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Muscle tissue engineering using gingival mesenchymal stem cells encapsulated in alginate hydrogels containing multiple growth factors

Sahar Ansari[1,#], **Chider Chen**[2,#], **Xingtian Xu**[3], **Nasim Annabi**[4,6], **Homayoun H. Zadeh**[3], **Benjamin M. Wu**[5], **Ali Khademhosseini**[6], **Songtao Shi**[2], and **Alireza Moshaverinia**[5,*]

[1]Division of Growth and Development, School of Dentistry, University of California, Los Angeles, CA.

[2]School of Dental Medicine, University of Pennsylvania, Philadelphia, PA.

[3]Ostrow School of Dentistry, University of Southern California, Los Angeles, CA.

[4]Department of Chemical Engineering, Northeastern University, Boston, MA.

[5]Weintraub center for reconstructive biotechnology, Division of Advanced Prosthodontics, School of Dentistry, University of California, Los Angeles, CA.

[6]Biomaterials Innovation Research Center, Department of Medicine, Brigham and Women's Hospital Harvard Medical School, Cambridge, MA.

Abstract

Repair and regeneration of muscle tissue following traumatic injuries or muscle diseases often presents a challenging clinical situation. If a significant amount of tissue is lost the native regenerative potential of skeletal muscle will not be able to grow to fill the defect site completely. Dental-derived mesenchymal stem cells (MSCs) in combination with appropriate scaffold material, present an advantageous alternative therapeutic option for muscle tissue engineering in comparison to current treatment modalities available. To date, there has been no report on application of gingival mesenchymal stem cells (GMSCs) in three-dimensional scaffolds for muscle tissue engineering. The objectives of the current study were to develop an injectable 3D RGD-coupled alginate scaffold with multiple growth factor delivery capacity for encapsulating GMSCs, and to evaluate the capacity of encapsulated GMSCs to differentiate into myogenic tissue *in vitro* and *in vivo* where encapsulated GMSCs were transplanted subcutaneously into immunocompromised mice. The results demonstrate that after 4 weeks of differentiation *in vitro*, GMSCs as well as the positive control human bone marrow mesenchymal stem cells (hBMMSCs) exhibited muscle cell-like morphology with high levels of mRNA expression for gene markers related to muscle regeneration (MyoD, Myf5, and MyoG) *via* qPCR measurement. Our quantitative PCR analyses revealed that the stiffness of the RGD-coupled alginate regulates the

*Corresponding author: Alireza Moshaverinia, DDS, MS, PhD, FACP, Assistant Professor, Diplomate, American Board of Prosthodontics, UCLA School of Dentistry, 10833 Le Conte Ave, B3-023 CHS, Los Angeles, California 90095-1668, ; Email: amoshaverinia@ucla.edu, Tel: (310) 794-6324

#These authors contributed to this work equally.

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myogenic differentiation of encapsulated GMSCs. Histological and immunohistochemical/fluorescence staining for protein markers specific for myogenic tissue confirmed muscle regeneration in subcutaneous transplantation in our *in vivo* animal model. GMSCs showed significantly greater capacity for myogenic regeneration in comparison to hBMMSCs ($P<0.05$). Altogether, our findings confirmed that GMSCs encapsulated in RGD-modified alginate hydrogel with multiple growth factor delivery capacity is a promising candidate for muscle tissue engineering.

Keywords

Tissue engineering; Muscle regeneration; Dental mesenchymal stem cells; RGD-coupled alginate hydrogel

Introduction

Traumatic injuries, congenital defects and muscular diseases, such as muscular dystrophy, usually cause significant muscle tissue loss necessitating application of regenerative treatment modalities to rehabilitate the affected patients. However, larger defects are challenging due to difficulties to find appropriate supply of muscle tissues.[25, 45] An advantageous and promising treatment modality for muscle tissue engineering is the application of mesenchymal stem cells (MSCs). MSCs are multipotent cells that can differentiate into multiple lineages, depending on the nature of the environmental signals they receive. Specifically with regards to their capacity to form muscle, studies have shown that direct implantation of MSCs functionally improves the capability of muscle regeneration.[2,4,9] However, a vital parameter for muscle tissue regeneration is the identification of an optimal source of cell with suitable muscle differentiation capacity. Studies have shown the presence of satellite cells (SCs) in mature muscles that are identified to be committed to myogenic tissue regeneration.[22,27] However, the main disadvantages associated with the application of these SCs are the invasive isolation procedure and difficulty in the purification process. In addition, the low expansion capacity of satellite cells makes them a questionable cell source for repair of large muscle defects.[15, 21, 22,27] Alternatively, it has been shown that MSCs can be identified and isolated from a wide range of post-natal tissue types, including the craniofacial structures.[10,17, 19, 43] One of the attractive sources of MSCs in the craniofacial region is gingiva.[53] Gingival mesenchymal stem cells (GMSCs) are of particular interest as they are easily harvested in the oral environment. GMSCs are obtained as discarded biological samples in dental clinics for many common dental procedures.[30, 31] Moreover, the donor site in the oral cavity tends to heal faster than all other human dermal tissues, and completes healing without scarring. Our previous studies and others have confirmed the multilineage differentiation capabilities of these stem cells *in vitro* and *in vivo.*[30–34, 53] Therefore, human GMSC-mediated tissue regeneration can be considered as a promising cellular-based treatment for muscle tissue engineering.

One of the challenges in the stem cell mediated tissue regeneration is development of a suitable microenvironment for stem cells to proliferate and differentiate them to the desired

lineage/phenotype. It is well known that scaffolds have important role in tissue engineering.[18, 39, 40, 49] It has been reported that stem cell-mediated tissue regeneration is partially controlled by the recipient local microenvironment, including the presence of growth factors, immune cells and cytokines.[24, 11] To that end, exogenous growth factors have been delivered along with stem cells to differentiate the stem cells to desired lineage/phenotype. Several studies have demonstrated that the mechanical properties (e.g. elasticity) of the scaffold control the survival and determine the fate of the stem cells.[13,14,40] Therefore, a scaffold with tunable physiochemical properties, to regulate the fate of encapsulated stem cells, seems to be very important and practical in developing stem cell based therapies.

To develop a promising microenvironment for muscle regeneration based on GMSCs, we sought to engineer a microenvironment with the physiochemical characteristics of the extracellular micro-milieu. We utilized RGD-coupled alginate hydrogel to modify the niche properties and to direct the cell phenotype through differentiation. Alginate microspheres have been used extensively for controlled delivery of growth factors and have an excellent track record for safety.[3, 38, 39, 14] Presence of cell-binding peptides, such as RGD (arginine-glycine-aspartic acid tripeptide), in the structure of the alginate scaffold could be advantageous because these peptides mimic the cell-matrix interaction typical of the ECM.[26, 34] Studies have shown that alginate hydrogels have multiple growth factor delivery capacity, which make the scaffold of choice for complex tissue regeneration purposes.[8]

Therefore, in the current study, we focus on the potential of the delivery of multiple growth factors (a cocktail of Forskolin (FSK), MeBIO (6-Bromo-1-methylindirubin-3'-oxime), and basic-FGF) to drive GMSCs differentiation into muscle tissue.[1,7,16] Studies have shown that FSK induces cell differentiation via direct activation of adenylate cyclase and the stimulation of cyclic AMP (cAMP) production, promoting muscle regeneration in animal models.[1,167, 23, 42] Moreover, MeBIO (an analog of 6-bromoindirubin-30 - oxime) is a specific inhibitor of glycogen synthase kinase-3 (GSK-3).[47] It has been shown that this small molecule can support self-renewal and pluripotency potential in human and mouse embryonic stem cells (ESCs) leading to maintenance of stem cell properties.[36] Furthermore, it has been demonstrated that MeBIO has the ability to control the muscle regeneration and proliferation of mammalian cardiomyocytes.[47, 48] Basic fibroblast growth factor (bFGF) plays an important role in the survival and differentiation of MSCs in muscle regeneration and repair.[43, 36]

A literature search failed to reveal any reports evaluating the application of GMSCs encapsulated in RGD-coupled alginate microspheres, with multiple growth factor delivery capacity, in muscle repair and regeneration. Therefore, in the current study, we developed an injectable and 3D RGD-coupled alginate hydrogel cell encapsulation architecture with multiple growth factor delivery capability. Considering the fact that GMSCs can be easily accessible from the oral cavity, and given the rapidity of healing without scar formation, they can be considered ideal for stem cell banking purposes provided they show promise in MSC-based tissue regeneration. This approach was designed to optimize muscle regeneration for potential application in the repair of the skeletal or cardiac muscle regeneration.

