



Increased Survival and Function of Mesenchymal Stem Cell Spheroids Entrapped in Instructive Alginate Hydrogels

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ABSTRACT

Mesenchymal stem cell (MSC)-based therapies are under broad investigation for applications in tissue repair but suffer from poor cell persistence and engraftment upon transplantation. MSC spheroids exhibit improved survival, anti-inflammatory, and angiogenic potential *in vitro*, while also promoting vascularization when implanted *in vivo*. However, these benefits are lost once cells engage the tissue extracellular matrix and migrate from the aggregate. The efficacy of cell therapy is consistently improved when using engineered materials, motivating the need to investigate the role of biomaterials to instruct spheroid function. In order to assess the contribution of adhesivity on spheroid activity in engineered materials and promote the bone-forming potential of MSCs, we compared the function of MSC spheroids when entrapped in Arg-Gly-Asp (RGD)-modified alginate hydrogels to nonfouling unmodified alginate. Regardless of material, MSC spheroids exhibited reduced caspase activity and greater vascular endothelial growth factor (VEGF) secretion compared with equal numbers of dissociated cells. MSC spheroids in RGD-modified hydrogels demonstrated significantly greater cell survival than spheroids in unmodified alginate. After 5 days in culture, spheroids in RGD-modified gels had similar levels of apoptosis, but more than a twofold increase in VEGF secretion compared with spheroids in unmodified gels. All gels contained mineralized tissue 8 weeks after subcutaneous implantation, and cells entrapped in RGD-modified alginate exhibited greater mineralization versus cells in unmodified gels. Immunohistochemistry confirmed more diffuse osteocalcin staining in gels containing spheroids compared with dissociated controls. This study demonstrates the promise of cell-instructive biomaterials to direct survival and function of MSC spheroids for bone tissue engineering applications. *STEM CELLS TRANSLATIONAL MEDICINE* 2016;5:773–781

SIGNIFICANCE

Mesenchymal stem cell (MSC) spheroids exhibit improved therapeutic potential *in vitro* compared with dissociated MSCs, yet spheroids are directly injected into tissues, ceding control of cell function to the extracellular matrix and potentially limiting the duration of improvement. Cell delivery using adhesive biomaterials promotes cell retention and function. These studies explored the role of adhesion to the surrounding matrix on spheroid function. When entrapped in an adhesive biomaterial, MSC spheroids exhibited improved survival and proangiogenic growth factor secretion *in vitro* and bone formation *in vivo* compared with cells in nonadhesive hydrogels. These findings demonstrate the value of deploying MSC spheroids in instructive biomaterials to improve cell function.

INTRODUCTION

The delivery of mesenchymal stem cells (MSCs) into hypoxic, poorly vascularized tissue defects commonly induces cellular apoptosis, translating to poor cell survival or impaired engraftment [1, 2]. Thus, there is a compelling need for improved methods to promote cell survival and function. The vast majority of cell therapies deploy dissociated cells expanded in monolayer, trypsinized from the culture dish, and injected or seeded on a biomaterial. However, trypsinization of the cell monolayer disrupts critical cell-cell communication

established during culture. Although cell-cell interactions are crucial in spheroid culture, the cell-extracellular matrix (cell-ECM) interaction is equally important. MSCs are anchorage-dependent cells that require cell adhesion to matrix proteins via integrin engagement to prevent anoikis [3]. Furthermore, cell-cell contact by cadherin binding activates other mechanosensitive transmembrane proteins that can participate in cell and tissue homeostasis [4].

Cells cultured as three-dimensional (3D) aggregates, also known as spheroids, yield a multicellular mass mediated by cadherins that more

accurately mimics the physiological cellular niche [5, 6]. Others have hypothesized that spheroid culture preconditions cells residing in the inner core to an ischemic environment, making them more resilient when implanted into hypoxic conditions [7] and enhancing endogenous secretion of growth factors and cytokines necessary for cell survival [8], angiogenesis [1, 9–12], and immunomodulation [13, 14]. Despite the benefits observed when cells are formed as aggregates, spheroids are commonly injected directly into the tissue [1, 7, 15, 16]. Thus, cells are allowed to immediately interact with adhesion sites within the native (ECM) and migrate from the aggregate, potentially limiting the benefit conferred by 3D culture.

Engineered biomaterials are valuable platforms for interrogating the role of integrin activation and cell adhesion, and they may serve as cell carriers for use in tissue engineering. Alginate hydrogels are particularly promising because of their biocompatibility, high water content, low cost, and tailorability [17–19]. In addition, the nonfouling native alginate is easily modified to present specific binding sites for cell adhesion. The cell recognition motif arginine-glycine-aspartic acid (RGD), a tripeptide found in fibronectin and other adhesive ECM proteins, is one of the most widely used peptide sequences for triggering integrin-stimulated cell adhesion within biomaterials [20]. Cell spreading and formation of focal adhesions initiate the survival and proliferative pathways of anchorage-dependent cells [21, 22]. Therefore, alginate is an ideal material for probing the role of cell adhesion in spheroid culture.

This study sought to address two important questions. (a) Would MSC spheroids exhibit improved functionality compared with an equal number of dissociated MSCs once entrapped in a cytocompatible material designed as an implant? (b) Do MSC spheroids function differently when allowed to adhere to the material? We hypothesized that MSC spheroids would exhibit increased survival and enhanced osteogenesis when entrapped in RGD-modified alginate gels compared with unmodified alginate lacking adhesion motifs. To test this hypothesis, we entrapped MSC spheroids in RGD-modified or unmodified alginate gels and compared their survival and function to equal numbers of dissociated cells in the same materials. We then characterized the capacity of spheroids entrapped in these gels to create bone in a murine ectopic site.

