in 10% formalin solution and dehydrated in an ascending series of ethanol and embedded in paraffin. Six-micrometer sections were cut using a microtome and mounted on glass slides. Four randomly selected cross-sections from each implant were stained with hematoxylin & eosin (H&E). Additionally, sections were stained with toluidine blue and with Safranin-O to identify newly synthesized cartilage ECM glycosaminoglycans (GAGs) Five areas were randomly selected from each sample, and the positive area in the field was calculated with Image-J software and shown as a percentage of the area over total field area.

2.10. Immunohistochemical and Immunofluorescence staining

For immunohistochemical analysis, the animals were sacrificed three weeks after transplantation and harvested specimens were prepared according to the abovementioned method. The de-paraffinized sections were washed, and non-specific endogenous peroxidase activity was quenched by immersing in 3% H₂O₂/methanol for 15 min. Sections were incubated with primary antibody (1:200–1:300 dilution) for 1 h. Immunohistochemistry examination was performed on sections using anti-BMP4 (Abcam, 1:200 dilution) and anti-FGF-2 (Abcam, 1:100 dilution) and counterstaining with hematoxylin.

For immunofluorescence staining, samples (retrieved after eight weeks of transplantation) were treated with 3% H₂O₂, followed by a blocking buffer (1% BSA and 0.25% Triton X-100 in PBS). Samples were then incubated with mouse anti-rabbit collagen II (Calbiochem, San Diego, CA) and Sox 9 (Abcam) antibodies at a dilution of 1:100 and detected using Alexa Fluor conjugated secondary antibody (1:200 dilution; Invitrogen). The samples were then counterstained with DAPI.

2.11. Statistical Analysis

Data were analyzed using an independent two-tailed Student's *t*-test or analysis of variance (ANOVA) ($p < 0.05$). The numerical data are presented as the mean \pm SD.

3. Results

3.1. *In vitro* characterization of dental-derived MSCs

In this study the CFU-F assay was performed to assess the colony-forming ability of the newly isolated stem cells. PDLSCs and GMSCs showed significantly higher numbers of single-colony clusters (CFU-F) compared to hBMSCs (Fig. 1a). Next, in order to identify whether the isolated cells were MSC-like, FACS analysis was performed and demonstrated that human PDLSCs and GMSCs display specific MSC markers such as CD 73, CD 105 CD 146, and CD 166, while not expressing hematopoietic lineage markers such as CD 34 and CD 45 (Fig. 1b). These data confirmed the stem cell properties for each of these types of dental-derived MSCs.

3.2. Alginate microspheres as a drug delivery vehicle that maintains MSC viability

An alginate microencapsulation system was developed in this study in order to induce cartilage differentiation of PDLSCs and GMSCs. As a positive control, hBMSCs encapsulated in alginate were used. In the current study, RGD-coupled alginate microcapsules containing stem cells had an average diameter of 327 μ m (Fig 2a). Microscopic images of stem cells loaded into microcapsules showed a uniform microsphere size and cell distribution. Fig. 2b represents the cumulative release profile of TGF- β 1 from the RGD-coupled alginate scaffold compared to the alginate scaffold without RGD tripeptide. The results show sustained release of TGF- β 1 for up to 14-days. Also, there was

no significant difference in release rates from the two scaffolds as a function of time (p -value >0.05), which both rates showing first-order kinetics.

A live-dead assay showed a high degree of viability of MSCs in alginate microspheres after four weeks of *in vitro* culturing (Fig. 2c). Furthermore, an MTT assay was utilized to quantitatively measure cytotoxicity and MSC viability in the alginate microencapsulation system *in vitro*. All the MSCs-alginate constructs showed high MTT absorbance, indicating high metabolic activity and viability of the encapsulated stem cells after up to two weeks of culturing in regular media (Fig. 2d). These results show that the viability of PDLSCs, GMSCs as well as hBMMSCs were favorably supported in our biodegradable alginate microspheres. Furthermore, MSCs encapsulated in RGD containing alginate microspheres showed significantly higher degrees of viability in comparison to MSCs encapsulated in non-RGD containing alginate microspheres after 14 days of culturing in regular culture media (supplementary Fig. 1a).