Materials and methods

Animals

Female immunocompromised nude (Beige nu/nu XIDIII) mice were used in this study. All the animal experiments were performed in accordance with IACUC-approved small animal protocols at the University of Southern California.

Progenitor cell isolation, and culture

Human GMSCs were isolated and cultured according to previously published procedures.[51,52] Human bone marrow mesenchymal stem cells (BMMSCs), purchased from Lonza (Gaithersburg, MD).

GMSCs, and hBMMSCs (as a positive control) were separately cultured in a regular culture media containing alpha-MEM (Invitrogen) with 15% FBS, 2 mM L-glutamine (Invitrogen), 100 nM Dex, 100 μM ascorbic acid (Sigma), 2 mM sodium pyruvate (R&D Systems Inc, Minneapolis, MN), 100 U/mL penicillin, and 100 μg/mL streptomycin (Sigma). Passage 4 cells were used in the experiments and hBMMSCs were used as the positive control group.

Flow cytometric analysis

Approximately 2×10^5 of either GMSCs or hBMMSCs from passages 4 were incubated with specific phycoerythrin conjugated mouse monoclonal antibodies for human CD34, and CD45 (as negative hematopoietic stem cell marker), CD73, CD105, and CD146 (as positive MSC marker) (BD Biosciences), or isotype-matched control immunoglobulin Gs (IgGs; Southern Biotechnology Associates) and subjected to flow cytometric analysis using a FACSCalibur with CellQuest software (BD Biosciences) according to methods previously reported.[34]

Biomaterial fabrication and characterization

RGD-coupled alginate (NovaMatrix FMC Biopolymer, Norway) was used as the scaffold material. Alginate was purified and partially oxidized (2%) to increase its degradability according to published methods in the literature.[30,31] The prepared alginate was concentrated, freeze-dried under reduced pressure, and mixed with a cocktail containing 10 μM forskolin (FSK) (Sigma), 10 μM MeBIO (Santa Cruz Biotechnology), and 10 ng/ml recombinant human bFGF (R&D Systems).

To study the effects of elasticity of the alginate hydrogel on myogenic differentiation capacity of encapsulated GMSCs the elastic modulus (E) of alginate hydrogels in presence of different concentrations of $CaCl_2$ (10, 25, 50, 75, and 100 mM) was measured using an Instron mechanical testing machine (Norwood, PA) at a compression rate of 0.5 $mm.min^{-1}$ according to the methods already in literature.[18]

To characterize the release profile of all the components of the cocktail, the hydrogel microspheres were loaded at the abovementioned concentrations and at each selected time interval (1, 3, 5, 7, 10, 12, and 14 days), the amounts of medium were collected and analyzed for released of FSK and MeBIO, using a UV spectrophotometer at 320 nm

(Beckman, Brea, CA). Moreover, the release profile of bFGF was characterized using human b-FGF Immunoassay kit (BioSource International Inc. Camerillo, CA).

Cell encapsulation and Live/dead staining

GMSCs and hBMMSCs (as a positive control) were encapsulated separately in alginate loaded with cocktail of 10 μM forskolin (FSK), MeBIO (10 μM), and 10 ng/ml recombinant human bFGF. Cells were encapsulated at a density of 2×10^6 cells/mL of alginate solution. Microsphere formation was accomplished by adding the MSC-alginate mixture dropwise to 100 mM $CaCl_2$ solution. The resulting microspheres were incubated at 37°C for 45 min to complete cross-linking and then washed three times in non-supplemented DMEM. RGD coupled alginate hydrogel without cells (and not loaded with cocktail) was used as the negative control in this study.

Following 14 days of incubation in culture medium, the cell viability of the encapsulated MSCs was measured as described according to methods in the literature[32, 233] using calcein AM to stain live cells and ethidium bromide homodimer-1 to stain dead cells (Invitrogen). The percentage of live cells was measured using NIH ImageJ software (NIH, Bethesda, MD).

Moreover, to evaluate stem cell viability in the alginate microspheres loaded with myogenic differentiation cocktail, MTT assay was utilized according to previously published protocols.[34] The MTT absorbance was obtained at different time intervals (1, 7, and 14 days) and normalized to the absorbance of alginate containing the same type of stem cells measured at day 1. For each experimental group tested five independent specimens were analyzed at each time interval.

In vitro myogenic differentiation

To induce myogenic differentiation, encapsulated GMSCs and hBMMSCs (2×10^6 cells) in 1 mL alginate microspheres containing the growth factors cocktail were cultured in a medium containing alpha- MEM with 15% FBS, 2 mM L-glutamine, 100 nM Dex, 100 μM ascorbic acid, 2 mM sodium pyruvate (R&D Systems Inc), 100 U/mL penicillin, 100 μg/mL streptomycin, and a cocktail of 10 μM forskolin (FSK), MeBIO, and 10 ng/ml recombinant human bFGF. Cell free RGD-alginate microspheres without any growth factor were used as the control group. Four weeks after induction, the samples were fixed with 4% PFA, and paraffin sections were made. Sections were immunolabeled using antibodies against MF20 (R&D Systems Inc), Myf5, and MyoD (Santa Cruz Biotechnology Inc, Dallas, TX), at 4°C overnight, detected using Alexa fluor conjugated secondary antibody (1:200 dilution; Invitrogen), and counterstained with DAPI.

RNA isolation, reverse transcription and real time PCR

Following 2 weeks of myogenic differentiation RNA was extracted from the encapsulated cells.[32, 33] Data were analyzed by the $2^{-\Delta\Delta Ct}$ method, with normalization to the Ct of the housekeeping gene GAPDH (glyceraldehyde 3-phosphate dehydrogenase). Primer sequences are described in Table 1. Additionally, to study the effects of the elasticity of the hydrogel biomaterial on the myogenic differentiation capacity of encapsulated cells; GMSCs

were encapsulated in alginate hydrogels with different modulus of elasticity and after 2 weeks of myogenic differentiation, the myogenic genes expression levels were analyzed using qPCR.

Western blot analysis

After two weeks of myogenic differentiation *in vitro*, Western blot analysis was performed to analyze the differentiation of encapsulated MSCs. Primary antibody (mouse monoclonal anti-MyoD antibody) was used at 1:500. Western blot analyses were carried out as previously reported.[33]

In vivo model for muscle regeneration

All animals in our in vivo experiments were treated according to the approved Guidelines and Regulations for the Use and Care of Animals (IACUC) at the University of Southern California. 4×10^6 of GMSCs or hBMMSCs (passage 4) were encapsulated in RGD-coupled alginate microspheres loaded with myogenic differentiation cocktail and approximately 500 μl (10 alginate microspheres) were implanted subcutaneously into the dorsal surface of 5-month-old Beige nude mice (Harlan, Livermore, CA; N=4 for each group). After 8 weeks of implantation the mice were sacrificed, and the microspheres and the surrounding tissue were surgically removed and analyzed using histological and immunohistochemical staining. Cell free RGD-alginate microspheres were used as the negative control group.

Histological, Immunohistochemical and Immunofluorescence staining

Harvested specimens were fixed in 4% PFA solution, dehydrated in an ascending series of ethanol, and embedded in paraffin. 6-μm sections were cut and glass slides were prepared. Five randomly selected cross-sections from each group were stained with Hematoxylin & Eosin (H&E).