MATERIALS AND METHODS

Cell Culture

Human bone marrow-derived MSCs from a single donor were purchased from Lonza (Walkersville, MD, <http://www.lonza.com>) and used without further characterization. Cells were cultured in α -modified Eagle's medium (α -MEM) (Invitrogen, Carlsbad, CA, <http://www.invitrogen.com>) supplemented with 10% fetal bovine serum (JR Scientific, Woodland, CA, <http://www.jrscientific.com>), 100 units/ml penicillin, and 100 μ g/ml streptomycin (Mediatech, Manassas, VA, <http://www.cellgro.com>) under standard culture conditions until use at passage 6.

Fabrication of MSC Spheroids

MSC spheroids were formed by using the hanging-drop technique with 15,000 MSCs in a 25- μ l droplet as described [11]. Droplets were added to a nontissue culture-treated petri dish (CellTreat Scientific, Shirley, MA, <https://www.celltreat.com>) by using a

multichannel pipette, and phosphate-buffered saline was added to the bottom of the dish to prevent evaporative loss. After MSCs were allowed to aggregate for 48 hours, spheroids were collected via pipette for entrapment in alginate.

Preparation of RGD-Modified Alginate

RGD-modified alginate was prepared as described previously [23–25]. Briefly, G₄RGDSP (Commonwealth Biotechnologies, Richmond, VA, <http://www.cbi-biotech.com>) was covalently coupled to irradiated UltraPure MVG sodium alginate (ProNova, Lysaker, Norway, <http://pronovasolutions.com>) by using standard carbodiimide chemistry, yielding hydrogels with 0.32 mM RGD. The resulting RGD-alginate was sterile-filtered and lyophilized for 4 days. Lyophilized alginate was reconstituted in serum-free α -MEM to obtain a 2% (wt/vol) solution. Unmodified alginate was reconstituted in serum free α -MEM to obtain a 2% alginate solution and sterile-filtered.

Entrapment of Spheroids Within Alginate Gels

Current methods that use CaSO₄ to cross-link alginate hydrogels involve mixing components with two syringes, causing loss of polymeric and cellular material for a specific volume. To ensure we entrapped equivalent numbers of MSCs as dissociated cells or spheroids, we applied a diffusion-mediated method of alginate cross-linking similar to that described for entrapping individual spheroids [26]. Briefly, alginate gels containing a known spheroid concentration were pipetted into wells of a silicone mold adhered to a glass plate to yield 2% alginate gels with a consistent volume of 80 μ l. A dialysis membrane (MWCO 3500, Spectrum Laboratories, Rancho Dominguez, CA, <http://www.spectrumlabs.com>) was overlaid and flattened onto the mold. Molds were then immersed in CaCl₂ solution to cross-link the alginate. The optimal parameters for alginate cross-linking, specifically CaCl₂ concentration and exposure time, were determined by characterizing changes in rheological properties and viability of entrapped cells. Subsequently, all remaining studies were conducted with a single CaCl₂ concentration and exposure duration.

Assessment of Hydrogel Mechanical Properties

We measured the viscoelastic properties of spheroid-containing alginate gels cross-linked in varying CaCl₂ concentrations and exposure durations by using a Discovery HR2 Hybrid Rheometer (TA Instruments, New Castle, DE, <http://www.tainstruments.com>). An 8.0-mm-diameter peltier plate geometry was used for 80- μ L gels with corresponding 8.0-mm diameter. Gels were cross-linked and then maintained in medium under standard culture conditions for 24 hours before measurement. An oscillatory strain sweep ranging from 0.004% to 4% strain was performed on each gel to obtain the linear viscoelastic region (LVR) before gel failure. A minimum of 10 data points were collected for the LVR and averaged to obtain gel shear storage modulus.

Live/Dead Staining of MSCs Within Alginate Gels

Calcein acetoxymethyl (calcein AM) ester and propidium iodide (Invitrogen) were added to α -MEM to create a 2 μ M and 5 μ M solution of each reagent, respectively. Gels were fully immersed for 30 minutes before imaging. Gels collected for

