Adipose Stem Cell Microbeads as Production Sources for Chondrogenic Growth Factors

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Microencapsulating stem cells in injectable microbeads can enhance delivery and localization, but their ability to act as growth factor production sources is still unknown. To address this concern, growth factor mRNA levels and production from alginate microbeads with encapsulated human adipose stem cells (ASC microbeads) cultured in both growth and chondrogenic media (GM and CM) were measured over a two week period. Human ASCs in microbeads were either commercially purchased (Lonza) or isolated from six human donors and compared to human ASCs on tissue culture polystyrene (TCPS). The effects of crosslinking and alginate compositions on growth factor mRNA levels and production were also determined. Secretion profiles of IGF-I, TGF-β3 and VEGF-A from commercial human ASC microbeads were linear and at a significantly higher rate than TCPS cultures over two weeks. For human ASCs derived from different donors, microencapsulation increased pthlh and both IGF-I and TGF-β3 secretion. CM decreased fgf2 and VEGF-A secretion from ASC microbeads derived from the same donor population. Crosslinking microbeads in $BaCl_2$ instead of $CaCl_2$ did not eliminate microencapsulation's beneficial effects, but did decrease IGF-I production. Increasing the guluronate content of the alginate microbead increased IGF-I retention. Decreasing alginate molecular weight eliminated the effects microencapsulation had on increasing IGF-I secretion. This study demonstrated that microencapsulation can enhance chondrogenic growth factor production and that chondrogenic medium treatment can decrease angiogenic growth factor production from ASCs, making these cells a potential source for paracrine factors that can stimulate cartilage regeneration.

Key Words: Microencapsulation, Adipose stem cells, Alginate, Growth factor delivery, Cartilage

Introduction

Adult stem cell therapies such as adipose stem cells (ASCs) are an attractive option for various clinical applications because of their accessibility and ability to differentiate into multiple cell types[1]. However, challenges related to their delivery, such as high injection pressures[2], low retention in the desired site[3] and low viability *in vivo* when injected or delivered on scaffolds[4, 5], may limit their effectiveness in clinical studies[6]. To address these problems, we have recently developed a microencapsulation technology where ASC microbeads under 200 microns can be injected under low injection pressures without any changes in shape, can maintain greater than 80% cell viability for at least 3 weeks post-injection and can be localized in the desired site *in vivo* for at least 3 months[2].

Although ASCs can be used for a variety of clinical applications, repairing cartilage focal lesions is an attractive option because of the tissue's limited regenerative capacity and the lack of an effective treatment[7, 8]. Current cell therapies, such as autologous chondrocyte implantation aim to directly regenerate cartilage by providing a source of cells that can then synthesize new tissue[9]. However, this strategy has had limited clinical adoption mainly due to high variability in

cartilage quality and functional outcomes[10, 11]. Therefore, a new paradigm has emerged in using stem cells as growth factor production sources to stimulate diseased or damaged musculoskeletal tissues like cartilage to regenerate themselves[12, 13].

We have previously showed that ASC microbeads consisting of alginate can be preconditioned with different medium treatments to secrete factors that affect chondrocyte proliferation and differentiation *in vitro* and cartilage regeneration *in vivo*[14, 15]. Additionally, we showed that the degradation rate of these microbeads can be controlled to affect cell release rates and growth factor production[16]. To further optimize ASC microbeads as growth factor production sources for regenerating cartilage, a few unanswered issues need to be addressed. First, even though the mass transfer properties of soluble compounds and growth factors in alginate have been characterized[17-19], it is unknown how microencapsulation affects growth factor production and secretion from ASCs derived from multiple donors. Additionally, it is unknown if chondrogenic medium can also affect growth factor production and secretion from microbeads encapsulated ASCs derived from multiple human donors. Finally, although the mass transfer properties of alginate hydrogels are dependent on the divalent

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crosslink[20, 21] and the type of alginate polymer used[17], the effects these parameters have on growth factor expression and production from ASCs is unknown. Therefore, the objectives of this study were to determine the time course of growth factor production and secretion from microencapsulated commercially available human ASCs cultured in growth and chondrogenic media, to determine the effects of microencapsulation and chondrogenic medium treatments on human ASCs derived from different donors and to determine the effect microbead composition has on growth factor expression and production.

Materials and Methods

Cell Isolation and Passaging

To determine the extent of growth factor production and secretion from ASC microbeads over time, first passage human ASCs from a 28 year old male donor were commercially obtained (Lonza, Basel, Switzerland) and cultured up to third passage in Lonza Mesenchymal Stem Cell Growth Medium (GM, Lonza, PT-3001) prior to microencapsulation. To test whether chondrogenic medium and microencapsulation had consistent effects on ASCs from different donors, adipose tissue was collected from six female patients ranging from 18 to 49 years of age (mean age ± standard error = 32.7±4.1) under an approved IRB protocol at Georgia Institute of Technology, Northside Hospital (Atlanta, GA) and Children's Healthcare of Atlanta (Atlanta, GA). Fat was obtained by breast reduction from five donors and abdominoplasty from one donor. All patients gave written consent to both the procedure and handling of fat thereafter. ASCs were isolated using a collagenase and dispase digestion cocktail as previously described[22]. Cells were then seeded at 5,000 cells/cm^2 and cultured in GM. When primary ASCs from three of the donors were at 90% confluence (P1 ASCs), they were trypsinized and microencapsulated immediately. Cells from the other 3 donors were pelleted at 500 g for 10 minutes, resuspended in GM with 5% DMSO at , cooled from room temperature to -80°C at 1°C/min, stored at -80°C overnight and transferred to liquid nitrogen for later use. After 2 to 8 months, these cryopreserved cells were then cultured up to third passage and microencapsulated (P3 ASCs). To determine the effects microbead composition had on growth factor production, cryopreserved ASCs from a 33 year old donor's breast tissue were cultured up to third passage and microencapsulated. Prior to microencapsulation, all ASCs were positive for CD73 and CD271 and negative for CD45, indicating that these cells still maintained the surface phenotype characteristic of multipotent ASCs.