Immunofluorescence staining was utilized and specimens were treated with 3% H_2O_2, followed by a blocking buffer (1% BSA and 0.25% Triton X-100 in PBS). Then, sections were incubated with anti-MyoD (Santa Cruz Biotechnology, Inc, 1:100 dilution), anti- Myf5 (Santa Cruz Biotechnology, Inc, 1:100 dilution) and detected using Alexa Fluor conjugated secondary antibody (1:200 dilution; Invitrogen). The samples were then counterstained with DAPI.

Moreover, to study the degree of local angiogenesis immunohistochemical analysis was used. De-paraffinized sections were washed, and non-specific endogenous peroxidase activity was quenched by immersing in 3% H_2O_2/methanol for 15 min. Immunohistochemistry examination was performed on sections using anti-CD31 (Abcam, 1:200 dilution) and counterstaining with hematoxylin.

To identify the origin of the cells immunohistochemical staining was utilized. The retrieved specimens were incubated with antibody directed against human mitochondria (mouse antibody anti-human mitochondria; Chemicon, Billerica, MA) at 1:200 dilution and detected

using the universal immunoperoxidase (HRP) ABC kit (Vector Laboratories). Subsequently, sections were counterstained with hematoxylin.

Statistical Analysis

The Kruskal–Wallis rank sum test was utilized to analyze the data at a significance level of $\alpha = 0.05$. Also, two-tailed Student's t-test and two-way ANOVA were utilized for comparisons whenever needed. Quantitative data are expressed as mean ± standard deviation (SD).

Results

RGD coupled alginate microsphere fabrication and release profile characterization Our results demonstrated that both GMSCs and hBMMSCs after two weeks of culturing in myogenic condition media changed their morphology towards myogenic cells (Fig. 1a). However, GMSCs exhibited morphological changes more rapidly and extensively in comparison to hBMMSCs. In addition, the stem cells were expanded in vitro until passages 4 for further experiments. Our flow cytometric analysis revealed that the GMSCs as well as hBMMSCs expressed specific MSC markers CD73, CD105, and CD146, but not the hematopoietic lineage markers CD34 and CD45 (Fig. 1b) with similar expression profiles confirming the stem cell properties of GMSCs. Moreover, the light microscopic imaging confirmed that the fabricated alginate microspheres were of uniform size and showed even cell distribution (Fig. 1c). Approximately, 10 microspheres were fabricated from each 1 mL of alginate/MSC solution with average diameter of 650 micrometer for each microsphere containing 10^5 loaded cells in each sphere. Our SEM analysis showed that the alginate hydrogel had a porous morphology with encapsulated MSCs dispersed within the hydrogel matrix (Fig. 1d).

In addition, live/dead assays exhibited high cell viability of encapsulated MSCs up to two weeks of in vitro culturing (Fig. 2a). Additionally, our MTT assay confirmed that the presence of the myogenic cocktail did not show any significant effects on MSC viability (Fig. 2c), while MSCs encapsulated in RGD-containing alginate microspheres showed significantly higher degrees of viability in comparison to MSCs encapsulated in non-RGD coupled alginate microspheres (Fig. 2b). Moreover, MTT assay further confirmed the cell viability data. The cytotoxicity of the alginate microencapsulation system loaded with myogenic differentiation cocktail was quantitatively analyzed in vitro using MTT assay. GMSCs- and hBMMSCs-alginate constructs showed high MTT absorbance, indicating high metabolic activity and cell viability after up to 2 weeks of culturing in regular media (Fig. 2c).

Next, the possibility of utilization of alginate hydrogel as a scaffold with multiple growth factor delivery capacity was analyzed and the release profile of FSK, MeBIO, and bFGF from the RGD-coupled alginate scaffold was characterized for two weeks (Fig. 2d). The cumulative release profile revealed the sustained release of all the components of the differentiation cocktail for up to 14 days confirming the capability of our utilized cell delivery system with multiple growth factor delivery capacity.

Myogenic differentiation of GMSCs in vitro and the effects of hydrogel matrix elastic modulus

Myogenic differentiation of encapsulated MSCs was confirmed after four weeks of differentiation of encapsulated GMSCs and hBMMSCs *in vitro* by positive immunofluorescence staining with antibodies against MF20 (also known as Myosin II), MyoD, and Myf5. (Fig. 3a).[29, 46] MyoD is one of the key regulators of muscle differentiation.[37] Myf5 is another protein member of this family It is a protein with a key role in regulating muscle differentiation or myogenesis.[37, 21] Without Myf5 and MyoD, myogenic cells will fail to progress normally during the determination stage of myogenesis.[50, 51, 15, 46] Semi-quantitative analysis of the specimens confirmed that engrafted GMSCs expressed higher amounts of myogenic related genes (MF20, Myf5, and MyoD) than engrafted hBMMSCs (P-value < 0.05) (Fig. 3b).

In the next step, the molecular mechanism underlying the myogenic differentiation was evaluated. Gene expression analyses were performed using several markers that are associated with myogenic differentiation of MSCs, including MyoG, MyoD, and Myf5 (Fig. 4a). MyoG or myogenin is a member of the basic helix-loop-helix (bHLH) family of transcription factors, which also includes MyoD and Myf5.[49, 50]

The expression levels of these myogenic genes were analyzed and compared via quantitative PCR. Our results revealed that GMSCs as well as hBMMSCs expressed abundant MyoG, MyoD, and Myf5 after cultured in myogenic cocktail (Fig. 4a). Specifically, RGD-coupled alginate hydrogel markedly elevated myogenic gene expression when compared to non-RGD alginate hydrogel (Fig. 4a). GMSCs showed significantly higher expression levels for all the tested genes compared to hBMMSC ($p<0.05$). Moreover, encapsulation of GMSCs in alginate hydrogel significantly enhanced the expression level of these myogenic genes ($P<0.05$) in comparison to GMSCs cultured without any encapsulation (Figure 4b) suggesting elasticity of alginate hydrogel may contribute to myogenesis of MSCs. Additionally, our immunofluorescence staining (Figs. 5a and 5b) and PCR (Fig. 5c) analysis confirmed that the modulus of RGD-coupled alginate hydrogel regulates the myogenic differentiation of encapsulated MSCs. Our data obtained from quantitative PCR analysis showed that MSC encapsulated in alginate hydrogel with intermediate modulus of elasticity (10–16 kPa) exhibited the highest capacity for myogenic differentiation in comparison to softer (<5kPa) or stiffer (>20kPa) hydrogels (Fig. 5c). Our findings are in good correlation with data reported in the literature.[10, 13,35]

Furthermore, Western blot analysis showed increased expression levels of myogenesis-related gene, MyoD, in specimens encapsulated in RGD-coupled alginate microspheres with middle range elasticity containing myogenic differentiation cocktail (Fig. 5d). The results of Western blot analysis correlated well with the data from the immunostaining and PCR analyses.

Ectopic myogenic tissue regeneration *in vivo*

Encapsulated GMSCs in alginate microspheres loaded with myogenic differentiation cocktail were subcutaneously implanted in immunocompromised mice. H&E staining

showed the formation of small islands of muscle-like structures, while unresorbled alginate hydrogel was still observed after 8 weeks of implantation (Fig. 6). Immunohistochemical and immunofluorescent staining was used to detect newly formed tissue by gene expression analysis. Immunofluorescence staining for MF20 and MyoD antigens after 8 weeks of transplantation revealed extensive production and deposition of these markers within the regenerated muscle tissues (Fig. 6a). Interestingly, engrafted GMSCs showed significantly higher MF20 and MyoD expression levels in comparison to engrafted hBMMSCs ($p < 0.05$) (Fig. 6b).

