

## Role of Sox9 in Growth Factor Regulation of Articular Chondrocytes

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### ABSTRACT

Chondrogenic polypeptide growth factors influence articular chondrocyte functions that are required for articular cartilage repair. Sox9 is a transcription factor that regulates chondrogenesis, but its role in the growth factor regulation of chondrocyte proliferation and matrix synthesis is poorly understood. We tested the hypotheses that selected chondrogenic growth factors regulate sox9 gene expression and protein production by adult articular chondrocytes and that sox9 modulates the actions of these growth factors. To test these hypotheses, we delivered insulin-like growth factor-I (IGF-I), fibroblast growth factor-2 (FGF-2), bone morphogenetic protein-2 (BMP-2) and/or bone morphogenetic protein-7 (BMP-7), or their respective transgenes to adult bovine articular chondrocytes, and measured changes in sox9 gene expression and protein production. We then knocked down sox9 gene expression with sox9 siRNA, and measured changes in the expression of the genes encoding aggrecan and types I and II collagen, and in the production of glycosaminoglycan, collagen and DNA. We found that FGF-2 or the combination of IGF-I, BMP-2, and BMP-7 increased sox9 gene expression and protein production and that sox9 knockdown modulated growth factor actions in a complex fashion that differed both with growth factors and with chondrocyte function. The data suggest that sox9 mediates the stimulation of matrix production by the combined growth factors and the stimulation of chondrocyte proliferation by FGF-2. The mitogenic effect of the combined growth factors and the catabolic effect of FGF-2 appear to involve sox9-independent mechanisms. Control of these molecular mechanisms may contribute to the treatment of cartilage damage. *J. Cell. Biochem.* 116: 1391–1400, 2015. © 2015 Wiley Periodicals, Inc.

**KEY WORDS:** sox9; GROWTH FACTORS; ARTICULAR CHONDROCYTES; SMALL INHIBITORY RNA

Articular cartilage provides a gliding surface that enables pain-free motion of diarthrodial joints. Articular cartilage formation, maintenance, and repair depend on the chondrocytes embedded in the cartilage extracellular matrix. These cells have a poor intrinsic healing capability after cartilage damage or loss from injury or osteoarthritis [Buckwalter and Mankin, 1998]. As a result, the tissue loss is generally persistent and progressive. Polypeptide growth factors play a central role in articular cartilage function [Trippel, 1995]. Several growth factors have been identified that promote mitogenic and anabolic activities by articular chondrocytes. These growth factors include insulin-like growth factor-I (IGF-I), fibroblast

growth factor-2 (FGF-2), bone morphogenetic protein-2 (BMP-2) and/or bone morphogenetic protein-7 (BMP-7). IGF-I is considered a candidate for articular cartilage repair because it stimulates both cell proliferation and the synthesis of the key cartilage matrix components, aggrecan and type II collagen [Luyten et al., 1988; Sah et al., 1994; Fortier et al., 2002; Loeser et al., 2003]. FGF-2 is a potent mitogen for chondrocytes and modulates both chondrocyte anabolic and catabolic activities [Bradham et al., 1994; Sah et al., 1994; Fujimoto et al., 1999; Madry et al., 2004; Henson et al., 2005; Im et al., 2007; Shi et al., 2012]. BMP-2 [Sailor et al., 1996; Grunder et al., 2004] and BMP-7 [Flechtenmacher et al., 1996; Chubinskaya

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et al., 2007] each promote chondrocyte matrix synthesis. All these factors have been shown to augment articular cartilage repair in ex vivo and in vivo models [Madry et al., 2001, 2005; Yokoo et al., 2005; Goodrich et al., 2007].

Sox9 is a master transcription factor that is required for cartilage formation during embryogenesis [Akiyama and Lefebvre, 2011] and contributes to articular cartilage development postnatally [Henry et al., 2012]. The *sox9* gene (*sox9*) is expressed in chondroprogenitor cells in mouse embryos [Wright et al., 1995] and mouse *sox9*<sup>-/-</sup> embryonic stem cells are unable to differentiate into chondrocytes [Bi et al., 1999]. Expression of *sox9* is detected in mouse articular cartilage up to nine months of age and *sox9* protein up to 15 months [Salminen et al., 2001] but its gene expression is completely shut off in mature hypertrophic chondrocytes [de Crombrughe et al., 2000]. Sox9 is essential for the expression of the type II collagen gene (*col2a1*) [Bell et al., 1997; Zhou et al., 1998], a marker of the chondrocyte phenotype. In human articular chondrocytes, transfer of the human *sox9* gene (*SOX9*) increased the synthesis of type II collagen and proteoglycan [Cucchiari et al., 2007], and both the rate and capacity of the synthesis of proteoglycan glycosaminoglycans [Tew et al., 2008].