Microencapsulation

For all studies, alginate powders (FMC BioPolymer, Sandvika, Norway) were UV light sterilized overnight and dissolved in 0.22 µm sterile filtered (Thermos Fisher Scientific, Rochester, NY, USA) saline (Ricca Chemical, Arlington, TX, USA) at 20 mg/mL. ASCs were then suspended in the alginate solution at 10^7 cells/mL. Microbeads containing the commercial ASCs seeded homogenously throughout the hydrogel were created using a Nisco Encapsulator VAR V1 LIN-0043 (Nisco Engineering AG, Zurich, Switzerland) at a 10 mL/hr flow rate, 0.12 mm inner diameter nozzle and 6kV/cm electrostatic potential[2, 23]. Microbeads were imaged with an inverted microscope (Motic, Richmond, British Columbia , Canada) and had a mean

diameter ± standard deviation of 122±15 µm (Motic Images Plus 2.0).

In studies determining growth factor production and secretion from ASC microbeads over time and in studies determining the effect of chondrogenic medium and microencapsulation on ASCs from multiple donors, microbeads with 130 kDa, 44% guluronate alginate (LVM, low viscosity high mannuronate) were crosslinked in a solution of 50 mM CaCl$_2$ (Sigma, St. Louis, MO, USA), 150 mM glucose (Sigma) and 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Sigma) at pH 7.3 for at least 15 minutes. To determine the effect Ca^{++} crosslinks had on growth factor production and secretion, ASC microbeads consisting of LVM were also crosslinked in a solution of 20 mM BaCl$_2$ (Sigma, St. Louis, MO, USA), 150 mM glucose and 15 mM HEPES at pH 7.3. To determine the effect alginate molecular weight and chemistry had on growth factor production and secretion, ASC microbeads were also made with the following alginates: 170 kDa, 66% guluronate (LVG, low viscosity high guluronate); <50 kDa, 44% guluronate (VLVM, very low viscosity high mannuronate); and 220kDa, 43% guluronate (MVM, medium viscosity high mannuronate). These ASC microbeads were crosslinked in the CaCl$_2$ solution as described above. For all studies, microbeads were washed three times in GM to remove any excess crosslinking solution. ASCs were also plated in 6-well tissue culture polystyrene (TCPS) plates (BD Biosciences, San Jose, CA, USA) for comparison.

Chondrogenic Treatment

Once ASCs in the 6-well plates reached 90% confluence, TCPS and microbead cultures were treated with either GM or chondrogenic medium (CM) consisting of Dulbecco's modified Eagle medium (DMEM) with 4.5g/L glucose and 1 mM sodium pyruvate (Mediatech, Manassas, VA, USA), 40 µg/ml proline (Sigma), 50 µg/ml ascorbate-2-phosphate (Sigma), 1% ITS+ (Sigma), 100 nM dexamethasone (Sigma), 10 ng/ml recombinant human transforming growth factor beta-1 (TGF-β1, R&D Systems, Minneapolis, MN, USA) and 100 ng/ml recombinant human bone morphogenic protein 6 (BMP-6, PeproTech, Rocky Hill, NJ, USA). For studies determining cumulative growth factor production and secretion from ASC microbeads over time, media were collected with each media change at 3, 5, 7, 10, 12, 14 and 15 days. For all subsequent studies, treatments lasted 7 and 14 days, RNA was collected 6 hours after the last media change as described below and media and ASC and lysates in 0.05% Triton X-100 (Sigma) were collected 24 hours after the last media change.

RNA Isolation

Alginate microbeads crosslinked in CaCl$_2$ (Ca microbeads) were uncrosslinked in 82.5 mM sodium citrate (Sigma) whereas alginate microbeads crosslinked in BaCl$_2$ (Ba microbeads) were uncrosslinked in 30 mM ethylenediaminetetraacetic acid (EDTA, Sigma) and 135 mM NaCl (Sigma). Released cells were then pelleted and washed two more times in their respective uncrosslinking solution to remove any residual alginate. TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was added to the cell pellet, which was fed through a QIAshredder (QIAGEN, Valencia, CA, USA) and RNA was isolated using chloroform and an RNeasy Kit (QIAGEN) as previously described[24]. A high capacity reverse transcription cDNA kit (Applied Biosystems, Carlsbad, CA, USA) was used to reverse transcribe 1 µg RNA to cDNA.

Quantifying mRNA Levels and Growth Factor Production

mRNA levels and secretion of multiple signaling molecules were measured to assess the therapeutic potential of ASC microbeads. FGF-2, IGF-I and PTHrP were investigated because of their ability to increase chondrocyte proliferation and regulate hypertrophy[3, 6, 7, 25]. TGF-β3 secretion was investigated since TGFs are potent stimulators of proteoglycan synthesis[8, 10]. However, TGF-β1 secretion was not investigated due to its large concentration in chondrogenic medium. Noggin was investigated because of its role in cartilage differentiation[26]. VEGF-A was investigated because of its detrimental effects on cartilage regeneration[14, 27]. Sox9,aggrecan, type II collagen, type X collagen and cartilage oligomeric matrix protein (COMP) were investigated since they are well-characterized markers for the different stages of chondrogenesis[28]. mRNA levels were normalized to that of ribosomal protein subunit 18 (Rps18), a common housekeeping gene[29].

Messenger RNA levels for the genes listed in Table 1 were quantified using real-time PCR with gene-specific primers using the Step One Plus Real-time PCR System and Power Sybr® Green Master Mix (Applied Biosystems) as previously described[30]. All primers were designed with Beacon Designer software (Premier Biosoft, Palo Alto, CA, USA) and synthesized by Eurofins MWG Operon (Huntsville, AL, USA) unless otherwise noted (Table 1). Growth factor production was quantified using ELISA (R&D Systems) and normalized