In addition, the human origin of cellular components of the transplants was confirmed through immunostaining with specific antibodies against human mitochondria (Fig. 7a). Furthermore, immunohistochemical staining with CD-31 endothelial cell marker was utilized to analyze the angiogenesis levels in the regenerated tissues. Our data showed that implanted GMSCs in alginate hydrogel delivery system loaded with the myogenic differentiating cocktail significantly increased the capillary density based on CD-31 endothelial cell marker staining and H & E staining in comparison to the control group (Figs. 7a and 7b) confirming the contribution of engrafted GMSCs to enhanced local vasculature. To examine the ability of implanted MSCs to form vascular networks in vivo, microvessel density was determined, based on the H & E staining, by counting erythrocyte-containing luminal structures in the retrieved specimens. Our data (Fig. 7b) confirmed that implanted GMSCs formed significantly higher number of perfused blood vessels in comparison to hBMMSCs ($P<0.05$).

Discussion

Studies have exploited the potential of cell-based therapies to promote muscle regeneration. It has been shown the possibility of application of stem cells from muscle tissues, satellite cells or entire myofibres for muscle tissue engineering. However, none of these strategies have shown promising results and low expansion capacity (low regenerative capacity) of these stem cell sources have been reported.[15, 21, 22,27] In addition, the isolation procedure for these type of stem cells is invasive and they require a difficult purification procedure.[22,27] It has been confirmed that pluripotent embryonic stem cells (ESCs) have great potential for cell-based therapies. However, their applications are limited due to ethical issues, heterologous immuno-rejection possibility, and the risk of teratoma formation.[41] As an alternative to ESCs, induced pluripotent stem cells (iPSCs) have been successfully utilized for muscle tissue engineering. GMSCs are easily accessible by harvesting from the oral cavity or cells can often be obtained as discarded biological tissue samples. Therefore, gingiva can be considered as an ideal source for stem cell banking purposes provided they show promise in MSC-based tissue regeneration.[41] A thorough review of the literature search revealed no report on application of gingival mesenchymal stem cells for muscle tissue engineering. Therefore, in the current study we demonstrate that GMSCs encapsulated in an injectable and biodegradable RGD-modified alginate hydrogel can effectively be differentiated into muscle tissue *in vitro* and *in vivo* in the presence of appropriate inductive signals. Alginate hydrogel coupled with RGD tripeptide was utilized as the cell delivery system and loaded with a myogenic differentiation cocktail containing FSK, MeBIO, and

recombinant human bFGF to optimize the microenvironment for GMSCs to be differentiated into muscle tissue.

Our *in vitro* analyses demonstrate that RGD coupled alginate hydrogel encapsulation system supported the viability of GMSCs and their myogenic differentiation capacity. GMSCs exhibited significantly higher levels of MyoG, MyoD, and Myf5 mRNA expression in comparison to hBMMSCs as the gold standard of MSC therapy.

In the current study, we utilized RGD-coupled alginate microspheres as a delivery vehicle for GMSCs and we confirmed the important role of microenvironment of the viability and myogenic differentiation of encapsulated GMSCs. It was revealed that the elasticity of the alginate hydrogel controls the fate of the encapsulated GMSCs. Our biomechanical analysis showed that GMSCs encapsulated in alginate hydrogel with intermediate modulus of elasticity (10–16 kPa) exhibited the highest capacity for myogenic differentiation in comparison to softer (<5kPa) or stiffer (>20kPa) hydrogels. Additionally, encapsulation of GMSCs in alginate hydrogel in the presence of the myogenic differentiation cocktail (*in vitro*) doubled the expression level of myogenic related genes (MyoG, MyoD, and Myf5) in comparison to GMSCs that were not encapsulated in alginate.

Moreover, in our previous studies we have shown that alginate hydrogels possess a porous microstructure.[16] The presence of porosities in the structure of alginate hydrogel enables the diffusion of oxygen, nutrients and growth factors (e.g. FSK, MeBIO, and recombinant human bFGF). This porous microstructure in the presence of RGD tripeptides synergistically facilitates GMSC adhesion and availability of oxygen, nutrients and desirable growth factors. These properties in combination with optimized matrix elasticity make alginate hydrogel a highly suitable scaffold biomaterial for muscle tissue engineering.

Furthermore, histological analysis of regenerated tissues in our *in vivo* studies confirmed the formation of muscle-like organizations. Interestingly, GMSCs showed greater capacity for muscle tissue regenerating in comparison to hBMMSCs. In addition, the implanted GMSCs in alginate hydrogel delivery system loaded with the myogenic differentiating cocktail showed increased capillary density and improved neo-vascularization and local angiogenesis in comparison to the control group (hBMMSCs). Taking into account the availability and the high capacity for muscle regeneration of GMSCs, it can be concluded that GMSCs are a unique and promising candidate for muscle (skeletal and cardiac) tissue engineering in the presence of appropriate microenvironment containing the suitable inductive signals. It has to be emphasized that GMSCs were not cultured in myogenic induction media prior to their application *in vivo* studies. These findings of the current study confirm the important role of the microenvironment, as well as the value of presenting inductive signals necessary to support the viability and differentiation of MSCs along a desired phenotype. More importantly, considering the neural crest origin of GMSCs they might be utilized as a unique platform for functional regeneration of smooth muscles (e.g. cardiac regeneration) or tongue muscle tissues.

Here, in a proof of concept study, we demonstrate the potential of GMSCs to be utilized in repair/regeneration of muscular tissues when encapsulated in an appropriately designed

delivery vehicle loaded with myogenic growth factors. Additionally, the GMSC/alginate hydrogel construct can be considered a promising candidate for vascularized tissue engineering. Considering the promising results of the current study it can be envisioned that GMSCs might be promising alternative for cardiac tissue engineering or tongue muscle repair and regeneration.

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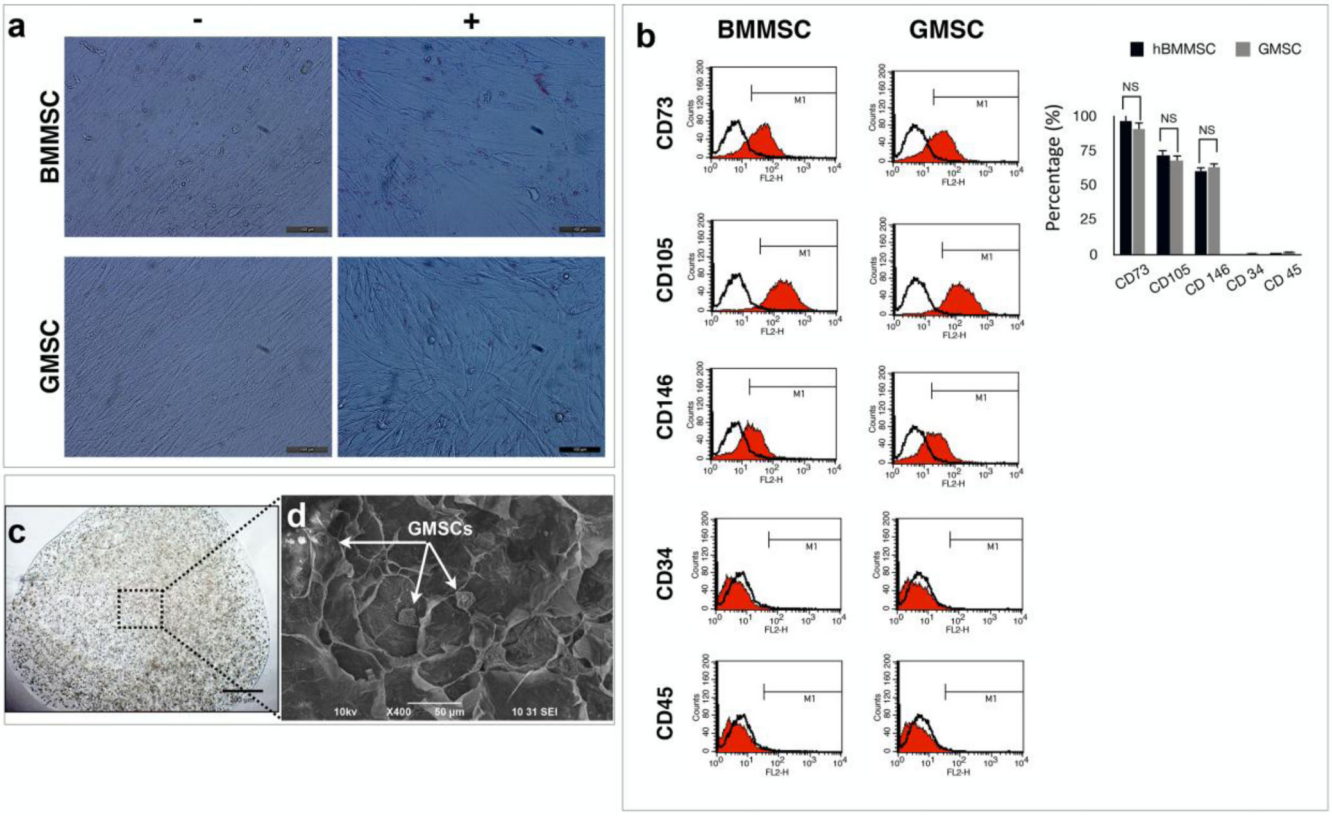

