Regulation of articular chondrocyte catabolic genes by growth factor interaction

Shuiliang Shi[1,#], **Scott Mercer**[1,†], **George J. Eckert**[2], **Stephen B. Trippel**[1,3,4,*]

[1]Department of Orthopaedic Surgery, Indiana University School of Medicine.

[2]Department of Biostatistics, Indiana University School of Medicine.

[3]Department of Anatomy and Cell Biology, Indiana University School of Medicine.

[4]Department of Biomedical Engineering, Indiana University Purdue University Indianapolis

Abstract

Osteoarthritis is characterized by a loss of articular cartilage homeostasis in which degradation exceeds formation. Several growth factors have been shown to promote cartilage formation by augmenting articular chondrocyte anabolic activity. This study tests the hypothesis that such growth factors also play an anti-catabolic role. We transferred individual or combinations of the genes encoding insulin- like growth factor I, bone morphogenetic protein-2, bone morphogenetic protein-7, transforming growth factor-β1 and fibroblast growth factor-2, into adult bovine articular chondrocytes and measured the expression of catabolic marker genes encoding A disintegrin and metalloproteinase with thrombospondin motifs-4 and −5, matrix metalloproteinases-3 and −13, and interleukin-6. When delivered individually, or in combination, these growth factor transgenes differentially regulated the direction, magnitude and time course of expression of the catabolic marker genes. In concert, the growth factor transgenes regulated the marker genes in an interactive fashion that ranged from synergistic inhibition to synergistic stimulation. Synergistic stimulation prevailed over synergistic inhibition, reaching maxima of 15.2-fold and 2.7-fold, respectively. Neither the magnitude nor the time course of the effect of the transgene combinations could be predicted on the basis of the individual transgene effects. With few exceptions, the data contradict our hypothesis. The results demonstrate that growth factors that are traditionally viewed as chondrogenic tend also to promote catabolic gene expression. The competing actions of these potential therapeutic agents add an additional level of complexity to the selection of regulatory factors for restoring articular cartilage homeostasis or promoting repair.

*To whom correspondence should be addressed: Stephen B. Trippel: Department of Orthopaedics, Indiana University School of Medicine, Indianapolis, IN 46202; strippel@iupui.edu; Tel. (317) 278-0085; Fax. (317) 274-3702.
Present address: Apt. 102, Unit 4, Bldg 8, Jinping Xiaoqu,Pinghu, Zhejiang 314200, China
† 2863 West 39th Street #313, Indianapolis, IN 46228

Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

Author Contributions

Conception and design: SBT, SS

Methodology: SS, SM

Statistical analysis: GJE

Manuscript writing and review: SBT, SS, SM, GE

Keywords

articular chondrocytes; growth factor; cytokines; gene transfer; cellular regulation

Introduction

Articular cartilage loss in osteoarthritis reflects a disruption of normal homeostasis. The balanced matrix turnover of healthy cartilage is replaced by greater matrix degradation than formation, resulting in a net loss of cartilage tissue and impaired joint function. One approach to articular cartilage repair is to reverse this imbalance (Trippel, 1995). This approach may be accomplished by augmenting chondrocyte anabolic and mitotic activity, or suppressing chondrocyte catabolic activity, or both. Several polypeptide growth factors have been shown to augment articular chondrocyte anabolic or mitotic activity (Chopra and Anastassiades, 1998; Guerne et al., 1994; Trippel, 1997). These include insulin-like growth factor-I (IGF-I) (Sah et al., 1994; Fortier, Mohammed et al., 2002; Luyten et al., 1988), fibroblast growth factor-2 (FGF- 2) (Sah et al., Kato et al., 1987; Henson et al., 2005), bone morphogenetic protein-2 and −7 (BMP-2, BMP-7) (Grunder et al., 2004; Reddi, 2003; Chubinskaya et al., 2007; Flechtenmacher et al., 1996), and transforming growth factor beta1 (TGF-β1) (Morales and Roberts, 1988; Rosier, O'Keefe et al., 1989). Delivered as exogenous proteins or as endogenous proteins by gene transfer *in vitro*, each of these growth factors is anabolic and/or mitogenic. *In vivo*, each of these factors improves the repair of articular cartilage damage (Sellers et al., 1997; Chubinskaya et al., 2007; Fujimoto et al., 1999; Cucchiarini et al., 2005; Goodrich et al., 2007; Madry et al., 2005; Nixon et al., 1999; Yokoo et al., 2005; Rey-Rico et al., 2017). These growth factors also selectively interact to synergistically augment articular chondrogenic functions (Shi et al., 2012; Shi et al., 2013).

An anabolic or mitogenic stimulus that simultaneously decreases catabolic activity could be more beneficial than a factor that acts only to promote repair or to inhibit degradation. This study tests the hypothesis that growth factors known to be anabolic for articular chondrocytes are also anti-catabolic.

Articular cartilage degradation in osteoarthritis is thought to be mediated by catabolic enzymes and cytokines. Prominent among these are disintegrin and metalloproteinase with thrombospondin motifs-4 and −5 (ADAMTS-4 and ADAMTS- 5), also known as aggrecanase-1 and aggrecanases-2 (Tortorella et al., 1999; Abbaszade et al., 1999), matrix metalloproteinase-3 and −13 (MMP-3 and MMP-13) (Flannery et al., 1992;Goldring et al., 2011; Mitchell et al., 1996; Tetlow et al., 2001), and interleukin-6 (IL-6) (Qu et al., 2015).

ADAMTS-4 and ADAMTS-5 cleave aggrecan at the ADAMTS cleavage site in the interglobular domain of its core protein, and cleavage products from this site are prevalent in OA. Removal of ADAMTS-5 (but not ADAMTS-4) by gene knock-out in the mouse protects against the development of cartilage damage. Additional data suggest that, in contrast to the mouse, ADAMTS-4 is at least as important as ADAMTS-5 in human OA (Dancevic and McCulloch, 2014).

MMP-3 and MMP-13 are increased in OA cartilage (Tetlow et al., 2001) and, like ADAMTS-4 and ADAMTS-5, are upregulated by inflammatory cytokines. They cleave aggrecan at a site in the interglobular domain that is distinct from that of the aggrecanases (Hughes et al., 1998). Unlike the aggrecanases, MMP-3 and MMP-13 also both cleave type II collagen. MMP-13 is the principal collagenase for type II collagen in joints, and may be more important in OA pathology than MMP-3 (Goldring et al., 2011).

IL-6 is an inflammatory cytokine that is elevated in the serum, synovial fluid (Gobezie et al., 2007; Kokebie et al., 2011), and articular cartilage (Qu et al., 2015) of patients with OA and the synovial fluid of patients with articular cartilage defects (Tsuchida et al., 2012). IL-6 serum levels predict radiographic knee OA (Livshits et al., 2009) and *IL6* gene expression is increased in knee articular cartilage in OA patients (Qu et al., 2015).

We assessed the growth factor regulation of the gene expression of these catabolic mediators by transfecting adult articular chondrocytes with the genes encoding IGF-I, BMP-2, BMP-7, TGF-β1 and FGF-2. To our knowledge, this is the first systematic analysis of the regulation of catabolic factor gene expression by chondrogenic growth factors. Because the genes encoding these growth factors, and the growth factors themselves, are under consideration as therapeutic agents for articular cartilage repair, these data are important to their further evaluation and development.

Materials and Methods

Construction of IGF-I, FGF-2, BMP-2, BMP-7 and TGF-β1 pAAV vectors

The vectors pAAV-IGF-I, pAAV-FGF-2, pAAV-BMP-2, pAAV-BMP-7 and pAAV-TGF-β1 were generated as previously described (Shi et al., 2012). Briefly, the human growth factor cDNA coding regions were generated by PCR and, after confirming the sequences, were subcloned into pAAV-MCS (Stratagene, La Jolla, CA) to obtain the pAAV-based vectors. To improve the readability of the data presented, the transgenes carried by pAAV- IGF-I, pAAV-FGF-2, pAAV-BMP-2, pAAV-BMP-7 and pAAV-TGF-β1 are here designated tIGF-I, tFGF-2, tBMP-2, tBMP-7 and tTGF-β1.

Chondrocyte cell culture and transfection

Basal medium was prepared with DMEM, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Invitrogen, Carlsbad, CA) and 50 μg/ml ascorbic acid (Sigma, St. Louis, MO). Complete medium was prepared by supplementing basal medium with 10% FBS (Invitrogen). Bovine articular chondrocytes were isolated and placed in primary culture as previously described (Shi et al., 2010). Briefly, chondrocytes were isolated from the carpal joints of skeletally mature (growth plates closed) bovids, cultured at 3 × 105 cells/well in complete medium for 3 days, and transfected using FuGENE 6 (Roche Applied Science, Indianapolis, IN) and plasmid DNA. For single transfections, 2 μg of each plasmid DNA per well was used. For multiple transfections, 2 μg of each plasmid DNA per well was used together. Control cells received 2 μg, 4 μg, or 6 μg empty vector DNA for 1-, 2-, and 3-transgene transfections respectively. Transfection was stopped by replacing the medium with fresh complete medium. On days 2 and 4 after transfection, conditioned medium (CM) was

collected and replaced by basal medium. On day 6 after transfection, CM was collected and cell culture was terminated. At daily intervals, triplicate samples of chondrocytes were lysed with lysis buffer RLT (RNeasy Mini kit, Qiagen, Valencia, CA), homogenized by passing 6 times through a 20- gauge needle and stored at −80°C for total RNA purification.