Table 1. Primer Sequences

Gene	Direction	Sequence
Acan	Sense	TCAGCGGTTCCTTCTCCA G
	Antisense	GCAGTTGTCTCCTCTTCTACG
Col2	Sense	CTGCTCGTCGCCGCTGTCCTT
	Antisense	AAGGGTCCCAGGTTCTCCATC
Col10	Sense	TTACGCTGAACGATACCAAATG
	Antisense	GCTCTCCTCTTACTGCTATACC
Comp	Sense	CCTGCGTTCTTCTGCTCAC
	Antisense	GCGTCACACTCCATCACC
Fgf2	Sense	GCGACCCTCACATCAAGC
	Antisense	AGCCAGTAATCTTCCATCTTCC
Igf1	Sense	GGAGGCTGGAGATGTATTG
	Antisense	GACTTCGTGTTCTTGTTGG
Nog	Sense	Global Gene Sequence (Qiagen)
	Antisense	
Pthlh	Sense	CTCGCTCTGCCTGGTTAG
	Antisense	CAATGCCTCCGTGAATCG
Rps18	Sense	CGTCTGCCCTATCAACTTTCG
	Antisense	CTTGGATGTGGTAGCCGTTTC
Sox9	Sense	GAGCAGCGAAATCAACGAGAAAC
	Antisense	ACAAAGTCCAAACAGGCAGAGAG
Tgfb1	Sense	CAACAATTCCTGGCGATACCTC
	Antisense	CCACGGCTCAACCACTGC
Tgfb2	Sense	CGAGAGGAGCGACGAAGAG
	Antisense	TAGAAAGTGGGCGGGATGG
Tgfb3	Sense	CAACGAACTGGCTGTCTG
	Antisense	CTCTGCTCATTCCGCTTAG
Vegfa	Sense	CTTGCCTTGCTGCTCTACC
	Antisense	TTCTGCCCTCCTCCTTCTG

to DNA content measured by a Quant-iT PicoGreen kit (Invitrogen). For the study investigating growth factor secretion over time, all values were normalized to the final DNA concentration at day 15. Growth factor retention within the microbead were measured as previously described[15]. Quantified mRNA levels are referred to by the name of the gene whereas quantified protein levels were referred to by the name of the growth factor.

Statistical Analysis

All experiments had six independent cultures per treatment group and were conducted multiple times to confirm the findings. For studies involving six donors, all data are presented in the form of treatment (i.e. CM or microencapsulation [μB]) over control (i.e. GM or TCPS). For these studies, the sample sizes were 6 donors (i.e. the mean of six samples from each of the six donors) and statistical differences between the treatment and control groups and among the different groups at 1 and 2 weeks were determined via a Mann-Whitney-Wilcoxon test (GraphPad Prism, La Jolla, CA, USA). The significance of medium, microencapsulation, culture time, passage number and their interactions on ASC growth factor mRNA levels and production were determined via a 4-way ANOVA (MATLAB, MathWorks, Natick, MA, USA). For studies that did not investigate ASCs from six donors, only data from a single representative experiment are shown and are expressed as means ± standard errors. Statistical differences among these experimental groups were determined via ANOVA with a post hoc Tukey test (GraphPad Prism). Linear regression was used to assess cumulative growth factor secretion profiles from ASC microbeads and monolayers (GraphPad Prism). All differences and effects were considered to be statistically significant if the p-value was less than 0.05.
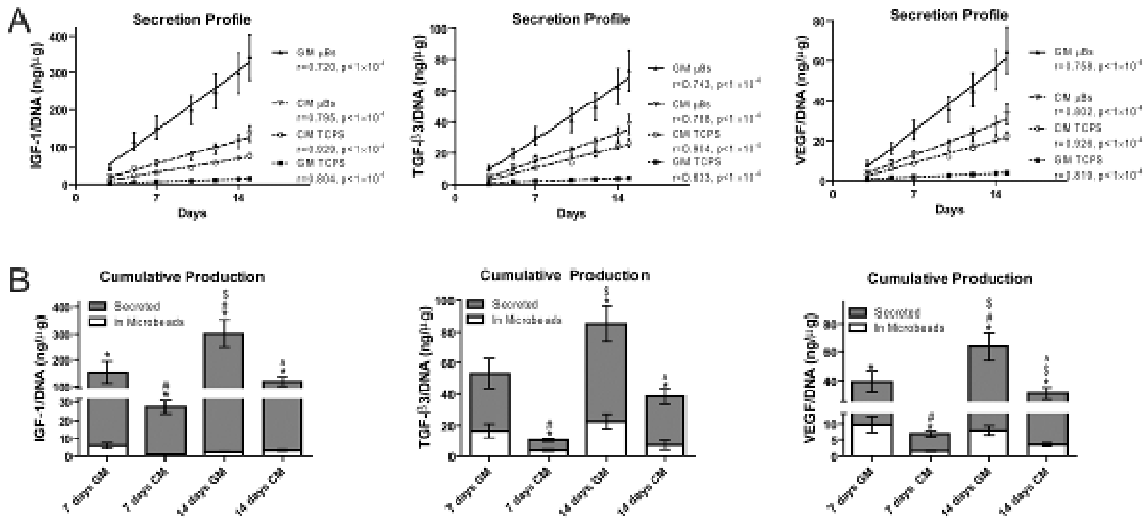
Results

Growth Factor Production and Secretion from ASC Microbeads over Time

Linear regressions of the cumulative secretion profiles of IGF-I, TGF-β3 and VEGF-A from all ASC cultures were linear over a 2 week period (Figure 1A). Specifically, microencapsulation was able to increase the rate of all measured growth factor secretion in both GM and CM and the rates of IGF-I, TGF-β3 and VEGF-A secretions from all ASC cultures were significantly different compared to each other (Figure 1A, $p<1\times10^{-4}$). Microbeads in CM secreted less IGF-I, TGF-β3 and VEGF-A than those in GM, but TCPS cultures in CM secreted more of the same growth factors than TCPS cultures in GM.

In GM, ASC microbeads secreted 25 fold more IGF-I, 2.2 fold more TGF-β3 and 3 fold more VEGF-A than what was retained within the microbead after 7 days (Figure 1B). In CM, ASC microbeads secreted 27 fold more IGF-I, 1.9 fold more TGF-β3 and 3.7 fold more VEGF-A than what was retained within the microbead after 7 days. Although cumulative production and secretion of growth factors from microbeads increased between 7 and 14 days in both GM and CM, the amount of growth factor retained within the microbead did not change after 7 days.

Effect of Chondrogenic Medium and Microencapsulation on ASCs from Multiple Donors

For ASCs derived from 6 different donors, CM had the most apparent effect on fgf2, vegfa and nog (Figure 2A). CM decreased fgf2 in TCPS cultures by 8.8 to 9.6 fold and microbeads by 3.7 to 4.2 fold at day 7 and 14 (Figure 2A). CM treatment for 7 and 14 days also decreased vegfa in the TCPS cultures and microbeads by 4.3 to 6.5 fold. 7 and 14 days of CM treatment increased nog in TCPS cultures by 66 and 146 fold respectively, but nog in microbeads only increased by 7.2 and 12.8 fold.