FIGURE 1. Development of RGD-coupled alginate hydrogel microenvironment containing myogenic cocktail for encapsulation of MSCs

(a) Characterization and comparison of the cellular morphology of GMSCs and hBMMSCs before and during myogenic differentiation. (b) Evaluation and quantification of the percentage of cells that express stem cell surface markers (passage 4) through flow cytometric analysis. (c) Bright field image of translucent alginate microspheres showing their retained spherical shape with a uniform cell distribution (average microsphere diameter 650 micrometer). (d) SEM image of the alginate hydrogel-MSC construct showing encapsulated MSCs within porous alginate hydrogel microspheres after two weeks of culturing in regular media. NS=not significant.

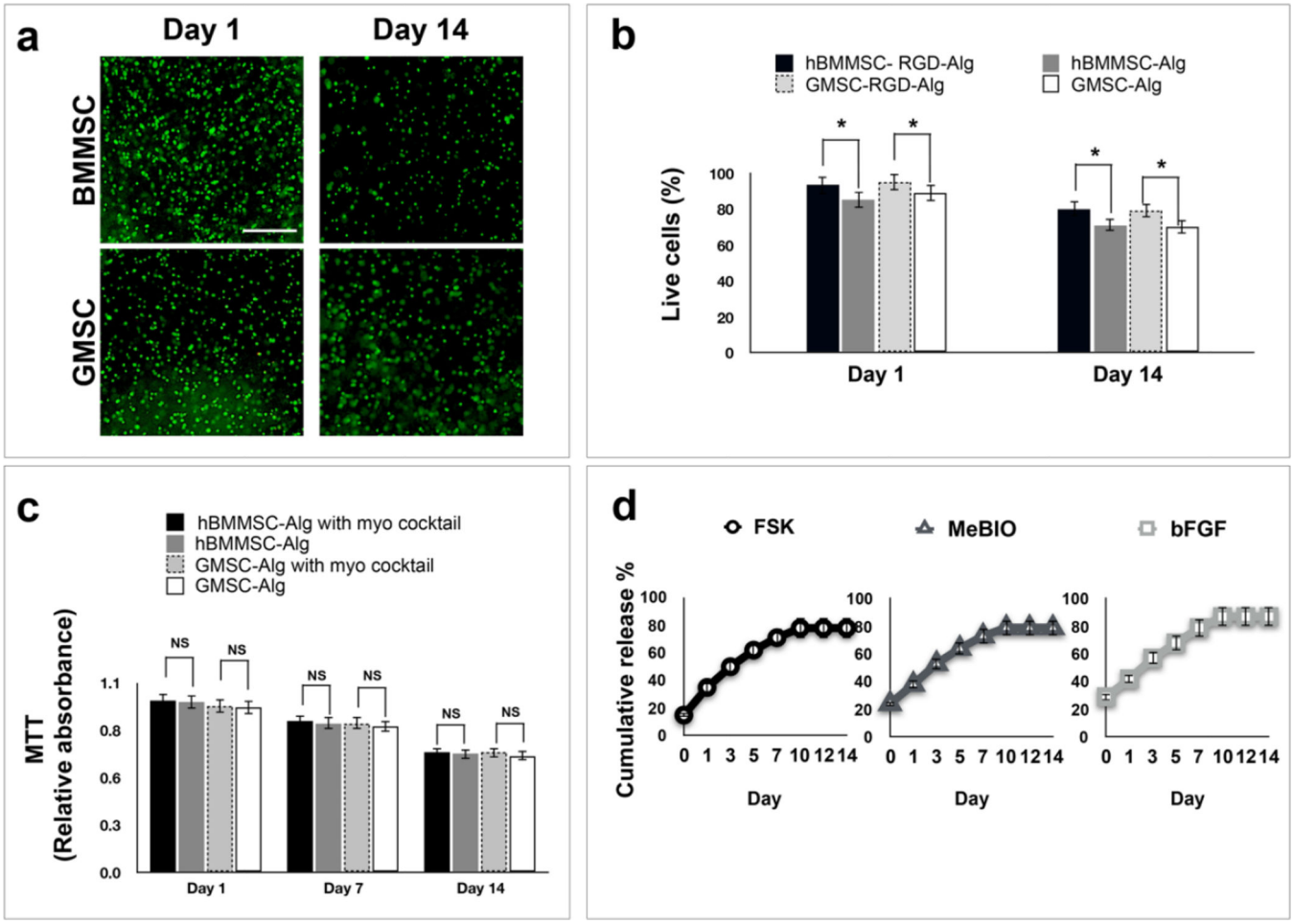

FIGURE 2. MSC viability and release profile characterization of the alginate microencapsulation system

(a, b) Live/dead staining of the encapsulated MSCs in alginate microspheres after one day and two weeks of culturing (scale bar = 200 mm). Viability of the encapsulated MSCs was measured as a percentage of live cells in either RGD-coupled alginate or non-RGD coupled alginate microspheres after two weeks of culturing in regular media. (c) 3-(4,5-Di-methylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay of metabolic activity of encapsulated MSCs. No significant difference was observed between GMSCs and hBMMSCs at each time interval. Also, the myogenic cocktail had no adverse effects of the metabolic activity of the encapsulated MSCs. (d) Characterization of the *in vitro* release profile of different components of the myogenic differentiation cocktail form the alginate hydrogel microspheres showing the multiple growth factor delivery capacity of the alginate microencapsulation system. Sustained release of FSK, MeBIO, and b-FGF was observed from alginate microsphere for up to 2 weeks. *$p < 0.05$.

