Tailoring Adipose Stem Cell Trophic Factor Production with Differentiation Medium Components to Regenerate Chondral Defects

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Recent endeavors to use stem cells as trophic factor production sources have the potential to translate into viable therapies for damaged or diseased musculoskeletal tissues. Adipose stem cells (ASCs) can be differentiated into chondrocytes using the chondrogenic medium (CM), but it is unknown if this approach can optimize ASC growth factor secretion for cartilage regeneration by increasing the chondrogenic factor production, while decreasing angiogenic and hypertrophic factor production. The objective of this study was to determine the effects the CM and its components have on growth factor production from ASCs to promote cartilage regeneration. ASCs isolated from male Sprague-Dawley rats and cultured in monolayer or alginate microbeads were treated with either the growth medium (GM) or the CM for 5 days. In subsequent studies, ASC monolayers were treated with either the GM supplemented with different combinations of 50 $\mu\text{g}/\text{mL}$ ascorbic acid-2-phosphate (AA2P), 100 nM dexamethasone (Dex), 10 ng/mL transforming growth factor (TGF)- β 1, and 100 ng/mL bone morphogenetic protein (BMP)-6 or with the CM excluding different combinations of AA2P, Dex, TGF- β 1, and BMP-6. mRNA levels and growth factor production were quantified at 8 and 24 h after the last media change, respectively. The CM increased chondrogenic factor secretion (TGF- β 2, TGF- β 3, and insulin-like growth factor [IGF]-I) and decreased angiogenic factor production (the vascular endothelial growth factor [VEGF]-A, the fibroblast growth factor [FGF]-2). Microencapsulation in the GM increased production of the chondrogenic (IGF-I, TGF- β 2) and angiogenic (VEGF-A) factors. AA2P increased secretion of chondrogenic factors (IGF-I, TGF- β 2), and decreased angiogenic factor (VEGF-A) secretion, in addition to decreasing mRNA levels for factors associated with chondrocyte hypertrophy (FGF-18). Dex increased mRNA levels for hypertrophic factors (BMP-2, FGF-18) and decreased angiogenic factor secretion (VEGF-A). TGF- β 1 increased angiogenic factor production (FGF-2, VEGF-A) and decreased chondrogenic factor mRNA levels (IGF-I, PTHrP). BMP-6 increased hypertrophic mRNA levels (FGF-18) and chondrogenic factor production (TGF- β 2). When ASC microbeads preconditioned with the CM were implanted in a focal cartilage defect and immobilized within an RGD-conjugated hydrogel, tissue infiltration from the edges of the defect and perichondrium was observed. These results show that differentiation media components have distinct effects on ASC's production of angiogenic, chondrogenic, and hypertrophic factors and that AA2P may be the most beneficial CM component for preconditioning ASCs to stimulate cartilage regeneration.

Introduction

ADIPOSE STEM CELLS (ASCs) have been considered a promising candidate for cartilage repair because of their easy accessibility and chondrogenic potential.^{1,2} More recently, ASCs have demonstrated the ability to secrete trophic factors that can potentially stimulate endogenous cartilage regeneration,³ eliminating the need for ASCs to directly replace damaged chondrocytes or synthesize new cartilagi-

nous tissue. However, ASCs secrete other factors that can delay or even inhibit cartilage repair. Specifically, the vascular endothelial growth factor (VEGF)-A, an angiogenic growth factor that is secreted in large quantities by ASCs,^{4,5} has been shown to increase matrix metalloproteinase expression in chondrocytes⁶ and is highly expressed in osteoarthritic cartilage.⁷ Additionally, prolonged exposure to hypertrophic growth factors like the fibroblast growth factor (FGF)-18 and the bone morphogenetic protein (BMP)-2 can

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lead to hypertrophic differentiation, calcification, skeletal vascularization, and subsequent bone formation.^{8–10} Growth factors, such as the FGF-2, insulin-like growth factor [IGF]-I, and PTHrP can increase chondrocyte proliferation and regulate hypertrophy,^{11–14} while growth factors, such as the transforming growth factor (TGF)- β 1 and TGF- β 2, can stimulate proteoglycan synthesis.^{15,16} Furthermore, BMP inhibitors like noggin have an important role in regulating cartilage differentiation and endochondral ossification.¹⁷ Therefore, well-defined methods that increase ASC secretion of factors that promote chondrocyte proliferation and cartilaginous tissue synthesis, decrease secretion of angiogenic factors, and limit secretion of hypertrophic factors from ASCs are needed to treat chondral defects effectively.

Although viral and nonviral genetic manipulations of ASCs can be used to increase or decrease secretion of specific trophic factors, their ability to target only one gene at a time limits their therapeutic potential since cartilage formation is orchestrated by numerous growth factors and other signaling molecules.^{8,18} Furthermore, the potentially harmful side effects of genetic manipulations have hindered the clinical potential of these techniques.^{18,19} Therefore, preconditioning stem cells *in vitro* to obtain a desired secretory profile has also been suggested. While hypoxic exposure, heat induction, and biophysical stimulation have previously been used to augment the paracrine actions of stem cells,^{18,20,21} pharmacological treatments (chemical compounds) and biological treatments (growth factors and hormones) may provide the most precision in controlling the intensity and time of preconditioning. Additionally, providing different structural microenvironments (three-dimensional [3D] scaffolds, cell-cell interactions), such as microencapsulation, may further control the secretome of stem cell therapies *in vitro* and *in vivo*. Microencapsulation of ASCs may also provide a delivery method to localize these therapies.

The effects of differentiation media, such as the chondrogenic medium (CM), on multiple stem cell sources under varying structural environments are well defined.^{1,22,23} However, the effect of short-term exposure to these media on trophic factor production by ASCs is unknown. To decide if the CM and its individual components could be used to effectively precondition ASCs to secrete factors for regenerating cartilage, the objectives of this study were to determine the effect the CM has on ASC growth factor secretion in different structural environments, to determine the effect different CM components have on ASC growth factor production, and to determine if ASCs delivered *in vivo* via microbeads could promote cartilage regeneration.

Materials and Methods

Cell isolation

ASCs were isolated from the inguinal fat pads of six 125g male Sprague-Dawley rats (Harlan Laboratories, Indianapolis, IN) as previously described^{24,25} and cultured in the Lonza Mesenchymal Stem Cell (MSC) Growth Medium (GM, Lonza, Walkersville, MD). After one passage, these cells were pooled together and were negative for CD45 and positive for CD73 and CD271.²⁵ Costochondral chondrocytes from the ribs of 125g male Sprague-Dawley rats were isolated as described previously.^{26,27} Primary resting zone cells were cultured in the Dulbecco's modified Eagle's medium

(DMEM) containing 10% fetal bovine serum (FBS) and 50 μ g/mL ascorbic acid (Invitrogen, Carlsbad, CA) until fourth passage before experimental analysis. These cells continued to express type II collagen, aggrecan, and the cartilage oligomeric matrix protein (COMP).²⁶

Microencapsulation

When primary ASCs reached 90% confluence, they were harvested by trypsinization and microencapsulated in low molecular weight ($\sim$ 150 kDa) alginate with a high mannuronate to guluronate ratio (40% guluronate; FMC BioPolymer, Sandvika, Norway) as previously described,^{28,29} resulting in cell viabilities over 80% for at least 2 weeks postmicroencapsulation. The alginate (20 mg/mL) was dissolved in sterile-filtered saline (Ricca Chemical, Arlington, TX). Cells were suspended in the alginate solution at a concentration of 25×10^6 cells/mL. Microbeads were formed using a NiscoEncapsulator VAR V1 LIN-0043 (Nisco Engineering AG, Zurich, Switzerland) and a crosslinking solution of 50 mM CaCl₂, 150 mM glucose, and a 15 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer, pH=7.3; Sigma, St. Louis, MO). Microbeads were washed three times in the GM before cell culture studies. First passage ASCs were also plated in six-well plates.