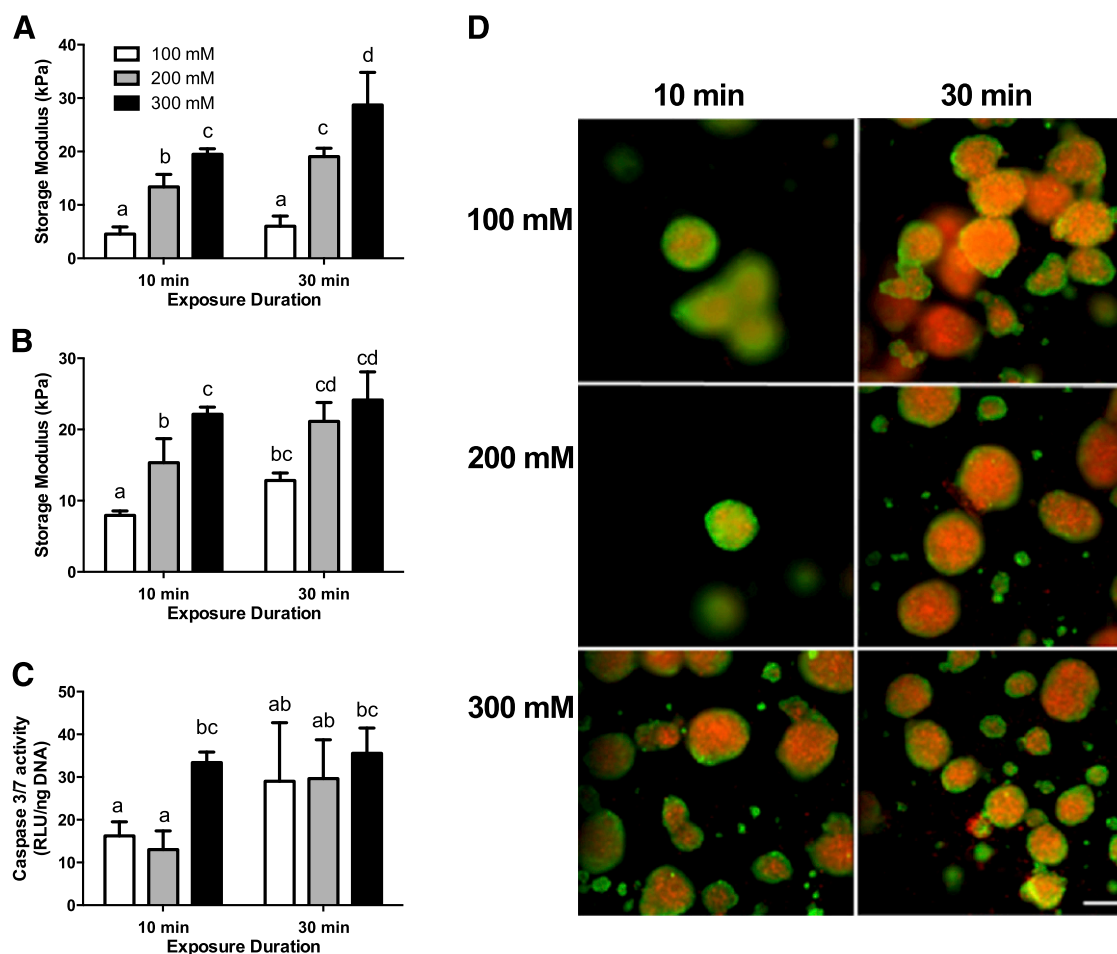


Figure 1. Rheological properties and survival of entrapped mesenchymal stem cell spheroids correlate with calcium concentration and duration of exposure. **(A):** Shear storage moduli of acellular arginine-glycine-aspartic acid (RGD)-modified gels at day 1 ($n = 3$ per group). **(B):** Shear storage moduli of RGD-modified gels containing spheroids ($n = 3$ per group). **(C):** Caspase-3/7 activity as an indicator of apoptosis. **(D):** Live/dead staining of spheroids entrapped in RGD-modified gels cross-linked with varying CaCl_2 concentrations and for 10 and 30 minutes at day 1. Live/dead images at $\times 10$ magnification using inverted fluorescence microscopy; scale bar = $200 \mu\text{m}$. Different letters above bars indicate significance of at least $p < .05$. Bars sharing the same letters indicate no significance. Abbreviation: RLU, relative luminescence units.

confocal microscopy were processed in the same manner and imaged by using an Olympus Fluoview FV1000 confocal laser scanning system with a $\times 20$ objective (Olympus, Tokyo, Japan, <http://www.olympus.co.jp>).

Biochemical Analysis of MSC Spheroid Function Within Alginate Gels

MSCs, either dissociated cells or spheroids, were entrapped in alginate gels at a concentration of 40×10^6 cells per milliliter. Gels were cultured in α -MEM under standard culture conditions for 5 days, and collected, minced, and lysed in passive lysis buffer (Promega, Madison, WI, <http://www.promega.com>). Apoptosis was quantitatively measured by analyzing $100 \mu\text{l}$ of lysate per sample using a Caspase-Glo 3/7 assay (Promega) as we reported [27, 28]. Luminescence was detected on a microplate reader and normalized to DNA content determined from the same lysate by using the Quant-iT PicoGreen DNA Assay Kit (Invitrogen). To measure vascular endothelial growth factor (VEGF) secretion, medium was refreshed 24 hours before collection, and VEGF concentration was determined by using a human-specific VEGF enzyme-linked immunosorbent assay (ELISA) kit

(R&D Systems, Minneapolis, MN, <https://www.rndsystems.com>) according to the manufacturer's instructions [11]. Quantification of proangiogenic proteins within conditioned medium was performed by using a Human Angiogenesis Antibody Array (RayBiotech, Norcross, GA, <http://www.raybiotech.com>) [27, 29]. Protein quantification was performed with a GenePix 4000B scanner and associated GenePix Pro software (Molecular Devices, Sunnyvale, CA, <http://www.moleculardevices.com>) to calculate median minus background fluorescent intensity at 532 nm for each cytokine. Data are normalized to internal positive controls.

To quantify osteogenic potential of entrapped spheroids, gels prepared as described above were cultured in α -MEM overnight and then moved to osteogenic medium containing standard osteogenic supplements (10 mM β -glycerophosphate, 50 $\mu\text{g/ml}$ ascorbate-2-phosphate, and 100 nM dexamethasone; all from Sigma-Aldrich, St. Louis, MO, <http://www.sigmaaldrich.com>). Osteogenic potential was assessed by quantifying intracellular alkaline phosphatase (ALP) activity from a *p*-nitrophenyl phosphate assay, osteocalcin secretion via ELISA, and calcium deposition by *o*-cresolphthalein assay [24, 30, 31].