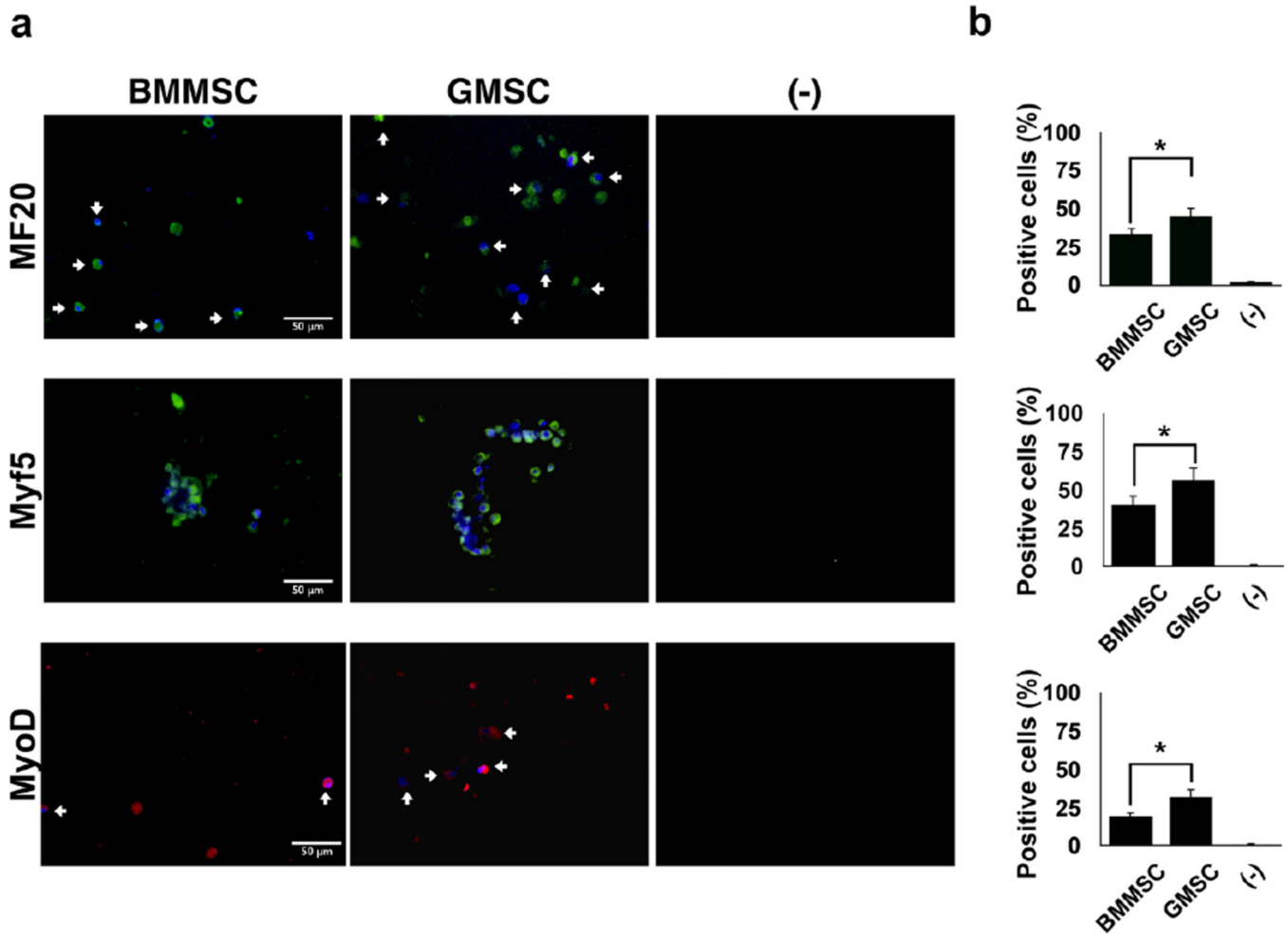

FIGURE 3. ***In vitro*** **myogenic differentiation of GMSC**

(a) Immunofluorescence staining against MF20 (Myosin Heavy Chain), Myf5 (Myogenic factor 5), and MyoD (myogenic differentiation protein) antibodies after four weeks of *in vitro* culturing in myogenic differentiation. Both GMSCs and hBMMSCs positively immunostained with antibodies against MF20, Myf5, and MyoD. Results confirmed that both hBMMSCs and GMSCs were positively stained for myogenic markers (white arrows), while the negative control (−), cell-free alginate hydrogel microspheres failed to express any of these myogenic markers. (b) Analysis of the percentage of cells positive for anti-MF20, Myf5, and MyoD antibodies, showing higher expression levels of myogenic markers in GMSCs in comparison to hBMMSCs (positive control) and negative control groups. *$p < 0.05$.

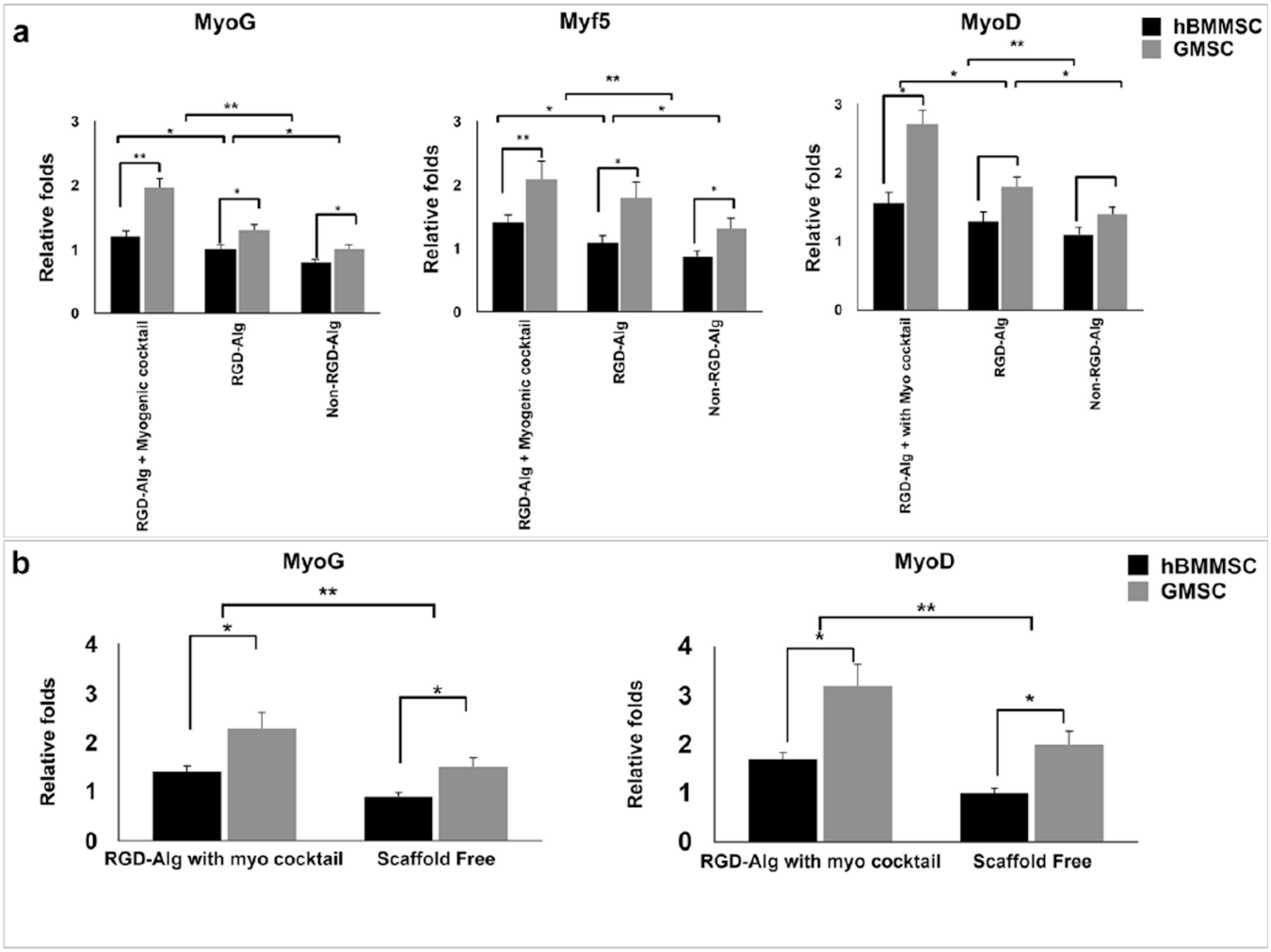

FIGURE 4. Molecular analysis of myogenic differentiation of MSCs *in vitro*

(a) RT-PCR analysis demonstrate significantly greater expression level (in fold changes) of MyoG, Myf5, and MyoD genes for encapsulated GMSCs after 4 weeks of culturing in myogenic differentiation media *in vitro* in comparison to hBMMSCs. The obtained data were normalized by the Ct of the housekeeping gene GAPDH and expressed relative to the expression level for the same gene at day 1. (b) The expression level of MyoG and MyoD for encapsulated hBMMSCs and GMSCs in alginate hydrogel in comparison to scaffold-free MSC cultures after two weeks of myogenic differentiation *in vitro* containing the myogenic cocktail. Data confirmed the important role of the microenvironment and the presence of an encapsulating scaffolds, as the encapsulated MSC expressed greater levels ($p<0.05$) of expressions for examined myogenic genes in comparison to scaffold free MSC groups. *$P<0.05$, **$P<0.01$.