Cell culture

Once first passage ASCs reached 90% confluence, ASC monolayers and microbeads were then treated for 5 days with either the GM or the CM consisting of the DMEM containing 4.5g/L glucose with 1 mM sodium pyruvate (Mediatech, Manassas, VA), 40 μ g/mL proline (Sigma), 50 μ g/mL ascorbic acid 2-phosphate (AA2P; Sigma), 1% ITS+ (Sigma), 100 nM dexamethasone (Dex, Sigma), 10 ng/mL recombinant human transforming growth factor beta-1 (TGF- β 1; R&D Systems, Minneapolis, MN), and 100 ng/mL of the recombinant human bone morphogenic protein 6 (BMP-6; PeproTech, Rocky Hill, NJ). TGF- β 1 and BMP-6 were included in the medium based on the observation that this formulation is able to effectively induce chondrogenesis.¹ Moreover, BMP-6 increases TGF- β receptor 1 expression, enhancing the ability of ASCs to respond to TGF- β treatment.³⁰ A lower concentration of BMP-6 was used in the present study, since it was previously shown to support chondrocyte differentiation of ATDC5 cells.³¹ In subsequent experiments, ASC monolayers were treated for 5 days with either the GM that was supplemented with different combinations of AA2P, Dex, TGF- β 1, and BMP-6 or with the CM that lacked different combinations of AA2P, Dex, TGF- β 1, and BMP-6. Once media were changed on the fifth day, RNA was collected after 8 h as described below. Conditioned media and ASCs were collected after 24 h, and ASCs were lysed in 0.05% Triton X-100. Monolayer fourth passage chondrocytes cultured in the DMEM, 10% FBS, and 50 μ g/mL ascorbic acid, and Sprague-Dawley-derived clone 9 liver cells (ATCC, Manassas, VA) cultured in the F12K medium and 10% FBS served as a reference. All media contained 1% penicillin and streptomycin.

RNA isolation and reverse transcription

Alginate microbeads were uncrosslinked in 82.5 mM sodium citrate (Sigma), pelleted at 500 g for 10 min, and washed two more times in sodium citrate to remove any

residual alginate. The TRIzol reagent (Invitrogen) was added to the resulting cell pellet, homogenized using a QIAshredder (QIAGEN, Valencia, CA), and RNA was isolated using chloroform and an RNeasy Kit (QIAGEN) as previously described.³² 1 µg RNA was then reverse transcribed to cDNA using a High Capacity Reverse Transcription cDNA kit (Applied Biosystems, Carlsbad, CA).

Microarray analysis

cDNA was converted into cRNA using a RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Biotin-labeled cRNA was cleaned up using a GeneChip Sample Cleanup Module (Affymetrix, Inc., Santa Clara, CA) and fragmented at 94°C in the fragmentation buffer for 35 min. Following fragmentation, 15 µg biotinylated cRNA was hybridized to an Affymetrix Rat Genome GeneChip (Rat 230_2.0) at 45°C for 16 h, washed, stained with streptavidin phycoerythrin (Fluidics Station 400, Affymetrix), and scanned according to the manufacturer's guidelines. The GeneChips were then assessed for data quality using Affymetrix-developed quality controls. Data analysis was performed using GeneSifter (Geospiza, Seattle, WA) with significant differences in mRNA levels being defined as a threefold difference. Significant differences in mRNAs of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways were defined as z-scores greater than 2.0. RNA isolated from rat liver tissue served as a reference control.

Growth factor mRNA levels and production

Because chondrogenesis is a complex process orchestrated by a wide array of growth factors, mRNA levels and production of paracrine factors involved in chondrocyte proliferation, proteoglycan synthesis, hypertrophic differentiation, and vascular invasion were quantified. Along with these paracrine factors, mRNAs for chondrocytic markers were also quantified as previously described 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).³³ All primers were designed using Beacon Designer software (Premier Biosoft, Palo Alto, CA) and synthesized by Eurofins MWG Operon (Huntsville, AL), unless otherwise noted (Table 1). Growth factor production over the last 24 h of culture was quantified using ELISA (R&D Systems) and normalized to the DNA content measured with a Quant-iTPicoGreen kit (Invitrogen). Baseline levels of growth factors detected in the different media were subtracted from groups with cells. To measure growth factor retention within the microbeads, cultures were uncrosslinked in 82.5 mM sodium citrate and both supernatants and cells were frozen at -80°C. Samples were then lyophilized for 24 h and the resulting dried constructs were digested in 1 unit/mL alginate lyase (Sigma) for 1 h and measured with ELISA. TGF-β1 production and secretion was not measured because of the large concentration of the recombinant human TGF-β1 in the CM. Quantified mRNA levels were referred to by the name of the gene, whereas quantified protein levels were referred to by the name of the growth factor.

Xiphoid defect

To assess the effects of ASCs on cartilage regeneration, 2 mm cylindrical defects were made in the xiphoids of 125 g

TABLE 1. PRIMER SEQUENCES

Gene	Direction	Sequence
<i>Acan</i>	Sense	GCTTCGCTGTCCTCAATGC
	Antisense	AGGTGTCACCTCCCAACTATCC
<i>Bmp2</i>	Sense	TGTGAGGATTAGCAGGTCTTTG
	Antisense	CTCCGCTGTTTGTGTTTGG
<i>Bmp6</i>	Sense	CCGCAGCAGCAACAATCG
	Antisense	ATCCTCTTCGTCGTCCTTGG
<i>Col2</i>	Sense	CGAGTATGGAAGCGAAGG
	Antisense	GCTTCTTCTCCTTGCTCTTGC
<i>Comp</i>	Sense	AGTGACAGCGATGG GATGG
	Antisense	TCCCCGCTCCTGGTCTTGG
<i>Fgf2</i>	Sense	Global Gene Sequence (Qiagen)
	Antisense	
<i>Fgf18</i>	Sense	CTTCCAGGTTCAAGGTGTTG
	Antisense	GCTTCCGACTCACATCATC
<i>Igf1</i>	Sense	GGTTCCTTATCTCCATTCTTCC
	Antisense	CCCAGTGTCTATTGCTTTCC
<i>Nog</i>	Sense	TAAGCCATCCAAGTCTGTG
	Antisense	AGCAGGAACACTTACACTC
<i>Pdgfa</i>	Sense	GAGGAGACGGATGTGAGG
	Antisense	ACGGAGGAGAAACAAAGACC
<i>Pthlh</i>	Sense	TGGTCGCAGGCTAAAACG
	Antisense	TGTGGATCTCCGCAATCAG
<i>Rps18</i>	Sense	TCGCTATCACTGCCATTAAGG
	Antisense	TGTATTGTCTGTTGGTTCTGC
<i>Sox9</i>	Sense	GTGGGAGCGACAACCTTACC
	Antisense	ATCGGAGCGGAGGAG GAG
<i>Tgfb1</i>	Sense	AGCTGCTTCTTGAGTCC
	Antisense	AAGTGGGGTGTCTTAAATAGG
<i>Tgfb2</i>	Sense	AGCCTGCTTCTTGAGTCC
	Antisense	CTCAGAGGAAGGGATGGG
<i>Tgfb3</i>	Sense	AAGGAGTGGACAACGAAG
	Antisense	CGGTGTGGAGGAATCATC
<i>Vegfa</i>	Sense	GGACATCTTCCAGGAGTACC
	Antisense	CGTCTTGCTGAGGTAACC