RNA purification, reverse transcription and real-time PCR analysis

RNA purification and reverse transcription were performed as previously described (Shi et al., 2009). Briefly, total RNA was prepared using the RNeasy Mini kit (Qiagen). On-column DNase digestion was performed to remove any residual DNA. Reverse transcription was performed using the High-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) and random primer. Reverse transcription was terminated by heating at 95°C for 20 minutes. cDNA samples were used for real-time PCR analysis. Real-time PCR was performed as previously described (Shi et al., 2012). Briefly, ADAMTS-4 and −5, MMP-3 and 13, and IL-6 transcripts, and 18S rRNA content were measured by real-time PCR using SYBR Green master mix (Life Technologies) and CFX96 real-time PCR detection system (Bio-Rad). Primers were synthesized by Life Technologies (Table 1). The standard curve method was used to calculate the expression of target genes encoding ADAMTS-4 (*ADAMTS4),* ADAMTS-5 *(ADAMTS5),* MMP-3 (*MMP3)*, MMP-13 *(MMP13)*, and IL-6 (*IL6),* and the content of 18S rRNA. Target gene mRNA levels were normalized to 18S rRNA levels. Fold changes of target gene expression were measured as the ratio of expression by growth factor gene transfected cells to that of cells transfected with empty vector (mock-transfected) control. Three independent experiments were performed using articular chondrocytes obtained from different bovine joints at different times. Data are presented as the average of fold changes.

Statistical Analysis

The effects of IGF-I, TGF-β1, BMP-2, BMP-7 and FGF-2 gene transfer on outcome variables (*ADAMTS4, ADAMTS5, MMP3,MMP13* and *IL6* expression) were evaluated using repeated measures ANOVA. The ANOVAs used terms for group, day, and the group-by-day interaction, as well as a random effect to correlate data within each of the three experimental runs. An unstructured variance/covariance matrix was used for the variances and correlations within a sample across the six days. In the case of *MMP13* expression, the values were through day 5 because day 6 values were below PCR detection. The natural log transformation of the measurements was used for the analyses to address skewing of the data. No adjustments were made for multiple comparisons. Using the ANOVAs, tests were performed to 1) compare the effects of individual growth factor transgenes, 2) compare the effects of combinations of multiple, simultaneously delivered transgenes with the effects of individual transgenes and with the effects of the other transgene combinations, and 3) determine whether multiple gene transfer generated synergistic or inhibitory effects compared to the transfer of individual genes. The interaction data are expressed as comparisons of ratios of combined to separate effects (C/S ratios), where combined effect is the value of the dependent variable in response to the transgene combination, and separate effects is the sum of the values of the dependent variable in response to the two independent variables. Four types of results were obtained from the interaction analysis: 1) Additive effects were those for which the result from the combined transgenes was not significantly

different from the sum of the results from the individual transgenes (absence of interaction). 2) Synergistic effects were those for which the result from the combined transgenes was greater than the sum of the results from the individual transgenes (C/S > 1). 3) Inhibitory effects were those for which the result from the combined transgenes was reduced compared to the sum of the results from the individual transgenes (C/S < 1.0). Inhibition occurred when a.) C and S were both greater than control (positive values), b.) C was less than S (the combined transgenes had a reduced increase compared to the sum of the individual transgenes), c.) C and S were both less than control (negative values), or d.) the absolute value of the decrease by C was less than that of S (the combined transgenes had less of a decrease compared to the sum of the individual transgenes). 4) Synergistically inhibitory effects were those for which the sum of the results from the individual transgenes was less than control, and the result from the combined transgenes was less than this sum (Shi et al., 2013). A 5% significance level was used for all comparisons. The term inhibitory is used only to describe interactions (C/S ratios) and not to describe direct effects (changes compared to controls).

Results

ADAMTS4 Expression:

All individual growth factor transgenes stimulated *ADAMTS4* expression compared to mock-transfected controls. The IGF-I and FGF-2 transgenes (tIGF-1 and tFGF-2) each increased *ADAMTS4* expression more than two-fold and maintained stimulation for the 6-day duration of the experiments. Stimulation by the other individual transgenes was transient and was never greater than 2-fold (Figure 1A). All combinations of growth factor transgenes generated a biphasic time course, characterized by peak stimulation at day 3 or 4 and followed by a partial return toward baseline. Maximal stimulation of *ADAMTS4* was 15.6-fold ($p<0.0001$) by the combination [tIGF-I + tFGF-2]. No transgene combination reduced *ADAMTS4* below control levels at any time point (Figure 1B).

The growth factor transgenes interacted in a complex fashion, ranging from inhibitory to synergistic. In all cases, this interaction followed a time course from no interaction to inhibition to synergistic stimulation of *ADAMTS4* expression. The duration of the synergistic stimulation varied from the full remainder of the study period, as for the combination [tIGF-I + tFGF-2], to only one day, as for [tIGF-I + tFGF-2+ tBMP-7], after which the interactions became inhibitory again. Maximum synergy was obtained by combining tIGF-I and tFGF-2($C/S=4.39$, $p<0.0001$). Maximum inhibition occurred when tBMP-7 was added to [tIGF-I + tFGF-2] ($C/S=0.54$, $p<0.0001$) (Tables 2 and 3).

ADAMTS5 Expression:

All individual growth factor transgenes decreased *ADAMTS5.* The time course of the decrease in *ADAMTS5* expression varied from progressive decrease over time for tTGF-β1, to minimal change for tIGF-I, to diminishing effect over time for tBMP-2 and tBMP-7 (Figure 2A). Of the transgene combinations, [tIGF-I + tBMP-2 + tBMP-7] caused the greatest reduction (96%, $p<0.0002$) in *ADAMTS5* expression compared to control. Only

tFGF-2 increased *ADAMTS5*, a transient increase to 1.3 fold control on day 3 (p = 0.0161) (Figure 2B).

Several growth factor transgenes interacted in inhibiting *ADAMTS5* expression. Notably, when tIGF-I was added to either tFGF-2 or tTGF-β1, the combination [tIGF-I + tFGF-2] or [tIGF-I + tTGF-β1] synergistically inhibited *ADAMTS5* expression at five of the six time points tested. Maximum synergistic inhibition (C/S = 2.68, p<0.0001) was generated by combining tIGF-I and tFGF-2 (Tables 2 and 3).

MMP3 Expression:

Most individual transgenes had little effect on MMP-3 expression. An exception, tFGF-2, increased *MMP3* expression to 7.7-fold (p=0.001) on day 4 and maintained a greater than 4-fold stimulation throughout the remainder of the culture period. Only tIGF-I and tTGF-β1 reduced *MMP3* expression below control levels, tIGF-I transiently to 75% (p=0.0203) and tTGF-β1 progressively to 54% (p=0.0258) of control (Figure 3A). Transgene combinations generally followed a biphasic time course characterized by increasing stimulation for 3–4 days, followed by a return toward control levels. In contrast, combinations that included both tIGF-I and tFGF-2 produced a sustained, >6-fold increase in *MMP3* expression. The only transgene combination that reduced *MMP3* expression to less than control was [tIGF-I + tTGF-β1] (Figure 3B).

Interactions among [tIGF-I + tBMP-2] in regulating *MMP3* expression led to the largest synergistic stimulation observed in these studies (C/S=15.2, p<0.0001). Interestingly, because the individual effects of these transgenes were minimal, this interaction generated only a 3.22-fold stimulation compared to control. The addition of tIGF-I as a second transgene delayed, but did not abrogate, the pronounced *MMP3* stimulation by tFGF-2. No growth factor transgene overcame the stimulation *of MMP3* expression by tFGF-2, and only tTGF-β1 as a second transgene succeeded in reducing this stimulation (Tables 2 and 3).

MMP13 expression:

Treatment with tBMP-2, tBMP-7 or tFGF-2 each increased *MMP13* expression. The 71-fold (p<0.0001) increase in *MMP13* expression by tFGF-2 compared to control was the largest change observed in these studies. Treatment with tBMP-2 or tBMP-7 increased *MMP13* expression up to 2.3-fold and 5.3-fold respectively. Treatment with tTGF-β1 decreased *MMP13* expression to 40% of control (p=0.0088). This was the maximum reduction in gene expression by an individual growth factor transgene observed in these studies. Treatment with tIGF-I initially decreased *MMP13* expression, but subsequently increased it 2.3 fold (p=0.0213) compared to control (Figure 4A). All growth factor transgene combinations that did not include tFGF-2 reduced *MMP13* expression to less than control levels by day 4 (Figure 4B).