3.3. Chondrogenic differentiation of dental MSCs *in vitro*

After four weeks of culturing in chondrogenic induction media, the deposition of proteoglycans by either PDLSCs or GMSCs *in vitro* was detected using safranin-O and toluidine blue staining. Safranin-O staining was positive for PDLSCs and GMSCs, as well as hBMMSCs (Fig 3a). All the MSCs were also positive for toluidine blue (Fig. 3b). As expected, MSCs encapsulated in RGD immobilized alginate microspheres exhibited statistically greater amounts of proteoglycan deposition (by toluidine blue staining) in comparison to non-RGD immobilized counterparts (supplementary Figs. 1b and 1c). Additionally, the results of immunofluorescent staining correlated well with the histochemical staining, showing that all the tested MSCs expressed type II collagen (*Col II*) genes as shown by positive immunostaining (Figs. 3c). Interestingly, the semi-quantitative analysis of the specimens revealed that PDLSCs showed higher amounts of chondrogenesis than hBMMSCs and GMSCs (P -value <0.05) (Fig. 3e). No significant difference was observed between GMSCs and hBMMSCs ($P>0.05$). Subsequently, the expression levels of the chondrogenic genes (*Sox9* and type II collagen) were confirmed and compared *via* RT-PCR. *Sox9* is an essential factor for the initiation of the chondrogenic differentiation pathway leading to a subsequent upregulation of Col II. *Sox9* shows the greatest amount of activity in proliferating chondrocytes. The results showed that all the MSC groups expressed abundant *Col II* and *Sox 9* (Figs. 4a and b). PDLSCs showed significantly greater expression levels of *Sox9* and *Col II* than GMSCs and hBMMSC ($p<0.05$). However, *Sox 9* and *Col II* gene expression levels were similar between hBMMSCs and GMSCs ($P>0.05$). Further supplementation of alginate microspheres with BMP-4 or FGF2 in combination with TGF- β 1, slightly increased the expression levels of *Sox9* and *Col II* in comparison to samples loaded with only TGF- β 1 (supplementary Fig. 2).

3.4. Dental MSCs contributed to cartilage tissue regeneration *in vivo*

The ability of RGD-coupled alginate microspheres loaded with TGF- β 1 to support MSC chondrogenic differentiation was evaluated in an ectopic site after subcutaneous implantation into nude mice. Toluidine blue and safranin-O staining of matrix confirmed the chondrogenic differentiation of all the tested MSCs. The cells in these constructs exhibited the round morphology of committed chondrocytes. By contrast, the control group, with implants consisting of alginate hydrogel alone, failed to show any of the signs positive for chondrogenesis (Figs. 5a and b). Immunofluorescent staining for type-II collagen and *Sox 9* antibodies after 8-weeks of transplantation revealed extensive production and deposition of chondrogenic tissues (Figs. 5c and 5d). The production of collagen type II was more pronounced for PDLSCs than for GMSCs and hBMMSCs ($P<0.05$) (Fig. 5e), while GMSCs and hBMMSCs showed the same amounts of chondrogenesis ($P<0.05$). H&E staining

results confirmed the presence of large islands of rounded cells with human chondrocytes morphology in MSC-seeded constructs, which is not found in the cell-free transplants (Fig. 6a). To further characterize MSC-mediated chondrogenesis *in vivo*, immunohistochemical staining was utilized. Results revealed detection of BMP-4 and FGF-2 growth factors in the early stages of chondrogenic differentiation (after three weeks of transplantation), suggesting the important role of TGF- β 1 in the process of chondrogenesis (Figs. 6b and 6c).

4. Discussion

It is well known that the cell delivery vehicle has an important role in the *in vivo* performance of stem cells and the success of regenerative therapy [25]. In this study, we developed an injectable RGD-coupled alginate-hydrogel microsphere as a stem cell delivery system for potential application in cartilage tissue engineering. We demonstrated that this system supported the viability, metabolic activity, and chondrogenic differentiation of encapsulated PDLSCs and GMSCs *in vitro* and *in vivo*. More importantly, in the current study we are introduced PDLSCs and GMSCs for the first time as promising candidates for optimized cartilage regeneration with potential applications for reconstruction of the temporomandibular joint (TMJ) disk.

To our knowledge, the chondrogenesis of dental MSC has not been developed and reported in an RGD-coupled alginate microencapsulation system before. From a practical perspective, dental MSCs such as PDLSCs and GMSCs are a superior source of stem cells considering their accessibility and suitability for autologous transplantation. Studies have confirmed that both PDL and gingiva contain populations of multipotent postnatal stem cells that can readily be isolated and expanded *in vitro* [10,11]. As a result, human PDLSC- or GMSC-mediated tissue regeneration can be considered as a promising cellular-based treatment for cartilage tissue engineering. More importantly, considering the neural crest origin of both dental MSCs and TMJ disk cartilage [26], they can be used as a platform for functional regeneration of the TMJ disk.

The RGD-coupled-dental MSC system developed in the current study was observed strongly positively stained by Safranin-O and toluidine blue *in vitro* and *in vivo*. We observed the *in vitro* positive staining 4 weeks after culturing in chondrogenic culturing media. Furthermore, immunofluorescent staining demonstrated strong positive reactivity for *Col II* and *Sox 9* antibodies around the cells both *in vitro* and *in vivo*. The synthesis of the two main matrices of articular cartilage, proteoglycan and type II collagen, demonstrates that dental MSCs embedded in alginate microspheres and induced with the addition of TGF- β 1 in the medium, can effectively undergo chondrogenic differentiation. Furthermore, we report that PDLSCs have greater chondrogenic differentiation capacity than hBMMSCs.

