Published in final edited form as:

J Biomed Mater Res A. 2010 June 1; 93(3): 994–1003. doi:10.1002/jbm.a.32589.

The effects of BMP6 overexpression on adipose stem cell chondrogenesis: interactions with dexamethasone and exogenous growth factors

Brian O. Diekman[†], Bradley T. Estes[†], and Farshid Guilak^{*}

Departments of Surgery and Biomedical Engineering, Duke University Medical Center, Durham, North Carolina 27710 USA

Abstract

Adipose-derived stem cells (ASCs) are multipotent progenitors that can be chondrogenically induced by growth factors such as bone morphogenetic protein 6 (BMP-6). We hypothesized that non-viral transfection of a BMP-6 construct (pcDNA3-BMP6) would induce chondrogenic differentiation of ASCs encapsulated in alginate beads and that differentiation would be enhanced by the presence of the synthetic glucocorticoid dexamethasone (DEX) or the combination of epidermal growth factor (EGF), fibroblast growth factor-2 (FGF-2), and transforming growth factor beta-1 (TGF- β 1), collectively termed expansion factors (EFs). Chondrogenesis was assessed using quantitative real-time polymerase chain reaction for types I, II, and X collagen, aggrecan, and BMP6. Immunohistochemistry was performed with antibodies for types I, II, and X collagen and chondroitin-4-sulfate. BMP6 overexpression alone induced a moderate chondrogenic response. The inclusion of EFs promoted robust type II collagen expression but also increased type I and X collagen deposition, consistent with a hypertrophic chondrocyte phenotype. Early gene expression data indicated that DEX was synergistic with BMP-6 for chondrogenesis, but immunohistochemistry at 28 days showed that DEX reduced glycosaminoglycan accumulation. These results suggest that chondrogenic differentiation of ASCs depends on complex interactions among various growth factors and media supplements, as well as the concentration and duration of growth factor exposure.

Keywords

adult stem cells; chondrogenesis; tissue engineering; bone-morphogenetic protein 6; dexamethasone

INTRODUCTION

Adipose-derived stem cells (ASCs) are an abundant source of multipotent progenitor cells, with estimates of stem cell yield in the range of 400,000 stem cells per milliliter of adipose tissue¹. ASCs exhibit multipotent differentiation abilities under prescribed conditions^{2–4}, including a significant potential for chondrogenic differentiation^{5,6}. In three-dimensional (3D) culture, high concentrations of soluble bone morphogenetic protein 6 (BMP-6, bone morphogenetic protein 6 gene will be abbreviated BMP6) have been shown to upregulate expression of cartilage-specific matrix components, namely type II collagen (COL2A1) and

^{*}Corresponding Author: Farshid Guilak, Orthopaedic Research Laboratories, Department of Surgery, Division of Orthopaedic Surgery, 375 MSRB, Box 3093, Duke University Medical Center, Durham, North Carolina 27710, Phone: (919) 684-2521, Fax: (919) 681-8490, guilak@duke.edu.

[†]Brian O. Diekman and Bradley T. Estes are co-first authors for this work.

aggrecan (AGC1), while inhibiting the expression of the hypertrophic chondrocyte marker, type X collagen (COL10A1)^{7,8}. An alternate strategy for delivery of BMP-6 for chondrogenic differentiation is non-viral transfection, a method of gene delivery that has been shown to be effective in human ASCs⁹. Ultimately, it is expected that the interaction of multiple growth factors and/or bioactive molecules will be required to induce the desired phenotype. Thus, the potential of other chondroinductive agents to enhance BMP-6 mediated chondrogenesis was investigated.

In monolayer, expansion medium containing transforming growth factor beta-1 (TGF- β 1), epidermal growth factor (EGF), and basic fibroblast growth factor (bFGF, or FGF-2) promotes rapid proliferation of ASCs and may act to either prime or initiate chondrogenesis while still in monolayer expansion¹⁰. We hypothesized that the continued use of these growth factors during chondrogenic differentiation would further support chondrogenesis over long term 3D culture. TGF- β has been used often for chondrogenic differentiation of ASCs and bone-marrow derived MSCs^{11–15} and has been shown to promote chondrogenesis of ASCs when used in conjunction with low concentrations of BMP-6¹³. FGF-2 has been shown to induce a chondrogenic phenotype in ASCs as noted by significant increases in the chondrogenic transcription factor, SOX9, and increases in total glycosaminoglycan (GAG) composition, while inhibiting osteogenesis^{16,17}.

In addition to these growth factors, the synthetic glucocorticoid dexamethasone (DEX) has been used previously as a supplement in both MSC and ASC cultures to promote chondrogenesis^{11–15}. Up to 5 days in culture, we have previously shown DEX to suppress sulfated biosynthesis rates in ASCs cultured in alginate, though this effect had disappeared by day 9 of culture¹⁸. DEX has been hypothesized to augment the responsiveness of MSCs to the differentiation agents of multiple lineages including chondrogenic¹⁹ and has demonstrated the ability to regulate cartilage specific genes in MSC culture^{20–22}. However, the potential interaction between DEX and BMP-6 is not well characterized. As direct glucocorticoid regulation of the BMP6 promoter occurs in other cell types²³, we proposed that DEX will enhance the chondrogenesis of ASCs, in part by upregulating BMP6 transcription. While it appears that the use of glucocorticoids may expedite chondrogenesis in a mesenchymal progenitor cell population, it is also well known that glucocorticoids can act as inhibitors of chondrocyte cell growth, specifically inhibiting the production of type II collagen and proteoglycans^{24–29}.