In Vivo Subcutaneous Ectopic Bone Assay

Treatment of all experimental animals was in accordance with the University of California, Davis animal care guidelines and all National Institutes of Health animal handling protocols. Eight-week-old nonobese diabetic/severe combined immunodeficient γ (NSG, NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice (Jackson Laboratory, Bar Harbor, ME, <https://www.jax.org>) were anesthetized and maintained under a 2% isoflurane/O₂ mixture delivered through a nose cone. Two longitudinal incisions were made in the dorsum, and subcutaneous pockets were created on each side of the incision to create four spaces for biomaterial implantation, after which surgical sites were closed with staples. Animals were euthanized after 8 weeks by CO₂ inhalation. Alginate gels were explanted and fixed in phosphate-buffered formalin for 24 hours, then maintained in 70% ethanol ($n = 4$ per group) for analysis.

Analysis of Bone Formation in Gel Explants

The formation of mineralized tissue was detected in explants by radiography at a voltage of 22 kVp for 2 minutes. Explants were subsequently demineralized in Calci-Clear Rapid (National Diagnostics, Atlanta, GA, <https://www.nationaldiagnostics.com>), processed, paraffin-embedded, and sectioned at 5- μ m thickness. Sections were stained with hematoxylin and eosin (H&E) and imaged by using a Nikon Eclipse TE2000U microscope (Nikon, Tokyo, Japan, <http://www.nikon.com>) and Andor Zyla 5.5 scientific complementary metal-oxide semiconductor (sCMOS) digital camera (Andor, Belfast, Northern Ireland, <http://www.andor.com>). To visualize cells undergoing osteogenic differentiation, we performed immunohistochemistry (IHC) on sections by using a primary antibody against osteocalcin (1:200, ab13420, Abcam, Cambridge, MA, <http://www.abcam.com>) [32] and a mouse-specific horseradish peroxidase/3,3'-diaminobenzidine detection kit (ab64259, Abcam).

Statistical Analysis

Data are presented as means \pm SD. Statistical analysis was performed by using a two-way analysis of variance with Bonferroni correction for multiple comparisons in Prism 6 software (GraphPad Software Inc., La Jolla, CA, <http://www.graphpad.com>); $p < .05$ was considered statistically significant. Significance is denoted by alphabetical letterings; groups with no significance are linked by the same letters, whereas groups with significance do not share the same letters.

RESULTS

Cross-Linking Alginate Hydrogels by Dialysis for Entrapping MSC Spheroids

The rheological properties of alginate hydrogels were initially characterized without cells to directly ascertain the role of CaCl₂ concentration and duration. Alginate hydrogels cross-linked by CaCl₂ diffusion exhibited increasing shear storage modulus with increasing calcium concentration (Fig. 1A). Additionally, shear storage modulus increased when prolonging the exposure duration to 30 minutes for gels exposed to 200 and 300 mM CaCl₂. When spheroids were entrapped in alginate hydrogels, we observed similar relationships between shear storage modulus, CaCl₂ concentration, and duration of exposure (Fig. 1B). Compared with acellular gels, the entrapment of MSC spheroids

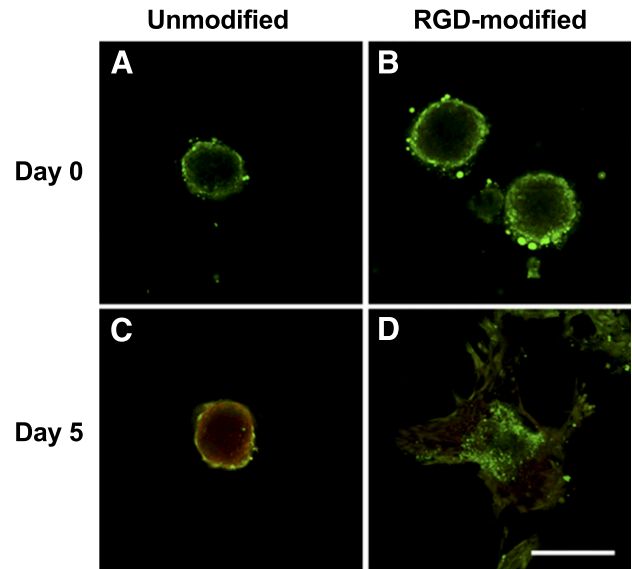


Figure 2. Mesenchymal stem cell spheroids entrapped in RGD-modified gels exhibit increased cell viability and migration. **(A, B):** Live/dead stain reveals comparable viability and spherical morphology for spheroids at day 0 in unmodified **(A)** and RGD-modified **(B)** gels when visualized with confocal microscopy. **(C, D):** Live/dead stain demonstrates increase in dead cells and retained spherical morphology for spheroids at day 5 in unmodified alginate **(C)**, whereas spheroids in RGD-modified alginate gels have increased viability and migration from the aggregate **(D)**. Scale bar = 200 μ m. Abbreviation: RGD, arginine-glycine-aspartic acid.

resulted in increased storage modulus for most conditions. Quantification of cell apoptosis was performed in tandem with Live/dead imaging by using inverted fluorescence microscopy. A concentration of 300 mM CaCl₂ at 10 minutes of exposure significantly increased caspase 3/7 activity compared with 100 and 200 mM concentrations (Fig. 1C). The viability of entrapped spheroids was dependent upon the calcium concentration and exposure time (Fig. 1D). We observed increasing cell death as CaCl₂ concentration increased after 10 minutes of exposure. After 30 minutes of exposure, all groups exhibited high levels of cell death, regardless of CaCl₂ concentration. Similar trends were observed after 4 and 7 days in culture (data not shown). In order to achieve the biomaterial with the highest mechanical properties while limiting cell death, we cross-linked alginate gels in subsequent experiments with 200 mM CaCl₂ for 10 minutes.