male Sprague-Dawley rats as previously described³⁴ under a protocol approved by the Institutional Animal Care and Use Committee of the Georgia Institute of Technology. ASC microbeads preconditioned with the GM or CM were implanted into the defect and immobilized with a hydrogel mixture consisting of 25 mg/mL RGD-conjugated alginate (NOVATACH M RGD, FMC BioPolymer) in the DMEM to promote cell infiltration and 20 mg/mL CaSO₄ (Sigma) mixed at a 4:1 volume ratio. Empty defects with the hydrogel mixture and reimplanted excised cartilage (autografts) served as controls. Each treatment regimen was tested in seven rats and each control group had seven rats.

The xiphoids were excised at 35 days postoperation, and radiographic images in the coronal plane were obtained at a voltage of 22 mV and exposure time of 16 s. Four blind observers with experience examining radiographic images scored the presence of soft tissue penetration with a score of 0 representing no healing, a score of 0.5 representing partial healing, and a score of 1 representing full healing. The average score each observer gave for each group was used for statistical analysis ($n=4$ scores). Samples were then fixed in 10% phosphate-buffered formalin (Sigma) for 48 h and embedded in paraffin. Sagittal sections of the defect were stained with safranin-O using a fast green counter stain to evaluate the proteoglycan present.

TABLE 2. GENE ARRAY OF SIGNALING PATHWAYS

Signaling pathways different than control (liver)	Treatment	# Different genes/# Total genes	# Higher genes	# Lower genes	z-score (Higher)	z-score (Lower)
TGF- β signaling pathway	monolayer	27/52	21	6	3.5	-2.08
	microbeads	18/52	14	4	1.65	-1.99
MAPK signaling pathway	monolayer	90/185	56	34	3.27	-1.75
	microbeads	71/185	45	26	2.26	-1.53
Wnt signaling pathway	monolayer	40/87	30	10	3.18	-2.71
	microbeads	32/87	23	9	2.04	-1.94
PPAR signaling pathway	monolayer	31/58	7	24	-1.67	3.22
	microbeads	32/58	8	24	-0.88	4.63
Jak-STAT signaling pathway	monolayer	38/84	14	24	-0.97	1.08
	microbeads	26/84	18	8	0.79	-2.1

Statistical analysis

Statistical differences among all experimental groups were determined via ANOVA with a *post hoc* Tukey's test (GraphPad Prism, La Jolla, CA). Statistical differences between control and experimental groups were determined via the Student's t-test. Differences in means were considered to

be statistically significant if the p-value was less than 0.05. All *in vitro* experiments had six independent cultures per treatment group to ensure sufficient power to detect statistically significant differences and were conducted multiple times to validate the observations. However, only data from a single representative experiment are shown and are expressed as means $\pm$ standard errors.

FIG. 1. The effect of the chondrogenic medium (CM) on adipose stem cell (ASC) monolayers and microbeads. **(A)** Trophic factor gene expression of ASCs in the CM or microbeads (μ B) with chondrocytes (Chond) and liver cells (Liv) serving as controls. **(B)** Growth factor secretion from ASCs in the CM or microbeads (μ B) with chondrocytes (Chond) serving as controls ($n=6$, mean $\pm$ SE, * $p < 0.05$ vs. Chond, # $p < 0.05$ vs. ASCs, ^ $p < 0.05$ vs. ASC μ B).

Results

Growth factor signaling pathways with high and low mRNA levels in ASC cultures

mRNAs for 21 out of 52 proteins associated with the TGF- β signaling pathway were higher in ASC monolayers compared to liver RNA (Table 2). These higher mRNAs included paracrine factor genes *bmp2*, *nog*, *tgfb1*, *tgfb2*, and *tgfb3*; receptor genes *tgfb2*, *tgfb3*, and *bmpr2*; and secondary messenger genes *smad2*, *smad3*, *smad4*, and *p300* (data not shown). mRNAs for proteins associated with the mitogen-activated protein kinase (MAPK) signaling pathway were also higher in ASC monolayers and microbeads compared to liver RNA with z-scores of 3.27 and 2.26, respectively (Table 2). Higher mRNAs included numerous *fgfs* and *pdgfa*, *pdgfra*, *pdgfrb*, *fgfr*, *mapk3*, *rras*, *prkcc*, and *rac1* (data not shown). mRNAs for proteins associated with the Wnt signaling pathway were higher in both ASC monolayers and microbeads (Table 2) compared to the liver. However, higher mRNA levels were mainly due to secondary messengers of the Wnt canonical pathway; Wnt2 and Wnt4 were the only Wnt proteins with higher mRNA levels. mRNAs for proteins associated with the PPAR signaling pathway were significantly lower in both ASC monolayers and microbeads with z-scores of 3.22 and 4.63, respectively. Based on these results, paracrine factors associated with TGF- β and MAPK were further investigated.

Effect of CM on ASC cultures

The CM increased *pthlh*, *bmp2* (Fig. 1A), *igf1*, *tgfb2*, and *nog* by 1.8 to 110-fold (Table 3) in ASC monolayers. ASC microbeads experienced a similar increase in *igf1* and *tgfb2* when treated with the CM (Table 3). The CM decreased *fgf2* and *vegfa* in both ASC monolayers and microbeads by three-

to four-fold (Table 3), while microencapsulation alone increased *pthlh*, *bmp2* (Fig. 1A), *igf1*, and *tgfb2* (Table 3). The CM also increased *acan*, *sox9*, and *comp* in both ASC monolayers and microbeads. Compared to chondrocytes, ASC cultures treated with the CM had similar amounts of *tgfb2* and *vegfa* and higher amounts of *igf1*, *pthlh*, and *tgfb2* when compared to liver cells.

The CM had similar effects on protein secretion as it increased IGF-I, TGF- β 2, and TGF- β 3 secretion in ASC monolayer cultures by 6.3- to 30-fold (Fig. 1B). The CM also increased the secretion of IGF-I, TGF- β 2, and TGF- β 3 from ASC microbeads by 2- to 37-fold. Microencapsulation alone increased IGF-I, TGF- β 2, VEGF-A, and FGF-2 secretions by 3- to 40-fold (Fig. 1B). The CM decreased VEGF-A secretion from ASC monolayers and microbeads by 15- to 20-fold and decreased FGF-2 secretion from ASC microbeads by 2.4-fold. ASC monolayers treated with the CM had higher secretion levels of TGF- β 2 and TGF- β 3, similar secretion levels of VEGF-A and FGF-2, and lower secretion levels of IGF-I compared to chondrocytes. Detectable amounts of FGF-2, IGF-I, TGF- β 2, and VEGF-A were maintained within the microbeads as the CM decreased the amounts of FGF-2, IGF-I, and VEGF-A, but increased the amount of TGF- β 2 inside the microbeads (Table 3).