Despite the shared roles of chondrogenic growth factors and *sox9* on cartilage, few studies have investigated their potential interdependence, particularly in adult articular chondrocytes. Some of the growth factors that regulate chondrocyte activities also regulate *sox9*. IGF-I has been shown to increase *SOX9* expression during redifferentiation of dedifferentiated articular chondrocytes and to up-regulate *SOX9* expression [Shakibaei et al., 2006]. In adult canine articular chondrocytes, IGF-I increased *sox9* protein production and countered the inhibitory effect of IL-1 on type II collagen production [Montaseri et al., 2011]. Recently, IGF-I-mediated *COL2a1* expression in human articular chondrocytes has been shown to be associated with increased binding of *sox9* to its *cis* element in a *COL2a1* enhancer [Renard et al., 2012]. FGF-2 has been reported to increase *sox9* expression in mouse primary costal chondrocytes and to increase the activity of a *sox9*-dependent chondrocyte-specific enhancer in *col2a1* [Murakami et al., 2000]. When human osteoarthritic chondrocytes were treated with the *sox9* and/or the FGF-2 transgenes, the combination preserved the mitogenic properties of FGF-2 alone and added the anabolic effects of *sox9* alone [Cucchiari et al., 2009]. BMP-2 has been found to stimulate *sox9* expression through a CCAAT box in the *sox9* promoter region in mouse embryonic fibroblasts during BMP-2 induced differentiation to chondrocytes [Pan et al., 2008]. To our knowledge, the role, if any, of *sox9* in mediating the diverse actions of the growth factors that regulate articular chondrocytes remains unknown.

In prior studies, we found that delivery of the genes encoding IGF-I, FGF-2, BMP-2, and BMP-7, individually and in combination, to adult articular chondrocytes both differentially and similarly regulated chondrocyte reparative activities [Sailor et al., 1996; Shi et al., 2012, 2013]. These differences and similarities were particularly marked between the FGF-2 transgene and the combination of the IGF-I, BMP-2, and BMP-7 transgenes. Specifically, overexpression of FGF-2 inhibited, and the combined overexpression of IGF-I, BMP-2, and BMP-7 stimulated, type II collagen gene expression and protein production. Both treatments augmented

aggrecan gene expression, but only overexpression of IGF-I, BMP-2, and BMP-7 increased its deposition as matrix. Both treatments similarly stimulated chondrocyte proliferation [Shi et al., 2013]. Due to the separation and overlap of their actions, FGF-2 and the combination of IGF-I, BMP-2, and BMP-7 were chosen to test the hypothesis that *sox9* is involved in determining the specific actions of these growth factors. We found that *sox9* plays widely divergent roles in mediating the growth factor regulation of articular chondrocytes.

## MATERIALS AND METHODS

### CHONDROCYTE CULTURE

Dulbecco's minimum essential medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and glutamine were from Life Technologies (Carlsbad, CA). Ascorbic acid and bovine serum albumin (BSA) were from Sigma (Saint Louis, MO). Basal medium was prepared with DMEM, 50  $\mu$ g/ml ascorbic acid, 100 U/ml penicillin, 100  $\mu$ g/ml streptomycin and 2 mM glutamine. Complete medium was prepared with basal medium supplemented with 10% FBS. Recombinant human IGF-I was from Peptotech, Inc. (Rocky Hill, NJ). Recombinant human FGF-2, BMP-2 and BMP-7 were from R&D Systems (Minneapolis, MN). Articular chondrocytes from skeletally mature (physes closed) bovinds were isolated and placed in primary culture as previously described [Shi et al., 2012].

### DELIVERY OF GROWTH FACTOR TRANSGENES

Growth factor transgenes were delivered using the plasmid vectors pAAV-FGF-2, pAAV-IGF-I, pAAV-BMP-2, and pAAV-BMP-7 as previously described [Shi et al., 2012]. To improve the readability of the data presented, the transgenes carried by these vectors are designated tFGF-2, tIGF-I, tBMP-2, and tBMP-7 respectively. After 3 days in culture, chondrocytes were transfected using FuGENE 6 (Roche Applied Science) and pAAV plasmid DNA expressing the designated growth factors. Empty vector pAAV-MCS was used as control. For single transfections, 2  $\mu$ g of plasmid DNA per well was used. For two- and three-transgene combinations, 2  $\mu$ g of each plasmid DNA per well was used together. After 4–6 h, transfection was stopped by replacing the medium with 4 ml fresh complete medium. On days 2 and 4 after transfection, conditioned medium (CM) was collected and replaced by 4 ml basal medium. On day 6, CM was collected, and cell culture was terminated and cell layer was harvested.

### DELIVERY OF SOX9 SIRNA AND EXOGENOUS GROWTH FACTOR PROTEINS

Bovine *sox9* siRNA (ON-TARGETplus Custom SMARTpool) was synthesized by Dharmacon (Table I). To determine whether this *sox9* siRNA efficiently silences *sox9* expression, adult bovine articular chondrocytes were transfected with *sox9* siRNA or non-targeting siRNA (Dharmacon) according to manufacturer's instructions. After 3 days in culture, the medium was replaced with 2 ml complete medium and cells were transfected using 4  $\mu$ l/well DharmaFECT3 (Dharmacon) and 10  $\mu$ l of 20  $\mu$ M *sox9* siRNA. ON-TARGETplus Non-targeting siRNA was used for mock-transfection. After 16 h, siRNA transfection was stopped by replacing the medium with 4 ml of fresh