MSCs From Spheroids Adhere, Spread, and Exhibit Enhanced Survival in RGD-Modified Alginate

We used confocal microscopy to discern changes in spheroid morphology and cell survival over time. Immediately after entrapment at day 0, spheroids exhibited similar, spherical morphology with high viability in unmodified or RGD-modified alginate gels (Fig. 2A, 2B). After 5 days in culture, spheroid morphology in unmodified alginate remained compact and spherical, but with significant cell death (Fig. 2C). However, spheroids entrapped in RGD-modified gels demonstrated a loss in spherical structure, with cells migrating from the spheroid and becoming more elongated (Fig. 2D). Furthermore, we observed a significant increase in cell viability for spheroids in RGD-modified gels compared with cells entrapped in unmodified alginate.

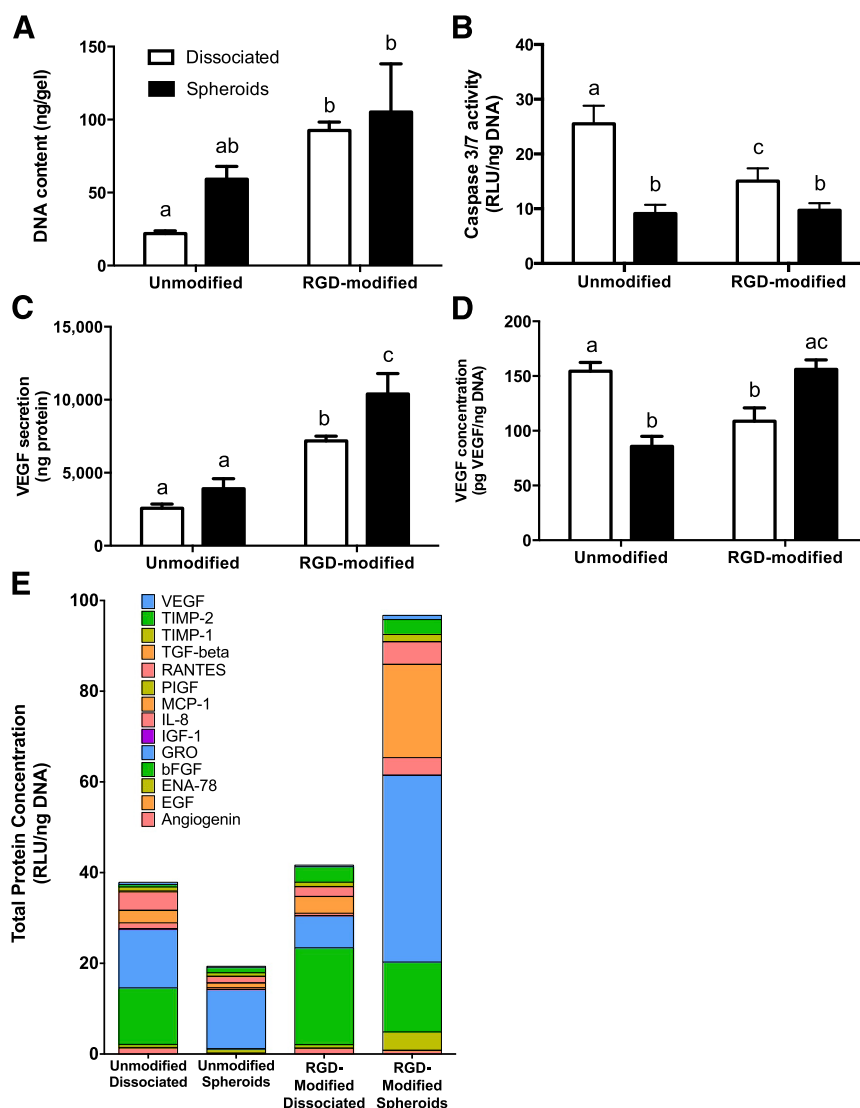


Figure 3. Mesenchymal stem cell (MSC) spheroids entrapped in RGD-modified alginate hydrogels have increased survival and proangiogenic potential compared with dissociated MSCs. **(A):** Total DNA content in gel scaffold. **(B):** Caspase-3/7 activity as an indicator of apoptosis. **(C):** VEGF secretion measured by enzyme-linked immunosorbent assay (ELISA). **(D):** Secreted VEGF normalized to DNA. Gels loaded with an equal number of dissociated cells are represented by open bars, and hydrogels entrapping spheroids are represented by filled bars ($n = 4$ for all groups). **(E):** Total angiogenic proteins normalized to DNA measured by angiogenic microarray. Different letters above bars indicate significance of at least $p < .05$. Bars sharing the same letters indicate no significance. Abbreviations: RGD, arginine-glycine-aspartic acid; RLU, relative luminescence units; VEGF, vascular endothelial growth factor.

MSCs in RGD-Modified Gels Demonstrate Reduced Apoptosis and Increased VEGF Secretion

We entrapped equal numbers of MSCs in both gel formulations to assess the role of adhesivity on cell proliferation, apoptosis, and VEGF secretion in dissociated cells and MSC spheroids. We detected increased DNA content, an indicator of cell number, when entrapping MSCs in RGD-modified alginate gels compared with cells in unmodified gels. The entrapment of dissociated MSCs in RGD-modified gels resulted in a 4.2-fold increase in DNA content compared with cells in unmodified gels (Fig. 3A). We measured significant reductions in caspase-3/7 activity, an indicator of apoptosis, for MSCs entrapped as spheroids versus dissociated cells, regardless of gel formulation (Fig. 3B). Spheroids in unmodified gels exhibited a 2.8-fold reduction in caspase activity compared with dissociated controls, while caspase-3/7