Effect of ascorbic acid-2-phosphate, Dex, and growth factors in GM

Adding AA2P to the GM decreased *fgf18* (Fig. 2A), *bmp6*, *nog*, and *pdgfa* by 2.9- to 10.9-fold (Table 4), while adding Dex to the GM increased *bmp2*, *fgf18*, *igf1*, *tgfb2*, *nog*, *pdgfa*, and *tgfb3* by 1.5- to 10.2-fold (Fig. 2A and Table 4). AA2P also increased IGF-I, FGF-2, and TGF- β 3 secretion by 2.7- to 8.9-fold (Fig. 2B). Adding Dex decreased *pthlh*, *fgf2*, and *vegfa* by 1.3- to 5.2-fold

TABLE 3. EFFECT OF CHONDROGENIC MEDIUM ON ASC MONOLAYER AND MICROBEAD CULTURES

	Chond	ASCs	+CM	+Microbead	+CM +Microbead	Liv
<i>mRNA levels</i>						
<i>Bmp6/Rps18</i>	11.8 $\pm$ 4.0	14.9 $\pm$ 2.0	9.5 $\pm$ 1.7	^a 26.7 $\pm$ 5.1	^a 1.6 $\pm$ 0.4	^a 35.7 $\pm$ 5.0
<i>Fgf2/Rps18</i>	0.6 $\pm$ 0.2	0.8 $\pm$ 0.1	0.3 $\pm$ 0.1 ^a	0.8 $\pm$ 0.2	0.2 $\pm$ 0.0	0.4 $\pm$ 0.0
<i>Igf1/Rps18</i>	90.6 $\pm$ 20.7	1.5 $\pm$ 0.2 ^a	32.7 $\pm$ 3.6 ^a	7.3 $\pm$ 1.3 ^a	39.4 $\pm$ 10.6 ^a	0.1 $\pm$ 0.0 ^a
<i>Nog/Rps18</i>	55.8 $\pm$ 19.6	17.5 $\pm$ 2.3	1995.3 $\pm$ 442.4 ^a	100.3 $\pm$ 16.0	589.3 $\pm$ 156.0 ^a	69.7 $\pm$ 6.1
<i>Pdgfa/Rps18</i>	1.7 $\pm$ 0.1	1.2 $\pm$ 0.2	1.5 $\pm$ 0.2	0.7 $\pm$ 0.1	0.5 $\pm$ 0.1 ^a	1.2 $\pm$ 0.2
<i>Tgfb1/Rps18</i>	6.0 $\pm$ 1.8	3.5 $\pm$ 0.5	7.7 $\pm$ 0.8	4.7 $\pm$ 0.8	11.0 $\pm$ 2.9	3.1 $\pm$ 0.3
<i>Tgfb2/Rps18</i>	18.0 $\pm$ 6.3	0.3 $\pm$ 0.0 ^a	12.2 $\pm$ 1.5	0.7 $\pm$ 0.2 ^a	8.7 $\pm$ 3.3	1.3 $\pm$ 0.3 ^a
<i>Tgfb3/Rps18</i>	207.9 $\pm$ 57.5	17.4 $\pm$ 1.8 ^a	265.3 $\pm$ 29.4	9.5 $\pm$ 1.3 ^a	106.1 $\pm$ 21.9	17.2 $\pm$ 1.6
<i>Vegfa/Rps18</i>	1.6 $\pm$ 0.4	8.0 $\pm$ 0.3 ^a	1.9 $\pm$ 0.2	8.2 $\pm$ 0.6 ^a	1.2 $\pm$ 0.3	0.7 $\pm$ 0.1 ^a
<i>Acan/Rps18</i>	7.5 $\pm$ 2.2	0.0 $\pm$ 0.0 ^{a,b}	46.0 $\pm$ 6.3 ^a	0.4 $\pm$ 0.1 ^a	54.7 $\pm$ 8.9 ^a	1.4 $\pm$ 0.3 ^a
<i>Col2/Rps18</i>	13.5 $\pm$ 1.6	0.2 $\pm$ 0.0 ^a	0.2 $\pm$ 0.0 ^a	0.0 $\pm$ 0.0 ^{a,b}	0.1 $\pm$ 0.0 ^a	0.0 $\pm$ 0.0 ^{a,b}
<i>Comp/Rps18</i>	14.0 $\pm$ 4.4	0.2 $\pm$ 0.0 ^a	31.5 $\pm$ 5.3 ^a	0.8 $\pm$ 0.2 ^a	164.2 $\pm$ 70.4 ^a	0.3 $\pm$ 0.1 ^a
<i>Sox9/Rps18</i>	8.4 $\pm$ 2.4	14.0 $\pm$ 1.4	35.4 $\pm$ 7.1 ^a	9.6 $\pm$ 1.7	44.1 $\pm$ 6.8 ^a	4.7 $\pm$ 0.6 ^a
<i>Growth factor in microbeads</i>						
FGF-2/DNA ^c	n.m.	n.m.	n.m.	2.1 $\pm$ 0.4	0.9 $\pm$ 0.1	n.m.
IGF-I/DNA ^c	n.m.	n.m.	n.m.	1.3 $\pm$ 0.2	0.3 $\pm$ 0.1	n.m.
TGF- β 2/DNA ^c	n.m.	n.m.	n.m.	0.0 $\pm$ 0.0 ^b	0.0 $\pm$ 0.0 ^b	n.m.
TGF- β 3/DNA ^c	n.m.	n.m.	n.m.	34.2 $\pm$ 2.6	63.4 $\pm$ 7.9	n.m.
VEGF-A/DNA ^c	n.m.	n.m.	n.m.	12.0 $\pm$ 0.7	4.0 $\pm$ 0.7	n.m.

^a*p* < 0.05 versus Chond, *n* = 6, mean $\pm$ SE.

^bValue less than 0.05.

^cUnits of ng/ μ g.

n.m., not measured; ASC, adipose stem cells; CM, chondrogenic medium; FGF, fibroblast growth factor; IGF, insulin-like growth factor; TGF, transforming growth factor; VEGF, vascular endothelial growth factor.

FIG. 2. The effect of ascorbic acid 2-phosphate, dexamethasone, and growth factors in the growth medium (GM). **(A)** Trophic factor gene expression of ASCs in the GM with ascorbic acid 2-phosphate (AA2P), dexamethasone (Dex), or TGF-β1 and BMP-6 (GFs). **(B)** Growth factor secretion from ASCs in the GM with ascorbic acid 2-phosphate (AA2P), dexamethasone (Dex), or TGF-β1 and BMP-6 (GFs) ($n=6$, mean $\pm$ SE, $*p < 0.05$ vs. GM, $\#p < 0.05$ vs. + AA2P, $^{\wedge}p < 0.05$ vs. + Dex, $\%p < 0.05$ vs. + AA2P + Dex).

(Fig. 2A and Table 4) and VEGF-A secretion by 87-fold (Fig. 2B). Adding both AA2P and Dex to the GM was not as effective in increasing *igf1*, *bmp2*, *fgf18*, and *nog* as adding only Dex to the GM. The combination of adding both AA2P and Dex in the GM effectively increased *acan*, but not *col2* or *comp*.