Figure 1. Growth Factor Production and Secretion from ASC Microbeads over Time. (A) Linear regressions of cumulative growth factor secretion profiles from ASC cultures with measurements at 3, 5, 7, 10, 12, 14 and 15 days and normalized to DNA content at day 15. ASCs were cultured in growth medium (GM) and chondrogenic medium (CM) on tissue culture polystyrene (TCPS) or within alginate microbeads (µB). (B) Cumulative growth factor production/DNA content secreted and maintained within ASC microbeads after 7 and 14 days in GM and CM. (n = 6 samples, mean±SE, *p<0.05 vs. in microbead, #p<0.05 vs. wk1 GM, $p<0.05 vs. wk1 CM, ^p<0.05 vs. wk2 GM).
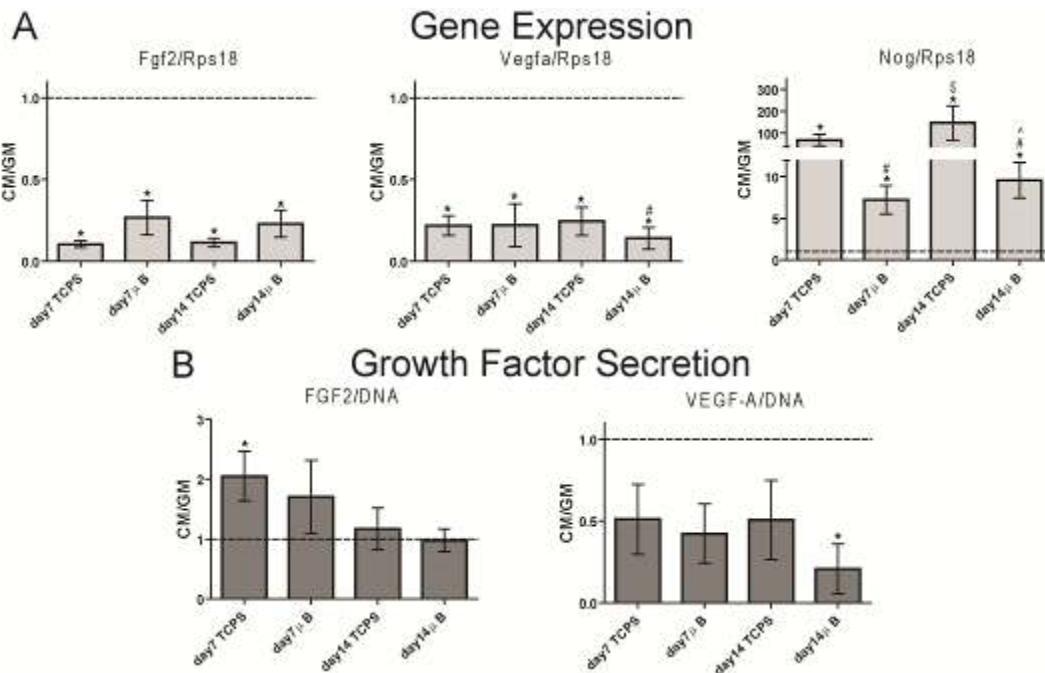

Figure 2. Effect of Chondrogenic Medium on Growth Factor mRNA Levels and Secretion from ASCs Isolated from Different Donors. (A) mRNA levels and (B) growth factor secretion/DNA content on day 7 and day 14 from ASCs cultured in chondrogenic medium (CM) on tissue culture polystyrene (TCPS) and within alginate microbeads (µB) normalized to matching 2-D and 3-D cultures in growth medium (GM) (n = 6 donors, mean±SE, *p<0.05 CM vs. GM, #p<0.05 vs. wk1 TCPS, $p<0.05 vs. wk1 µB, ^p<0.05 vs. wk2 TCPS). mRNA and secretion levels of other genes and growth factors can be found in Table 2.

CM did not decrease FGF-2 secretion on day 7 or 14 (Figure 2B) as FGF-2 production from TCPS cultures in CM was 2 fold higher than TCPS cultures in GM on day 7. VEGF-A secretion from microbeads in CM was 4.9 fold lower than microbeads in GM on day 14. CM also increased *acan*, *col2*, *col10*, *comp*, *igf1*, *sox9* and *tgfb3* and decreased *pthlh* in ASC cultures (Table 2).

Microencapsulation had differential effects on chondrocyte phenotypic mRNA levels of ASCs. After 7 days in CM, microencapsulation decreased *acan* and *comp* by 3.3 and 5.3 fold respectively but increased *col2* by 6.2 fold (Figure 3).

Table 2. Effect of Chondrogenic Medium and Microencapsulation on ASCs from Multiple Donors

	CM/GM			
	wk1 TCPS	wk1 µB	wk2 TCPS	wk2 µB
mRNA Levels				
Acan/Rps18	*94.5±41.3	*9.9±4.0	*184.7±95.6	*18.3± 5.6
Col2/Rps18	3.6± 1.4	2.4±1.5	2.5± 0.8	10.0± 4.5
Col10/Rps18	*48.8±33.8	*14.9±4.2	*11.7± 7.9	*23.4±11.2
Comp/Rps18	*8.7± 2.1	*4.1±1.0	*8.0± 1.9	*8.2± 2.4
Igf1/Rps18	*2.8± 0.8	1.6±0.6	4.6± 1.3	*3.3± 0.6
Pthlh/Rps18	*0.6± 0.2	*0.1±0.0	0.5± 0.1	*0.1± 0.0
Sox9/Rps18	1.8± 0.3	*1.9±0.3	*2.2± 0.6	*2.4± 0.2
Tgfb1/Rps18	*2.0± 0.2	1.5±0.4	*1.6± 0.2	1.0± 0.2
Tgfb2/Rps18	1.4± 0.3	1.0±0.5	1.5± 0.3	1.1± 0.3
Tgfb3/Rps18	*8.4± 1.8	0.6±0.2	*5.0± 1.2	0.6± 0.2
Growth Factor Secretion				
IGF-I/DNA	*4.7± 2.4	1.9±0.3	6.0± 2.9	2.0± 0.7
TGF-β3/DNA	1.2± 0.1	1.0±0.1	1.1± 0.2	1.0± 0.3
	µB/TCPS			
	wk1 GM	wk1 CM	wk2 GM	wk2 CM
mRNA Levels				
Fgf2/Rps18	*0.3±0.1	*0.5±0.1	0.6±0.3	0.7±0.3
Nog/Rps18	1.1±0.6	*0.1±0.0	1.4±0.7	*0.2±0.1
Tgfb1/Rps18	1.9±0.3	1.5±0.4	1.8±0.3	1.0±0.2
Tgfb2/Rps18	6.1±1.7	2.0±0.5	2.4±0.6	1.6±0.3
Vegfa/Rps18	2.2±0.7	0.9±0.3	2.0±0.7	1.3±0.5
Growth Factor Secretion				
FGF-2/DNA	0.8±0.4	1.6±1.4	*6.1±2.3	5.0±2.4
VEGF-A/DNA	0.5±0.2	1.1±0.7	1.3±0.5	2.1±1.1