Growth factor transgene interactions markedly altered *MMP13* expression. When tIGF-I was added to tFGF-2, it reduced the stimulation by tFGF-2 from 71-fold to 5.4-fold (C/S = 0.06, p=0.0397). The addition of tFGF-2 to tTGF-β1 overcame the reduction in *MMP13* expression by tTGF-β1 alone. Conversely, tTGF-β1 reduced the stimulation by tFGF-2 from 71-fold to 22-fold by day five following transfection (C/S=0.30, p<0.0001) (Tables 2 and 3).

IL6 Expression:

Treatment with tBMP-7 progressively decreased *IL6* expression to 41% of control ($p=0.0155$). Treatment with tBMP-2 tended to be inhibitory, but this effect was transient. Treatment with tFGF-2, tIGF-I, or tTGF-β1 increased *IL6* expression in a time-dependent manner to peak values of 10.8 fold, 3.0 fold and 2.4 fold (all $p<0.0001$) (Figure 5A). All growth factor transgene combinations that included tFGF-2 stimulated *IL6* expression, while all combinations that included a BMP transgene inhibited this expression. (Figure 5B).

Interaction between tIGF-I and tFGF-2 generated a synergistic stimulation of *IL6* expression to C/S=8.26 ($p<0.0001$) by day 4, corresponding to a >40-fold increase compared to control. In contrast, the addition of any other transgene to tIGF-I was inhibitory, or had no effect, at all-time points tested (Tables 2 and 3).

Discussion

We hypothesized that the anabolic and mitogenic actions of chondrogenic growth factors would be complemented by a reduction in the expression of chondrocyte genes encoding catabolic factors. Our results generally contradict this hypothesis. While certain growth factor transgenes and transgene combinations did inhibit catabolic marker genes, most had the opposite effect. The data also demonstrate considerable diversity among the five tested growth factor transgenes in regulating the expression of the five articular chondrocyte catabolic genes investigated in this study. These differences among the treatments included their magnitude, time course and interactions. The growth factors were selected on the basis of prior demonstration of chondrogenic properties and possible interactions due to differences in signal transduction pathways. In addition, the selected growth factor transgenes had been previously shown to differentially regulate anabolic functions in articular chondrocytes and to interact in doing so (Shi et al., 2013)

All five growth factor transgenes upregulated *ADAMTS4* expression and down-regulated *ADAMTS5* expression at one or more time points. Although the time course differed among the growth factor transgenes, no transgene downregulated *ADAMTS4* or, with the exception of tFGF-2, upregulated *ADAMTS5* at any time point. This shared action of all the growth factor transgenes was observed only for the *ADAMTSs*. The finding that all the growth factor transgenes and transgene combinations oppositely regulated *ADAMTS4* and *ADAMTS5* expression suggests that ADAMTS-4 and ADAMTS-5 play distinct roles in mediating growth factor regulation of articular cartilage homeostasis. The observation that ADAMTS 5 knockout mice are resistant to OA while ADAMTS 4 knockout mice remain susceptible to OA (Glasson et al., 2004; Glasson et al. 2005) are consistent with such a difference. A recent report suggests that the action of these two aggrecanases in OA may differ between mice and humans (Dancevic and McCulloch, 2014). It is unknown whether their regulation by growth factors is also species- specific. The finding that tFGF-2 upregulated *ADAMTS4* expression, but generally had no significant effect on *ADAMTS5* expression, differs from the inhibition of both *ADAMTS4* and *ADAMTS5* expression by exogenous FGF- 2 noted in a prior report (Sawaji et al., 2008). This difference may reflect differences in experimental design, including the use in that study of an IL-1α - stimulated human primary chondrocyte model.

Treatment with tFGF-2 generated a much greater increase in both MMP3 and MMP13 gene expression than any of the other growth factor transgenes. The effect of tFGF-2 on *MMP13* expression is consistent with prior studies, but the 71-fold stimulation observed in this study is greater than the previously reported 5-fold (Im et al., 2007) and 20-fold (Im et al., 2009) stimulation by 100ng/ml exogenous bFGF.

The data reveal an antagonistic relationship between tTGF-β1 and tFGF-2 in the regulation of *MMP13* expression. Among the individual transgenes tested in this study, tFGF-2 maximally stimulated, and tTGF-β1 maximally reduced, *MMP13* expression. The addition of tTGF-β1 to tFGF-2 produced a marked, sustained reduction in the stimulation by tFGF-2 (C/S=0.3). The finding that the combination [tFGF-2 and tTGF-β1] increased *MMP13* expression 22.4 fold suggests that tFGF-2 is dominant over tTGF-β1 in regulating *MMP13*. Paradoxically, although tIGF-I and tBMP-7 each increased *MMP13* expression, each also markedly reduced the strong stimulation by tFGF-2 of *MMP13* expression, and the addition of both tIGF-I and tBMP-7 to tFGF-2 further reduced this expression.

Modulation of *IL6* expression in this study included upregulation at multiple time points by tTGF-β1, but never downregulation. In contrast, tBMP-7 downregulated, but never upregulated *IL6* expression. Further, the downregulation of IL6 expression by tBMP-2 was transient, while that of tBMP-7 persisted for the duration of the experiments. These data indicate that, while these growth factors share membership in the tTGF-β superfamily, they do not share all of their regulatory actions on *IL6* expression. Treatment with tIGF-I and tFGF-2 together generated a striking synergistic stimulation of *IL6* expression to C/S=8.26. However, the addition of tBMP-7 as a third transgene abrogated this interaction, such that *IL-6* stimulation by [tFGF-2 + tIGF-I + tBMP-7] was similar to that by tFGF-2 alone.

IGF-I is unusual in the breadth of its cartilage reparative actions. It stimulates both chondrocyte proliferation and matrix synthesis, reduces the effect of pro-catabolic and anti-anabolic agents, and protects cartilage matrix from endogenous degradation (Sah et al., 1994; Sah et al., 1996; Tyler, 1989; Montaseri et al., 2011) . The present finding that tIGF-I interactions reduced the expression of several catabolic genes supplements existing evidence for IGF-I anti- catabolic activity. However, the pro-catabolic stimulation of both *ADAMTS4* and *IL6* expression by tIGF-I suggests a potentially more complex role for this growth factor in articular cartilage homeostasis.

BMP-2 has been reported to transiently increase aggrecan degradation simultaneously with its stimulation of proteoglycan synthesis and aggrecan gene expression in murine knee joints (Blaney Davidson et al., 2007). To our knowledge, BMP-7 has not been shown to promote catabolic activity by articular chondrocytes. The present studies suggest that tBMP-2 and tBMP-7 can each stimulate the expression of *ADAMTS4*, *MMP3* and *MMP13*, though to only a modest degree. They can also interact with tIGF-I to increase *ADAMTS4* and *MMP3*.

As hypothesized, several growth factor transgenes and transgene combinations did reduce the expression of the tested catabolic marker genes. All individual growth factor transgenes, except tFGF-2, decreased *ADAMTS5* expression. Similarly, all transgene combinations, except [tFGF-2 + tTGF-β1], inhibited *ADAMTS5* expression. Treatment with tTGF-β1

generated a sustained decrease in *MMP13* expression, but this was the only individual growth factor transgene to do so. All interactions among transgenes inhibited *MMP13* expression, even when the effect of the combination was positive compared to controls. Among individual growth factor transgenes, only tBMP-7 caused a sustained decrease in *IL6* expression. It also produced a marked inhibition of *IL6* expression (C/S ratios 0.14 – 0.32) when delivered in combination with tIGF-I and tFGF-2, though the tBMP-7 did not overcome the stimulatory effect of tIGF-I and tFGF-2 compared to controls (Tables 2 and 3). In general, the hypothesis tended to be supported most by tTGF-β and tBMP-7 and least by tFGF-2.

These data suggest a mechanism by which these growth factor transgenes regulate chondrocyte matrix production. A previous study (Shi et al., 2013) found that these growth factor transgenes regulated not only the synthesis of cartilage matrix molecules, but also their distribution. Specifically, the growth factor transgenes differentially regulated the proportion of matrix molecules deposited in the cell layer compared to those disbursed into the medium. This ratio of retained to released glycosaminoglycan (GAG) and collagen is important to cartilage repair because the retained GAG and collagen contribute to new tissue formation, while the released molecules are lost. The present data may lend insight into those findings. Among the tested growth factor transgenes, tFGF-2 generated the greatest stimulation of *ADAMTS4*, *MMP3*, *MMP13* and *IL6* expression, and tFGF-2 plus tIGF-I synergistically stimulated *ADAMTS4* and *IL6* expression. In the prior studies, of all these transgenes, tFGF-2 generated the lowest ratio of retained to released GAG and collagen, and this ratio was further lowered by interaction between tFGF-2 with tIGF-I (Shi et al., 2013). These data suggest that the reduced retention of newly synthesized matrix molecules reflects a stimulation by FGF-2 and FGF-2 + IGF-I of catabolic gene expression. Further, in the present study, the combination [tIGF-I + tBMP-2 + tBMP-7] reduced the expression of three of the five chondrocyte genes (*ADAMTS5*, *MMP13* and *IL6*) to a greater degree than any of the other growth factor transgenes or transgene combinations. In the prior studies, the combination [tIGF-I + tBMP-2 + tBMP-7] generated the highest GAG ratio and highest collagen ratio of any growth factor transgene or transgene combination tested (Shi et al., 2013). Taken together, these data suggest that growth factor regulation of chondrocyte catabolic activities may help determine the retention or loss of the matrix molecules whose production is regulated by the growth factors. In this event, the regulation of catabolic chondrocyte genes may contribute to the efficacy of the growth factors in articular cartilage engineering and repair, where matrix deposition is a key determinant of success.