Adding TGF-β1 and BMP-6 to the GM had the opposite effect of AA2P and Dex on *igf1*, *fgf2*, and *vegfa* (Table 4). The exogenous TGF-β1 decreased *igf1* by 48-fold and increased *fgf2* and *vegfa* by 11- and 3.8-fold, respectively. The TGF-β1 also decreased *pthlh* by fourfold and increased *bmp2*, *fgf18*, *tgfb2*, and *pdgfa* by 1.6- to 6.3-fold (Fig. 3A, Table 4). BMP-6 decreased *pthlh* and increased *fgf18*, *tgfb2*, *fgf2*, and *nog*. Growth factor secretion by ASCs in the GM was also influenced as the exogenous TGF-β1 increased TGF-β2, VEGF-A, and FGF-2 secretion by 2.5- to 13.6-fold (Fig. 3B). BMP-6 increased TGF-β2 secretion by 3.5-fold.

Effect of effect of ascorbic acid-2-phosphate, Dex, and growth factors in CM

Removing AA2P from the CM decreased *igf1*, *tgfb2*, and *fgf2* mRNAs by 1.2- to 6.3-fold (Table 5). The absence of

AA2P also increased *fgf18* and *vegfa* by 2.5 to 2.8-fold (Fig. 4A and Table 5). Removing Dex from the CM decreased *pthlh*, *bmp2*, *fgf18* (Fig. 4A), *igf1*, *bmp6*, and *nog* by 2.1- to 9.2-fold (Table 5). The lack of Dex also increased *tgfb2*, *fgf2*, *vegfa*, *pdgfa*, and *tgfb1* by 1.2- to 3-fold. Removing both AA2P and Dex further decreased *igf1* mRNA, while increasing *vegfa* and *pdgfa* (Table 5). Individually removing AA2P and Dex from the CM decreased IGF-I secretion by 10.5- to 22-fold and increased VEGF-A secretion by 5.3- to 58-fold (Fig. 4B). Removing AA2P also decreased TGF-β2 secretion 2.5-fold, while removing Dex increased TGF-β2 secretion 2.4-fold. Removing AA2P decreased *acan*, *comp*, and *sox9* 1.8- to 3.1-fold, while leaving out Dex increased mRNAs for these same genes 1.5- to 23-fold (Table 5).

Removing both exogenous TGF-β1 and BMP-6 from the CM significantly decreased *pthlh* (Fig. 4A), *igf1*, and *vegfa* by 1.4- to 10.1-fold (Table 5); *nog* 1000-fold; and *tgfb3* 16-fold (Table 5). Specifically, the lack of TGF-β1 reduced *pthlh*, *fgf18*, *igf1*, *tgfb2*, *tgfb1*, and *tgfb3* 2.7- to 25-fold, while the lack of BMP-6 reduced *pthlh*, *bmp2*, *fgf18*, *tgfb2*, *vegfa*, and *nog* 2.2- to 220-fold (Fig. 5A and Table 5). Taking out the TGF-β1 also increased *bmp2*, *fgf2*, *bmp6*, and *nog* 1.8- to 4.3-fold, while the

TABLE 4. THE EFFECT OF ASCORBIC ACID 2-PHOSPHATE, DEXAMETHASONE, TGF- β 1, AND BMP-6 ON ASC mRNA LEVELS IN GROWTH MEDIUM

	GM	+AA2P	+DEX	+AA2P +DEX	+TGF- β 1 +BMP-6
<i>Bmp6/Rps18</i>	97.9 $\pm$ 12.0	33.3 $\pm$ 18.1 ^a	51.7 $\pm$ 3.9 ^a	49.6 $\pm$ 11.9 ^a	37.4 $\pm$ 9.7 ^a
<i>Fgf2/Rps18</i>	1.6 $\pm$ 0.1	1.3 $\pm$ 0.2	1.1 $\pm$ 0.1 ^a	1.0 $\pm$ 0.1 ^a	3.3 $\pm$ 0.3 ^a
<i>Igf1/Rps18</i>	1.8 $\pm$ 0.2	2.9 $\pm$ 0.8	6.0 $\pm$ 0.5 ^a	3.1 $\pm$ 0.4 ^a	0.1 $\pm$ 0.1 ^a
<i>Nog/Rps18</i>	32.3 $\pm$ 7.0	8.8 $\pm$ 2.0 ^a	52.9 $\pm$ 7.1 ^a	29.7 $\pm$ 5.3	414.6 $\pm$ 29.0 ^a
<i>Pdgfa/Rps18</i>	10.9 $\pm$ 1.2	4.4 $\pm$ 1.3 ^a	16.8 $\pm$ 2.0 ^a	25.7 $\pm$ 3.6 ^a	44.8 $\pm$ 3.8 ^a
<i>Tgfb1/Rps18</i>	28.2 $\pm$ 4.7	13.2 $\pm$ 3.2 ^a	5.2 $\pm$ 0.4 ^a	5.2 $\pm$ 1.0 ^a	60.7 $\pm$ 16.0 ^a
<i>Tgfb2/Rps18</i>	0.6 $\pm$ 0.3	0.4 $\pm$ 0.1	6.8 $\pm$ 1.4 ^a	5.0 $\pm$ 0.4 ^a	7.1 $\pm$ 3.0 ^a
<i>Tgfb3/Rps18</i>	51.6 $\pm$ 8.0	58.4 $\pm$ 8.8	158.2 $\pm$ 10.9 ^a	237.2 $\pm$ 53.2 ^a	47.3 $\pm$ 9.6
<i>Vegfa/Rps18</i>	2.7 $\pm$ 0.4	2.7 $\pm$ 0.6	1.1 $\pm$ 0.1 ^a	0.6 $\pm$ 0.0 ^a	8.2 $\pm$ 1.0 ^a
<i>Acan/Rps18</i>	0.2 $\pm$ 0.0	0.2 $\pm$ 0.1	0.2 $\pm$ 0.0	0.5 $\pm$ 0.1 ^a	2.8 $\pm$ 0.3 ^a
<i>Col2/Rps18</i>	0.0 $\pm$ 0.0 ^b	0.0 $\pm$ 0.0 ^b	0.0 $\pm$ 0.0 ^b	0.0 $\pm$ 0.0	0.5 $\pm$ 0.2
<i>Comp/Rps18</i>	0.1 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0 ^b	0.0 $\pm$ 0.0	0.2 $\pm$ 0.1
<i>Sox9/Rps18</i>	0.3 $\pm$ 0.1	0.1 $\pm$ 0.0 ^a	0.1 $\pm$ 0.0 ^a	0.1 $\pm$ 0.0	0.3 $\pm$ 0.1 ^a