activity in dissociated cells was reduced in RGD-modified gels. MSC spheroids secreted more total VEGF than an equivalent number of dissociated MSCs in each alginate formulation (Fig. 3C). VEGF secretion was significantly increased for MSC spheroids and dissociated cells when entrapped in RGD-modified versus unmodified alginate (2.7- and 2.8-fold increase, respectively). Furthermore, MSC spheroids suspended in RGD-modified gels secreted 1.4-fold more VEGF compared with dissociated controls. These increases in VEGF secretion were not solely a function of cell proliferation, because MSC spheroids in RGD-modified gels secreted more normalized VEGF compared with dissociated cells or MSC spheroids in unmodified alginate (1.4- and 1.8-fold more, respectively) (Fig. 3D). The angiogenic microarray demonstrated the greatest protein secretion from spheroids in RGD-modified alginate and the least in unmodified

groups (Fig. 3E). MSC spheroids in RGD-modified alginate secreted more protein than both dissociated cell and unmodified controls (2.5- and 5-fold increase, respectively). Of interest, spheroids in RGD-modified alginate exhibited an upregulation in growth-regulated oncogene (GRO), monocyte chemoattractant protein 1 (MCP-1), and interleukin 8 (IL-8), potent stimulators of angiogenesis, compared with both dissociated and unmodified control groups.

Spheroids in RGD-Modified Alginate Undergo Osteogenic Differentiation

We analyzed the osteogenic response of MSC spheroids to unmodified or RGD-modified alginate when cultured under osteogenic conditions. ALP activity was relatively constant among all groups over the first 7 days. After 14 days, MSC spheroids in RGD-modified gels exhibited a 4.8-fold increase in ALP activity compared with dissociated controls (Fig. 4A), and spheroids in unmodified alginate demonstrated further increases. Spheroids entrapped in unmodified alginate demonstrated elevated osteocalcin levels compared with dissociated controls at all time points, while spheroids in RGD-modified gels exhibited a significant increase only at 7 days (Fig. 4B). We quantified cell-secreted calcium, a late stage marker of osteogenic differentiation, to determine mineralization of the scaffold. At days 7 and 14, we observed higher calcium content in all spheroid groups compared with dissociated controls (Fig. 4C), demonstrating that MSC spheroids can undergo osteogenic differentiation when entrapped in alginate gels.

Spheroids in RGD-Modified Alginate Exhibit Enhanced Osteogenesis In Vivo

We analyzed the presence and distribution of mineral within explants containing MSC spheroids and dissociated MSCs after 8 weeks. Representative radiographs confirmed widespread mineralization in all groups (Fig. 5A), but mineralization appeared substantially more intense in the RGD-modified groups. Radiograph data indicated that spheroids retain their capacity to form bone when compared with dissociated controls. Furthermore, dissociated MSCs delivered in RGD-modified alginate gels yielded markedly more mineralized tissue compared with unmodified alginate hydrogels seeded with an equal number of dissociated cells. As revealed by H&E staining, alginate gels containing dissociated MSCs had markedly reduced cellularity with larger clumps of alginate remaining compared with MSC spheroids in either formulation (Fig. 5B). Additionally, hydrogels containing MSC spheroids reveal more diffuse and homogeneous osteocalcin staining compared with dissociated controls (Fig. 5C), confirming osteogenic differentiation of cells entrapped in alginate gels.

DISCUSSION

Cell therapies consist of transplanting dissociated or dispersed cell populations, either in the presence or absence of a biomaterial, to stimulate tissue repair. Once implanted, cells exhibit poor survival and increased apoptosis *in vivo*, perhaps due to the loss of cell-ECM and cell-cell contacts established during cell culture. Previous studies reported the proangiogenic and antiapoptotic advantages of spheroids compared with dissociated cells [1, 9, 11, 13]. However, spheroids are commonly transplanted through