The observation that anabolic growth factor gene overexpression increases catabolic gene expression may reflect a feedback mechanism. Alternatively, or in addition, these dual roles may contribute to tissue remodeling. The wide range of actions and interactions of these growth factor transgenes on each of the target genes, and the differences in the regulation of the different target genes by the different growth factor transgenes and transgene combinations, offer a potentially tunable system for modulating the balance between mitogenic, anabolic, and catabolic gene expression in these cells.

The observation that chondrocyte genes that serve similar functions (eg MMP3 and MMP13) are differentially regulated by these growth factors are not readily interpreted at a

mechanistic level. For example, the effect of FGF-2 on these enzymes may be mediated, at least in part, by the regulation by FGF-2 of IL-6. The mechanisms underlying the interactions identified in this study remain to be elucidated. Further studies will be needed to define the specific pathways and networks through which the growth factors act and interact in regulating the expression of these genes and their protein products.

A strength of the study is the relatively large number of growth factor and catabolic genes tested. The growth factors possess known reparative actions on articular chondrocytes, and the regulated genes encode catabolic factors that play a role in cartilage degradation in OA. However, a limitation of the study is that other anabolic and catabolic factors are also likely to be involved in the disease process and may have therapeutic potential. For example, vascular endothelial growth factor (VEGF) influences chondrocytes and may interact with FGF-2, with which it shares a vascular regulatory function that may be involved in OA pathogenesis.

A further limitation of this study is its focus on gene expression. Assessment of the amount, location and fate of the endogenous growth factors is beyond the scope of this study. While the regulation of gene expression is a key step in chondrocyte function, further studies will be required to elucidate the post- transcriptional processing events that influence their contribution to cartilage homeostasis.

Taken together, these data indicate that growth factors that are traditionally viewed as chondrogenic appear to also regulate catabolic activity. The complex interplay among these growth factor transgenes was not predictable based on their individual actions. For these reasons, the selection of regulatory factors for articular cartilage repair may be more complex than previously thought. Empirical studies will likely be needed to optimize growth factor therapy for clinical application.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

This work was supported in part by NIH National Institute of Arthritis and Musculoskeletal and Skin Disease (NIAMS) grant AR047702, Veterans Administration award I01 BX000447, and the Indiana University School of Medicine Research Infrastructure Fund. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

Abbaszade I, Liu R-Q, Yang F, Rosenfeld SA, Ross OH, Link JR, … Hollis JM (1999). Cloning and characterization of ADAMTS11, an aggrecanase from the ADAMTS family. J. Biol. Chem. 274, 23443–23450. [PubMed: 10438522]

Blaney Davidson EN, Vitters EL, van Lent PL, van de Loo FA, van den Berg WB, and van der Kraan PM (2007). Elevated extracellular matrix production and degradation upon bone morphogenetic protein-2 (BMP-2) stimulation point toward a role for BMP-2 in cartilage repair and remodeling. Arthritis Res Ther 9, R102 10.1186/ar2305 [PubMed: 17922907]

Chopra R, and Anastassiades T (1998). Specificity and synergism of polypeptide growth factors in stimulating the synthesis of proteoglycans and a novel high molecular weight anionic glycoprotein by articular chondrocyte cultures. J Rheumatol 25, 1578–1584. [PubMed: 9712104]

Chubinskaya S, Hakimiyan A, Pacione C, Yanke A, Rappoport L, Aigner T, … Loeser RF (2007). Synergistic effect of IGF-1 and OP-1 on matrix formation by normal and OA chondrocytes cultured in alginate beads. Osteoarthritis Cartilage 15, 421–430. 10.1016/j.joca.2006.10.004 [PubMed: 17126570]

Chubinskaya S, Hurtig M, and Rueger DC (2007). OP-1/BMP-7 in cartilage repair. Int Orthop 31, 773–781.10.1007/s00264-007-0423-9 [PubMed: 17687553]

Cucchiarini M, Madry H, Ma C, Thurn T, Zurakowski D, Menger MD, … Terwilliger EF (2005). Improved tissue repair in articular cartilage defects in vivo by rAAV-mediated overexpression of human fibroblast growth factor 2. Mol Ther 12, 229–238. 10.1016/j.ymthe.2005.03.012 [PubMed: 16043094]

Dancevic CM, and McCulloch DR (2014). Current and emerging therapeutic strategies for preventing inflammation and aggrecanase-mediated cartilage destruction in arthritis. Arthritis Res Ther 16, 429. [PubMed: 25606593]

Flannery CR, Lark MW, and Sandy J (1992). Identification of a stromelysin cleavage site within the interglobular domain of human aggrecan. Evidence for proteolysis at this site in vivo in human articular cartilage. J. Biol. Chem. 267, 1008–1014. [PubMed: 1730630]

Flechtenmacher J, Huch K, Thonar EJ, Mollenhauer JA, Davies SR, Schmid, … Kuettner KE (1996). Recombinant human osteogenic protein 1 is a potent stimulator of the synthesis of cartilage proteoglycans and collagens by human articular chondrocytes. Arthritis Rheum 39, 1896–1904. [PubMed: 8912513]

Fortier LA, Mohammed HO, Lust G, and Nixon AJ (2002). Insulin-like growth factor-I enhances cell-based repair of articular cartilage. J Bone Joint Surg Br 84, 276–288. [PubMed: 11922373]

Fujimoto E, Ochi M, Kato Y, Mochizuki Y, Sumen Y, and Ikuta Y (1999). Beneficial effect of basic fibroblast growth factor on the repair of full-thickness defects in rabbit articular cartilage. Arch Orthop Trauma Surg 119, 139–145. [PubMed: 10392506]

Glasson SS, Askew R, Sheppard B, Carito BA, Blanchet T, Ma HL, … Peluso D (2004). Characterization of and osteoarthritis susceptibility in ADAMTS-4–knockout mice. Arthritis & Rheumatology 50, 2547–2558. 10.1002/art.20558

Glasson SS, Askew R, Sheppard B, Carito B, Blanchet T, Ma H-L, … Yang Z (2005). Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. Nature 434, 644–648. 10.1038/nature03369 [PubMed: 15800624]

Gobezie R, Kho A, Krastins B, Sarracino DA, Thornhill TS, Chase M, … Lee DM (2007). High abundance synovial fluid proteome: distinct profiles in health and osteoarthritis. Arthritis Res Ther 9, R36 10.1186/ar2172 [PubMed: 17407561]

Goldring MB, Otero M, Plumb DA, Dragomir C, Favero M, Hachem El, … Marcu KB (2011). Roles of inflammatory and anabolic cytokines in cartilage metabolism: signals and multiple effectors converge upon MMP-13 regulation in osteoarthritis. Eur Cell Mater 21, 202–220. [PubMed: 21351054]

Goodrich LR, Hidaka C, Robbins PD, Evans CH, and Nixon AJ (2007). Genetic modification of chondrocytes with insulin-like growth factor-1 enhances cartilage healing in an equine model. J Bone Joint Surg Br 89, 672–685. 10.1302/0301-620X.89B5.18343 [PubMed: 17540757]

Grunder T, Gaissmaier C, Fritz J, Stoop R, Hortschansky P, Mollenhauer J, and Aicher WK (2004). Bone morphogenetic protein (BMP)-2 enhances the expression of type II collagen and aggrecan in chondrocytes embedded in alginate beads. Osteoarthritis Cartilage 12, 559–567. 10.1016/j.joca.2004.04.001 [PubMed: 15219571]

Guerne PA, Sublet A, and Lotz M (1994). Growth factor responsiveness of human articular chondrocytes: distinct profiles in primary chondrocytes, subcultured chondrocytes, and fibroblasts. J Cell Physiol 158, 476–484. 10.1002/jcp.1041580312 [PubMed: 8126071]

Henson FM, Bowe EA, and Davies ME (2005). Promotion of the intrinsic damage-repair response in articular cartilage by fibroblastic growth factor-2. Osteoarthritis Cartilage 13, 537–544. 10.1016/j.joca.2005.02.007 [PubMed: 15922188]

Hughes CE, Little CB, Buttner FH, Bartnik E, and Caterson B (1998). Differential expression of aggrecanase and matrix metalloproteinase activity in chondrocytes isolated from bovine and porcine articular cartilage. J Biol Chem 273, 30576–30582. [PubMed: 9804828]