To investigate the effects of EGF, FGF-2, TGF- β 1, and DEX to enhance ASC chondrogenesis, we performed 3 different studies. In study 1, the chondrogenic effect of BMP6 overexpression in the presence or absence of EGF, FGF-2, and TGF- β 1 was investigated. Within this study, we also tested the hypothesis that short term growth factor administration would be more beneficial than continuous growth factor administration for long term culture, as has recently been shown with primary chondrocytes³⁰. In study 2, we examined gene expression changes in early culture with DEX in the presence of varying concentrations of BMP-6. In study 3, based on what was learned in the first two studies, we probed the effects of various combinations of growth factors on the chondrogenic phenotype after 28 days of culture. In addition, short term administration of DEX during the first week only was also performed in order to assess the potential for catabolic effects of continuous glucocorticoid administration to ASC chondrogenesis.

MATERIALS AND METHODS

Cell culture

Human ASCs (Zen-Bio, Durham, NC) were obtained from the subcutaneous adipose tissue liposuction waste of non-smoking, non-diabetic female donors. A “superlot” of combined

cells from 3 donors was used for study 1 (average age of 39 and an average body mass index (BMI) of 24.9) and a superlot of combined cells from 7 donors was used for studies 2 and 3 (average age of 41 and an average BMI of 25.2). The cells were cultured through the appropriate passage in expansion medium containing 0.25 ng/mL TGF- β 1, 5 ng/mL EGF, and 1 ng/mL bFGF as well as 10% fetal bovine serum (FBS, Hyclone, Logan, UT for study 1 and Atlas Biologicals, Ft. Collins, CO for studies 2 and 3) as previously described⁷.

Transfection

ASCs were transfected using the Human MSC Kit (Amaya Inc., Gaithersburg MD) under the high efficiency protocol of the Amaya Nucleofector (Amaya) with either pcDNA3-BMP6³¹ (3 μ g of plasmid/ 500,000 cells) or the pMAX-GFP transfection control plasmid (1 μ g of plasmid/ 500,000 cells), both of which are driven by constitutively active promoters. After transfection, the cells were allowed to recover in Ca²⁺ free medium containing 10% FBS for 1 hour followed by further monolayer expansion and encapsulation in alginate.

Cell Seeding

Following monolayer expansion, the ASCs were suspended in 1.2% alginate (Pronova UP LVG, FMC Biopolymer, Oslo, Norway) at 5×10^6 cells/mL. Using a 1 mL pipette, the cell suspension was dropped into a 102 mM CaCl₂ solution to form three spherical alginate beads per well, each approximately 4.5 mm in diameter, and containing approximately 275,000 cells.

Chondrogenic Differentiation

Study 1—After passage 5, the cells were removed and encapsulated in alginate or transfected according to protocol. The culture medium consisted of a control medium containing DMEM-high glucose (Gibco), 10% FBS, 1% penicillin-streptomycin (Gibco), 37.5 μ g/mL L-ascorbic acid 2-phosphate (Sigma, St. Louis, MO), and 1% ITS+ Premix (Collaborative Biomedical, Becton Dickinson, Bedford, MA). ASC/alginate constructs were incubated in control medium in the presence or absence of 0.25 ng/mL TGF- β 1, 5 ng/mL EGF, and 1 ng/mL bFGF, hereto after referred to as expansion factors or EFs. Eight culture conditions were studied as noted in Table 1. The BMP-6-short term exposure (abbreviated BMP-6-ST) constructs were exposed to 500 ng/mL of BMP-6 (R&D Systems) for the first two medium exchanges and subsequently received the control medium for the remaining culture period. The transfected ASCs (both pcDNA3-BMP6 and pMAX-GFP) received control medium throughout the culture period. ASC/alginate constructs were cultured for 7 or 28 days. Day 7 and 28 constructs were evaluated with quantitative real time RT-PCR (qPCR) and day 28 constructs were also evaluated with immunohistochemistry (IHC) as described.

Study 2—At the end of passage 4, all cells, both transfected and control, were removed from culture plates and encapsulated in alginate for differentiation culture. In addition to the control medium, either 10 ng/mL or 500 ng/mL BMP-6, both with and without 100 nM DEX (Sigma), were added to non-transfected ASC culture. The pcDNA3-BMP6 constructs received the control medium also with and without DEX. As in study 1, a total of 8 groups consisting of control medium plus bioactive molecules were studied and are summarized in Table 1. Day 7 constructs were evaluated with qPCR.

Study 3—To assess long term culture of chondrogenically induced ASCs, 11 different culture conditions were evaluated involving BMP-6, overexpressed BMP-6 via pcDNA3-BMP6 transfection, EFs, and DEX. The control culture medium was the same as defined in

study 2 and was included in the 11 different conditions along with bioactive molecules as indicated in Table 1. Day 28 constructs were analyzed using immunohistochemistry (IHC).