The alginate hydrogel microencapsulation system provides a unique 3D cell delivery scaffold for cartilage tissue engineering. Studies confirm that macromolecules with molecular weights of less than 49 kDa can penetrate the pores of the alginate hydrogel microspheres to influence the cell behavior [27]. Therefore, different growth factors and proteins (e.g. TGF- β 1) can enter the microspheres and regulate the proliferation and differentiation of encapsulated MSCs [28]. This and other advantages make alginate hydrogel a more desirable scaffold than other polymeric materials, such as polylactic acid, for cartilage tissue engineering [29]. Alginate hydrogel can be considered a semisolid structure, where the MSCs assumed a polygonal-rounded morphology with increasing proteoglycan and type II collagen synthesis [30].

It has been shown that several growth factors and cytokines, such as members of transforming growth factor superfamily (TGF- β 1, and BMP-4), and FGF-2 have important role in chondrogenic differentiation of MSCs [31, 32]. The results of the current study,

confirmed the importance of presence of these cytokines specifically TGF- β 1 in the chondrogenesis of the encapsulated dental MSCs (Figs. 6b and c) showing that the provided micro milieu and the initiating signals from TGF- β 1 alone might be sufficient to cause endogenous expression of BMP 4 and FGF 2 that are required for chondrogenesis.

Re'em *et al.* in their studies, investigated the effects of RGD peptide immobilized onto alginate scaffolds, on the chondrogenesis of human MSCs in the presence of TGF- β 1. Their results confirmed that immobilized RGD did not interfere with progression of MSCs [19]. In addition, immobilized RGD considerably encouraged and accelerated the TGF- β 1-induced chondrogenesis of human MSC encapsulated in alginate scaffolds. We have previously shown that our prepared alginate scaffold has a porous structure [22] (data not shown here). This porous structure might have a critical role in the diffusion capacity of oxygen, nutrients and signaling molecules within the alginate microspheres. Therefore, one can conclude that cell matrix interactions facilitated by the immobilization of RGD tripeptide onto the alginate hydrogel scaffold with porous structure can enable the adhesion of encapsulated dental-derived MSCs to the matrix, and permit enhanced cell accessibility to the oxygen, nutrients and the growth factors (e.g. TGF β 1) [20].

In the current study, stem cells were not cultured in chondrogenic media prior to surgical transplantation, and encapsulated PDLSCs and GMSCs in alginate microspheres promoted cartilage regeneration *in situ*. Chai *et al.* reported that the articulating disc and TMJ originate from cranial neural crest (CNC) cells [25]. Taking into account the fact that neural crest is the same origin of the dental MSCs used in this study, cells recovered from these origins might be considered as excellent candidates for disc and TMJ regeneration. Therefore, in future studies, the possibility of utilizing PDLSCs and GMSCs in the repair of cartilage and disk defects in TMJ in more appropriate animal models will be evaluated. The results of these endeavors will be reported in due course. More importantly, dental derived MSCs encapsulated in alginate hydrogel can be considered a promising candidate for appendicular skeleton repair and regeneration.