Im HJ, Muddasani P, Natarajan V, Schmid TM, Block JA, Davis F, … Loeser RF (2007). Basic fibroblast growth factor stimulates matrix metalloproteinase-13 via the molecular cross-talk between the mitogen-activated protein kinases and protein kinase Cdelta pathways in human adult articular chondrocytes. J Biol Chem 282, 11110–11121. 10.1074/jbc.M609040200 [PubMed: 17311929]

Im H-J, Sharrocks AD, Lin X, Yan D, Kim J, Van Wijnen AJ, and Hipskind RA (2009). Basic fibroblast growth factor induces matrix metalloproteinase-13 via ERK MAP kinase-altered phosphorylation and sumoylation of Elk-1 in human adult articular chondrocytes. *Open Access Rheumatology*: research and reviews 1, 151. [PubMed: 27789988]

Kato Y, Iwamoto M, and Koike T (1987). Fibroblast growth factor stimulates colony formation of differentiated chondrocytes in soft agar. J Cell Physiol 133, 491–498. 10.1002/jcp.1041330309 [PubMed: 2826497]

Kokebie R, Aggarwal R, Lidder S, Hakimiyan AA, Rueger DC, Block JA, and Chubinskaya S (2011). The role of synovial fluid markers of catabolism and anabolism in osteoarthritis, rheumatoid arthritis and asymptomatic organ donors. Arthritis Res Ther 13, R50 10.1186/ar3293 [PubMed: 21435227]

Livshits G, Zhai G, Hart DJ, Kato BS, Wang H, Williams FM, and Spector TD (2009). Interleukin-6 is a significant predictor of radiographic knee osteoarthritis: The Chingford Study. Arthritis Rheum 60, 2037–2045. 10.1002/art.24598 [PubMed: 19565477]

Luyten FP, Hascall VC, Nissley SP, Morales TI, and Reddi AH (1988). Insulin-like growth factors maintain steady-state metabolism of proteoglycans in bovine articular cartilage explants. Arch Biochem Biophys 267, 416–425. [PubMed: 3214163]

Madry H, Kaul G, Cucchiarini M, Stein U, Zurakowski D, Remberger K, … Trippel SB (2005). Enhanced repair of articular cartilage defects in vivo by transplanted chondrocytes overexpressing insulin-like growth factor I (IGF-I). Gene Ther 12, 1171–1179. 10.1038/sj.gt.3302515 [PubMed: 15815701]

Mitchell PG, Magna HA, Reeves LM, Lopresti-Morrow LL, Yocum SA, Rosner PJ, … Hambor JE (1996). Cloning, expression, and type II collagenolytic activity of matrix metalloproteinase-13 from human osteoarthritic cartilage. J Clin Invest 97, 761–768. 10.1172/JCI118475 [PubMed: 8609233]

Montaseri A, Busch F, Mobasheri A, Buhrmann C, Aldinger C, Rad JS, and Shakibaei M (2011). IGF-1 and PDGF-bb suppress IL-1beta-induced cartilage degradation through down-regulation of NF-kappaB signaling: involvement of Src/PI-3K/AKT pathway. PLoS One 6, e28663 https://doi.org/10.137journal.pone.0028663 [PubMed: 22194879]

Morales TI, and Roberts AB (1988). Transforming growth factor beta regulates the metabolism of proteoglycans in bovine cartilage organ cultures. J Biol Chem 263, 12828–12831. [PubMed: 3166454]

Nixon AJ, Fortier LA, Williams J, and Mohammed H (1999). Enhanced repair of extensive articular defects by insulin-like growth factor-I-laden fibrin composites. J Orthop Res 17, 475–487. 10.1002/jor.1100170404 [PubMed: 10459752]

Qu XQ, Wang WJ, Tang SS, Liu Y, and Wang JL (2015). Correlation between interleukin-6 expression in articular cartilage bone and osteoarthritis. Genet Mol Res 14, 14189–14195. 10.4238/2015 [PubMed: 26600476]

Reddi AH (2003). Cartilage morphogenetic proteins: role in joint development, homoeostasis, and regeneration. Ann Rheum Dis 62 Suppl 2, ii73–78. [PubMed: 14532155]

Rey-Rico A, Venkatesan JK, Schmitt G, Concheiro A, Madry H, Alvarez-Lorenzo C, and Cucchiarini M (2017). rAAV-mediated overexpression of TGF-β via vector delivery in polymeric micelles stimulates the biological and reparative activities of human articular chondrocytes in vitro and in a human osteochondral defect model. International Journal of Nanomedicine 12, 6985 10.2147/IJN.S144579 [PubMed: 29033566]

Rosier RN, O'Keefe RJ, Crabb ID, and Puzas JE (1989). Transforming growth factor beta: an autocrine regulator of chondrocytes. Connect Tissue Res 20, 295–301. [PubMed: 2612160]

Sah RL, Chen AC, Grodzinsky AJ, and Trippel SB (1994). Differential effects of bFGF and IGF-I on matrix metabolism in calf and adult bovine cartilage explants. Arch Biochem Biophys 308, 137–147. 10.1006/abbi.1994.1020 [PubMed: 8311446]

Sah RL, Trippel SB, and Grodzinsky AJ (1996). Differential effects of serum, insulin-like growth factor-I, and fibroblast growth factor-2 on the maintenance of cartilage physical properties during long-term culture. J Orthop Res 14, 44–52. 10.1002/jor.1100140109 [PubMed: 8618165]

Sawaji Y, Hynes J, Vincent T, and Saklatvala J (2008). Fibroblast growth factor 2 inhibits induction of aggrecanase activity in human articular cartilage. Arthritis Rheum 58, 3498–3509. 10.1002/art.24025 [PubMed: 18975307]

Sellers RS, Peluso D, and Morris EA (1997). The effect of recombinant human bone morphogenetic protein-2 (rhBMP-2) on the healing of full-thickness defects of articular cartilage. J Bone Joint Surg Am 79, 1452–1463. [PubMed: 9378731]

Shi S, Mercer S, Eckert GJ, and Trippel SB (2009). Growth factor regulation of growth factors in articular chondrocytes. J. Biol. Chem. 284, 6697–6704. 10.1074/jbc.M807859200 [PubMed: 19136669]

Shi S, Mercer S, Eckert GJ, and Trippel SB (2012). Regulation of articular chondrocyte aggrecan and collagen gene expression by multiple growth factor gene transfer. J Orthop Res 30, 1026–1031. 10.1002/jor.22036 [PubMed: 22180348]

Shi S, Mercer S, Eckert GJ, and Trippel SB (2013). Growth factor transgenes interactively regulate articular chondrocytes. J Cell Biochem 114, 908–919. 10.1002/jcb.24430 [PubMed: 23097312]

Shi S, Mercer S, and Trippel SB (2010). Effect of transfection strategy on growth factor overexpression by articular chondrocytes. J Orthop Res 28, 103–109. 10.1002/jor.20945 [PubMed: 19637273]

Tetlow LC, Adlam DJ, and Woolley DE (2001). Matrix metalloproteinase and proinflammatory cytokine production by chondrocytes of human osteoarthritic cartilage: associations with degenerative changes. Arthritis Rheum 44, 585–594. 10.1002/1529-0131(200103)44:3<585::AID-ANR107>3.0.CO;2-C [PubMed: 11263773]

Tortorella MD, Burn TC, Pratta MA, Abbaszade I, Hollis JM, Liu R, … Arner EC (1999). Purification and cloning of aggrecanase-1: a member of the ADAMTS family of proteins. Science 284, 1664–1666. [PubMed: 10356395]

Trippel SB (1995). Growth factor actions on articular cartilage. J Rheumatol Suppl 43, 129–132. [PubMed: 7752116]

Trippel SB (1997). Growth factors as therapeutic agents. Instr Course Lect 46, 473–476. [PubMed: 9143989]

Tsuchida AI, Beekhuizen M, Rutgers M, van Osch GJ, Bekkers JE, Bot, … Creemers LB (2012). Interleukin-6 is elevated in synovial fluid of patients with focal cartilage defects and stimulates cartilage matrix production in an in vitro regeneration model. Arthritis Res Ther 14, R262 10.1186/ar4107 [PubMed: 23206933]

Tyler JA (1989). Insulin-like growth factor 1 can decrease degradation and promote synthesis of proteoglycan in cartilage exposed to cytokines. Biochem J 260, 543–548. [PubMed: 2788408]

Yokoo N, Saito T, Uesugi M, Kobayashi N, Xin KQ, Okuda K, … Koshino T (2005). Repair of articular cartilage defect by autologous transplantation of basic fibroblast growth factor gene-transduced chondrocytes with adeno-associated virus vector. Arthritis Rheum 52, 164–170. 10.1002/art.20739 [PubMed: 15641065]