RNA isolation and real-time quantitative RT-PCR (qPCR)

Following culture, ASCs were released from alginate using a solution of 150 mM NaCl and 55 mM Na Citrate. RNA from each well was isolated using the Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Inc., Hercules, CA), quantified with spectrophotometry (Nanodrop ND-1000, Wilmington, DE), and reverse transcribed using iScript reverse transcriptase (Bio-Rad). Using primer probes from Applied Biosystems (Foster City, CA), qPCR (iCycler, Bio-Rad) was used to compare transcript levels for up to 5 different genes of interest (GOI) in addition to a housekeeping gene: 18S ribosomal RNA (endogenous control; assay ID Hs99999901_s1), aggrecan (AGC1; assay ID Hs00153936_m1), type I collagen (COL1A1; assay ID Hs00164004_m1), type II collagen (COL2A1; custom assay: FWD Primer 5-GAGACAGCATGACGCCGAG-3; REV primer 5-GCGGATGCTCTCAATCTGGT-3; Probe 5-FAM-TGGATGCCACACTCAAGTCCCTCAAC-TAMRA-3)³², type X collagen (COL10A1; assay ID Hs00166657_m1), and BMP-6 (BMP6; assay ID Hs00233470_m1). Using plasmids containing the section of the GOI, the standard curve method was used to determine starting transcript quantity (SQ) for each gene in triplicate (n = 3/condition). Data were analyzed by determining the ratio of SQ for the GOI to SQ for 18S for each sample. This ratio was then normalized to this same quantity for the Day 7 control samples.

$$\text{Fold Difference} = \frac{\left(\frac{SQ_{GOI}}{SQ_{18S}} \right)_{\text{Day7 or 28}}}{\left(\frac{SQ_{GOI}}{SQ_{18S}} \right)_{\text{Day7 control}}}$$

Immunohistochemistry

The constructs were fixed overnight at 4°C in a solution containing 4% paraformaldehyde, 100 mM sodium cacodylate, and 50 mM BaCl₂ buffer (pH 7.4) and then washed overnight in a 100 mM sodium cacodylate, 50 mM BaCl₂ buffer (pH 7.4). The beads were then dehydrated in ethanol and embedded in paraffin. Immunohistochemistry analysis on 5 µm-thick sections was performed using monoclonal antibodies to type I collagen (C2456, Sigma, St. Louis, MO), type II collagen (II-II6B3; Developmental Studies Hybridoma Bank, University of Iowa, Iowa City), type X collagen (C7974, Sigma), and chondroitin 4-sulfate (2B6 antibody). Pepsin (Digest-All, Zymed, San Francisco, CA) was used for digestion on sections for types I, II, and X collagen. Sections to be labeled for chondroitin 4-sulfate were treated with trypsin for tissue digestion, then with soybean trypsin inhibitor, and then with chondroitinase (all from Sigma) to expose the epitope for binding of the 2B6 antibody. The Histostain-Plus ES Kit (Zymed) was used on all sections for blocking and linking the secondary antibody to horseradish peroxidase. An anti-mouse IgG antibody (Sigma, Product No. B7151) was used as the secondary antibody, and aminoethyl carbazole was used as the enzyme substrate/chromogen. Pig osteochondral plugs were used as positive controls and were examined to ensure antibody specificity. Negative controls were also prepared to rule out nonspecific labeling.

Statistical analysis

For the statistical analysis, samples were evaluated by one or two factor ANOVA and Fisher's PLSD post-hoc test (α=0.05) to determine significance between conditions.

RESULTS

pMAX-GFP was used to assess transfection efficiency. Representative field cell counting by three independent observers indicated a transfection efficiency of 48.4%.

Study 1

qPCR (Figure 1)—In general, the use of EFs in the medium to promote chondrogenesis had a significant effect on all genes studied ($p<0.05$). BMP6 expression was confirmed in the pcDNA3-BMP6 transfected cells throughout the 28 day culture period, although the EFs decreased BMP6 expression by approximately 50% at both day 7 and 28. Control culture for 28 days altered AGC1 gene expression as compared to 7 days (11 fold increase). The presence of the EFs downregulated AGC1 expression, with control, BMP-6-ST, and pcDNA3-BMP6 groups all showing significant downregulation at 4 weeks when compared to the same groups without EFs. In contrast to AGC1, the presence of EFs generally upregulated COL2A1 expression. COL2A1 upregulation was most pronounced in the day 28 BMP-6-ST plus EFs group, with a 9.9 fold increase over concurrent controls and 3.5 fold increase over day 7 controls (both $p<0.05$). Inclusion of EFs was associated with a trend of increased COL1A1 transcript compared to the same group without EFs, although this effect only reached significance in the transfected pcDNA3-BMP6 group. Short-term exposure to BMP-6 also upregulated COL1A1, averaging a 3-fold increase over control at day 7. At both day 7 and day 28 for BMP6 overexpression and at day 28 for BMP-6-ST, the EFs significantly upregulated COL10A1 expression as compared to the same group without EFs ($p<0.05$).

Immunohistochemistry (Figure 2)—The use of EFs diminishes accumulation of the glycosaminoglycan (GAG) chondroitin 4-sulfate. Chondroitin 4-sulfate was found most prominently in either the control culture or in the short-term BMP-6 exposure condition, both without the addition of the EFs. For type II collagen, the most accumulation in the matrix occurred in the BMP-6-ST and BMP6 overexpressed (pcDNA3-BMP6) conditions, with the addition of the EFs. This accumulation was noted intracellularly, pericellularly, and diffusely throughout the matrix in these two conditions. For type I collagen, the BMP-6-ST condition, both with and without EFs, resulted in the most deposition of type I collagen in the hydrogel. The addition of EFs caused more diffuse type X collagen staining throughout the hydrogel matrix as compared to the mostly intracellular and pericellular staining seen without the EFs, whereas the addition of BMP-6 decreases type X collagen at day 28.