6. Conclusions

Altogether, our findings demonstrate the important role of the microenvironment as well as the presentation of inductive signals (TGF- β 1) for viability and chondro-differentiation of dental MSCs in an RGD-modified alginate microencapsulation system. Our *in vitro* and *in vivo* studies confirmed that the proposed system comprises a promising model for high quality cartilage regeneration. We show that RGD-coupled alginate hydrogel can be used to encapsulate PDLSCs and GMSCs for chondrogenesis in an animal model. The system developed here has the advantages of providing a simple and readily manipulated means to encapsulate dental MSCs in RGD-coupled alginate hydrogel, and a 3D, injectable and biodegradable cell delivery scaffold for cartilage tissue engineering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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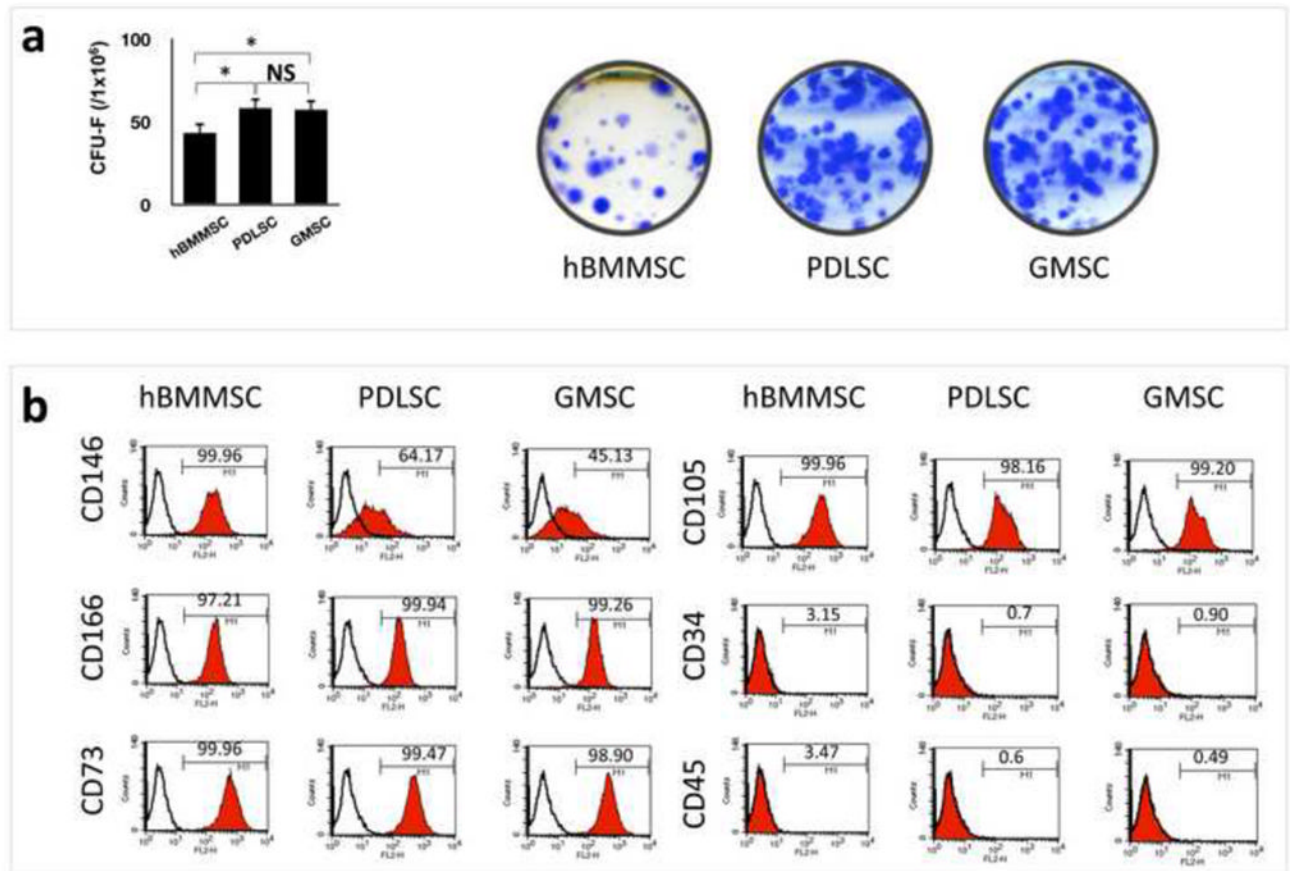


Figure 1.

Characterization of stem cells used in this study: (a) PDLSCs and GMSCs were able to form significantly higher numbers of single colonies than BMMSCs ($*P < 0.05$) when 1×10^6 cells were plated at a low density and cultured for 10 days. Single colonies were formed for PDLSCs, GMSCs as well as hBMMSCs after plating at low density and cultured for 10 days; (b) Expression of cell surface markers on PDLSCs, GMSCs, and hBMMSCs (passage 4) as determined by flow cytometric analysis.