TABLE I. siRNAs and Primers and Probes for Real Time PCR

Gene	ACC. No.	siRNA, primer or probe	Sequence
Sox9	AF278703	siRNA S1	5'-GCGTCAACGGCTCGAGCAA-3'
		siRNA S2	5'-TGACCGACGAGCAGGAGAA-3'
		siRNA S3	5'-GGAAGTCGGTGAAGAACGG-3'
		siRNA S4	5'-GACTGCTGAACGAGAGCGA-3'
Not applicable Sox9	Not applicable AF278703	Non-targeting siRNA	5'-TGGTTTACATGTCGACTAA-3'
		Forward primer	5'-AATCTCCTGGACCCCTCATG-3'
		Reverse primer	5'-GGCGGACAGGCCCTTCT-3'
		Probe	5'-AGATGACCGACGAGCAG-3'
Col1 $\alpha$ 1	NM_001034039	Forward primer	5'-AAAAGAGGCACGTCTGGTAC-3'
		Reverse primer	5'-CGGATTCAGTTCGAGTATGGCGG-3'
		Probe	5'-GTGGTAGGTGATGTTCTGGG-3'

complete medium supplemented with the designated growth factor(s), 50 ng/ml FGF-2 or the combination of 200 ng/ml IGF-1 with 100 ng/ml BMP-2 and 100 ng/ml BMP-7. Fresh complete medium without growth factors was used as control. On days 2 and 4 after transfection, conditioned medium was harvested and replaced with basal medium containing 0.1% BSA and the designated growth factor(s) as described above. Basal medium containing 0.1% BSA without growth factors was used as control. On day 6 after transfection, conditioned medium and cell layer were harvested separately.

#### PROTEIN SAMPLE PREPARATION AND WESTERN BLOTTING

The cell layer was harvested in 0.5 ml lysis buffer [20 mM Tris (pH 7.6), 120 mM NaCl, 10 mM EDTA, 10% glycerol, 1% NP-40, 100 mM NaF, 10 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM PMSF, 2 mM Na<sub>3</sub>VO<sub>4</sub>, 40  $\mu$ g/ml leupeptin, 1  $\mu$ M pepstatin A, and 10  $\mu$ g/ml aprotinin]. Cell lysates were sonicated on ice and protein concentration was determined by Bio-Rad Protein Assay (Bio-Rad). Cell lysates containing 20  $\mu$ g protein were electrophoresed on a 10% SDS-polyacrylamide gel under reducing condition, and the proteins were transferred to nitrocellulose membranes. The membranes were blocked with 5% fat-free milk (Bio-Rad) in Tris-buffered saline/Tween (TBST) for 1 h at room temperature and were then separately probed with primary antibodies to *sox9* (R&D Systems) and  $\beta$ -actin (Sigma) overnight at 4°C.

#### RNA ISOLATION AND REAL-TIME PCR ANALYSIS

Total RNA was prepared from the chondrocyte cell layer using the RNeasy Mini kit (Qiagen, MD) and real-time PCR was performed as previously described [Shi et al., 2012]. Briefly, *sox9* mRNA, aggrecan mRNA, *col1 $\alpha$ 1* mRNA, *col1 $\alpha$ 2* mRNA, *col2 $\alpha$ 1* mRNA, and 18S rRNA were measured using a Prism 7000 Sequence Detector System and TaqMan Universal Master Mix (Applied Biosystems, Foster City, CA). Primers and probes for aggrecan mRNA, *col1 $\alpha$ 2* mRNA, *col2 $\alpha$ 1* mRNA, and 18S rRNA analysis were previously described [Shi et al., 2012]. Primers and probes for *sox9* and *col1 $\alpha$ 1* mRNA analysis were synthesized by Invitrogen and Applied Biosystems respectively (Table I). Target gene mRNA levels were normalized to 18S rRNA levels. Changes of target gene expression are expressed as the ratio of growth factor gene transfected cells to cells transfected with empty vector (mock-transfected control) or as the ratio of growth factor treatment and or *sox9* siRNA transfected cells to the cells transfected by non-targeting siRNA. Three independent experiments were

performed using different batches of articular chondrocytes obtained from different bovine joints at different times. Data are presented as the average of fold changes.

#### GLYCOSAMINOGLYCAN, COLLAGEN AND DNA ANALYSIS

Glycosaminoglycan (GAG) and collagen were measured by dimethylmethylene blue assay and hydroxyproline assay respectively, and DNA content as an index of cell proliferation was assessed by Picogreen dsDNA assay (Invitrogen) as previously described [Shi et al., 2013]. GAG and collagen in the medium (released) and retained with the chondrocytes (cell-associated) were analyzed separately [Shi et al., 2013].

## RESULTS

#### EFFECT OF GROWTH FACTOR TRANSGENES ON SOX9 GENE EXPRESSION AND PROTEIN PRODUCTION

Delivery of tFGF-2 or [tIGF-I + tBMP-2 + tBMP-7] increased *sox9* expression to a greater degree than the other individual growth factors or growth factor combinations tested (Fig. 1). Growth factor transgene regulation of *sox9* protein production closely corresponded to *sox9* expression (Fig. 2). These findings supported the selection of these two treatments for further investigation.