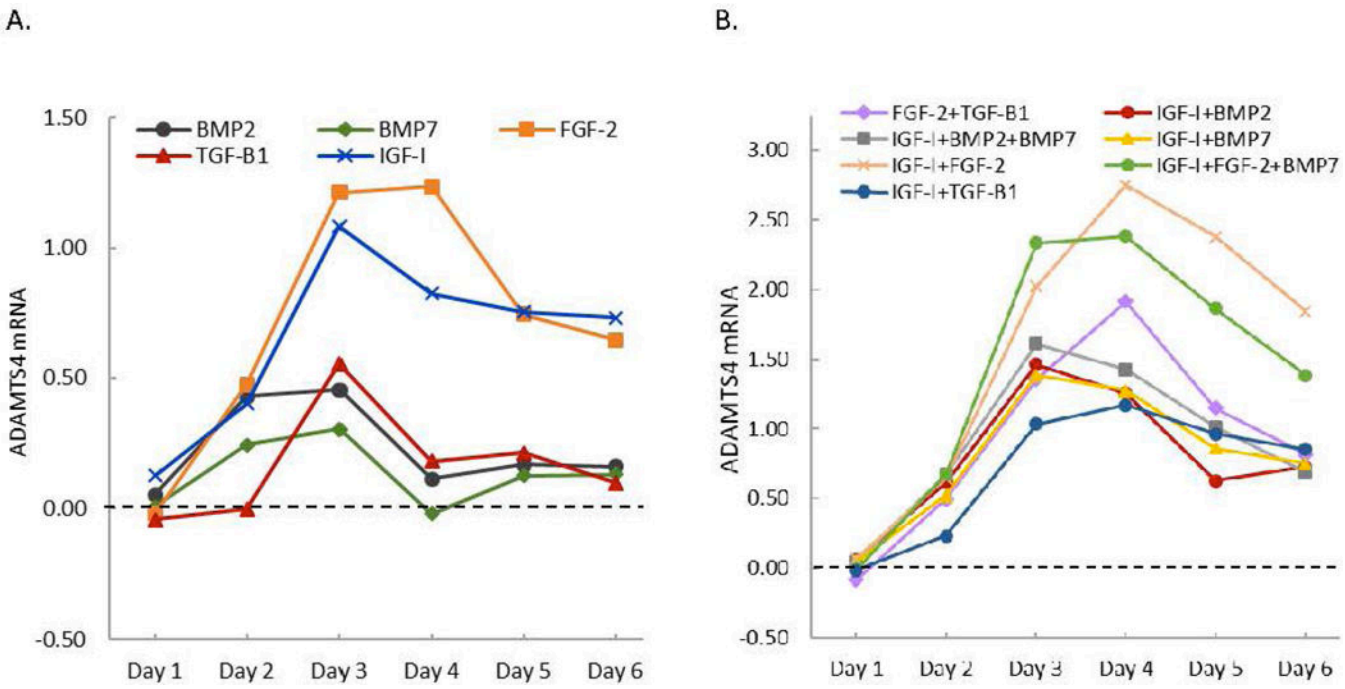

Figure 1:

Effect of individual **(A)** and multiple **(B)** growth factor gene transfer on the expression of *ADAMTS4* expression by adult articular chondrocytes over time. Values reflect the ratio of treated to control *ADAMTS4* mRNA levels in response to the designated transgene(s) at the designated time points. To illustrate ratios that are >1.0 and those that are <1.0, the data is presented as the natural log (ln) of the value and the y-axes are scaled to accommodate the magnitude of change. Data represent the mean of n=3 independent experiments. 95% confidence intervals and p-values compared to control are provided in Table S1. P-values for comparisons between groups and for comparisons between days are presented in STable 4 and STable 5 respectively.

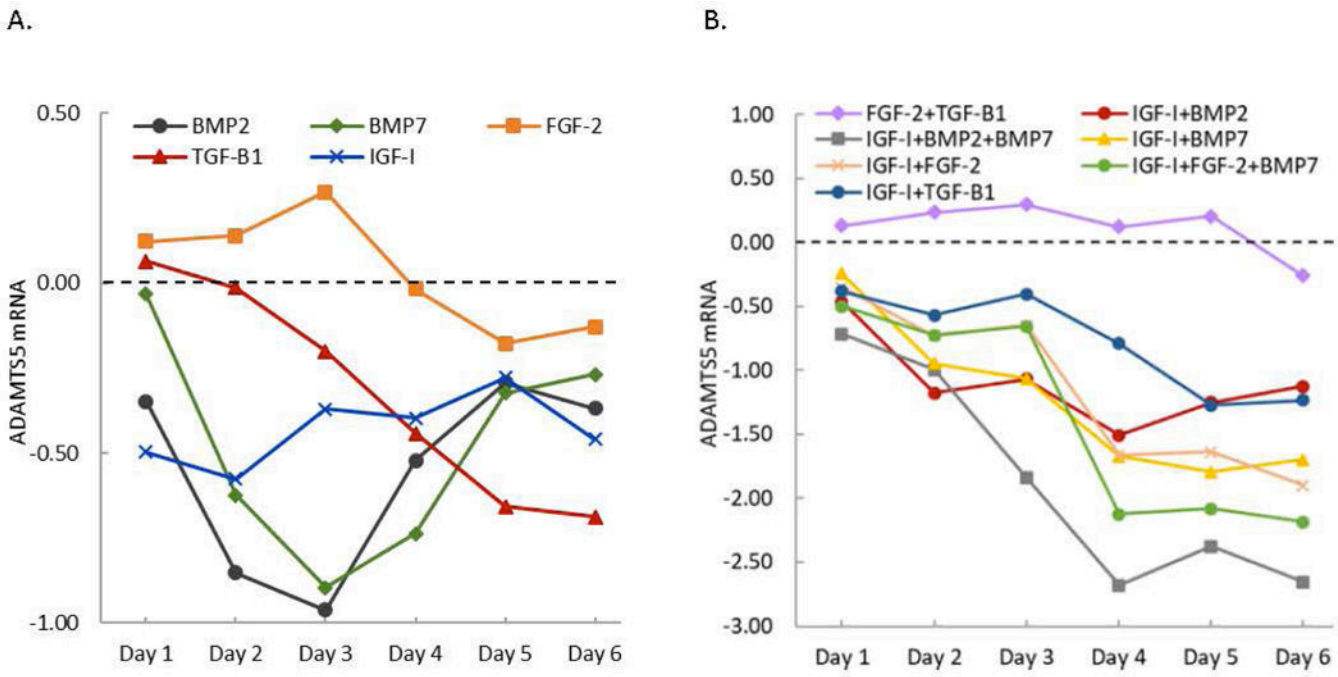

Figure 2:

Effect of individual **(A)** and multiple **(B)** growth factor gene transfer on the expression of *ADAMTS5* expression by adult articular chondrocytes over time. Values reflect the ratio of treated to control *ADAMTS5* mRNA levels in response to the designated transgene(s) at the designated time points. To illustrate ratios that are >1.0 and those that are <1.0, the data is presented as the natural log (ln) of the value and the y-axes are scaled to accommodate the magnitude of change. Data represent the mean of n=3 independent experiments. 95% confidence intervals and p-values compared to control are provided in STable 3. P-values for comparisons between groups and for comparisons between days are presented in STable 4 and STable 5 respectively.

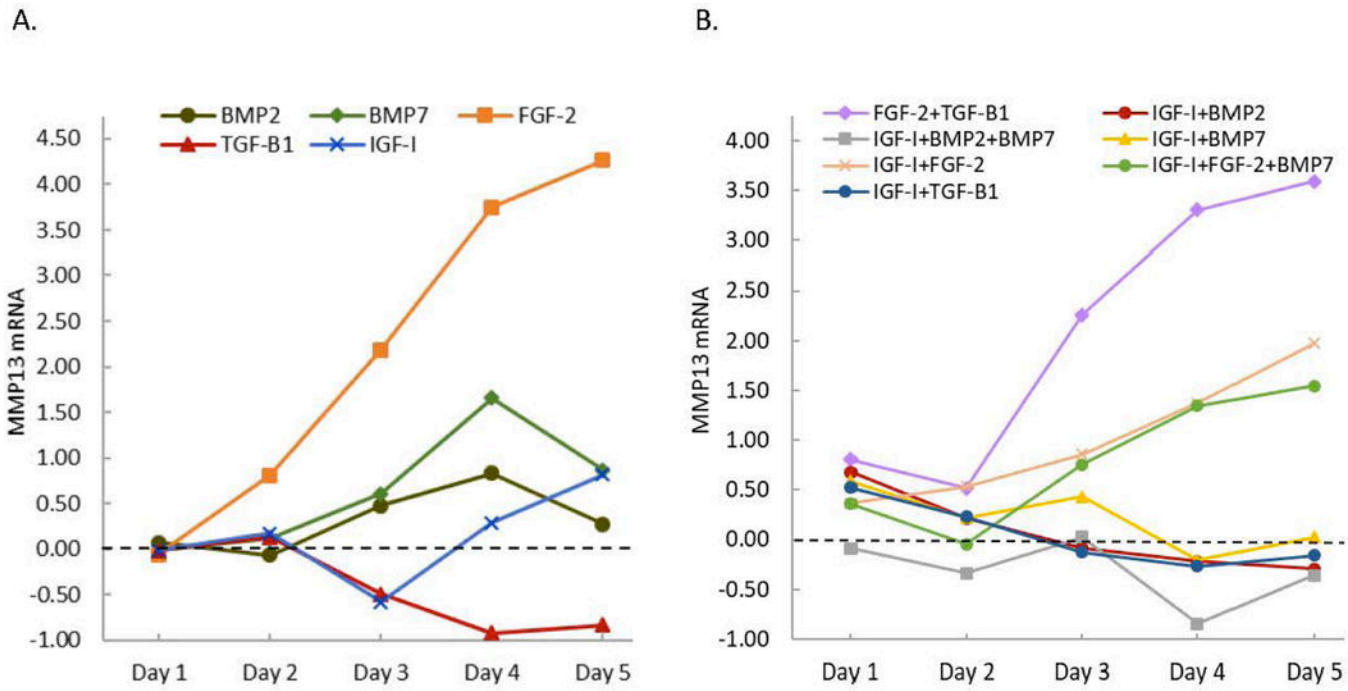

Figure 3:

