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# Growth factor regulation of growth factor production by multiple gene transfer to chondrocytes

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## Abstract

Of the many classes of molecules regulated by growth factors, growth factors themselves are not well investigated. We tested the hypothesis that combinations of endogenous growth factors interactively regulate the production of other growth factors. Growth factors have therapeutic potential for articular cartilage repair, and gene transfer is a promising approach to growth factor delivery. We tested the hypothesis using adult bovine articular chondrocytes treated with combinations of cDNAs encoding insulin-like growth factor I, bone morphogenetic protein-2 and protein-7, transforming growth factor β1, and fibroblast growth factor 2. We found that these growth factor transgenes regulated each other's growth factor production. This regulation ranged from stimulation to inhibition. Regulation by multiple transgenes was not predictable from the regulatory actions of the individual transgenes. Such interactions may be important for the selection of growth factor genes for cell-based therapies, including articular cartilage repair.

### Keywords

Insulin-like growth factor I; fibroblast growth factor 2; transforming growth factor β1; bone morphogenetic proteins; chondrocytes; gene therapy

## Introduction

Polypeptide growth factors play a central role in regulating the function of multiple cell types, including articular chondrocytes (Trippel et al. 1996). The quest for a source of locally produced growth factors has led to the application of gene transfer to cartilage repair (Trippel et al. 2004; Steinert et al. 2008). Due to the complexity of chondrocyte regulation, it is likely that more than one growth factor gene will be required to optimize chondrocyte function (Osborn et al. 1989; Loeser et al. 2003, 2005; Chubinskaya et al. 2007; Madry et al.



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Supplementary materials
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2010; Shi et al. 2010). However, in providing multiple growth factor genes, it is probable that the growth factors themselves will be among the regulated molecules (Elford and Lamberts 1990; Tsukazaki et al. 1994; Nixon et al. 2001; Shida et al. 2001; Shi et al. 2009). Currently, it is not known whether multiple growth factor gene delivery will be complicated by mutual regulation of the respective growth factor proteins. We tested the hypothesis that growth factor transgenes regulate the production of each other's encoded growth factors. We tested this hypothesis by delivering selected chondrotrophic growth factor genes to adult bovine articular chondrocytes and measuring their effect on growth factor production.

Damage to articular cartilage is the source of considerable disability in the form of arthritis and trauma (Centers for Disease Control Prevention 2010). Adult articular chondrocytes lack an effective intrinsic capacity for repair. Once articular cartilage substance is lost, damage is generally permanent and is often progressive (Buckwalter and Mankin 1998). Polypeptide growth factors play a central role in articular chondrocyte homeostasis (Trippel 1995). Several growth factors are anabolic and mitogenic for articular chondrocytes, raising the possibility that they may improve the reparative activities of these cells. Potentially therapeutic chondrotrophic growth factors include insulin-like growth factor I (IGF-I), bone morphogenetic protein-2 (BMP-2), bone morphogenetic protein-7 (BMP-7), transforming growth factor β1 (TGF-β1), and fibroblast growth factor 2 (FGF-2). IGF-I stimulates chondrocyte proliferation, glycosaminoglycan, and collagen synthesis, and inhibits endogenous catabolic activity (McQuillan et al. 1986; Luyten et al. 1988; Sandell and Dudek 1988; Sah et al. 1994; Nixon et al. 1999; Madry et al. 2005; Goodrich et al. 2007). TGF-β1 (Morales and Roberts 1988; Horton et al. 1989; Rosier et al. 1989; Shuler et al. 2000) and its superfamily members, BMP-2 (Reddi 2003; Grunder et al. 2004) and BMP-7 (Flechtenmacher et al. 1996; Chubinskaya et al. 2007), are pleiotropic factors that regulate chondrocyte differentiated functions. FGF-2 is a potent mitogen for chondrocytes which has diverse effects on matrix synthesis (Sah et al. 1994; Fujimoto et al. 1999; Cucchiarini et al. 2005; Henson et al. 2005; Yokoo et al. 2005). These growth factors were selected to represent a range of growth factor signaling pathways and biologic actions in articular chondrocytes.

## 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. 2010). Briefly, the human growth factor cDNA coding regions were generated by polymerase chain reaction (Table I) and, after confirming the sequences, were subcloned into pAAV-MCS (Stratagene, LaJolla, CA, USA) to obtain pAAV-based vectors.