Study 2

qPCR (Figure 3)—The presence of DEX in all conditions significantly increased BMP6 transcript as compared to the corresponding group without DEX ($p<0.05$), with a 5-fold difference in the overexpressed BMP6 group. The addition of exogenous BMP-6 to the media, at either 10 or 500 ng/mL did not alter BMP6 expression.

DEX strongly upregulated AGC1 expression ($p<0.001$), and the degree of upregulation was affected by the concentration of BMP-6 present ($p<0.001$). Overexpression of BMP6 in combination with DEX resulted in a 17-fold increase in AGC1 transcript as compared to control ($p<0.05$). COL2A1 expression showed a trend toward lower values in the groups treated with DEX in all conditions except the high dose (500 ng/mL) BMP-6 condition, although this effect was only significant in the absence of BMP-6 ($p<0.05$). High concentrations of BMP-6 (500 ng/mL), both in the presence and absence of DEX, resulted in a 1.6 fold increase on average in COL1A1 transcript values ($p<0.0001$). The presence of DEX in the culture medium, either without BMP-6 or with a low dose of BMP-6 (10 ng/mL), resulted in a significant 1.4-fold and 1.7-fold, respectively, downregulation of

COL1A1 transcript as compared to control ($p<0.05$). High concentrations (500 ng/mL) of BMP-6 downregulated COL10A1 as compared to control regardless of the presence of DEX (Figure 3). At every other concentration of BMP-6, DEX significantly suppressed COL10A1 transcript as compared to the same BMP-6 concentration without DEX ($p<0.05$).

Study 3

Immunohistochemistry (Figure 4)—Constitutive BMP6 expression by pcDNA3-BMP6 resulted in the most accumulation of chondroitin 4-sulfate. Marked intracellular and pericellular staining occurred regardless of the presence of DEX; however, the transfected group without DEX proved to promote the most robust matrix containing chondroitin 4-sulfate. The combination of high dose BMP-6, continuous DEX treatment, and EFs resulted in no labeling for this GAG molecule.

In direct contrast to that which was observed with chondroitin 4-sulfate labeling, DEX coupled with 500 ng/mL BMP-6 and EFs resulted in the most accumulation of type II collagen in the matrix. When DEX was removed from the medium along with the reduction from 500 ng/mL to 10 ng/mL of BMP-6, type II collagen production was present but not as strong as when DEX remained present throughout the 28 day culture. DEX without BMP-6 did not promote type II collagen production. For the pcDNA3-BMP6 groups, labeling was only seen in the absence of DEX.

Type I collagen production was most pronounced in response to a continuous dose of DEX in addition to high dose BMP-6, either delivered for the entire 28 days or only the first 7 days followed by low dose of BMP-6 for the remaining 3 weeks. However, the addition of EFs to the DEX, high dose BMP-6 culture attenuated type I collagen biosynthesis, resulting in much less type I collagen deposition. Removing DEX after the first 7 days also abrogated type I collagen production. BMP6 transfected cultures exhibited increases of type I collagen production, particularly in the pericellular regions, when DEX was omitted from the culture.

The addition of DEX to control cultures greatly increased the accumulation of type X collagen production, and this increased production was abrogated by continuous delivery of low but not high levels of BMP-6. All pcDNA3-BMP6 transfected ASC cultures demonstrated some labeling for type X collagen; though, qualitatively, 100 nM DEX for the entire culture period resulted in more intense staining for type X collagen relative to the transfected cultures without DEX or with DEX for only the first 7 days.

DISCUSSION

In this study, we examined the ability of TGF- β 1, EGF, FGF-2 (i.e., EFs), DEX, and BMP-6, either alone or in various combinations, to promote chondrogenesis in ASCs. BMP-6 has been previously demonstrated to exert potent chondrogenic effects on ASC culture^{7,8,13}, but continuous high dose BMP-6 results in a fibrocartilaginous phenotype after 28 days of culture⁸. We hypothesized that chondrogenesis in this cell population could be augmented by the addition of other pro-chondrogenic factors. The results indicate that both the overexpression of pcDNA3-BMP6 as well as BMP-6-ST treatment can enhance the production of cartilage specific matrix molecules in 3D culture, although neither was sufficient to induce an “ideal” chondrocytic phenotype either with or without the EFs or DEX.

In study 1, the EFs produced both beneficial and detrimental effects on ASC chondrogenesis as they increased COL2A1 production and subsequent type II collagen deposition in the matrix but also repressed AGC1 transcription and GAG accumulation. The expression of COL2A1 in the BMP-6 plus EFs groups is consistent with previous studies suggesting that

both TGF- β 1 and BMP-6 signaling are required to induce COL2A1 expression in ASCs¹³. The presence of EFs increased COL1A1 gene expression (Figure 1) and protein synthesis (Figure 2). The EFs interacted with BMP-6 to promote a hypertrophic phenotype as noted by increases in both type X and type I collagen production. Our results are in contrast to the reported ability of FGF-2 to both stimulate type II collagen and GAG production while inhibiting type I collagen production, although it is important to note that previously obtained results were dependent on FGF concentration and cell passage number^{16,33,34}. The contradictory effects of EFs on chondrogenesis underscore the need for further culture condition optimization to more fully understand ASC chondrogenesis.