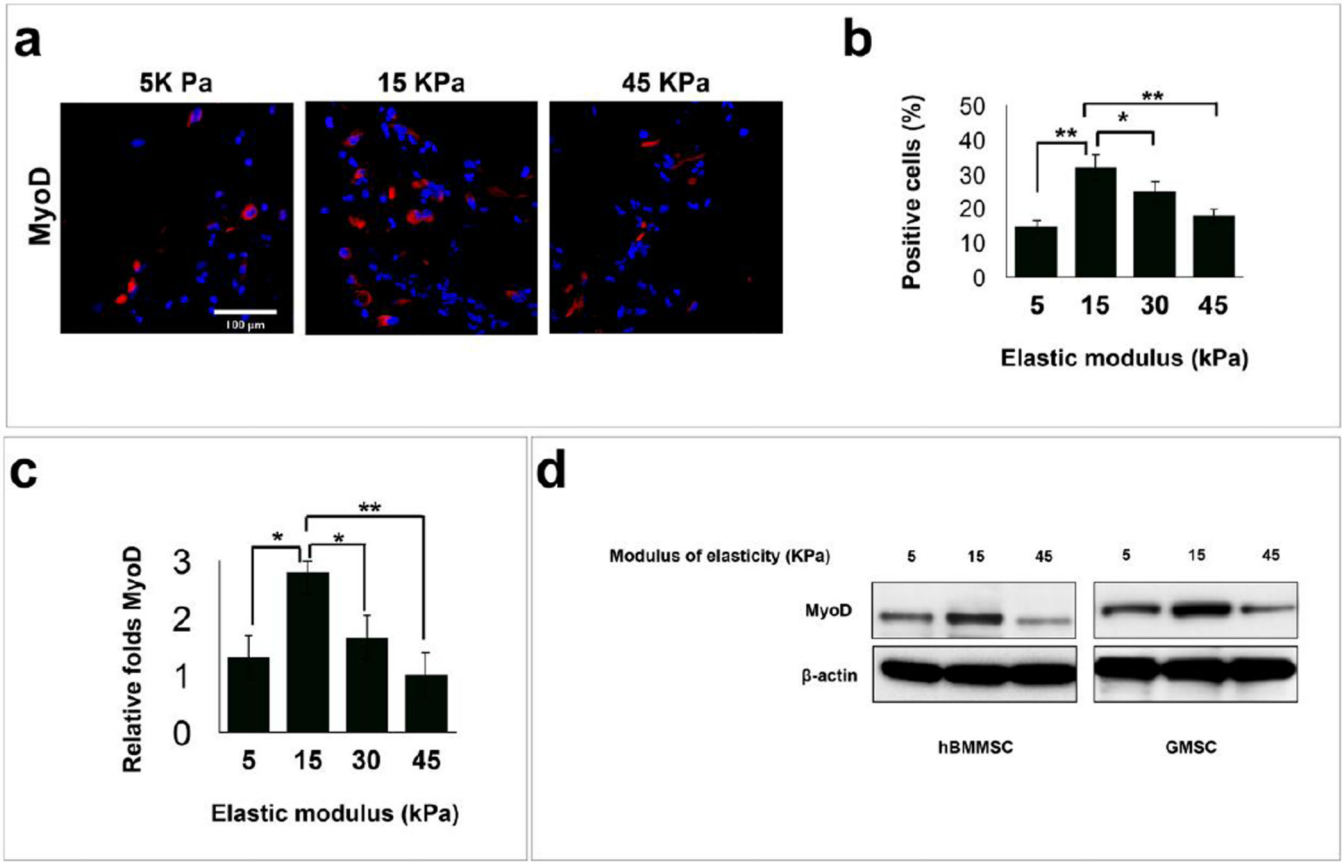

FIGURE 5. Fate determination and myogenic differentiation of MSCs encapsulated in RGD-coupled alginate hydrogel microspheres

(a) Immunofluorescence detection of MyoD protein localized to the GMSCs encapsulated in alginate microspheres with different modulus of elasticity after 4 weeks of culturing in myogenic differentiation media (counterstained with DAPI). (b) Semi-quantitative analysis of the percentage of cells positive for anti-MyoD antibodies via immunofluorescence staining images in panel a. (c) MyoD gene expression evaluation via RT-PCR for GMSCs encapsulated in RGD-coupled alginate hydrogel with different elastic moduli after 2 weeks of culturing in myogenic differentiation media. (d) Western blot analysis presented changes in the expression levels of regulators of myogenesis of GMSCs. The expression level of MyoD gene is elevated in the encapsulated GMSCs in RGD containing alginate microspheres with intermediate modulus of elasticity, while GMSCs encapsulated in alginate hydrogels with higher or lower elastic modulus showed decreased levels of MyoD gene expression conforming the important role of the mechanical properties of the matrix in fate determination of the encapsulated MSCs. *P <0.05, **P<0.01.