### Chondrocyte cell culture and transfection

Basal medium was prepared with Dulbecco's Modified Eagle Medium (DMEM), 100U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine (Invitrogen, Carlsbad, CA, USA), and 50 μg/ml ascorbic acid (Sigma, St. Louis, MO, USA). Complete medium was prepared with basal medium and 10% fetal bovine serum (FBS). Bovine articular chondrocytes were isolated as previously described (Shi et al. 2009). Briefly, carpal chondrocytes were isolated from skeletally mature (growth plates closed) bovids, placed in primary monolayer culture for 3 days, and transfected using FuGENE 6 (Roche Applied Science, Indianapolis, IN, USA) with 2 μg of each plasmid DNA per well. 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. CM was stored at − 20°C for growth factor analysis.

### Growth factor analysis by ELISA

IGF-I, BMP-7, and TGF-β1 were analyzed in CM by ELISA using DuoSet ELISA kits (R&D Systems, Minneapolis, MN, USA). BMP-2 was analyzed in CM by ELISA using BMP-2 Quantikine Immunoassay kit (R&D Systems). For TGF-β1 analysis, CM was pretreated with HCl to activate latent TGF-β1 and then neutralized with NaOH. Prior studies found that minimal FGF-2 was released into the medium (Shi et al. 2009) and was, therefore, not assayed.

### Statistical analysis

The effects of IGF-I, TGF-β1, BMP-2, BMP-7, and FGF-2 gene transfer on IGF-I, BMP-2, BMP-7, and TGF-β1 protein production were evaluated using repeated measures ANOVA. The ANOVAs used terms for growth factor, time (days 2, 4, and 6), and the growth factor—by—time interaction, as well as a random effect to correlate data from the same experimental run and random effects to correlate data from the same specimen over time. No adjustments were made for multiple comparisons. A 5% significance level was used for all comparisons. Details of the statistical significance of differences are provided in Supplementary Materials. 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, respectively.

## Results

### Effect of individual growth factor transgenes

Control chondrocytes transfected by empty vector produced low or undetectable levels of all tested growth factors. These data indicate that the growth production of that growth factor by singly transfected chondrocytes reflects almost entirely the expression of the respective transgene. Chondrocytes transfected with the individual transgenes tIGF-I, tBMP-2, tBMP-7, and tTGF-β1 produced physiologically relevant concentrations of the respective growth factor proteins (Figure 1).

### Effect of multiple growth factor genes

**IGF-I production—**The transgene combination (tFGF-2 + tIGF-I) did not change total IGF-I production compared with tIGF-I alone. Each of the other growth factor transgenes reduced IGF-I production. As the third transgene, tFGF-2 became an inhibitor, decreasing the effect of (tBMP-2 + tIGF-I) or (tBMP-7 + tIGF-I) on IGF-I production. Conversely, tBMP-2 or tBMP-7 reduced the effect of (tFGF-2 + tIGF-I). As the fourth transgene, tBMP-7 or tBMP-2 reduced IGF-I production while tFGF-2 had little effect (Figure 1A).

**BMP-2 production—**The addition of tFGF-2 increased BMP-2 production while tIGF-I, tBMP-7, and tTGF-β1 decreased it. The effect of adding a third or fourth transgene was highly transgene dependent. Specifically, the addition of tBMP-7 to (tIGF-I + tBMP-2), or of tIGF-I to (tBMP-7 + tBMP-2), reduced BMP-2 output. The addition of tBMP-7 or tIGF-I to (tFGF-2 + tBMP-2) virtually eliminated the stimulatory effect of tFGF-2 on BMP-2 release. As a fourth transgene, tFGF-2 increased BMP-2 production while tIGF-I or tBMP-7 further decreased it (Figure 1B).

**BMP-7 production—**All second transgenes inhibited BMP-7 production. All third transgenes further decreased BMP-7 output to a relatively uniform level that was no longer

transgene dependent. The addition of a fourth transgene had minimal or no effect on BMP-7 production compared with the three-transgene combinations (Figure 1C).

**TGF-β1 production—**The effect of a second transgene on TGF-β1 release was transgene specific, ranging from stimulatory to inhibitory. Specifically, tIGF-I or tFGF-2 increased TGF-β1 production while tBMP-2 or tBMP-7 decreased it (Figure 1D).