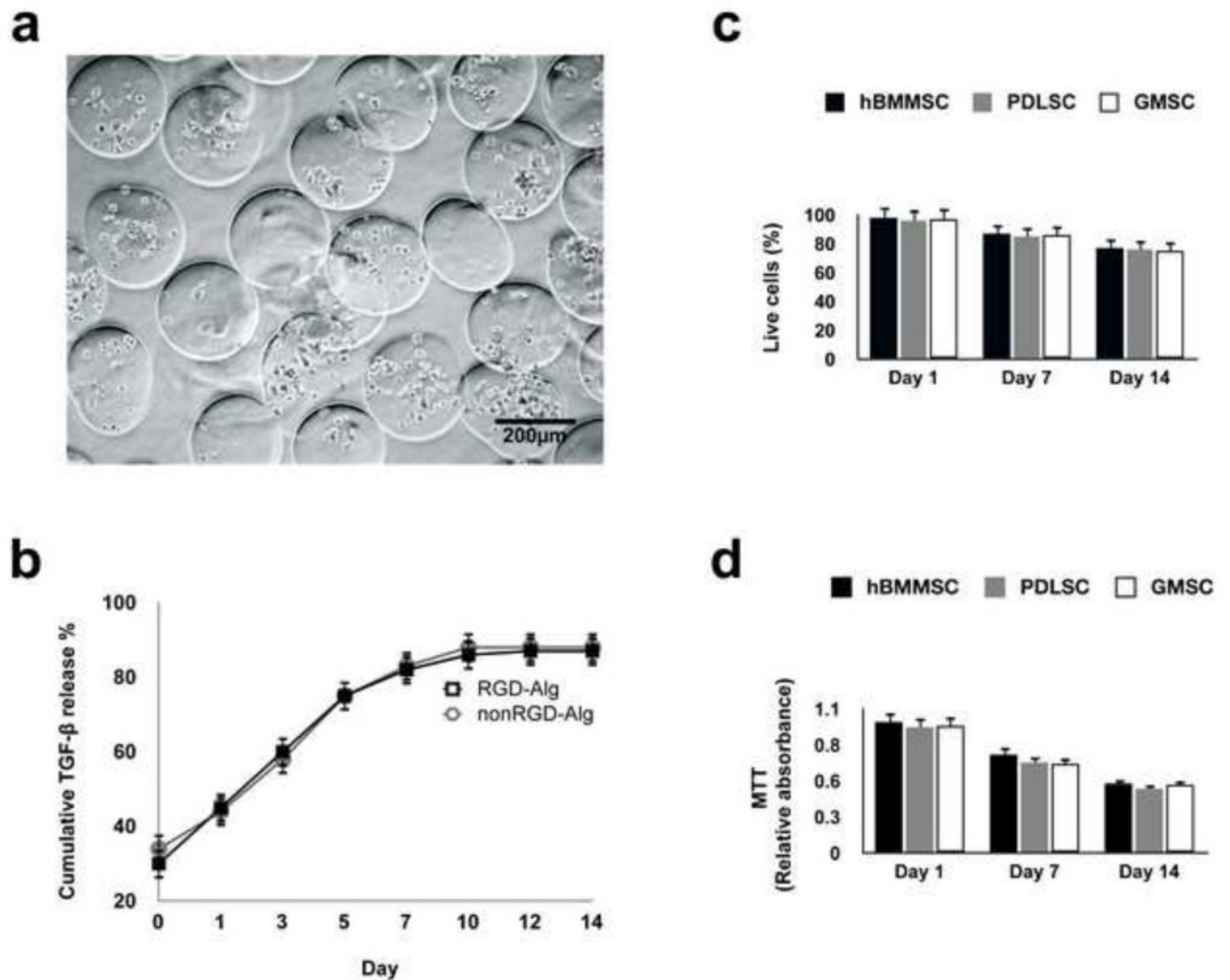


Figure 2.

Development of microenvironment based on TGF- β 1-loaded alginate hydrogel: (a) Microspheres produced by the microfluidic device. The diameter of the microspheres was between 196 μm and 581 μm (scale bar = 500 μm). (b) Characterization of the *in vitro* release profile of TGF- β 1 loaded alginate microspheres showing sustained release of TGF- β 1 from the RGD-modified alginate and the non-RGD modified alginate microspheres. Cumulative release is calculated from the total amount of TGF- β 1 released after 14-days. (c) Viability of the encapsulated MSCs: percentage of live cells in RGD-coupled alginate microspheres. (d) MTT assay to determine the metabolic activity of cells. No significant difference was observed between MSC groups at each time interval.