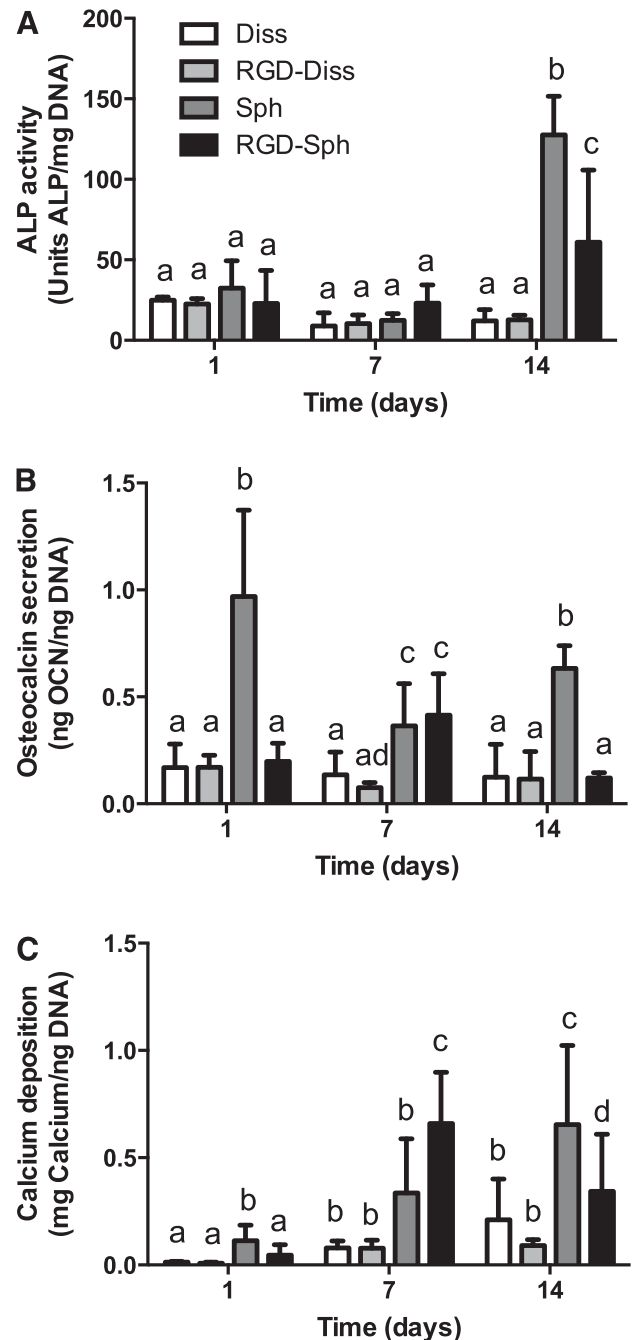


Figure 4. Mesenchymal stem cell (MSC) spheroids exhibit enhanced osteogenic potential in RGD-modified alginate gels over 14 days in culture compared with dissociated MSCs. **(A):** Intracellular ALP activity. **(B):** Secreted osteocalcin. **(C):** Secreted calcium in gels. $n = 5$ per group. Different letters above bars indicate significance of at least $p < .05$. Bars sharing the same letters indicate no significance. Abbreviations: ALP, alkaline phosphatase; Diss, dissociated; OCN, osteocalcin; RGD, arginine-glycine-aspartic acid; RGD-Diss, arginine-glycine-aspartic acid-dissociated; Sph, spheroids; RGD-Sph, arginine-glycine-aspartic acid-spheroid.

direct injection into the tissue site [1, 7, 15, 16], thereby ceding control of this improved cell function to the surrounding tissue ECM as cells migrate from the spheroid into the tissue in an uncontrolled manner. Hence, there is a critical need to understand the effect of controllable presentation of adhesion sites external

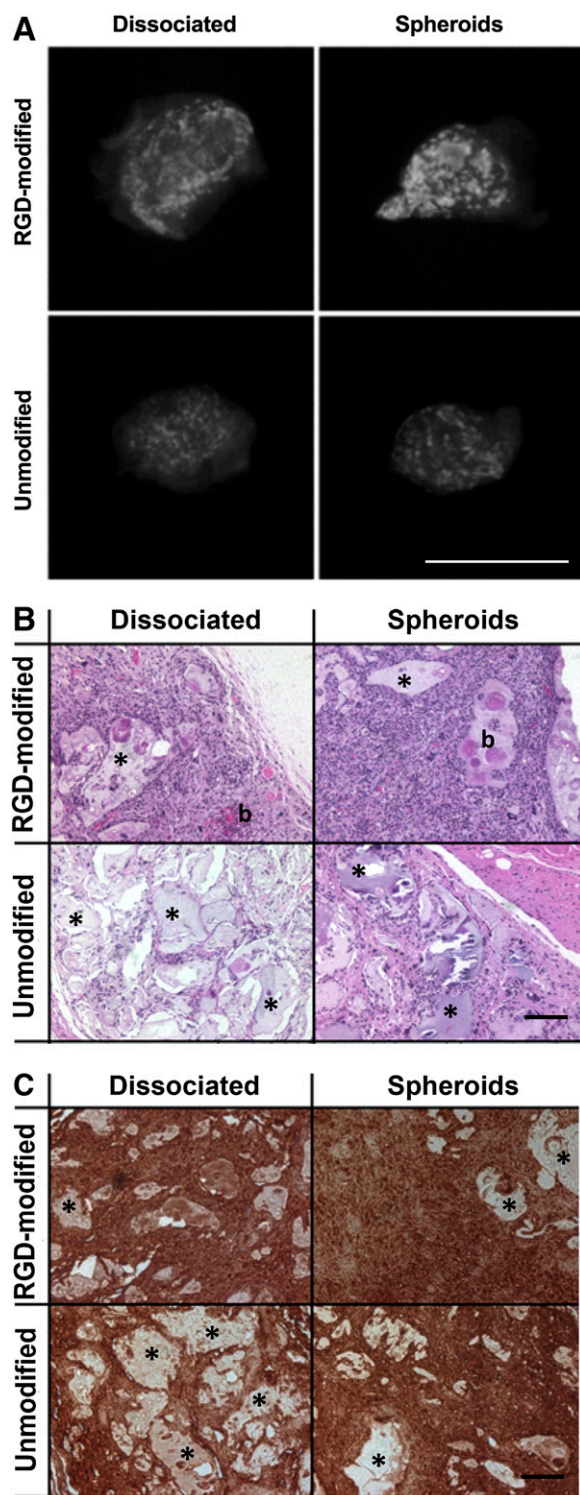


Figure 5. Mesenchymal stem cell spheroids exhibit robust bone-forming potential when deployed in RGD-modified alginate. **(A):** Representative radiograph illustrating mineral deposition in explants after 8 weeks. Image is not magnified; scale bar = 8 mm. **(B):** Representative H&E staining of explants at 8 weeks. Blue indicates cell nuclei, pink indicates matrix proteins, asterisk indicates residual alginate, and b represents new bone tissue. **(C):** Representative images of immunohistochemical staining of explants for osteocalcin. Asterisk indicates residual alginate. All stained images were taken under brightfield microscopy at a magnification of $\times 10$; scale bars = $100 \mu\text{m}$. Abbreviation: RGD, arginine-glycine-aspartic acid.

to the spheroid to develop improved delivery vehicles for preserving spheroid survival and function.