	GM	+TGF- β 1	+BMP-6	+TGF- β 1 +BMP-6
<i>Bmp6/Rps18</i>	43.7 $\pm$ 5.0	30.9 $\pm$ 6.9	45.8 $\pm$ 4.3	27.7 $\pm$ 9.2
<i>Fgf2/Rps18</i>	1.1 $\pm$ 0.1	12.1 $\pm$ 2.7 ^a	2.2 $\pm$ 0.4 ^a	12.5 $\pm$ 4.0 ^a
<i>Igf1/Rps18</i>	4.9 $\pm$ 0.7	0.1 $\pm$ 0.0 ^a	4.0 $\pm$ 0.6	0.1 $\pm$ 0.0 ^a
<i>Nog/Rps18</i>	2.6 $\pm$ 0.8	3.5 $\pm$ 0.9	30.3 $\pm$ 6.8 ^a	13.8 $\pm$ 4.8 ^a
<i>Pdgfa/Rps18</i>	6.9 $\pm$ 1.4	43.5 $\pm$ 7.0 ^a	7.8 $\pm$ 0.9	51.2 $\pm$ 1.6 ^a
<i>Tgfb1/Rps18</i>	4.7 $\pm$ 0.8	9.9 $\pm$ 2.2	5.9 $\pm$ 0.8	9.7 $\pm$ 1.6 ^a
<i>Tgfb2/Rps18</i>	0.7 $\pm$ 0.2	1.9 $\pm$ 0.4 ^a	1.6 $\pm$ 0.3 ^a	3.6 $\pm$ 1.2 ^a
<i>Tgfb3/Rps18</i>	0.1 $\pm$ 0.0	0.3 $\pm$ 0.1	0.3 $\pm$ 0.0 ^a	0.6 $\pm$ 0.2
<i>Vegfa/Rps18</i>	7.7 $\pm$ 1.3	29.1 $\pm$ 5.7 ^a	5.1 $\pm$ 1.2	31.0 $\pm$ 8.3 ^a
<i>Acan/Rps18</i>	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
<i>Col2/Rps18</i>	0.0 $\pm$ 0.0 ^b	0.0 $\pm$ 0.0 ^b	0.0 $\pm$ 0.0 ^b	0.0 $\pm$ 0.0
<i>Comp/Rps18</i>	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
<i>Sox9/Rps18</i>	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0	1.2 $\pm$ 0.6 ^a	0.1 $\pm$ 0.0

^a $p < 0.05$, vs. GM, $n = 6$, mean $\pm$ SE.^bValue less than 0.05.

ASC, adipose stem cell; TGF, transforming growth factor; BMP, bone morphogenetic protein; GM, growth medium; DEX, dexamethasone.

lack of BMP-6 increased *bmp6* 8.6-fold. Individually removing the exogenous TGF- β 1 and BMP-6 from the CM decreased IGF-I secretion 1.7- to 2.4-fold, TGF- β 2 secretion 8.3- to 25-fold, and VEGF-A secretion 1.3- to 1.6-fold (Fig. 5B). The absence of TGF- β 1 and BMP-6 had a similar effect on *acn* and *comp* reducing their mRNA levels 1.7- to 31-fold (Table 5).

Effect of ASC microbeads on cartilage regeneration

Defects with ASC microbeads preconditioned in the CM and autografts had higher radiographic scores than defects with just the hydrogel mixture (Fig. 6A). Defects with only the hydrogel mixture had no apparent cell infiltration, new extracellular matrix (ECM) deposition, or perichondrium formation as indicated by the lack of fast green staining (Fig. 6B). In general, defects treated with the ASC microbeads preconditioned in the GM had traces of fast green staining throughout the defect with cell infiltration and tissue deposition at the edges of the defect (Fig. 6C). Defects with ASC microbeads preconditioned in the CM consistently had ECM deposition throughout the defect with cell infiltration, tissue resembling a perichondrium, and initial proteoglycan deposition (Fig. 6D). Defects with the autograft had cell infiltration, a perichondrium that resembled that of the surrounding xiphoid and proteo-

glycan deposition between the edges of the defect and autograft (Fig. 6E).

Discussion

Stem cell therapies for cartilage regeneration have been investigated for nearly two decades, yet an effective stem cell treatment has yet to receive FDA approval.³⁵ Although it is evident that stem cells do have the capabilities for regenerating musculoskeletal tissues, using stem cells as a source to directly replace diseased or damaged cartilage may not be an effective method. However, using stem cells, such as ASCs, as trophic factor production sources to stimulate endogenous cartilage regeneration may provide a more potent approach. This is the first comprehensive study to show that microencapsulation and different components in the CM have distinct effects on growth factor production from ASCs and that ASC microbeads treated with these components can promote cartilage infiltration within a focal cartilage defect.

Based on this new paradigm of using chondrogenic treatments to precondition ASCs as trophic factor production sources, ascorbic acid 2-phosphate was most effective in increasing production of chondrogenic factors, while decreasing angiogenic and other factors that promote chondrocyte hypertrophy. This component regulated secretion of IGF-I and FGF-2, both potent stimulators of chondrocyte proliferation,¹¹

FIG. 3. The effect of TGF-β1 and BMP-6 in the GM. **(A)** Trophic factor gene expression of ASCs in the GM with TGF-β1 and BMP-6. **(B)** Growth factor secretion from ASCs in the GM with TGF-β1 and BMP-6. ($n=6$, mean $\pm$ SE, * $p < 0.05$ vs. GM, # $p < 0.05$ vs. + TGF-β1, ^ $p < 0.05$ vs. + BMP-6).

and decreased mRNA for FGF-18, a growth factor associated with hypertrophic differentiation.⁹ Furthermore, removing this component from the CM also increased VEGF-A mRNA levels and secretion. These results are consistent with previous findings where ascorbic acid 2-phosphate and other vitamin C derivatives stimulated cell proliferation of a variety of different cell types^{36–38} and stimulated IGF-I production from dermal papilla cells.³⁹ In contrast, Dex, an anti-inflammatory and immunosuppressant corticosteroid, decreased mRNA for the FGF-2 and secretion of VEGF-A and increased mRNAs for BMP-2 and FGF-18 in ASCs. These results are supported by the observation that Dex decreased VEGF-A production from hemangioma-derived stem cells, inhibiting tumor vasculogenesis in a murine model,⁴⁰ and upregulated the FGF-18 during osteogenesis in MSCs.⁴¹ Also in this study, TGF-β1 and BMP-6 increased mRNA levels and secretion of factors associated with chondrogenesis and hypertrophic differentiation, while TGF-β1 also increased secretion of factors associated with angiogenesis. Others have shown that TGF-β1 stimulated production of cartilaginous tissue from chondrocytes and progenitor cells,^{10,16,42} but also controlled endothelial cell proliferation, invasion, and ECM turnover.^{43,44} Likewise, BMP-6 has previously been shown to stimulate chondrocyte maturation⁴⁵ and bone nodule formation.⁴⁶

Interestingly, microencapsulation alone increased mRNA levels for PTHrP and production of IGF-I and TGF-β2 after 5 days in the GM, but not in the CM. This observation may be

due to the high density and round morphology that ASCs have in alginate microbeads, both of which have been shown to support chondrogenesis,^{47,48} and may explain why ASC microbeads just preconditioned in the GM promoted tissue ingrowth in focal cartilage defects. However, high cell density and round cell morphology may have less of an effect in the CM because of the overwhelmingly high concentrations of TGF-β1, BMP-6, and Dex. Microencapsulation also increased VEGF-A production, possibly due to hypoxia caused by the high cell density. Although hypoxic conditions inside the microbead were not assayed, increasing cell densities in hydrogel cultures has been shown to increase oxygen tension and gradients,⁴⁹ and hypoxic conditions can increase VEGF-A and FGF-2 secretion from ASCs.⁵⁰ The microenvironment can also influence the response of ASCs to Dex and TGF-β1 since they exert different chondrogenic effects on synovial MSC aggregates and synovial explants.⁵¹