Interestingly, short-term exposure to BMP-6, both with or without EFs, was associated with a large increase in the accumulation of type I collagen in the matrix, which was not observed in the transfected or control groups (Figure 2). As has been demonstrated with TGF- β 3 in primary chondrocytes³⁰, these data suggest that the temporal application of any of these agents alone or in combination may be exploited to enhance chondrogenesis in this stem cell population.

Our results from study 2 demonstrated that early gene expression of ASCs in 3-dimensional culture is strongly affected by the presence of DEX as well as BMP-6. Since DEX is known to be a common mesenchymal differentiation factor that is dependent on its concentration and interaction with other factors for successful differentiation³⁵, it is conceivable that other molecules will affect the ensuing phenotype³⁵. A recent study by Derfoul and colleagues showed that DEX has an overall positive effect on MSC chondrogenesis in the presence of TGF- β 3 as noted by the enhancement of cartilage specific transcript markers, namely aggrecan, type II collagen, and COMP²¹. Conversely, DEX in conjunction with BMP-2 significantly repressed these same markers in MSCs²².

In our study, DEX significantly increased AGC1 expression, and an interactive effect between DEX and BMP-6 was observed. COL2A1 was not expressed at high levels in any group as compared to control. The lack of COL2A1 transcript may not be indicative of failure to induce chondrogenesis, as many have reported that COL2A1 transcript is not observed during initial culture periods under chondrogenic stimuli^{13,36}. DEX also demonstrated the ability to repress COL10A1 expression, which is characteristic of a hypertrophic or mature chondrocyte phenotype. In this study (Figure 3) and previous studies⁷, a high dose of BMP-6 was required to downregulate COL10A1 in the absence of DEX.

Previous studies have shown that glucocorticoids act upstream of BMP6 and can upregulate BMP6 in various cell types^{19,23,37,38}. Accordingly, DEX was found to also upregulate BMP6 gene expression in human ASCs. Furthermore, this upregulation of BMP6 transcript correlated to the enhancement of AGC1 and repression of COL10A1 gene expression. These data suggest that transcriptional activation of BMP6 may be at least partially responsible for the effects of DEX on gene expression in ASCs. Also, the interactive effects between DEX and BMP-6 concentration indicate that chondrogenesis may be influenced by indirect mechanisms such as BMP and glucocorticoid signaling crosstalk.

Building on the first two parts of our study, 11 different culture conditions were selected to investigate the phenotype following 28 days of *in vitro* culture. Of particular interest were the long-term effects of continuous DEX treatment on ASC culture. Dexamethasone has been directly related to both enhancement and deterioration of the cartilage phenotype in both MSC and primary chondrocyte culture as a function of the cell type and specific growth factors employed^{20,22,25,26,29,39,40}. In general, early gene transcript data of the interaction

between BMP-6 and DEX from study 2 proved to be poor predictors of protein accumulation at day 28.

Even though DEX robustly enhanced AGC1 transcript at day 7 (Figure 3), it was clearly evident that DEX inhibited the accumulation of GAGs over 28 days in culture. The pcDNA3-BMP6 transfected ASCs, without DEX, produced the most GAG content compared across all culture conditions. Some chondroitin 4-sulfate labeling was seen with soluble BMP-6 when ASC constructs were cultured without DEX or when DEX was removed from the culture medium after the first week.

The additions of EFs to BMP-6 greatly enhanced biosynthesis of type II collagen (Figure 2). This effect was confirmed even when DEX was added to the culture (Figure 4). Without the presence of the EFs, type II collagen was inhibited by DEX alone or in combination with BMP-6. This trend towards decreased type II collagen production was reversed when the concentration of BMP-6 was reduced after the first 7 days in culture, suggesting that an initial high dose of BMP-6 followed by low doses may be more beneficial than continuous administration of either low or high doses of BMP-6.

DEX inhibited COL10A1 transcript levels at day 7; though, by day 28, DEX either alone or in combination with a high dose BMP-6 induced the accumulation of the cartilage hypertrophic marker type X collagen (Figure 4). It was noted that low doses of BMP-6 with DEX resulted in much less accumulation of type X collagen. The combination of DEX plus a high dose of BMP-6 also appeared to promote higher COLA1 expression and type I collagen production, suggesting that a synergistic relationship exists between DEX and BMP-6 in promoting a fibrocartilaginous phenotype.

The combined work of these studies shows that the effects of BMP6 overexpression are very sensitive to the addition of EFs and DEX. Further supporting that diverse effects are possible with BMP6 overexpression, Sheyn et al used non-viral BMP6 delivery to induce osteogenic differentiation *in vitro* by adding β -glycerophosphate to high density monolayer culture⁴¹. The same study demonstrated bone formation *in vivo* after injection of ASCs overexpressing rhBMP6 with a fibrin gel carrier⁴¹. The culture conditions and scaffold used may therefore be sufficient to drive transfected BMP-6 mediated differentiation of ASCs down different lineages, with the spherical phenotype exhibited by ASCs in alginate beads possibly being conducive to chondrogenesis¹¹.