### Time course of growth factor regulation

When treated with individual growth factor genes, chondrocyte production of the encoded growth factor decreased following distinct time courses. IGF-I production was maintained until it declined at the end of the culture period, whereas both BMPs declined early and rapidly. TGF-β1 production was biphasic, increasing early and decreasing later (Figure S1).

The addition of other growth factor transgenes generally prolonged the production of these growth factors. The time course of this effect varied with the growth factor being regulated and the transgenes used to regulate it. IGF-I production was increased early by all transgenes except tTGF-β1, creating a biphasic time course and a delayed subsequent decrease in production. BMP-2 production was prolonged by tIGF-I and tFGF-2, but not tBMP-7 or tTGF-β1. Treatment by tFGF-2 as a third or fourth transgene had the additional effect of reversing the decline in BMP-2 production over time. BMP-7 production progressively declined for all treatment groups, though transgene combinations that included tIGF-I or tFGF-2 prolonged its production. Unlike BMP-2 or BMP-7, the time course of TGF-β1 production was biphasic for all treatments. Both the magnitude and the duration of TGF-β1 production were increased by tIGF-I or tFGF-2, whereas tBMP-2 or tBMP-7 had no effect (Figure S1).

Although tIGF-I and tFGF-2 regulated the time course of production of the other growth factors in a similar fashion, the magnitude of tFGF-2 action was generally greater than that of tIGF-I. Conversely, tBMP-2 and tBMP-7 regulated the time course of growth factor production to a limited degree. The data indicate that growth factor transgene combinations differentially regulate both the duration and the magnitude of each other's growth factor protein production.

## Discussion

Articular chondrocytes have become an attractive target for growth factor gene therapy. Although individual transgenes have been well studied, little is known of the regulatory actions of multiple growth factor transgenes. To our knowledge, this is the first comprehensive analysis of multiple growth factor gene–transgene regulation of each other's growth factor production by articular chondrocytes.

The regulation of IGF-I by the other growth factor transgenes included both the magnitude and the duration of IGF-I production. For example, tFGF-2 preserved the amount and prolonged the duration of IGF-I production; tBMP-2 decreased IGF-I production but prolonged its duration; and tTGF-β both decreased the amount and duration of IGF-I production. Interestingly, the combination of tFGF-2 plus tBMP-2, but not that of tFGF-2 plus tBMP-7, also prolonged the duration of IGF-I production.

Regulation of the TGF-β superfamily members TGF-β1, BMP-2, and BMP-7 was complex. All three transgenes generally reduced the production of each other's growth factor proteins, suggesting a negative feedback among these related factors. Interestingly, tFGF-2 and tIGF-I each rapidly reversed the mutual inhibition of tBMP-2 and tBMP-7 on each other's protein production. These data suggest the presence of cross-talk between BMP signaling and that

of IGF-I and FGF-2, and raise the possibility that IGF-I and FGF-2 share some elements responsible for this interaction.

The TGF-β family members differed from each other in their regulation by tIGF-I and tFGF-2. The effect of tIGF-I was to increase the production of tTGF-β and to decrease that of BMP-2 and BMP-7. The addition of tFGF-2 increased the production of TGF-β and BMP-2 while decreasing that of BMP-7. Differences among TGF-β family members were particularly evident in their time courses of action. The amounts of both BMP-2 and BMP-7 fell markedly between 2d and 4d, whereas the production of TGF-β1 increased. This difference may indicate that transgene transcription and the subsequent translation may be downregulated earlier for the BMP-2 and BMP-7 transgenes than for the other transgenes tested.

Our data demonstrate a trend in each transgene's effect on the other transgene's growth factor production. The magnitude of stimulation generally followed the order: FGF-2 > IGF-I > BMP-2 > BMP-7 >TGF-β1. The data also demonstrate a trade-off between the number of growth factors produced and the amount of each growth factor produced. In general, increasing the number of growth factor transgenes decreased the amount of each growth factor protein. This relationship varied with time. At 2d, no transgene combination generated more growth factor protein than the corresponding individual transgenes. Differential regulatory effects of additional transgenes appeared at 4d and 6d, and some became pronounced. The effect of transgene number also decreased as the number of transgenes increased. These findings suggest that the therapeutic application of multiple gene transfer must weigh the benefits of more than one growth factor at relatively lower concentrations against a single growth factor at a higher concentration.