Effect of individual **(A)** and multiple **(B)** growth factor gene transfer on the expression of *MMP13* expression by adult articular chondrocytes over time. Values reflect the ratio of treated to control *MMP13* mRNA levels in response to the designated transgene(s) at the designated time points. To illustrate ratios that are >1.0 and those that are <1.0, the data is presented as the natural log (ln) of the value and the y-axes are scaled to accommodate the magnitude of change. Data represent the mean of n=3 independent experiments. 95% confidence intervals, and p-values compared to control are provided in STable 3. P-values for comparisons between groups and for comparisons between days are presented in STable 4 and STable 5 respectively.

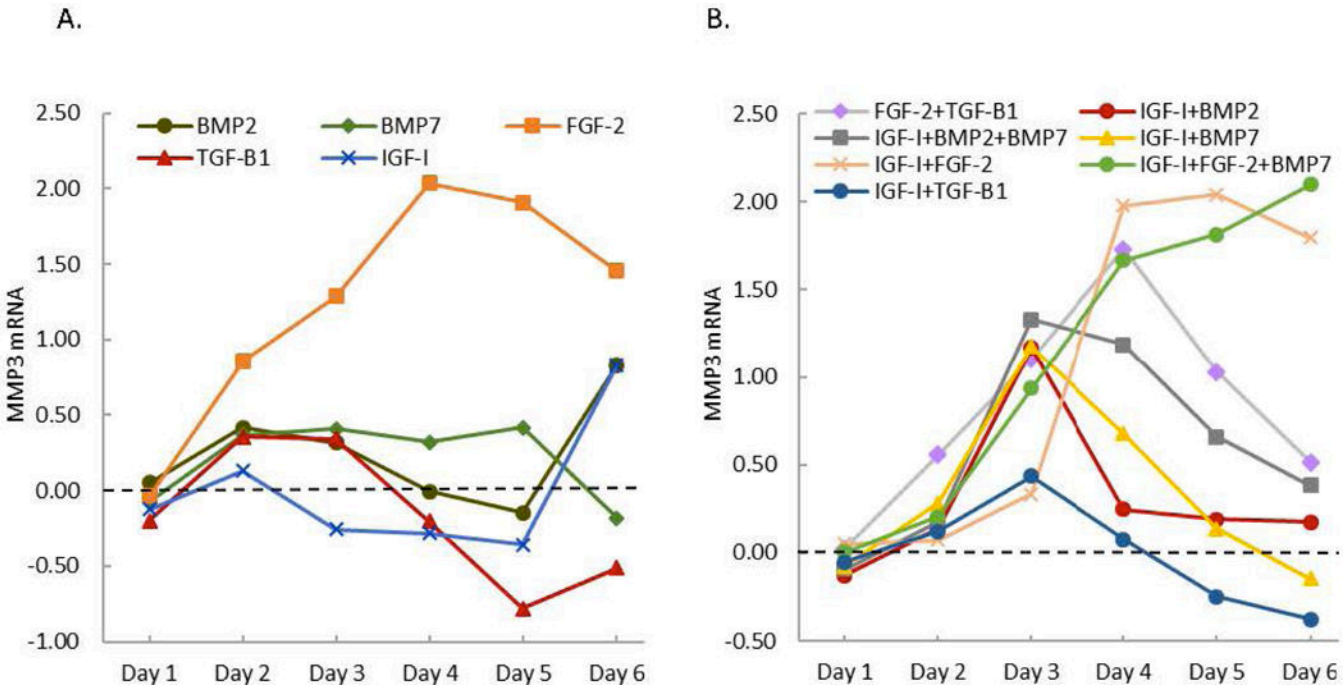

Figure 4:

Effect of individual **(A)** and multiple **(B)** growth factor gene transfer on the expression of *MMP3* expression by adult articular chondrocytes over time. Values reflect the ratio of treated to control *MMP3* mRNA levels in response to the designated transgene(s) at the designated time points. To illustrate ratios that are >1.0 and those that are <1.0, the data is presented as the natural log (ln) of the value and the y-axes are scaled to accommodate the magnitude of change. Data represent the mean of n=3 independent experiments. 95% confidence intervals and p-values compared to control are provided in STable 3. P-values for comparisons between groups and for comparisons between days are presented in STable 4 and STable 5 respectively.

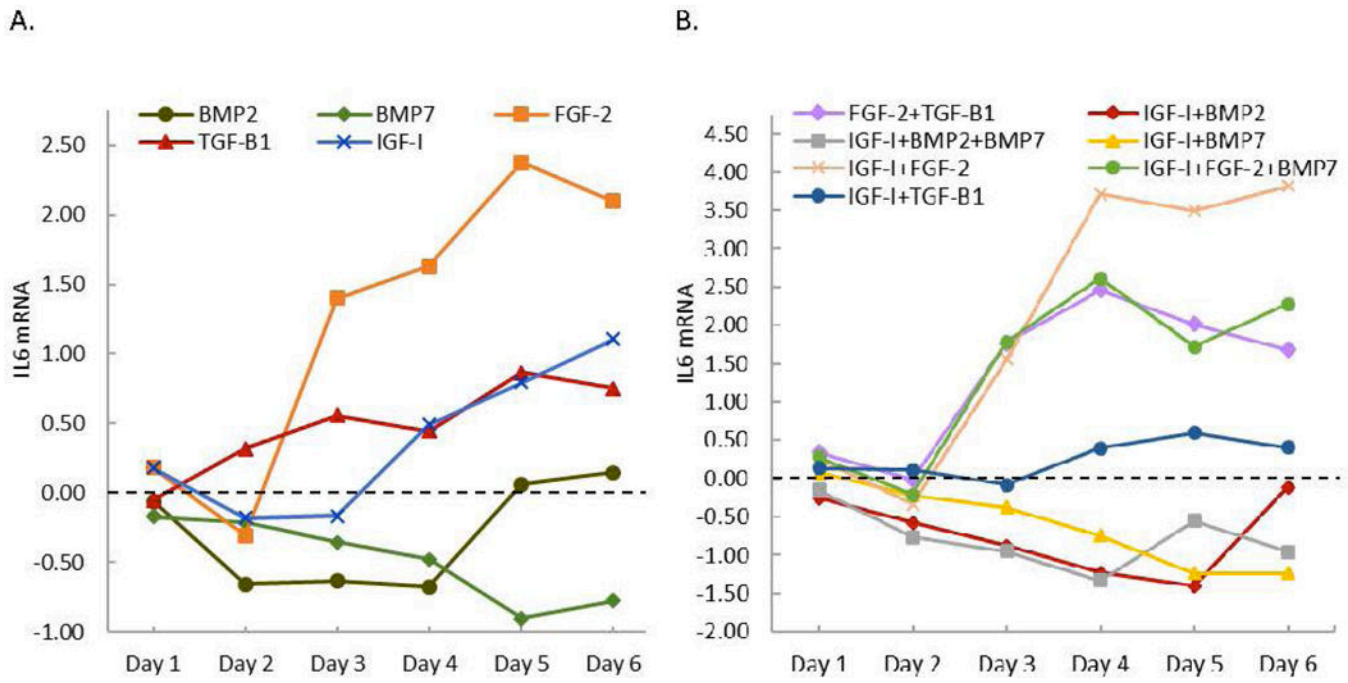

Figure 5:

Effect of individual **(A)** and multiple **(B)** growth factor gene transfer on the expression of *IL6* expression by adult articular chondrocytes over time. Values reflect the ratio of treated to control *IL6* mRNA levels in response to the designated transgene(s) at the designated time points. To illustrate ratios that are >1.0 and those that are <1.0, the data is presented as the natural log (ln) of the value and the y-axes are scaled to accommodate the magnitude of change. Data represent the mean of n=3 independent experiments. 95% confidence intervals and p-values compared to control are provided in STable 3. P-values for comparisons between groups and for comparisons between days are presented in STable 4 and STable 5 respectively.