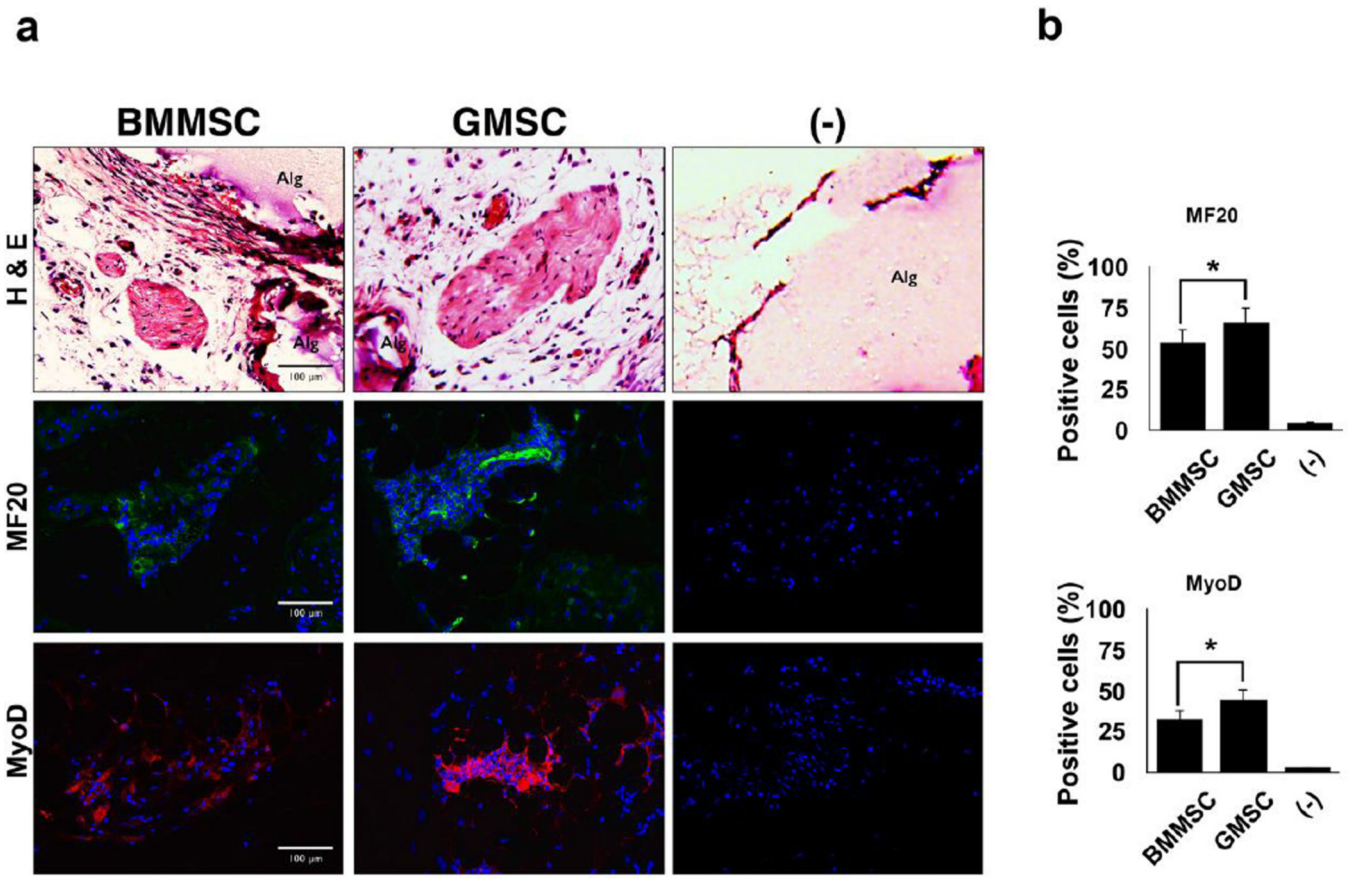

FIGURE 6. ***In vivo*** **myogenesis of encapsulated GMSCs after subcutaneous transplantation**

(a) GMSCs or hBMMSCs as the control group encapsulated in alginate hydrogel containing myogenic cocktail were subcutaneously transplantation into immunocompromised mice and myogenic tissue formation was analyzed after 8 weeks. Histological evaluation by hematoxylin and eosine staining (top panel) confirmed partial islands of muscle regeneration with typical myogenic morphology. Extensive positive staining in immunofluorescence analysis against MF20 (middle panel) and MyoD (lower panel) antibodies further confirmed myogenic differentiation of GMSC. The negative control (−) was cell-free alginate hydrogel scaffold failed to exhibit any positive staining or myogenic tissue regeneration. (b) Semi-quantitative analysis of the percentage of cells positive for anti-MF20 and MyoD antibodies via immunofluorescence staining images. *$p < 0.05$. Alg= unresorbed alginate hydrogel.

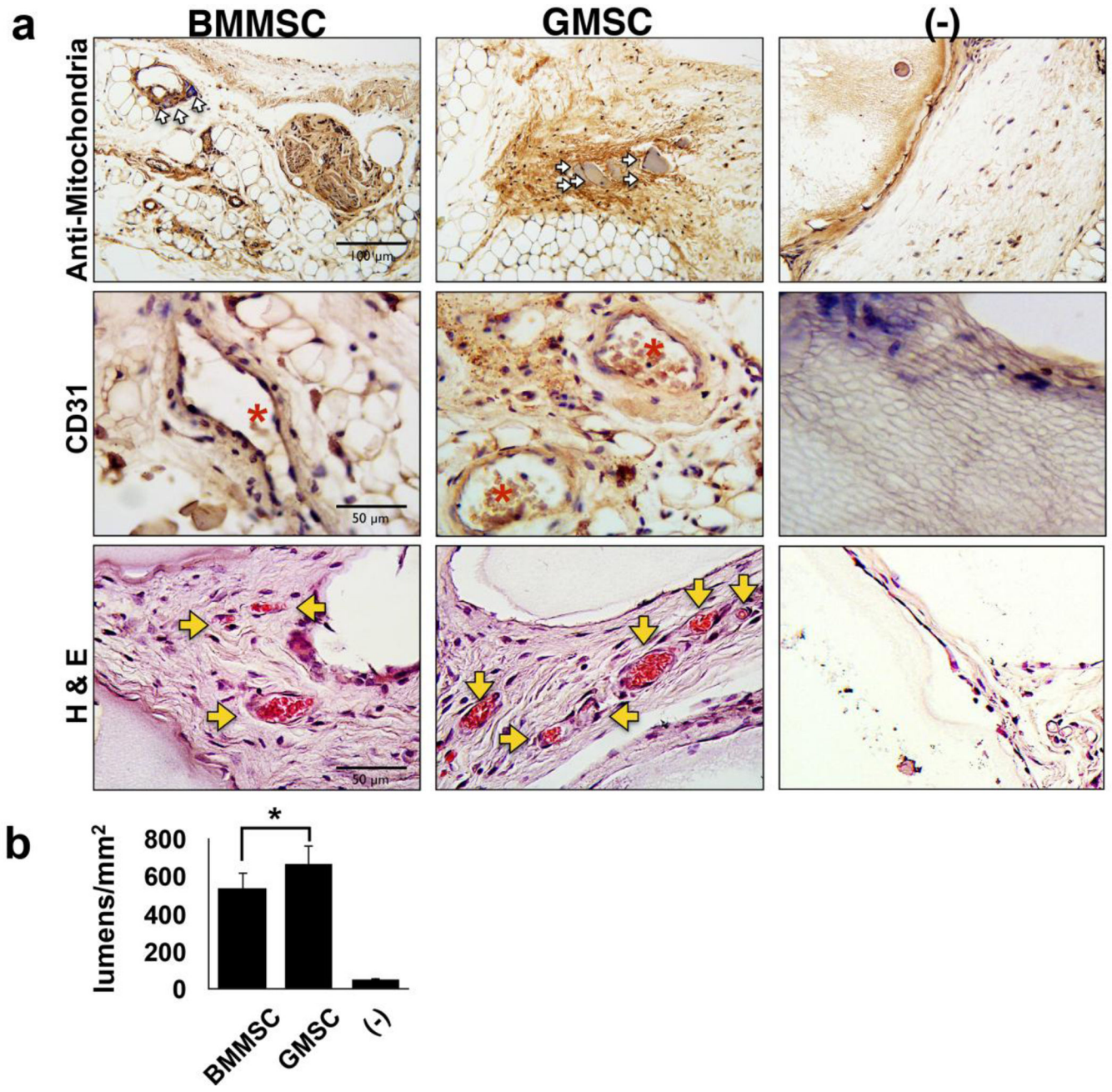

FIGURE 7. The origin of the implanted MSCs and their contribution to vascularization *in vivo*

(a) The human origin of the engrafted GMSCs and hBMMSCs was confirmed by immunohistochemical staining with an antibody specific for human mitochondria (white arrows) (upper panel). Endothelial cell marker was identified by immunohistochemistry using anti- CD31 antibody (middle panel). Histological evaluation by hematoxylin and eosine staining (top panel) (yellow arrows) (lower panel). (b) Semi quantitative analysis of microvessel density based on panel c data. *$p < 0.05$.

Table 1

Oligonucleotide primers used in RT-PCR analysis.

Gene	Sequence	Amplification (bp)
MyoD	5′ -GGGAAGAGTGCGGCGGTGTCGAG-3′ (forward) 5′-TCCGAGAAGGGTGCTGCGTGGAA-3′ (reverse)	448
MYF5 (Myogenic factor 5)	5′ -TTC TCC CCA TCC CTC TCG CT -3′ (forward) 5′ -AGC CTG GTT GAC CTT CTTCAG-3′ (reverse)	285
MyoG (Myogenin, MYF4)	5′ -CAG CTC CCT CAA CCA GGA -3′ (forward) 5′ -GCT GTG AGA GCT GCA TTC G-3′ (reverse)	245
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	5’-AGCCGCATCTTCTTTTGCGTC-3’ (forward) 5’-TCATATTTGGCAGGTTTTT CT-3’(reverse)	418