#### EFFECT OF SOX9 SIRNA ON GROWTH FACTOR REGULATION OF SOX9 GENE EXPRESSION

Growth factor proteins, rather than growth factor transgenes were selected for *sox9* knockdown studies because the *sox9* siRNA and growth factor transgenes required different transfection reagents, and we found that these failed to achieve satisfactory transfection efficiency when used in combination (data not shown).

Chondrocytes were treated with or without FGF-2 or [IGF-I + BMP-2 + BMP-7] in the presence or absence of *sox9* siRNA, and *sox9* mRNA levels were quantified 2, 4, and 6 days following treatment. In the absence of growth factor(s), *sox9* siRNA reduced *sox9* mRNA levels compared to non-targeting siRNA. FGF-2 stimulated *sox9* expression at all time points and *sox9* siRNA reduced these augmented *sox9* mRNA levels. The combination [IGF-I + BMP-2 + BMP-7] also stimulated *sox9* expression at all time points and *sox9* siRNA similarly reduced these augmented *sox9* mRNA levels (Fig. 3). These data indicate that *sox9* siRNA efficiently

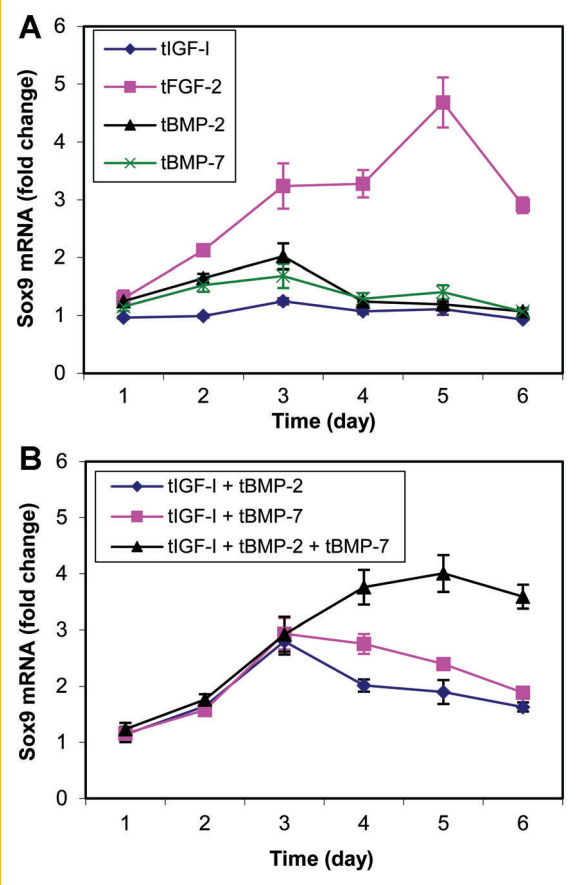


Fig. 1. Sox9 mRNA changes in response to the designated growth factor transgenes (A) or transgene combinations (B). Transfected chondrocytes were cultured for the designated time periods. Sox9 mRNA was normalized to 18S RNA. Data are expressed as sox9 mRNA fold change to the respective control for each time point  $\pm$  SD for three independent experiments. Maximal stimulation was 4.68-fold ( $P=0.0046$ ) and 4.00-fold ( $P=0.0039$ ) by tFGF-2 and [tIGF-I + tBMP-2 + tBMP-7], respectively.

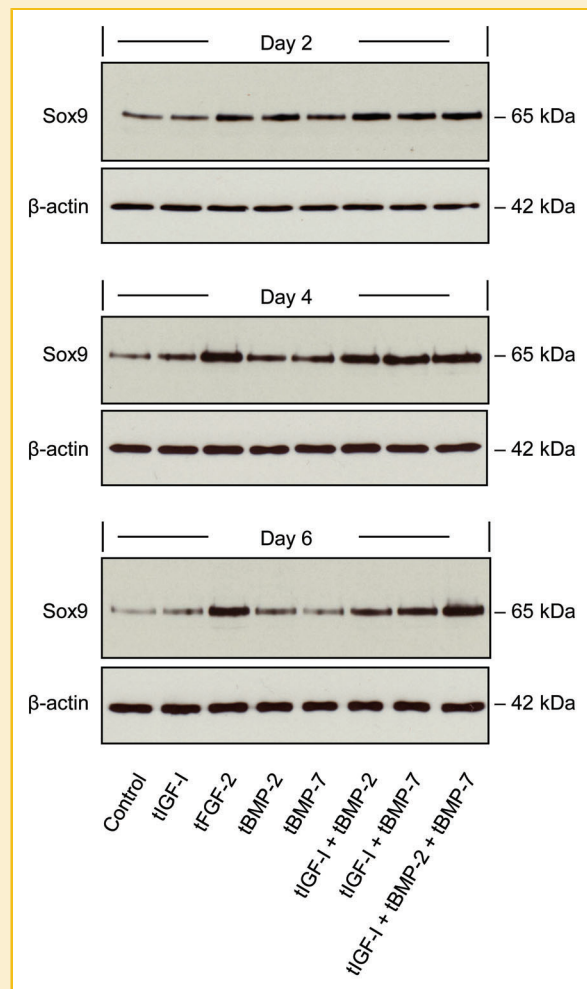


Fig. 2. Western blot of sox9 protein in response to the designated individual and combinations of growth factor transgenes. Cell lysate was prepared from transfected chondrocytes following culture for the designated time periods. Samples were separately probed for  $\beta$ -actin.

knocked down sox9 gene expression in chondrocytes with and without stimulation by FGF-2 or [IGF-I + BMP-2 + BMP-7].