CONCLUSIONS

BMP6 overexpression, in and of itself, did not produce a strong cartilage phenotype. However, control medium with pcDNA3-BMP6 transfected cells may have produced the best overall phenotype by day 28 as noted by the presence of chondroitin 4-sulfate and type II collagen labeling with minimal deposition of types I and X collagen. The EFs, both with and without DEX, promoted type II collagen deposition, although the EFs also resulted in increased production of two negative markers of chondrogenesis, types I and X collagen, and inhibited the accumulation of glycosaminoglycans. Culturing ASCs with DEX promoted positive chondrogenic gene expression results at day 7, which demonstrated a synergistic effect between DEX and BMP-6. However, the immunohistochemistry analysis suggests that continuous administration of 100 nM DEX could be problematic for ASC chondrogenesis. In future studies, consideration should be given to the short-term application of dexamethasone to maximize the benefit of this potent steroid without the potential negative effects. These data suggest a complicated interplay between BMP-6, FGF, EGF, TGF- β , and glucocorticoids, in which the correct growth factor concentrations and temporal application of the factors are critical to regulate the appropriate biosynthesis of

cartilage matrix proteins. As multiple agents may be necessary for successful chondrogenesis, future investigators should pay special attention to the interactive effects when characterizing novel culture conditions.

Acknowledgments

We are grateful to Dr. Gregory Helm of the University of Virginia for providing the pcDNA3-BMP6 construct and Dr. Virginia Kraus of Duke University Medical Center for donation of the 2B6 antibody. This study was supported in part by NIH grants AG15768, AR50245, AR48182, AR48852, and GM08555, the Coulter Foundation, the Duke Translational Research Institute (RR024128), and by a NSF Graduate Fellowship.

REFERENCES

1. Aust L, Devlin B, Foster SJ, Halvorsen YD, Hicok K, du Laney T, et al. Yield of human adipose-derived adult stem cells from liposuction aspirates. *Cytotherapy*. 2004; 6:7–14. [PubMed: 14985162]
2. Zuk PA, Zhu M, Ashjian P, De Ugarte DA, Huang JI, Mizuno H, et al. Human adipose tissue is a source of multipotent stem cells. *Mol Biol Cell*. 2002; 13:4279–4295. [PubMed: 12475952]
3. Guilak F, Lott KE, Awad HA, Cao Q, Hicok KC, Fermor B, et al. Clonal analysis of the differentiation potential of human adipose-derived adult stem cells. *J Cell Physiol*. 2006; 206:229–237. [PubMed: 16021633]
4. Mizuno H, Zuk PA, Zhu M, Lorenz HP, Benhaim P, Hedrick MH. Myogenic differentiation by human processed lipoaspirate cells. *Plast Reconstr Surg*. 2002; 109:199–209. discussion 10-1. [PubMed: 11786812]
5. Betre H, Ong SR, Guilak F, Chilkoti A, Fermor B, Setton LA. Chondrocytic differentiation of human adipose-derived adult stem cells in elastin-like polypeptide. *Biomaterials*. 2006; 27:91–99. [PubMed: 16023192]
6. Guilak F, Awad HA, Fermor B, Leddy HA, Gimple JM. Adipose-derived adult stem cells for cartilage tissue engineering. *Biorheology*. 2004; 41:389–399. [PubMed: 15299271]
7. Estes BT, Wu AW, Guilak F. Potent induction of chondrocytic differentiation of human adipose-derived adult stem cells by bone morphogenetic protein 6. *Arthritis Rheum*. 2006; 54:1222–1232. [PubMed: 16572454]
8. Estes BT, Wu AW, Storms RW, Guilak F. Extended passaging, but not aldehyde dehydrogenase activity, increases the chondrogenic potential of human adipose-derived adult stem cells. *J Cell Physiol*. 2006; 209:987–995. [PubMed: 16972251]
9. Zaragosi LE, Billon N, Ailhaud G, Dani C. Nucleofection is a valuable transfection method for transient and stable transgene expression in adipose tissue-derived stem cells. *Stem Cells*. 2007; 25:790–797. [PubMed: 17158239]
10. Estes BT, Diekman BO, Guilak F. Monolayer cell expansion conditions affect the chondrogenic potential of adipose-derived stem cells. *Biotechnol Bioeng*. 2008; 99:986–995. [PubMed: 17929321]
11. Awad HA, Wickham MQ, Leddy HA, Gimple JM, Guilak F. Chondrogenic differentiation of adipose-derived adult stem cells in agarose, alginate, and gelatin scaffolds. *Biomaterials*. 2004; 25:3211–3222. [PubMed: 14980416]
12. Erickson GR, Gimple JM, Franklin DM, Rice HE, Awad H, Guilak F. Chondrogenic potential of adipose tissue-derived stromal cells in vitro and in vivo. *Biochem Biophys Res Commun*. 2002; 290:763–769. [PubMed: 11785965]
13. Hennig T, Lorenz H, Thiel A, Goetzke K, Dickhut A, Geiger F, et al. Reduced chondrogenic potential of adipose tissue derived stromal cells correlates with an altered TGFbeta receptor and BMP profile and is overcome by BMP-6. *J Cell Physiol*. 2007; 211:682–691. [PubMed: 17238135]
14. Johnstone B, Hering TM, Caplan AI, Goldberg VM, Yoo JU. In vitro chondrogenesis of bone marrow-derived mesenchymal progenitor cells. *Exp Cell Res*. 1998; 238:265–272. [PubMed: 9457080]

15. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999; 284:143–147. [PubMed: 10102814]
16. Chiou M, Xu Y, Longaker MT. Mitogenic and chondrogenic effects of fibroblast growth factor-2 in adipose-derived mesenchymal cells. *Biochemical and Biophysical Research Communications*. 2006; 343:644–652. [PubMed: 16554022]
17. Quarto N, Longaker MT. FGF-2 inhibits osteogenesis in mouse adipose tissue-derived stromal cells and sustains their proliferative and osteogenic potential state. *Tissue Engineering*. 2006; 12:1405–1418. [PubMed: 16846339]
18. Awad HA, Halvorsen YD, Gimble JM, Guilak F. Effects of transforming growth factor beta1 and dexamethasone on the growth and chondrogenic differentiation of adipose-derived stromal cells. *Tissue Eng*. 2003; 9:1301–1312. [PubMed: 14670117]
19. Oshina H, Sotome S, Yoshii T, Torigoe I, Sugata Y, Maehara H, et al. Effects of continuous dexamethasone treatment on differentiation capabilities of bone marrow-derived mesenchymal cells. *Bone*. 2007; 41:575–583. [PubMed: 17690025]
20. Ochi K, Derfoul A, Tuan RS. A predominantly articular cartilage-associated gene, SCRG1, is induced by glucocorticoid and stimulates chondrogenesis in vitro. *Osteoarthritis Cartilage*. 2006; 14:30–38. [PubMed: 16188469]
21. Derfoul A, Perkins GL, Hall DJ, Tuan RS. Glucocorticoids promote chondrogenic differentiation of adult human mesenchymal stem cells by enhancing expression of cartilage extracellular matrix genes. *Stem Cells*. 2006; 24:1487–1495. [PubMed: 16469821]
22. Kurth T, Hedbom E, Shintani N, Sugimoto M, Chen FH, Haspl M, et al. Chondrogenic potential of human synovial mesenchymal stem cells in alginate. *Osteoarthritis Cartilage*. 2007; 15:1178–1189. [PubMed: 17502159]
23. Liu Y, Titus L, Barghouthi M, Vigneswarapu M, Hair G, Boden SD. Glucocorticoid regulation of human BMP-6 transcription. *Bone*. 2004; 35:673–681. [PubMed: 15336603]
24. Behrens F, Shepard N, Mitchell N. Alterations of rabbit articular cartilage by intra-articular injections of glucocorticoids. *J Bone Joint Surg Am*. 1975; 57:70–76. [PubMed: 1123374]
25. Farquhar T, Todhunter RJ, Fubini SL, Burton-Wurster N, Lust G. Effect of methylprednisolone and mechanical loading on canine articular cartilage in explant culture. *Osteoarthritis Cartilage*. 1996; 4:55–62. [PubMed: 8731396]
26. Miyazaki Y, Tsukazaki T, Hirota Y, Yonekura A, Osaki M, Shindo H, et al. Dexamethasone inhibition of TGF beta-induced cell growth and type II collagen mRNA expression through ERK-integrated AP-1 activity in cultured rat articular chondrocytes. *Osteoarthritis Cartilage*. 2000; 8:378–385. [PubMed: 10966845]
27. Nakamura M, Watanabe J, Ogawa R, Kanamura S. Immunohistochemical localization of type II and type I collagens in articular cartilage of the femoral head of dexamethasone-treated rats. *Histochem J*. 1997; 29:645–654. [PubMed: 9413737]
28. Celeste C, Ionescu M, Robin Poole A, Lavery S. Repeated intraarticular injections of triamcinolone acetonide alter cartilage matrix metabolism measured by biomarkers in synovial fluid. *J Orthop Res*. 2005; 23:602–610. [PubMed: 15885481]
29. Dechant JE, Baxter GM, Frisbie DD, Trotter GW, McIlwraith CW. Effects of dosage titration of methylprednisolone acetate and triamcinolone acetonide on interleukin-1-conditioned equine articular cartilage explants in vitro. *Equine Vet J*. 2003; 35:444–450. [PubMed: 12875321]
30. Lima EG, Bian L, Ng KW, Mauck RL, Byers BA, Tuan RS, et al. The beneficial effect of delayed compressive loading on tissue-engineered cartilage constructs cultured with TGF-beta3. *Osteoarthritis Cartilage*. 2007; 15:1025–1033. [PubMed: 17498976]
31. Laurent JJ, Webb KM, Beres EJ, McGee K, Li J, van Rietbergen B, et al. The use of bone morphogenetic protein-6 gene therapy for percutaneous spinal fusion in rabbits. *J Neurosurg Spine*. 2004; 1:90–94. [PubMed: 15291027]
32. Mehlhorn AT, Niemeyer P, Kaiser S, Finkenzeller G, Stark GB, Sudkamp NP, et al. Differential expression pattern of extracellular matrix molecules during chondrogenesis of mesenchymal stem cells from bone marrow and adipose tissue. *Tissue Eng*. 2006; 12:2853–2862. [PubMed: 17518654]