Several mechanisms may contribute to the growth factor regulation observed in these studies.

In this experimental design, a growth factor may be produced from either its native gene or its transgene. Additional growth factor transgenes alter the expression of the native gene, the transgene, or both. Prior studies indicate that at least some of these growth factors, delivered to chondrocytes as exogenous proteins, regulate the production of other growth factors via their native genes (Elford and Lamberts 1990; Villiger and Lotz 1992; Tsukazaki et al. 1994; Nixon et al. 2001; Shida et al. 2001; Shi et al. 2009), and as endogenous proteins via their transgenes (Shi et al. 2010). In this study, the endogenous growth factors may act, in part, by similarly regulating the expression of the chondrocytes' native growth factor genes. Alternatively, or in addition, the endogenous growth factors may differentially modulate transcriptor expression or activity via CMV promoter/enhancer transcription factor binding sites (Stinski and Isomura 2008). In addition, growth factor-regulated post-transcriptional mechanisms may contribute to the observed regulation of growth factor production. Further studies will be required to elucidate and distinguish between these possible mechanisms.

Two potential approaches to growth factor regulation of chondrocytes in cell-based therapeutic applications include the delivery of exogenous growth factors and the production of endogenous growth factors by gene transfer. These approaches differ in their use of growth factor proteins vs. growth factor genes as the therapeutic agents. The present data, taken together with prior studies, demonstrate both similarities and differences in growth factor regulation of the production of other growth factors by these two treatment methods. One difference is the time-dependent stimulation of IGF-I production by FGF-2 gene transfer observed in this study compared with the inhibition of IGF-I gene expression by exogenous FGF-2 (Shida et al. 2001; Shi et al. 2009). A more complex example is the inhibition by TGF-β1; gene transfer of IGF-I production. This differs from the stimulation of

IGF-I gene expression by exogenous TGF-β1 reported for rabbit and bovine articular chondrocytes (Tsukazaki et al. 1994; Reddi 2003), but is similar to the inhibition of IGF-I expression by exogenous TGF-β1 reported for rat articular chondrocytes (Tsukazaki et al. 1994). A similarity is the stimulation of TGF-β1 expression by both endogenous and exogenous FGF-2 (Shi et al. 2009).

These data address the regulation of growth factor production by multiple growth factor gene transfer. They do not answer the question whether multiple growth factor gene transfer regulates other chondrocyte functions. To address this question, the cell layer remaining after the final collection of culture medium for growth factor analysis was used to assess chondrocyte anabolic and mitotic activities by measuring DNA, glycosaminoglycan, and collagen content. Findings included complex interactions among growth factor transgenes that included synergistic stimulation and inhibition of these cell functions. Optimal stimulation was achieved by different gene combinations for different outcome measures. In addition, an inhibition of anabolic activity was associated with the increasing numbers of transgenes, a finding that corresponds to the similar trend observed for growth production (Shi et al. 2012). In other studies using similar experimental methods, multiple growth factor gene transfer was found to also differentially regulate chondrocyte aggrecan and collagen gene expression (Shi et al. 2011).

These studies employed a longitudinal experimental design in which the same groups of cells were used to generate the growth factors measured at each time point. Although this design is useful for time course studies, a limitation is the availability of DNA data only at the completion of the experiment. Coupled with the confounding effect of multiple growth factor gene co-transfection, this precludes the normalization of growth factor production to cell number. An additional limitation of the study is its focus on cell-based applications to therapy. It does not address the question whether similar results would be obtained with different culture models such as three-dimensional cultures or cartilage explants. An important limitation of such growth factor studies is the potential for negative effects from growth factor overexpression. These include uncontrolled cell proliferation, oncogenic, and off-target effects. To avoid these concerns, we employed a non-viral vector that is unlikely to integrate into the host cell genome and to minimize the risk of prolonged stimulation or oncogenesis. Should similar results be obtained when growth factor transgenes are delivered by other vectors in these or other cell types, these concerns would need to be addressed for each.