Alginate hydrogels can be cross-linked with the addition of divalent cations, commonly achieved via the addition of a supersaturated solution of CaSO_4 . This calcium solution enables slow and relatively homogenous gelation, but suffers from unpredictable gelation kinetics; distribution of large, potentially cytotoxic calcium nodules because of low solubility [18, 33]; and unpredictable loss of spheroids in the residual material. In these studies, we needed to entrap an exact number of spheroids to compare with equal numbers of dissociated MSCs, thus motivating the application of an alternative gelation method. CaCl_2 is widely used to rapidly cross-link alginate beads and cure gels [34, 35], and previous reports have used a similar diffusion-based strategy to entrap individual spheroids [26, 36]. We successfully engineered robust alginate gels by cross-linking with 200 mM CaCl_2 for 10 minutes, resulting in hydrogels with similar or greater mechanical properties as others used in bone formation [37] while retaining cell viability. This concentration is comparable to concentrations used to generate alginate microbeads [18] and higher than other dialyzing methods, but is used over shorter durations [38, 39].

These data confirm that alginate hydrogels seeded with equal numbers of MSCs as spheroids possess greater proangiogenic potential and decreased apoptosis compared with dissociated cells, data that are in agreement with other comparisons lacking a biomaterial [1, 9, 11, 13]. For dissociated MSCs, binding to the RGD ligand enhances MSC attachment and survival [40] and promotes osteogenic differentiation in a dose-dependent manner [41]. We selected a single, commonly used RGD concentration and polymer concentration to evaluate the response of MSC spheroids to adhesivity in alginate gels. In light of other studies reporting the interplay of RGD concentration and substrate stiffness on cell migration [42] and osteogenic differentiation [43], the role of polymer and RGD concentration on spheroid response to the delivery vehicle merits further investigation. Herein, we demonstrate that spheroids exhibit greater osteogenic differentiation compared with dissociated MSCs, regardless of adhesion to the gel. The underlying reason for why spheroids in unmodified gels exhibit higher levels of osteoblastic markers is unclear and merits further examination. This phenomenon may be due to relative differences in the contribution of cells within spheroids engaging adhesion ligands within the gel versus cells engaging endogenous matrix within the spheroid. These data suggest that cell-cell interactions have a similarly critical role as cell-ECM engagement to drive differentiation. Differences in osteogenic differentiation of spheroids with respect to RGD modification can be attributed to MSC population heterogeneity and differences in timing of cellular response. Although RGD improves cell engagement with the biomaterial, previous studies demonstrate that it does not affect angiogenesis in dissociated MSCs [44]. However, MSC spheroids entrapped in RGD-modified gels exhibited increased VEGF secretion and improved survival compared with hydrogels seeded with an equal number of dissociated MSCs or cells entrapped in unmodified alginate gels. Beyond VEGF, MSC spheroids in RGD-modified alginate secrete the most total proangiogenic factors. Of note, GRO, MCP-1, and IL-8 are important cytokines most highly upregulated in spheroids entrapped in RGD-modified alginate. These factors have critical angiogenic functions and, in addition to being secreted by MSCs, are also produced by cells of the innate immune system such as macrophages, neutrophils, and dendritic cells. These data indicate that RGD modification

enhances the proangiogenic potential of MSC spheroids in a manner previously unrecognized with dissociated cells, and manipulation of RGD concentration may further enhance the proangiogenic potential of entrapped spheroids. Together, these data motivate the use of RGD-modified alginate as a biomaterial vehicle to effectively balance survival, proangiogenic potential, and osteogenic differentiation of entrapped MSC spheroids.

When used as cell-delivery vehicles, all tested biomaterials facilitated mineralization *in situ*. MSCs in RGD-modified alginate, whether entrapped as dissociated cells or spheroids, exhibited more robust mineralization than either group in unmodified gels. Regardless of biomaterial, the implantation of spheroid-containing gels resulted in more diffuse osteocalcin staining compared with dissociated controls. Importantly, these data confirm that MSCs deployed as spheroids in alginate gels exhibit increased cell survival and promote host cell infiltration without compromising their potential for bone formation. We utilized a high cell density (40×10^6 cells per milliliter) of MSCs, facilitating mineralization even in unmodified alginate. The capacity of RGD-modified gels to instruct spheroid function and promote bone formation with lower cell concentrations merits further investigation to reduce the total number of required cells for therapy.

These studies demonstrate that MSC spheroids entrapped in an engineered biomaterial retain their advantages in survival and function compared with dissociated MSCs when used for bone tissue engineering. To our knowledge, this is the first report characterizing the response of MSC spheroids when entrapped in RGD-modified alginate hydrogels. The use of engineered biomaterials provides an opportunity to prolong the proangiogenic and anti-apoptotic advantages of spheroidal culture when cells are transplanted *in vivo*. Furthermore, increases in VEGF secretion observed with MSC spheroids in RGD-modified gels can be applied to any tissue-engineering strategy in need of vascularization.

CONCLUSION

The results of these studies confirm that MSC spheroids can be effectively entrapped in a biomaterial that subsequently enhances MSC survival and secretion of proangiogenic cues compared with dissociated cells. Future efforts to deploy spheroids *in vivo* should consider the use of biomaterials with desired adhesivity to regulate survival, angiogenesis, and tissue formation.

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AUTHOR CONTRIBUTIONS

S.S.H. and K.C.M.: conception and design, collection and/or assembly of data, data analysis and interpretation, manuscript writing; B.Y.K.B. and C.B.V.: collection and/or assembly of data, data analysis and interpretation, manuscript writing; J.K.L.: conception and design, data analysis and interpretation, manuscript writing, final approval of manuscript.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

J.K.L. is the president of a company to commercialize a hydrogel formulation from the University of California, Davis. The company is in the fundraising stage, there is no compensation for this work, and he is the inventor of the technology presented above. The other authors indicated no potential conflicts of interest.

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