Alginate hydrogels are known to bind different growth factors,^{52,53} and microbeads in the current study retained TGF-β2, VEGF-A, and FGF-2, which may also have a direct effect on subsequent growth factor production. To test whether this may affect the analysis of supernatants, a preliminary study showed that ASCs secrete at least 2- to 27-fold more of these growth factors from microbeads than what is retained within the beads over a 7-day period. Additionally, the differences in supernatant measurements of ASC microbeads cultured in the GM and the CM over the last 24 h

TABLE 5. THE EFFECT OF ASCORBIC ACID 2-PHOSPHATE, DEXAMETHASONE, TGF- β 1, AND BMP-6 ON ASC mRNA LEVELS IN CHONDROGENIC MEDIUM

	CM	–AA2P	–DEX	–AA2P –DEX	–TGF- β 1 –BMP-6
<i>Bmp6/Rps18</i>	67.0 $\pm$ 6.6	114.9 $\pm$ 24.9	27.3 $\pm$ 3.2	53.6 $\pm$ 5.1	62.2 $\pm$ 9.8
<i>Fgf2/Rps18</i>	0.9 $\pm$ 0.0	0.8 $\pm$ 0.1	1.4 $\pm$ 0.1 ^a	1.3 $\pm$ 0.1 ^a	0.2 $\pm$ 0.0 ^a
<i>Igf1/Rps18</i>	27.9 $\pm$ 3.1	7.9 $\pm$ 1.4 ^a	4.6 $\pm$ 0.7 ^a	1.4 $\pm$ 0.1 ^a	2.8 $\pm$ 0.6 ^a
<i>Nog/Rps18</i>	1746.1 $\pm$ 171.4	1382.8 $\pm$ 205.8	310.3 $\pm$ 33.7 ^a	826.7 $\pm$ 70.7 ^a	1.7 $\pm$ 0.4 ^a
<i>Pdgfa/Rps18</i>	12.2 $\pm$ 1.2	12.3 $\pm$ 2.6	22.2 $\pm$ 2.9 ^a	27.7 $\pm$ 2.5 ^a	2.1 $\pm$ 0.7 ^a
<i>Tgfb1/Rps18</i>	15.8 $\pm$ 2.6	15.6 $\pm$ 1.9	47.9 $\pm$ 11.5 ^a	47.3 $\pm$ 2.8 ^a	1.7 $\pm$ 0.3 ^a
<i>Tgfb2/Rps18</i>	17.4 $\pm$ 1.5	8.3 $\pm$ 2.4 ^a	21.5 $\pm$ 4.1 ^a	10.8 $\pm$ 2.1 ^a	16.4 $\pm$ 3.0
<i>Tgfb3/Rps18</i>	748.9 $\pm$ 50.2	656.0 $\pm$ 85.4	656.4 $\pm$ 82.4	604.2 $\pm$ 30.0 ^a	47.4 $\pm$ 8.5 ^a
<i>Vegfa/Rps18</i>	0.9 $\pm$ 0.1	2.2 $\pm$ 0.2 ^a	2.5 $\pm$ 0.3 ^a	5.0 $\pm$ 0.3 ^a	0.6 $\pm$ 0.1 ^a
<i>Acan/Rps18</i>	6.7 $\pm$ 0.4	3.6 $\pm$ 0.3 ^a	153.8 $\pm$ 19.7 ^a	95.0 $\pm$ 5.3 ^a	0.7 $\pm$ 0.2 ^a
<i>Col2/Rps18</i>	0.4 $\pm$ 0.2	0.1 $\pm$ 0.0	0.6 $\pm$ 0.1	10.1 $\pm$ 0.0	0.2 $\pm$ 0.1
<i>Comp/Rps18</i>	17.5 $\pm$ 2.2	5.7 $\pm$ 0.7 ^a	95.1 $\pm$ 26.5 ^a	146.6 $\pm$ 45.7 ^a	0.2 $\pm$ 0.1 ^a
<i>Sox9/Rps18</i>	5.2 $\pm$ 0.2	3.0 $\pm$ 0.4 ^a	7.8 $\pm$ 0.7 ^a	3.6 $\pm$ 0.2 ^a	0.0 $\pm$ 0.0 ^{a,b}

	CM	–TGF- β 1	–BMP-6	–TGF- β 1 –BMP-6
<i>Bmp6/Rps18</i>	12.2 $\pm$ 1.4	21.5 $\pm$ 3.4 ^a	103.9 $\pm$ 22.6 ^a	37.8 $\pm$ 5.7 ^a
<i>Fgf2/Rps18</i>	1.2 $\pm$ 0.2	2.7 $\pm$ 0.4 ^a	1.4 $\pm$ 0.2	0.8 $\pm$ 0.1
<i>Igf1/Rps18</i>	4.8 $\pm$ 1.1	0.9 $\pm$ 0.1 ^a	6.4 $\pm$ 1.9	1.6 $\pm$ 0.3 ^a
<i>Nog/Rps18</i>	18.5 $\pm$ 3.4	80.4 $\pm$ 17.7 ^a	0.1 $\pm$ 0.0	0.3 $\pm$ 0.1 ^a
<i>Pdgfa/Rps18</i>	18.9 $\pm$ 3.9	13.7 $\pm$ 2.1	26.1 $\pm$ 7.6	6.9 $\pm$ 0.8
<i>Tgfb1/Rps18</i>	4.8 $\pm$ 1.0	1.7 $\pm$ 0.3 ^a	4.0 $\pm$ 0.8	1.6 $\pm$ 0.4 ^a
<i>Tgfb2/Rps18</i>	16.7 $\pm$ 2.1	3.8 $\pm$ 0.8 ^a	2.1 $\pm$ 0.6 ^a	1.8 $\pm$ 0.5 ^a
<i>Tgfb3/Rps18</i>	7.0 $\pm$ 1.1	2.6 $\pm$ 0.6 ^a	4.5 $\pm$ 1.3	0.6 $\pm$ 0.1 ^a
<i>Vegfa/Rps18</i>	0.9 $\pm$ 0.1	1.0 $\pm$ 0.2	0.4 $\pm$ 0.1 ^a	0.4 $\pm$ 0.1 ^a
<i>Acan/Rps18</i>	3.0 $\pm$ 0.8	0.6 $\pm$ 0.2 ^a	0.1 $\pm$ 0.1 ^a	0.1 $\pm$ 0.0
<i>Col2/Rps18</i>	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
<i>Comp/Rps18</i>	0.4 $\pm$ 0.1	0.1 $\pm$ 0.0 ^a	0.1 $\pm$ 0.0	0.1 $\pm$ 0.0
<i>Sox9/Rps18</i>	0.8 $\pm$ 0.2	1.0 $\pm$ 0.2	0.5 $\pm$ 0.1	0.0 $\pm$ 0.0

^a $p < 0.05$ vs. CM, $n = 6$, mean $\pm$ SE.^bValue less than 0.05.

were consistent with differences in cumulative growth factor production between the two groups over the course of 7 days. Regardless, these retained factors may significantly influence tissue regeneration once the microbeads begin to degrade.

Along with the structural environment imparted by microencapsulation, exposure time to the CM and its individual components may have significant effects on growth factor production from ASCs. CM treatment lengths needed to induce optimal chondrogenesis of MSCs and ASCs in 3D cultures have varied between 3 and 12 weeks,^{1,23,54} significantly longer than the treatment times used in this study. This difference in treatment times may explain the minimal effect CM components had on type II collagen expression and may suggest that longer treatment times are needed to optimize the effects different medium components have on growth factor production. However, the differences in treatment times and subsequent effects on cell behavior between previous studies and this current work do not take into account the mass-transfer properties of larger 3D hydrogels.