*p<0.05 CM vs. GM for CM/GM values and µB vs.TCPS for µB/TCPS values.

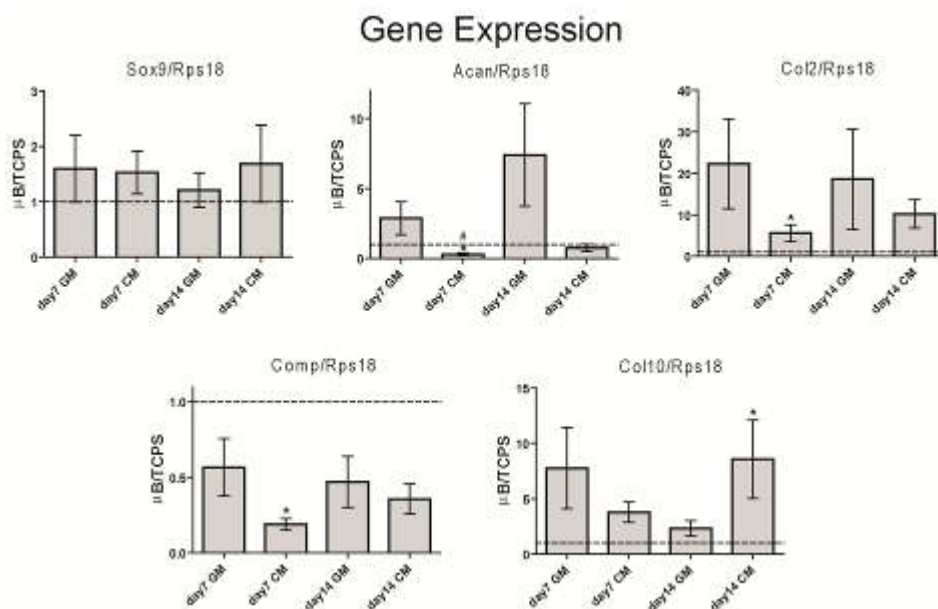Additional values for CM/GM and µB/TCPS can be found in Figures 2, 3 and 4.

Figure 3. Effect of Microencapsulation on Phenotypic mRNA Levels of ASCs Isolated from Different Donors. (A) mRNA levels on day 7 and day 14 of ASC microbeads cultured in growth medium or chondrogenic medium (CM) normalized to ASCs on tissue culture polystyrene (TCPS) cultured in matching media. (n = 6 donors, mean±SE, *p<0.05 µB vs. TCPS, #p<0.05 vs. wk1 GM, $p<0.05 vs. wk1 CM, ^p<0.05 vs. wk2 GM). mRNA levels of ASCs cultured in chondrogenic medium (CM) on tissue culture polystyrene (TCPS) and within alginate microbeads (µB) normalized to matching 2-D and 3-D cultures in growth medium can be found in Table 2.

After 14 days in CM, microencapsulation increased *col10* by 8.6 fold. Meanwhile, microencapsulation had no effect on *sox9* in either GM or CM. For growth factor mRNA levels, microencapsulation had the most apparent effect on *igf1, pthlh* and *tgfb3* in ASCs derived from 6 different donors (Figure 4A). Microencapsulation consistently decreased *igf1* for 6 different donors with a 4.2 fold decrease in CM after 7 days (Figure 4A). The effects of microencapsulation on *pthlh* and *tgfb3* were media dependent. Microencapsulation only increased *pthlh* in GM by 5.3 and 3.7 fold after 7 and 14 days respectively. Microencapsulation increased *tgfb3* in GM by 8.2 and 4.8 fold after 7 and 14 days respectively but decreased *tgfb3* in CM by 2.3 fold at day 7.

Microencapsulation increased IGF-I secretion at day 7 in GM

and CM by 11.9 and 7.6 fold respectively (Figure 4B). Microencapsulation also increased TGF-β3 secretion by 2.3 fold at day 7 in CM and by 4.6 fold on day 14 in GM. VEGF-A secretion from ASCs was not affected by microencapsulation, but microencapsulation did increase FGF-2 secretion at day 14 in GM and decreased *nog* expression after 7 and 14 days in CM (Table 2).

4-way ANOVA showed that media treatment significantly influenced *acan*, *col10*, *comp*, *fgf2*, *igf1*, *nog pthlh* and *sox9* as well as IGF-I and VEGF-A secretion (Table 3).Microencapsulation significantly affected *col2*, *col10*, *comp*, *fgf2*, *igf1*, *nog* and *tgfb2* as well as IGF-I and TGF-β3 secretion. Seven days versus 14 days in culture did not
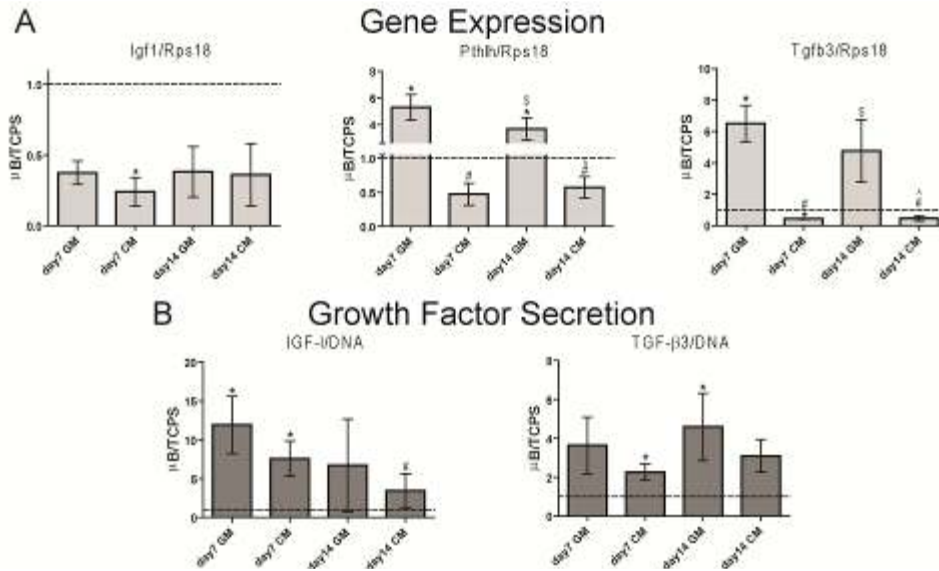