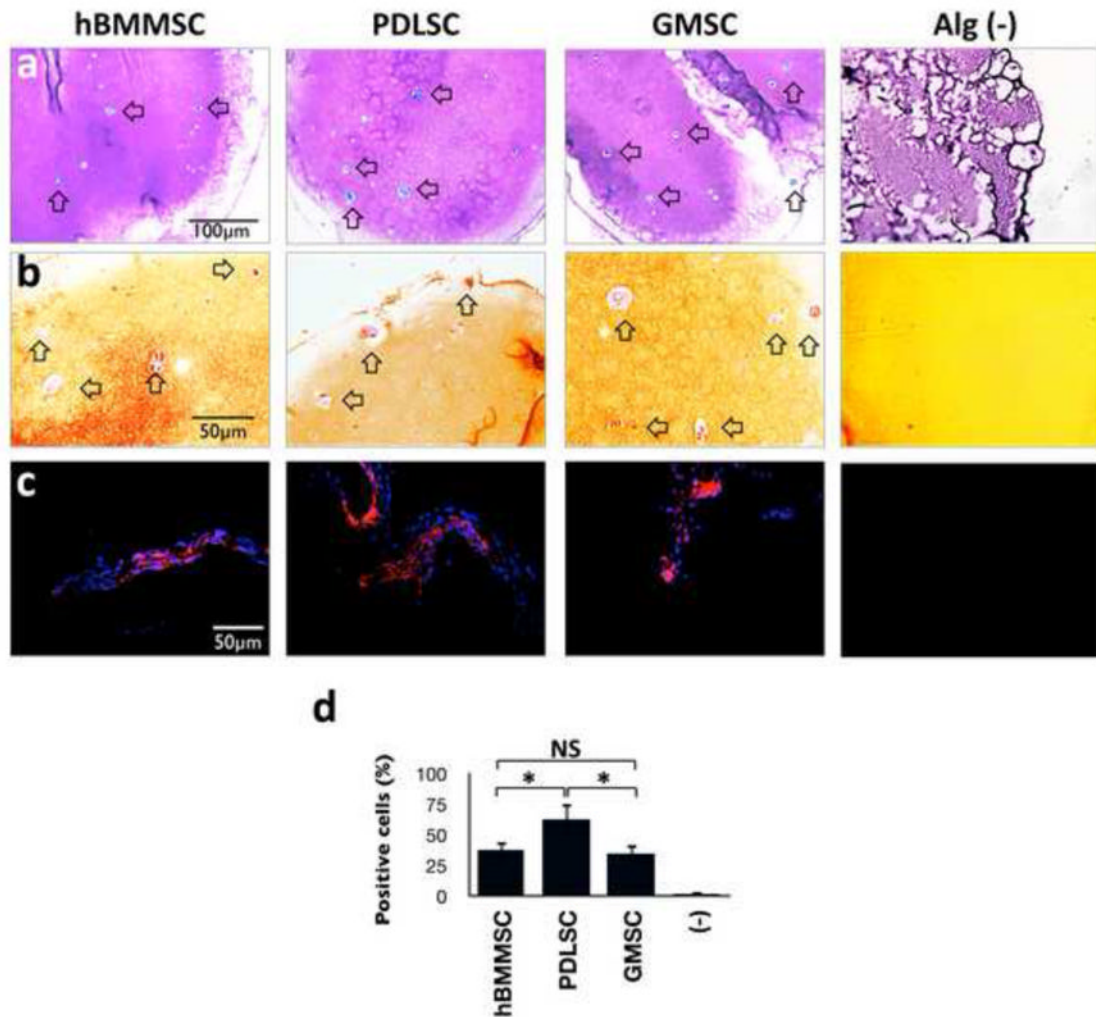


Figure 3. Histochemical analysis showed that RGD coupled alginate microspheres promoted spread-out cell morphology. Stem cells and cell aggregates are surrounded by toluidine blue (a) and Safranin-O (b) and strongly stained matrix, confirming the production and accumulation of cartilage extracellular matrix. (c) Immunofluorescence staining against collagen type II (Col II) antibodies confirmed the production and secretion of type-II collagen by PDLSCs and GMSCs. (d) Analysis of the percentage of cells positive for anti-Col II antibodies, showing that PDLSCs encapsulated in RGD coupled alginate presented the greatest amounts of production and secretion of collagen type II in comparison to GMSCs and hBMMSCs.

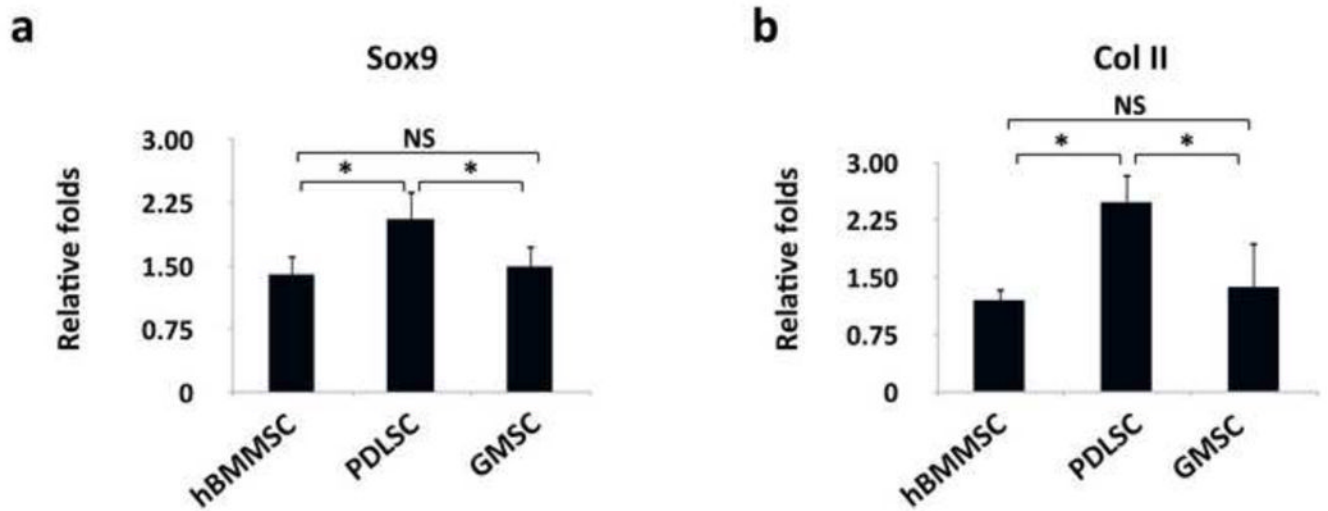


Figure 4.