## Conclusions

Taken together, the data indicate that adult articular chondrocytes can produce multiple chondrotrophic growth factors in response to multiple gene transfer, that these growth factor transgenes regulate each other's growth factor production, and that they differentially interact with each other in doing so. The data suggest that regulating the regulators of chondrocyte function creates a high level of complexity, and also provides a high level of versatility, to the use of growth factor genes in cell-based gene therapy for cartilage repair. These results pertain to a specific cell type and a selected set of growth factors. Further studies will be required to determine whether such findings are applicable to other cell types and other growth factors.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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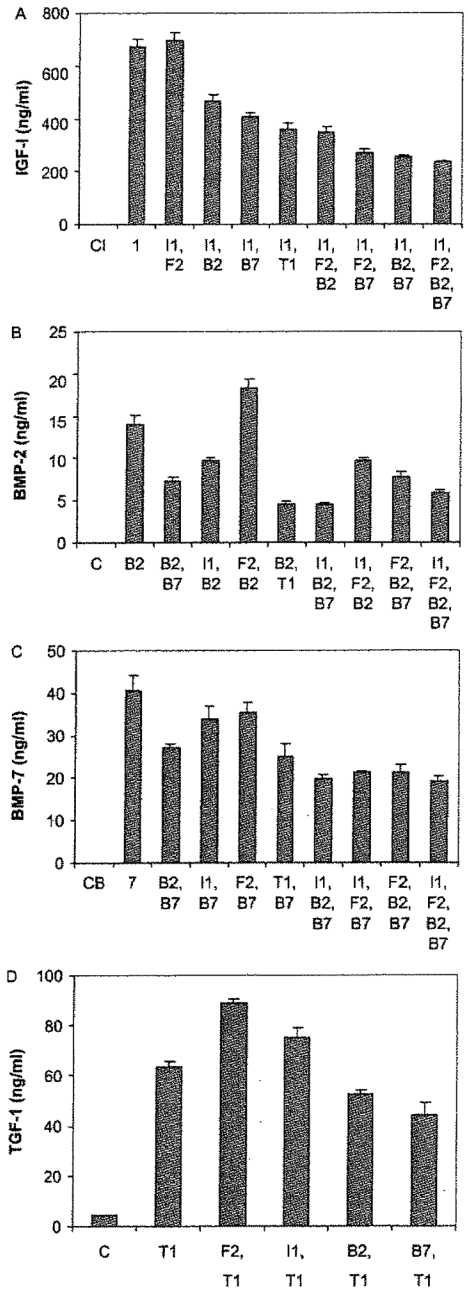


**Figure 1.**

Production by transfected articular chondrocytes of (A) IGF-I, (B) BMP-2, (C) BMP-7, and (D) TGF-β1. Articular chondrocytes were transfected with pAAV vectors carrying the genes encoding the designated growth factors, and growth factor production was measured by ELISA at 2-day intervals. Data represent the total amount of growth factor produced over the 6-day duration of the studies. Key: C, empty vector control; I1, IGF-1; B2, BMP-2; B7, BMP-7; T1, TGF-β1; F2, FGF-2. Data represent the mean of three ± SD independent experiments.

**Table I**

Primers used for cDNA cloning by RT-PCR and plasmid construction.

| | |
|---|---|
| IGF-I FW primer | 5′-CAGAATTCACAATGGGAAAAATCAGCAGTCTTCC-3′ |
| IGF-I RV primer | 5′-CTAGATCTCTACATCCTGTAGTTCTTGTTTCCTG-3′ |
| FGF-2 FW primer | 5′-TCGAATTCACCATGGCAGCCGGGAGCATCACCACG-3′ |
| FGF-2 RV primer | 5′-CTAGATCTTCAGCTCTTAGCAGACATTGGAAG-3′ |
| BMP-2 FW primer | 5′-ACGAATTCACCATGGTGGCCGGGACCCGCTGTCTTC-3′ |
| BMP-2 RV primer | 5′-ACAGATCTCTAGCGACACCCACAACCCTCCACAACC-3′ |
| BMP-7 FW primer | 5′-GAGGATCCGCGATGCACGTGCGCTCACTGCGAGC-3′ |
| BMP-7 RV primer | 5′-CAAGATCTAGCTAGTGGCAGCCACAGGCCCGGACC-3′ |
| TGF-β1 FW primer | 5′-ACGAATTCGCCATGCCGCCCTCCGGGCTGCGGCTGC-3′ |
| TGF-β1 RV primer | 5′-ACAGATCTTCAGCTGCACTTGCAGGAGCGCACGATC-3′ |