Figure 4. Effect of Microencapsulation on Growth Factor mRNA Levels and Secretion from ASCs Isolated from Different Donors. (A) mRNA levels and (B) growth factor secretion/DNA content on day 7 and day 14 from ASC microbeads cultured in growth medium or chondrogenic medium (CM) normalized to ASCs on tissue culture polystyrene (TCPS) cultured in matching media (n = 6 donors, mean±SE, *p<0.05 µB vs. TCPS, #p<0.05 vs. wk1 GM, $p<0.05 vs. wk1 CM, ^p<0.05 vs. wk2 GM). mRNA and secretion levels of other genes and growth factors can be found in Table 2.

Table 3. P-values of 4-Way ANOVA of Medium, Microencapsulation (µB), Culture Time (Time) and Passage Number (Passage)

	Medium	µB	Time	Passage	µB × Medium	µB × Time	µB × Passage
mRNA Levels							
Acan/Rps18	*<0.001	0.822	0.328	0.128	0.058	0.453	0.217
Col2/Rps18	0.180	*0.001	0.853	*0.006	0.638	0.962	0.101
Col10/Rps18	*<0.001	*<0.001	0.172	*0.003	0.524	0.546	0.673
Comp/Rps18	*<0.001	*0.001	0.339	0.198	0.580	0.720	0.299
Fgf2/Rps18	*<0.001	*<0.001	0.931	*0.070	0.074	0.544	0.831
Igf1/Rps18	*0.022	*<0.001	0.546	0.612	0.537	0.893	0.978
Nog/Rps18	*<0.001	*<0.001	0.795	*0.003	*0.019	0.588	0.232
Pthlh/Rps18	*<0.001	0.428	0.335	*<0.001	*<0.001	0.972	0.386
Sox9/Rps18	*0.002	0.352	0.818	0.654	0.707	0.787	0.151
Tgfb1/Rps18	0.465	0.456	0.590	*0.001	0.596	0.827	0.991
Tgfb2/Rps18	0.968	*0.001	0.328	*<0.001	0.496	0.130	0.703
Tgfb3/Rps18	0.141	0.393	0.814	*0.045	*<0.001	0.452	0.943
Vegfa/Rps18	*0.001	0.895	0.881	*0.017	0.626	0.931	0.463
Protein Secretion							
FGF-2/DNA	0.799	0.132	*0.007	0.250	0.730	0.931	*0.031
IGF-I/DNA	*0.026	*<0.001	0.184	*<0.001	0.379	*0.028	*0.023
TGF-β3/DNA	0.965	*<0.001	*0.001	*0.001	0.825	0.108	0.181
VEGF-A/DNA	*<0.001	*0.029	0.512	0.947	0.981	0.556	0.229

*p<0.05 CM vs GM for medium, µB vs.TCPS for µB, 1 week vs. 2 weeks for time, 1 passage vs. 3 passages for passage.

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influence baseline gene expression but did influence FGF-2 and TGF-β3 secretion. The interaction between medium and microencapsulation had significant effects on *nog*, *pthlh* and *tgfb3* but not on growth factor secretion. Although there were significant differences in baseline growth factor expression and secretion between freshly isolated P1 ASCs and cryopreserved P3 ASCs, the interaction between microencapsulation and cell passage did not affect mRNA levels but did affect FGF-2 and IGF-I secretion.

Effect of Microbead Composition on Growth Factor Production and Secretion

Igf1 in Ba microbeads was less than that of Ca microbeads (Figure 5A). Ba microbeads also secreted less IGF-I than Ca microbeads on day 7 (Figure 5B) and Ba microbeads maintained 10 fold less IGF-I within than Ca microbeads over 7 days (Figure 5C). However, both Ca microbeads and Ba microbeads secreted 10.9 and 4.4 fold greater IGF-I than TCPS cultures on day 7 (Figure 5B). *Pthlh* and *tgfb3* in Ba microbeads were not statistically different from Ca

microbeads and was 28 to 29 fold greater than TCPS cultures (Figure 5A). Ca and Ba microbeads secreted similar amounts of TGF-β3 on day 7 (Figure 5B) and although Ca microbeads maintained more TGF-β3 than Ba microbeads (Figure 5C), this difference was not significant.