The specific concentration of different components may also have a significant effect on growth factor production from ASCs. It is well known that TGF- β 1 has a biphasic effect: at concentrations ranging from 100 pg/mL to 1 ng/mL, TGF- β 1 increased endothelial cell proliferation⁴³ and the effect of FGF-2 and VEGF-induced invasion,⁴⁴ whereas at

concentrations ranging from 5 to 10 ng/mL, TGF- β 1 inhibited endothelial cell invasion and capillary formation⁴⁴ and induced ASC chondrogenesis.^{1,55} Although ascorbic acid 2-phosphate and Dex were also at their optimal concentrations for inducing chondrogenesis,^{1,23} the concentration of BMP-6 was only at 20% of its optimal chondrogenic dose for ASCs.⁵⁵ It is possible that higher BMP-6 concentrations would significantly affect its actions on ASC growth factor production, but the concentration used increased aggrecan mRNA levels in the current study and previously increased the alkaline phosphatase activity in a chondrogenic cell line.³¹ It is also possible that these components in the CM, as well as other soluble components in chondrogenic and growth media, may have interfered with ELISA measurements of growth factors secreted at low levels in the current study (e.g., FGF-2).

The effects of ascorbic acid 2-phosphate, Dex, TGF- β 1, and BMP-6 on certain growth factors in this study were dependent on the presence and concentration of each other and other CM components. For instance, TGF- β 1 decreased mRNAs for IGF-I and PTHrP in the GM, but increased these mRNAs in the CM. Additionally, TGF- β 1 was responsible for the increased VEGF-A secretion in the GM, but BMP-6 was also responsible for increased VEGF-A secretion in the CM. These different responses in these distinct media maybe due to the differences in glucose concentrations and the presence

FIG. 4. The effect of ascorbic acid 2-phosphate, dexamethasone, and growth factors in the CM. **(A)** Trophic factor gene expression of ASCs in the CM without ascorbic acid 2-phosphate (AA2P), dexamethasone (Dex), or TGF-β1 and BMP-6 (GFs). **(B)** Growth factor secretion from ASCs in the CM without ascorbic acid 2-phosphate (AA2P), dexamethasone (Dex), or TGF-β1 and BMP-6 (GFs) ($n=6$, mean $\pm$ SE, * $p < 0.05$ vs. CM, # $p < 0.05$ vs. + AA2P, ^ $p < 0.05$ vs. + Dex, % $p < 0.05$ vs. + AA2P + Dex).

or absence of ascorbic acid 2-phosphate, Dex, proline, ITS+ culture supplement, and serum. Although the effects of glucose, proline, ITS+ culture supplement, and serum were not directly investigated in this study, these components may also have significant effects on ASC growth factor production.

It is important to note that although ASC microbeads were able to promote cartilaginous tissue infiltration into focal defects, this study only disclosed methods to use CM components to affect the production of factors associated with angiogenesis, chondrogenesis, and hypertrophic differentiation. Determining the ideal ASC secretory profile for cartilage regeneration is a more complex problem due to the differential effects individual growth factors have. For instance, although VEGF-A is an angiogenic growth factor that is detrimental to chondrocytes and cartilage,^{6,7} FGF-2, another important facilitator for blood vessel formation,⁵⁶ is known to improve cartilage regeneration.⁵⁷ Additionally, factors associated with hypertrophic differentiation, like BMP-2, are important for progenitor cell proliferation and differentiation during early stage cartilage development⁸ and may be needed to initiate cartilage regeneration. Factors like IGF-I and TGF-β1 are essential for chondrogenesis, cartilage formation, and

homeostasis, but it is unknown whether long-term secretion of these factors from ASCs will lead to hypertrophic differentiation or other unforeseen side effects. In this study, ASC microbeads appeared to promote cell and tissue infiltration from the edges of the cartilage defect and surrounding perichondrium; however, it is unknown, which factors were involved in tissue repair, whether different preconditioning treatments would have further accelerated cell infiltration and ECM deposition, or whether ASC microbeads needed more time to enhance cartilage regeneration.

Conclusion

To use ASCs as trophic factor production sources to stimulate cartilage regeneration, ASCs may have to be preconditioned to increase the production of chondrogenic factors, while decreasing the production of angiogenic and hypertrophic factors. Compared to liver tissue, ASC cultures in 2D monolayer and 3D alginate microbeads had high mRNA levels of proteins associated with the TGF-β and MAPK signaling pathways. The CM affected these cultures by increasing mRNA levels and secretion of chondrogenic factors (IGF-I, TGF-β2, and TGF-β3) and decreasing mRNA

FIG. 5. The effect of TGF-β1 and BMP-6 in the CM. **(A)** Trophic factor gene expression of ASCs in the CM without TGF-β1 and BMP-6. **(B)** Growth factor secretion from ASCs in the CM without TGF-β1 and BMP-6. ($n=6$, mean $\pm$ SE, $*p<0.05$ vs. GM, $\#p<0.05$ vs. +TGF-β1, $^{\wedge}p<0.05$ vs. +BMP-6).

levels and secretion of angiogenic factors (VEGF-A, FGF-2). Microencapsulation alone increased the chondrogenic factor (PTHrP, IGF-I, and TGF-β2) and angiogenic factor (VEGF-A) mRNA levels and production in the GM, but not necessarily in the CM. In subsequent studies with ASC monolayers

cultured in growth and chondrogenic media, ascorbic acid 2-phosphate decreased mRNA levels and secretion of angiogenic (VEGF-A) and hypertrophic (FGF-18) factors and increased chondrogenic factor (IGF-I, TGF-β2) secretion. Dex increased mRNA levels for hypertrophic factors (BMP-2,

FIG. 6. The effect of ASC microbeads in a focal cartilage defect. **(A)** Scoring of radiographic images of 2 mm cylindrical xiphoid defects in the coronal plane 35 days postoperation ($*p<0.05$ vs. hydrogel, $\#p<0.05$ vs. ASC microbeads, $n=4$ scores, arrow points to defect). Sagittal sections of representative xiphoid defects with **(B)** RGD-conjugated hydrogel, **(C)** ASC microbeads preconditioned with the GM and RGD-conjugated hydrogel, **(D)** ASC microbeads preconditioned with the CM and RGD-conjugated hydrogel, and **(E)** autograft. Sections were stained with safranin-O and counter stained with fast green. Bar represents 1 mm. Color images available online at www.liebertpub.com/tea

FGF-18) and decreased production of angiogenic factors (FGF-2, VEGF-A) in growth and chondrogenic media. TGF- β 1 and BMP-6 increased secretion and mRNA levels of chondrogenic (TGF- β 2) and hypertrophic (FGF-18) factors, while TGF- β 1 also increased secretion of angiogenic factors (FGF-2, VEGF-A). When implanted in a focal cartilage defect, ASC microbeads preconditioned with the CM and immobilized in a RGD-conjugated hydrogel stimulated tissue infiltration from the defect edges and perichondrium, while promoting proteoglycan deposition. This study disclosed a new paradigm of using CM components to precondition ASCs as trophic factor production sources: different medium components have distinct effects on the secretome of stem cells, and ascorbic acid 2-phosphate may be the most important component for preconditioning ASCs to stimulate cartilage regeneration.

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Disclosure Statement

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