Expression level (in fold changes) of collagen type II (a) and Sox-9 (b) genes for each encapsulated stem cell population after 2-weeks of chondro-differentiation *in vitro* evaluated by RT-PCR. Data were normalized by the Ct of the housekeeping gene GAPDH and expressed relatively to gene expression level at day 1. * $P < 0.05$, NS: not significant.

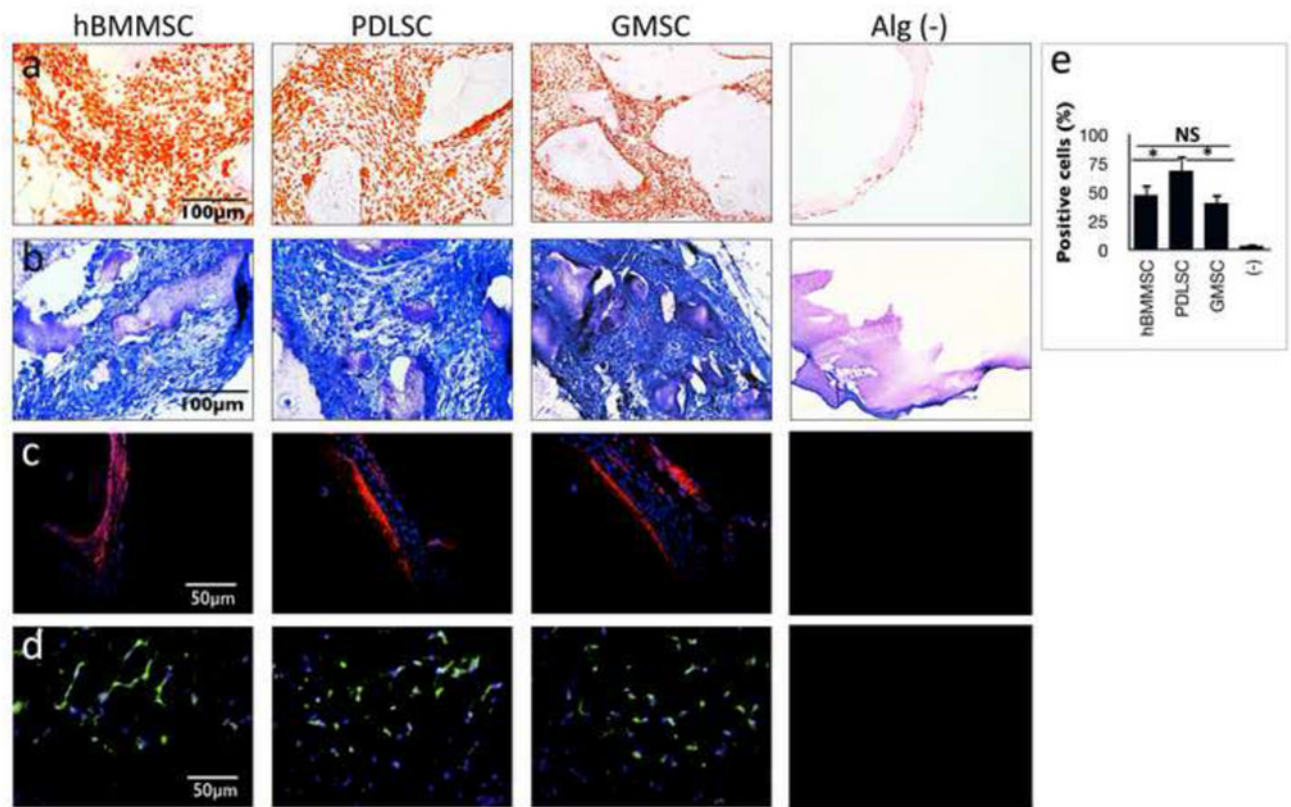


Figure 5.

Chondrogenesis in TGF- 1 loaded alginate subcutaneously transplanted in nude mice. MSC-constructs were removed eight weeks after transplantation, sectioned, and stained with Safranin-O (a) and toluidine blue (b). Immunofluorescence staining, using antibodies against collagen type II (c) and Sox-9 (d) antibodies confirmed the production and secretion of type-II collagen. (e) Semi-quantitative analysis of the percentage of cells positive for anti-Sox9 antibodies via immunofluorescence staining images. * $P < 0.05$, NS: not significant.