MVM microbeads had 2.3 fold lower *pthlh* than LVM microbeads, but all microbeads had at least 24 fold higher *pthlh* than TCPS cultures (Figure 6A). LVG microbeads had 2.9 fold higher *tgfb3* than LVM, but all microbeads still had at least 22 fold higher *tgfb3* than TCPS cultures. Although the different alginate polymers did not have a significant effect on *igf1* (Figure 6A), VLVM microbeads secreted 2.8 fold less IGF-I than LVM microbeads on day 7 (Figure 6B). Additionally, LVM, LVG and MVM microbeads secreted 2.9 to 4.3 fold more IGF-I than TCPS cultures, but VLVM microbeads secreted similar levels of IGF-I to TCPS cultures on day 7. LVG microbeads retained 2.4 fold more IGF-I than LVM microbeads over 7 days (Figure 6C). The molecular weight and guluronate content of the alginate polymer had no effect on TGF-β3 secreted or retained in the microbeads (Figure 6B, C).
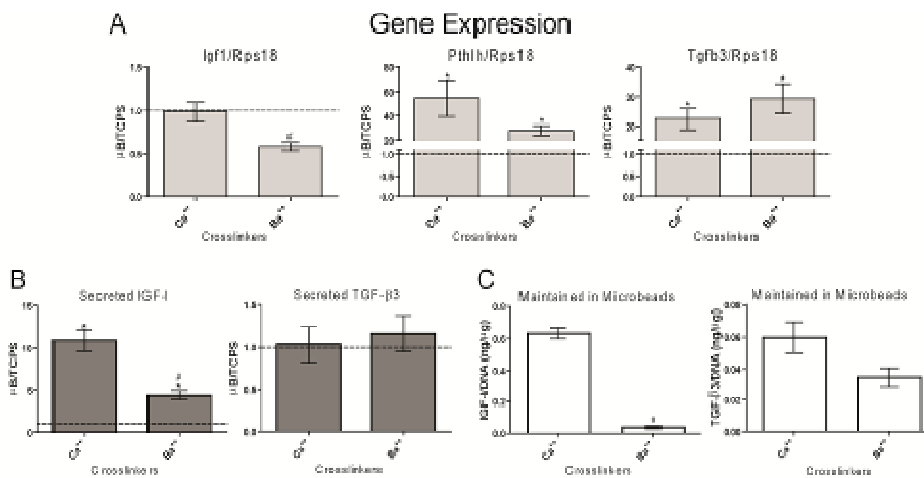

Figure 5. Effect of Divalent Crosslinks in ASC Microbead on Growth Factor mRNA Levels and Production. (A) mRNA levels and (B) growth factor secretion/DNA content on day 7 from ASC microbeads crosslinked in CaCl₂ Ca++) or BaCl₂ (Ba++), cultured in growth medium and normalized to ASCs on tissue culture polystyrene (TCPS). (C) Growth factor retained within microbeads normalized to DNA content (n = 6 samples, mean±SE, *p<0.05 μB vs. TCPS, #p<0.05 vs. Ca++).
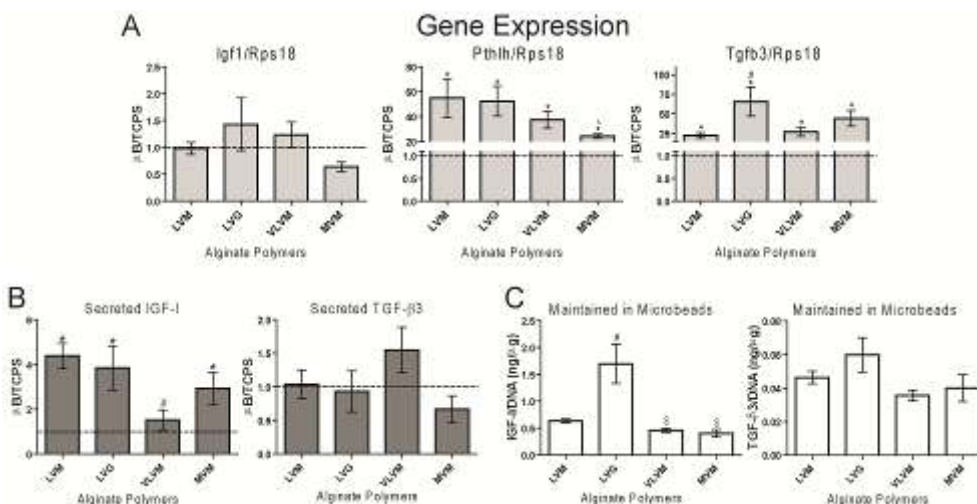

Figure 6. Effect of Alginate Molecular Weight and Chemistry in ASC Microbead on Growth Factor mRNA Levels and Production. (A) Growth factor mRNA and (B) growth factor secretion/DNA content on day 7 from ASC microbeads consisting of LVM, LVG, VLVM and MVM alginates; cultured in growth medium; and normalized to ASCs on tissue culture polystyrene (TCPS). (C) Growth factor retained in microbeads normalized to DNA content (n = 6 samples, mean±SE, *p<0.05 μB vs. TCPS, #p<0.05 vs. Ca++).

Discussion

Although alginate microbeads have long been viewed as a protective vehicle for cell therapies, they have a more promising end use in controlling the production, secretion and localization of growth factors synthesized by stem cells in order to repair damaged or diseased tissues. This study demonstrated that microencapsulation alone can enhance growth factor production by ASCs and that ASC microbeads can secrete different growth factors at a constant rate over a two week period. Important for cartilage regeneration, microencapsulation increased IGF-I and TGF-β3 secretion and chondrogenic medium decreased VEGF-A secretion from ASCs derived from multiple donors (Figure 7).

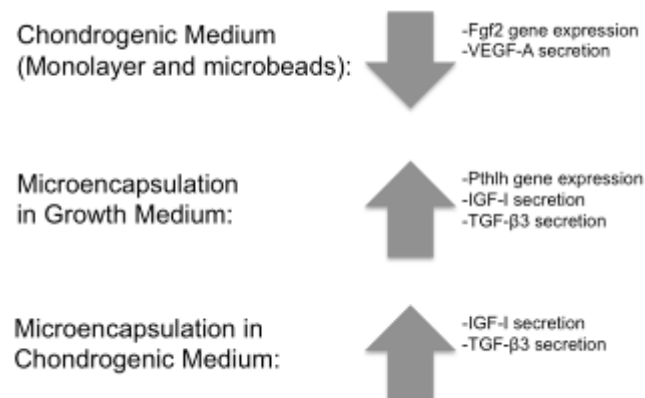Additionally, IGF-I production and secretion were higher for

Figure 7. Summative Affect of Chondrogenic Medium and Microencapsulation on ASCs

alginate microbeads with larger molecular weights and calcium crosslinks. These results suggest that a biomaterials-mediated approach using microbeads, in addition to medium induction can be used to tailor growth factor production for cartilage regeneration. This approach has been supported by other studies, which have shown barium crosslinks reducing protein secretion from alginate beads[21] and low guluronate content alginate hydrogels with a more flexible polymer backbone having greater solute diffusivity[17]. Furthermore, our findings are consistent with previous studies in which alginate microcapsules maintained growth factor gene expression and sustained growth factor release for at least 22 days while promoting bone and myocardial muscle regeneration[31, 32].