#### SOX9 KNOCKDOWN MODULATES GROWTH FACTOR REGULATION OF CHONDROCYTE MATRIX GENE EXPRESSION

**Aggrecan gene expression.** In the absence of growth factor(s), sox9 knockdown inhibited aggrecan gene (*acan*) expression at all time points. FGF-2 increased *acan* expression over time, but sox9 knockdown had minimal effect on this FGF-2 stimulation. The combination [IGF-I + BMP-2 + BMP-7] progressively stimulated *acan* expression and sox9 knockdown reduced this augmented expression at all time points (Fig. 4).

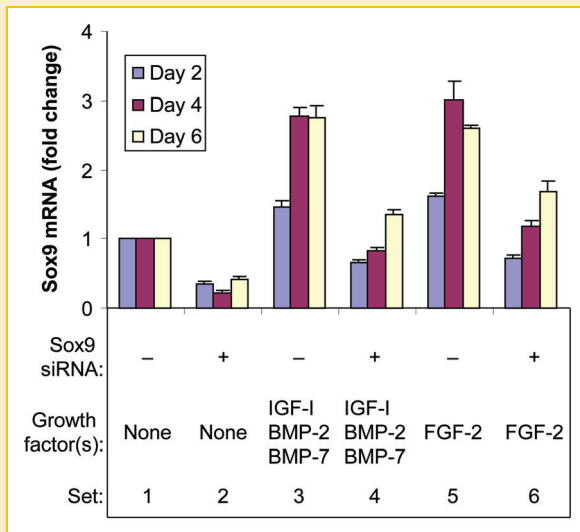
**Collagen gene expression.** *Col2 $\alpha$ 1* expression was inhibited by sox9 knockdown at all time points in the absence of growth factor(s). FGF-2 progressively decreased *col2 $\alpha$ 1* expression and sox9 knockdown further decreased this *col2 $\alpha$ 1* expression. In contrast to FGF-2, [IGF-I + BMP-2 + BMP-7] progressively stimulated *col2 $\alpha$ 1* expres-

sion. As for FGF-2, sox9 knockdown progressively decreased this *col2 $\alpha$ 1* expression (Fig. 5A). *Col1 $\alpha$ 1* expression was stimulated by sox9 knockdown at all time points in the absence of growth factor(s). FGF-2 decreased *col1 $\alpha$ 1* expression and sox9 siRNA partly reversed this inhibition at all time points. The combination [IGF-I + BMP-2 + BMP-7] initially decreased *col1 $\alpha$ 1* expression and then progressively increased it over time. Sox9 siRNA further stimulated this *col1 $\alpha$ 1* expression at all time points (Fig. 5B). Regulation of *col1 $\alpha$ 2* was similar to that of *col1 $\alpha$ 1* (Supplemental Fig. S1).

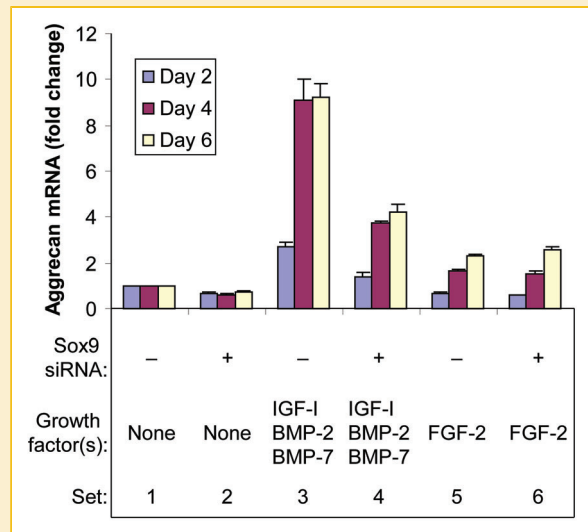
#### SOX9 KNOCKDOWN MODULATES GROWTH FACTOR REGULATION OF CHONDROCYTE EXTRACELLULAR MATRIX PRODUCTION

**Glycosaminoglycan production.** In the absence of growth factor(s), sox9 knockdown did not significantly affect released GAG production, and only modestly inhibited cell-associated GAG production. FGF-2 stimulated released GAG, but did not change cell-associated GAG. In the presence of FGF-2, sox9 knockdown inhibited both forms