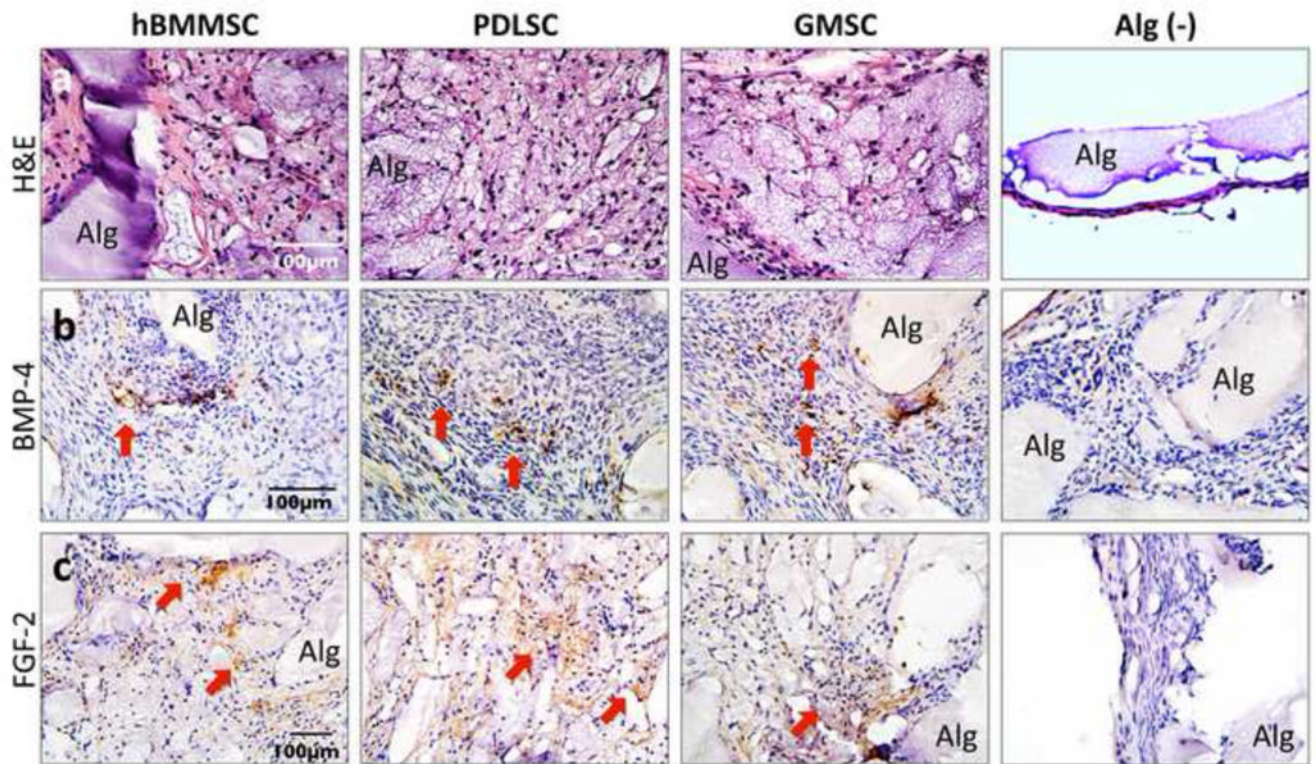


Figure 6.

In vivo chondrogenic differentiation after subcutaneous transplantation in nude mice: (a) H&E staining showing presence of large islands of rounded cells with human chondrocytes morphology in MSC-seeded constructs, while (-) is the unseeded alginate scaffold. Immunohistochemical staining in specimens retrieved three weeks after transplantation showing positive results (red arrows) for anti-BMP-4 (b) and anti-FGF-2 (c) antibody staining, while negative control (-) failed to express any positive staining (**Alg**: unresorbed alginate scaffold).

Table 1

Oligonucleotide primers used in RT-PCR analysis.

Gene	Sequence	Amplification (bp)
Sox 9	Sense: 5 -ACGCCGAGCTCAGCAAGA-3 Antisense: 5 -CACGAAGGGCCGCTTCT-3	289
Collagen II	Sense: 5 -GGCAATAGCAGGTTACGTACA-3 ; Antisense: 5 -CGATAACAGTCTTGCCCACTT-3	827
Glyceraldehyde 3-phosphate dehydrogenase (GADPH)	Sense: 5 -AGCCGCATCTTCTTTGCGTC-3 ; Antisense: 5 -TCATATTGGCAGGTTTT CT-3	418