Alginate encapsulation is well known to restore the chondrocytic phenotype of dedifferentiated chondrocytes and to enhance the differentiation of mesenchymal stem cells[33, 34] due to the round cell morphology and cell-cell interactions imparted by high density encapsulation[35, 36]. However, alginate microbeads may have an even more effective application in controlling the localization of chondrogenic growth factors secreted by ASCs. This study showed that alginate microbeads can retain endogenously produced IGF-I and TGF-β3. Although not directly observed in this study, alginate degradation and electrostatic interaction between the alginate and growth factor can influence growth factor diffusive properties and subsequent secretion from alginate microbeads[18, 37] and may partially explain the discrepancies between mRNA and secretion levels of certain growth factors. Moreover, although chondrogenic medium and

alginate chemistry were the main drivers for manipulating growth factor secretion from ASC microbeads in this study, controlled degradation of these hydrogels can further regulate the temporal release of these growth factors[16, 38-40]. Incorporating biomimetic peptides, such as RGD, within the microbeads can also manipulate the expression of certain growth factors[32]. Therefore, these diverse attributes imparted by the hydrogel's microenvironment make ASC microbeads ideal candidates as trophic factor production sources for cartilage regeneration.

Interestingly, microencapsulation increased mRNA levels for both PTHrP and TGF-β3 in ASCs in growth medium but decreased mRNA and the secretion profile of TGF-β3 in chondrogenic medium. Calcium has been shown to increase secretion of PTHrP from human cells[41], but this current study showed that increases in PTHrP and TGF-β3 mRNA levels were independent of the calcium crosslinks. Previous studies have shown that soluble components of serum can hinder chondrogenesis[42] whereas TGF-β1 and BMP-6 in chondrogenic medium can increase chondrocytic phenotype in ASCs[1, 34, 43]. Although the effects of individual serum components on growth factor production were not directly measured in this study, microencapsulation may have hindered the mass transport of these inhibitory soluble factors in growth medium and stimulatory soluble factors (e.g., TGF-β1, BMP-6) in chondrogenic medium. This potential explanation is supported by the detectable amounts of TGF-β3 maintained within the microbead, but disproved by the even larger amounts of TGF-β3 that were secreted over time. Regardless, no combination of microencapsulation or medium treatement optimized the ideal growth factor secretion profile of increased chondrogenic factor production (e.g., TGF-β3, IGF-I) along with decreased angiogenic factor production (e.g., VEGF-A). Therefore, subsequent studies may have to investigate different media treatments in addition to microbead formulations as we have previously explored[15].

Before the therapeutic benefit of ASC microbeads as growth factor production sources for cartilage regeneration is fully known, the effect of donor and isolation variability on ASC growth factor secretion and the subsequent effects these secreted factors have in vivo need to be explored. Several studies have investigated whether donor age or body mass index affect ASC density in tissue and their chondrogenic potential, but the results have been conflicting[44-46]. These discrepancies may be due to differences in tissue harvesting techniques, anatomical location, passage number and donor sex, all of which have been shown to affect ASC yield and differentiation potential[36, 44, 47, 48].

The variability in these parameters in this current study may have caused the inconsistencies among the different cell culture experiments, specifically in the effect of microencapsulation on growth factor secretion between different studies. Despite these discrepancies, ASCs and ASC microbeads implanted in focal cartilage defects have shown promise for cartilage regeneration in multiple studies[15, 49] and support the clinical potential of ASC microbeads as growth factor production sources for cartilage regeneration.

Conclusion

Human ASC microbeads secreted IGF - I, TGF - β3 and

VEGF-A at a constant rate in both growth and chondrogenic over the course of 7 days, ASC microbeads secreted 2 to 27 fold more of these growth factors than what was maintained within the alginate. Microencapsulation consistently increased mRNAs for PTHrP and secretion of IGF-I and TGF-β3 from ASCs from multiple donors. Meanwhile, chondrogenic medium consistently decreased mRNAs for FGF-2 and secretion of VEGF-A from ASC microbeads derived from multiple donors. Crosslinking microbeads in $BaCl_2$ instead of $CaCl_2$ did not eliminate the beneficial effects of microencapsulation, but did decrease IGF-I mRNA and production. Increasing the guluronate content of the alginate microbead increased TGF-β3 mRNA and IGF-I retained within the microbead. Decreasing the molecular weight of the alginate used eliminated the beneficial effects microencapsulation had on IGF-I secretion while increasing the molecular weight of alginate used decrease PTHrP mRNA. This study, in conjunction with prior findings, demonstrated that ASC microbeads may be a reliable source for delivering multiple growth factors to facilitate cartilage regeneration.

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Abbreviations:

ACAN:	Aggrecan
ANOVA:	Analysis of Variance
ASCs:	Adipose Stem Cells
Ba++:	Barium Ion
BaCl$_2$:	Barium Chloride
BMP:	Bone Morphogenetic Protein
cDNA:	Complementary Deoxyribonucleic Acid
Ca++:	Calcium Ion
CaC$_{l2}$:	Calcium Chloride
CM:	Chondrogenic Medium
COL2:	Type-II Collagen
COL10:	Type-X Collagen
COMP:	Cartilage Oligomeric Matrix Protein
DMEM:	Dulbecco's Modified Eagle's Medium
DMSO:	Dimethyl Sulfoxide
ELISA:	Enzyme-Linked Immunosorbent Assay
FGF:	Fibroblast Growth Factor
GM:	Growth Medium
IGF:	Insulin-like Growth Factor
LVG:	Low Viscosity High Guluronate
LVM:	Low Viscosity High Mannuronate
mRNA:	Messenger Ribonucleic Acid
MVM:	Medium Viscosity High Mannuronate
Nog:	Noggin
μB:	Microbead
PCR:	Polymerase Chain Reaction
PTHrP:	Parathyroid Hormone-related Peptide
PTHLH:	Parathyroid Hormone Linked Hormone
RGD:	Arginine-Glycine-Aspartic Acid
RPS18:	Ribosomal Protein S18
SE:	Standard Error
SOX9:	Sex Determining region Y-Box containing Gene 9
TCPS:	Tissue Culture Polystyrene
TGF-β:	Transforming Growth factor-Beta
UV:	Ultraviolet
VEGF-A:	Vascular Endothelial Growth factor-A
VLVM:	Very Low Viscosity High Mannuronate

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