**Fig. 3.** Knockdown of *sox9* gene expression by *sox9* siRNA. Chondrocytes were transfected by *sox9* siRNA (+) or by non-targeting siRNA (-) and treated with no growth factor (None), FGF-2 or the combination of IGF-I, BMP-2, and BMP-7 proteins, then cultured for the designated time periods. Sox9 mRNA was normalized to 18S RNA. Data are expressed as *sox9* mRNA change compared to the respective control for each time point  $\pm$  SD for three independent experiments. Comparison groups are illustrated as data sets. Ranges in values are over the three time points. Sets 1 versus 2: range 59–77% (all  $P \leq 0.0027$ ). Sets 1 versus 3: range 1.47- to 2.77-fold (all  $P \leq 0.0106$ ). Sets 3 versus 4: range 51–70%, (all  $P < 0.0001$ ). Sets 1 versus 5: range 1.61- to 3.02-fold (all  $P \leq 0.0054$ ), Sets 5 versus 6: range 35–61%, (all  $P < 0.0001$ ).



**Fig. 4.** Effect of *sox9* knockdown on aggrecan gene (*acan*) expression. Chondrocytes were transfected by *sox9* siRNA (+) or by non-targeting siRNA (-) and treated with no growth factor (None), FGF-2 or the combination of IGF-I, BMP-2 and BMP-7 proteins, then cultured for the designated time periods. Aggrecan mRNA was normalized to 18S RNA. Data are expressed as *sox9* mRNA change compared to the respective control for each time point  $\pm$  SD for three independent experiments. Comparison groups are illustrated as data sets. Ranges in values are over the three time points. Sets 1 versus 2: range 29–38%, (all  $P < 0.012$ ). Sets 1 versus 3: range 2.72- to 9.23-fold ( $P \leq 0.0040$ ). Sets 3 versus 4: range 49–59% (all  $P \leq 0.0001$ ). Sets 1 versus 5: 32% ( $P = 0.0062$ ) at day 2, 1.68-fold ( $P = 0.0016$ ) at day 4 to 2.33-fold ( $P = 0.0007$ ) at day 6. Sets 5 versus 6: range 16%, ( $P = 0.0015$ ) at day 2, no significant difference (NS) at day 4 or day 6.

of GAG. In contrast, [IGF-I + BMP-2 + BMP-7] stimulated both forms of GAG and this effect was greater for cell-associated (15.00-fold) than for released (4.14-fold) GAG. Sox9 knockdown did not change the effect of [IGF-I + BMP-2 + BMP-7] on released GAG, but reduced the stimulation of cell associated GAG (Fig. 6A). To quantify the effect of these treatments on the fate of the GAG produced by the cells, the ratio of cell-associated to released GAG (GAG distribution ratio) was calculated for each treatment condition. These results demonstrate that, although FGF-2 increased total GAG production, it decreased the proportion of GAG that led to matrix formation. In contrast, [IGF-I + BMP-2 + BMP-7] increased both total GAG production and the GAG distribution ratio (Fig. 6B). Sox9 knockdown did not influence the action of FGF-2, but decreased the GAG distribution ratio in the presence of [IGF-I + BMP-2 + BMP-7].

**Collagen production.** In absence of growth factor(s), *sox9* knockdown stimulated released collagen and inhibited cell-associated collagen. FGF-2 inhibited both released and cell-associated collagen and *sox9* knockdown further inhibited both of them. In contrast, the combination [IGF-I + BMP-2 + BMP-7] stimulated both released and cell associated collagen. Sox9 knockdown further increased released collagen, but reduced the stimulation of cell associated collagen (Fig. 7A). In addition to decreasing the total amount of collagen, FGF-2 decreased the proportion of that collagen that was retained by the cells, while [IGF-I + BMP-2 + BMP-7] increased both the amount of collagen and its distribution ratio (Fig. 7B). Sox9 knockdown had minimal effect on the action of FGF-2, but decreased

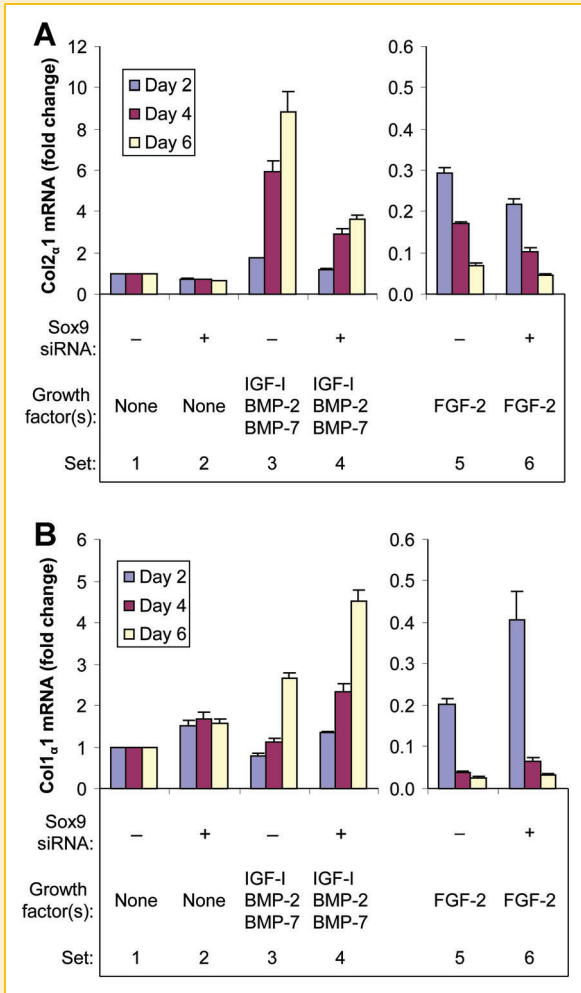
the collagen distribution ratio in the presence of [IGF-I + BMP-2 + BMP-7].