33. Tsutsumi S, Shimazu A, Miyazaki K, Pan H, Koike C, Yoshida E, et al. Retention of multilineage differentiation potential of mesenchymal cells during proliferation in response to FGF. *Biochem Biophys Res Commun*. 2001; 288:413–419. [PubMed: 11606058]
34. Solchaga LA, Penick K, Porter JD, Goldberg VM, Caplan AI, Welter JF. FGF-2 enhances the mitotic and chondrogenic potentials of human adult bone marrow-derived mesenchymal stem cells. *J Cell Physiol*. 2005; 203:398–409. [PubMed: 15521064]
35. Grigoriadis AE, Heersche JN, Aubin JE. Differentiation of muscle**fat**cartilage, and bone from progenitor cells present in a bone-derived clonal cell population: effect of dexamethasone. *J Cell Biol*. 1988; 106:2139–2151. [PubMed: 3384856]
36. Mushtaq T, Farquharson C, Seawright E, Ahmed SF. Glucocorticoid effects on chondrogenesis, differentiation and apoptosis in the murine ATDC5 chondrocyte cell line. *J Endocrinol*. 2002; 175:705–713. [PubMed: 12475381]
37. Boden SD, Liu Y, Hair GA, Helms JA, Hu D, Racine M, et al. LMP-1, a LIM-domain protein, mediates BMP-6 effects on bone formation. *Endocrinology*. 1998; 139:5125–5134. [PubMed: 9832452]
38. Yoon ST, Park JS, Kim KS, Li J, Attallah-Wasif ES, Hutton WC, et al. ISSLS prize winner: LMP-1 upregulates intervertebral disc cell production of proteoglycans and BMPs in vitro and in vivo. *Spine*. 2004; 29:2603–2611. [PubMed: 15564908]
39. Sekiya I, Koopman P, Tsuji K, Mertin S, Harley V, Yamada Y, et al. Dexamethasone enhances SOX9 expression in chondrocytes. *J Endocrinol*. 2001; 169:573–579. [PubMed: 11375127]
40. Chen CW, Tsai YH, Deng WP, Shih SN, Fang CL, Burch JG, et al. Type I and II collagen regulation of chondrogenic differentiation by mesenchymal progenitor cells. *J Orthop Res*. 2005; 23:446–453. [PubMed: 15734261]
41. Sheyn D, Pelled G, Zilberman Y, Talasazan F, Frank JM, Gazit D, et al. Nonvirally Engineered Porcine Adipose Tissue-Derived Stem Cells: Use in Posterior Spinal Fusion. *Stem Cells*. 2008

Figure 1.

Study 1 qPCR results for BMP6, AGC1, COL2A1, COL1A1, and COL10A1. Data presented as fold difference to week 1 control without EFs transcript values. EFs = 5 ng/ml EGF, 1 ng/ml FGF, and .25 ng/ml TGF- β 1. Groups not sharing the same letter are statistically different from each other, ANOVA, Fisher's PLSD, $\alpha=0.05$. No significant differences were observed between the pMAX-GFP transfection control and the non-transfected control group. Therefore, the transfection control is not presented.

Figure 2.
Study 1 immunohistochemistry results for chondroitin 4-sulfate types II, I, and X collagen.
Scale bar = 20 μm.

Figure 3.

Study 2 qPCR results for BMP6, AGC1, COL2A1, COL1A1, and COL10A1. Data presented as fold difference to Day 7 Control without DEX transcript values. "X" indicates transfection with the pcDNA3-BMP6 plasmid. Groups not sharing the same letter are statistically different from each other, ANOVA, Fisher's PLSD, $\alpha=0.05$.

Figure 4.

Study 3 immunohistochemistry results for chondroitin 4-sulfate and types II, I, and X collagen. If one line of text is present under image, these are the conditions for the entire 28 day culture. If a 2nd line is present, the 1st line under image are the conditions for the first 7 days of culture; the 2nd line are the conditions for the last 21 days of culture. Scale bar = 20 μ m.

Table 1

Culture conditions given for each study are the additions to the control medium of DMEM-high glucose, 10% FBS, 1% penicillin-streptomycin, 37.5 µg/mL L-ascorbic acid 2-phosphate, and 1% ITS+ Premix. Expansion factors (EFs) represent 0.25 ng/mL TGF-β1, 5 ng/mL EGF, and 1 ng/mL bFGF. BMP-6-short term exposure (BMP-6-ST) constructs received 500 ng/mL of BMP-6 for only the first four days. The transfected ASCs (both pcDNA3-BMP6 and pMAX-GFP) received control medium throughout the culture period. In study 3, as noted, some groups had different culture conditions for the final 21 days.

Study 1 <i>day 7 and day 28 qPCR, day 28 IHC</i>	Study 2 <i>day 7 qPCR</i>	Study 3 <i>day 28 IHC</i>
Control	Control	Control
EFs	DEX	DEX
BMP-6-ST	10 ng/ml BMP-6	10 ng/ml BMP-6 + DEX
BMP-6-ST + EFs	10 ng/ml BMP-6 + DEX	pcDNA3-BMP6 + DEX
pMAX-GFP	500 ng/ml BMP-6	500 ng/ml BMP-6 + DEX
pMAX-GFP + EFs	500 ng/ml BMP-6 + DEX	500 ng/ml BMP-6 + DEX (7 days), then 10 ng/ml BMP-6 + DEX (21 days)
pcDNA3-BMP6	pcDNA3-BMP6	500 ng/ml BMP-6 + DEX (7 days), then 10 ng/ml BMP-6 (21 days)
pcDNA3-BMP6 + EFs	pcDNA3-BMP6 + DEX	pcDNA3-BMP6 + DEX (7 days), then control (21 days)
		Control + DEX (7 days), then control (21 days)
		500 ng/ml BMP-6 + DEX + EFs
		pcDNA3-BMP6