Table 1:

Primers used for real-time PCR

Gene	*ACC. No.*	*Primer (5' to 3')*
18S	AF176811	Forward: CTGAGAAACGGCTACCACATC
		Reverse: GCCTCGAAAGAGTCCTGTATTG
ADAMTS-4	NM_181667	Forward: GAAGCAATGCACTGGTCTGA
		Reverse: CTAGGAGACAGTGCCCGAAG
ADAMTS-5	NM_001166515	Forward: TGCAGATTCTTGCCACAGAC
		Reverse: CTTTTGGAGCCGACTTCTTG
MMP-3	NM_001206637	Forward: TGTGCTCAGCCTATCCACTG
		Reverse: AGCTTTCCTGTCACCTCCAA
MMP-13	NM_174389	Forward: AGGCCTTCAGAAAAGCCTTC
		Reverse: CAACAGACCAGAGGGTCCAT
IL-6	EU276071	Forward: CAGCTATGAACTCCCGCTTC
		Reverse: TTCGGTTTTCTCTGGAGTGG

Table 2.

Summary of interactions among two growth factor transgenes in regulating articular chondrocyte gene expression. Chondrocytes were transfected with the designated individual transgenes or transgene combinations, and expression of the designated chondrocyte genes after 1 to 6 days were compared. C/S ratio: the change in gene expression in response to the combined treatments (C) divided by the sum of the changes in gene expression in response to the separate treatments(s). Types of interaction (Int) are designated as follows. + + : synergistic stimulatory interaction in which the result from the combined treatments was greater than the sum of the results from the individual treatments (C/S > 1); — : inhibitory interaction in which the result from the combined treatments reduced the results from the individual treatments (C/S < 1.0); — — : synergistically inhibitory interaction in which the sum of the results from the individual treatments was less than control, and the result from the combined treatment was less than this sum. * or **: C/S is indeterminate because (*) the sum of the individual treatments is less than control but the combined treatments are greater than control or (**) the sum of the individual treatments factors is greater than control but the combined treatments are less than control. Empty cell: interactive effect did not achieve statistical significance (P ≥ 0.05). All designated interactions are statistically significant (P < 0.05). Values represent the mean of three independent experiments. Detailed data are presented in STable 1.

Growth Factor Transgene Comparisons	Day	ADAMTS 4		ADAMTS 5		MMP 3		MMP13		IL-6	
		C/S Ratio	Int	C/S Ratio	Int	C/S Ratio	Int	C/S Ratio	Int	C/S Ratio	Int
FGF2+TGFB1 v FGF2TGFB1	1	1.33		**		*		*		2.65	+ +
	2	1.07	+ +	1.53	+ +	0.41	−	0.39	−	**	
	3	0.92	−	0.21		0.67	−	0.66	−	1.26	+ +
	4	2.19	+ +	0.18		0.72	−	0.41	−	2.23	+ +
	5	1.59	+ +	0.25	−	0.34	−	0.30	−	0.59	−
	6	1.23	+ +	0.56	−	0.23	−			0.53	−
IGF1+BMP2 v IGF1BMP2	1	0.34		0.84	−	1.98	− −	2.16		**	−
	2	0.83	−	0.76	−	0.24		0.03		0.68	−
	3	1.32	+ +	0.94	−	15.24	+ +	**		0.96	−
	4	1.79	+ +	1.18	− −	*		**		**	
	5	0.69	−	1.66	− −	*		**		**	
	6	0.85	−	1.23	− −	0.07				**	
IGF1+BMP7 v IGF1BMP7	1	0.40		1.02	− −	0.38		*		1.85	
	2	0.89	−	0.75	−	0.53	−	0.36		0.56	−
	3	1.32	+ +	0.95	−	8.02	+ +	0.39		0.74	
	4	2.04	+ +	1.07	− −	6.57	+ +	**		**	
	5	1.11	+ +	1.78	− −	0.68		**		**	
	6	0.92	−	1.51	− −	**				**	
IGF1+FGF2 v IGF1FGF2	1	0.63		1.83	− −	*		*		0.78	−
	2	0.82	−	1.82	− −	0.05		0.34	−	0.68	−
	3	1.51	+ +	**	−	0.17	−	0.15	−	1.29	+ +
	4	3.93	+ +	2.68	− −	0.97	−	0.06		8.26	+ +
	5	4.39	+ +	2.20	− −	1.22	+ +	0.06	−	2.93	+ +
	6	2.66	+ +	1.91	− −	1.08	+ +			4.83	+ +

Growth Factor Transgene Comparisons	Day	ADAMTS 4		ADAMTS 5		MMP 3		MMP13		IL-6	
		C/S Ratio	Int	C/S Ratio	Int	C/S Ratio	Int	C/S Ratio	Int	C/S Ratio	Int
IGF1+TGFB1 v IGF1TGFB1	1	**		1.32	– –	0.17		*		0.98	–
	2	0.53	–	1.02	– –	0.23		0.29		0.53	
	3	0.67	–	1.11	– –	3.03	+ +	0.43		*	
	4	1.50	+ +	1.06	– –	*		2.34		0.38	
	5	1.20	+ +	1.09	– –	0.25		**		0.31	
	6	1.14	+ +	0.95	–	**				0.16	

Table 3.

Summary of interactions among three growth factor transgenes in regulating articular chondrocyte gene expression. Chondrocytes were transfected with the designated individual transgenes or transgene combinations, and expression of the designated chondrocyte genes after 1 to 6 days was compared. Data represent the mean of the three independent experiments as described in Table 2. Results are expressed as in Table 2. Detailed data are presented in STable 2.

Growth Factor Transgene Comparisons	Day	ADAMTS 4		ADAMTS 5		MMP 3		MMP13		IL-6	
		C/S Ratio	Int	C/S Ratio	Int	C/S Ratio	Int	C/S Ratio	Int	C/S Ratio	Int
IGF1+BMP2+BMP7 v IGF1BMP2BMP7	1	0.25		0.80	–	0.67	–	**		14.52	– –
	2	0.73	–	0.52	–	0.18	–	**	–	0.64	–
	3	1.39	+ +	0.61	–	4.24	+ +	**		0.70	–
	4	2.29	+ +	0.77	–	13.61	+ +	**		4.32	
	5	1.21	+ +	1.24	– –	6.20	+ +	**		**	
	6	0.71	–	1.09	– –	0.20	–			**	
IGF1BMP2+BMP7 v IGF1BMP2BMP7	1	0.70		0.94	–	0.46	–	**		0.37	–
	2	0.85	–	0.63	–	0.32	–	**	–	0.86	–
	3	1.09	+ +	0.63	–	1.02	+ +	**		0.72	–
	4	1.27	+ +	0.70	–	3.09	+ +	**		0.67	
	5	1.70	+ +	0.87	–	1.22	+ +	**		0.30	
	6	0.81	–	0.93	–	12.18	+ +			0.96	
IGF1BMP7+BMP2 v IGF1BMP2BMP7	1	0.44		0.79	–	5.61	– –	**		**	–
	2	0.79	–	0.62	–	0.24	–	**	–	0.79	–
	3	1.11	+ +	0.63	–	1.05	+ +	**		0.80	–
	4	1.17	+ +	0.74	–	2.30	+ +	**		0.78	
	5	1.11	+ +	0.82	–	12.79	+ +	**		0.67	
	6	0.76	–	0.82	–	0.40	–			1.11	
IGF1+FGF2+BMP7 v IGF1FGF2BMP7	1	**		1.58	– –	*		*		1.35	+ +
	2	0.70	–	0.88	–	0.12	–	**	–	0.31	–
	3	1.99	+ +	1.26	– –	0.54	–	0.09	–	1.86	+ +
	4	2.66	+ +	1.08	– –	0.63	–	0.03		2.82	+ +
	5	2.29	+ +	1.36	– –	0.86	–	0.04		0.44	–
	6	1.39	+ +	1.37	– –	1.60	+ +			1.01	+ +
IGF1FGF2+BMP7 v IGF1FGF2BMP7	1	**		0.91	–	*		*		2.13	+ +
	2	0.82	–	0.67	–	0.41	–	**	–	0.40	–
	3	1.35	+ +	0.59	–	1.72	+ +	0.40	–	1.41	+ +
	4	0.67	–	0.65	–	0.65	–	0.24		0.32	–
	5	0.54	–	0.80	–	0.71	–	0.54		0.14	–
	6	0.55	–	0.85	–	1.47	+ +			0.20	–
IGF1BMP7+FGF2 v IGF1FGF2BMP7	1	**		1.53	– –	*		0.95		1.17	+ +
	2	0.75	–	1.25	– –	0.14	–	**	–	0.42	–
	3	1.72	+ +	1.36	– –	0.32	–	0.10	–	1.79	+ +
	4	1.96	+ +	1.01	– –	0.56	–	0.04		3.39	+ +

Growth Factor Transgene Comparisons	Day	ADAMTS 4		ADAMTS 5		MMP 3		MMP13		IL-6	
		C/S Ratio	Int	C/S Ratio	Int	C/S Ratio	Int	C/S Ratio	Int	C/S Ratio	Int
	5	2.16	+ +	0.86	−	0.87	−	0.04		0.50	−
	6	1.46	+ +	0.96	−	2.24	+ +			1.36	+ +