#### EFFECT OF SOX9 KNOCKDOWN ON GROWTH FACTOR REGULATION OF CELL PROLIFERATION

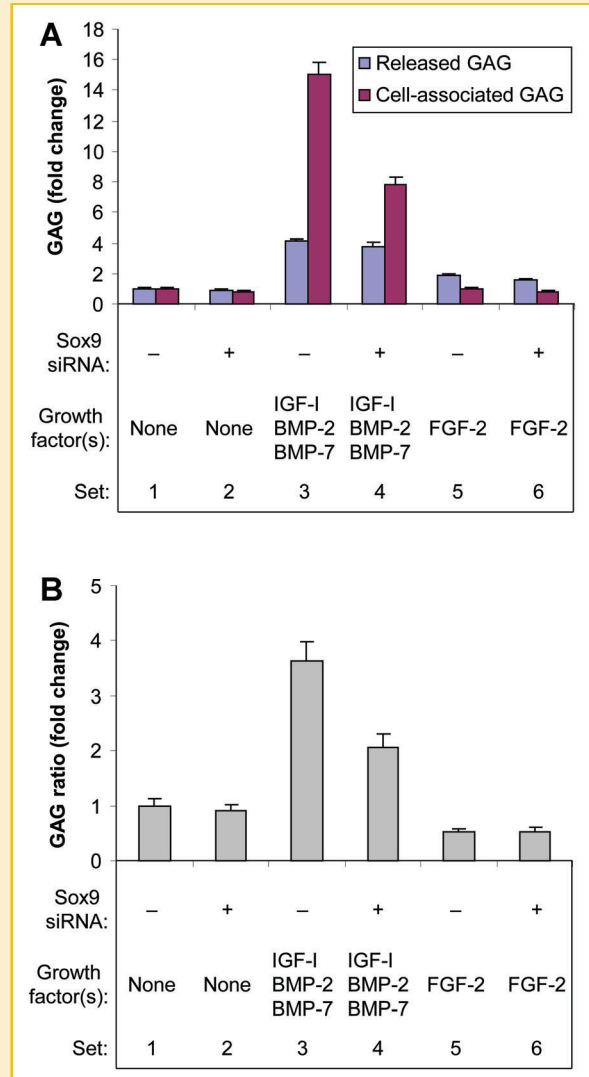
In the absence of growth factor(s), *sox9* knockdown had little effect on chondrocyte proliferation. FGF-2 increased DNA content, and *sox9* siRNA attenuated this effect. The combination [IGF-I + BMP-2 + BMP-7] increased DNA content, but *sox9* siRNA did not significantly influence this action (Fig. 8).

#### DISCUSSION

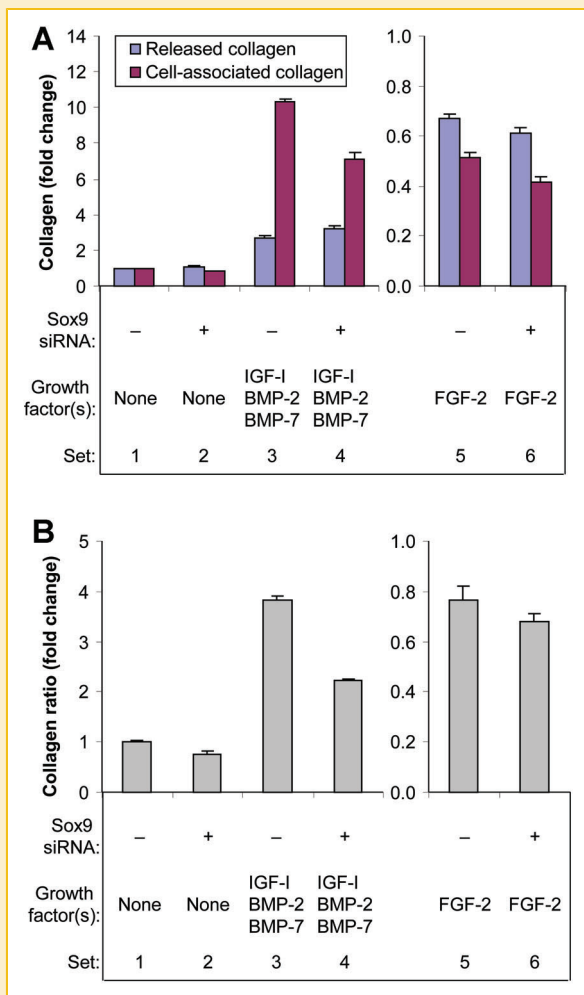
To our knowledge, this is the first demonstration that *sox9* plays distinct roles in the growth factor regulation of adult articular chondrocytes. The present data are consistent with the prior observation that tFGF-2 modestly increased aggrecan gene expression and GAG production, while markedly inhibiting type II collagen and type I collagen gene expression and collagen protein production, and that [tIGF-I + tBMP-2 + tBMP-7] markedly stimulated aggrecan and type II collagen gene expression, and the production of both GAG and collagen protein. These data contrast with the current findings that FGF-2 and [IGF-I + BMP-2 + BMP-7], delivered either as transgenes or as proteins stimulated *sox9* expression and protein



**Fig. 5.** Effect of *sox9* knockdown on type II collagen gene (*col2α1*) A: and type I collagen α1 gene (*col1α1*) B: expression. Chondrocytes were transfected by *sox9* siRNA (+) or by non-targeting siRNA (-) were treated with no growth factor (None), FGF-2 or the combination of IGF-I, BMP-2 and BMP-7 proteins, then cultured for the designated time periods. *Col2α1* mRNA and *col1α1* mRNA were normalized to 18S RNA respectively. Data are expressed as *col2α1* mRNA (A) and *col1α1* mRNA (B) changes compared to the respective control for each time point ±SD for three independent experiments. Comparison groups are illustrated as data sets. Ranges in values are over the three time points. A: Sets 1 versus 2: range 29–37%, (all  $P \leq 0.0097$ ). Sets 1 versus 3: range 1.76-fold ( $P = 0.0018$ ) to 8.85-fold ( $P = 0.0050$ ). Sets 3 versus 4: range 32% ( $P < 0.0001$ ) to 59% ( $P < 0.0001$ ). Sets 1 versus 5: range 71% ( $P = 0.0001$ ) to 93% ( $P \leq 0.0001$ ). Sets 5 versus 6: range 25–41% (all  $P \leq 0.0001$ ). B: Sets 1 versus 2: range 1.49– to 1.68-fold (all  $P \leq 0.0238$ ). Sets 1 versus 3: range 22% ( $P = 0.0222$ ) at day 2, NS at day 4 to 2.67-fold ( $P = 0.0015$ ) at day 6. Sets 3 versus 4: range 1.70– to 2.09-fold (all  $P < 0.0001$ ). Sets 1 versus 5: range 80% ( $P = 0.0001$ ) to 97% ( $P < 0.0001$ ). Sets 5 versus 6: range 1.21– to 2.00-fold (all  $P \leq 0.0147$ ).



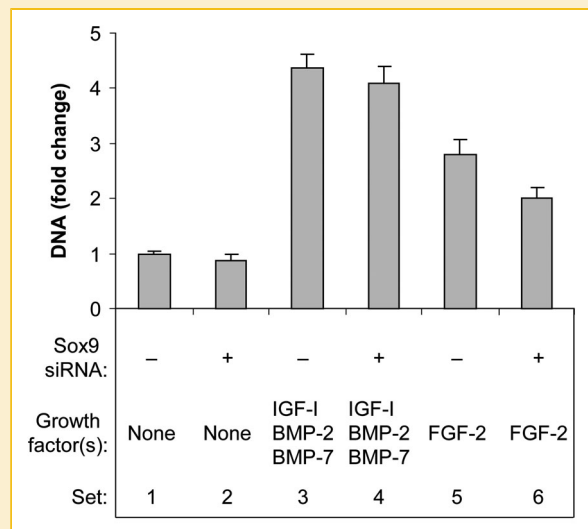
**Fig. 6.** Effect of *sox9* knockdown on glycosaminoglycan production (A) and glycosaminoglycan distribution between the medium and the cells (B). Chondrocytes were transfected by *sox9* siRNA (+) or by non-targeting siRNA (-) and treated with no growth factor (None), FGF-2 or the combination of IGF-I, BMP-2, and BMP-7 proteins. Culture medium was harvested at the designated time points and the cells harvested at 6 days. Glycosaminoglycan in the medium (released GAG) and cell layer (cell-associated GAG) were measured separately. Data are expressed as changes in released GAG and cell-associated GAG (A), and change in the ratio of cell-associated GAG to released GAG (B) for the treatment group compared to the respective control (GAG distribution ratio) ±SD for three independent experiments. Comparison groups are illustrated as data sets. A: Sets 1 versus 2: released GAG (NS), cell-associated GAG 18% ( $P = 0.0067$ ). Sets 1 versus 3: released GAG 4.14-fold ( $P < 0.0001$ ), cell associated GAG 15.00-fold ( $P < 0.0001$ ). Sets 3 versus 4: released GAG (NS), cell associated GAG 48% ( $P < 0.0001$ ). Sets 1 versus 5: released GAG 1.91-fold ( $P < 0.0001$ ), cell-associated GAG (NS). Sets 5 versus 6: released GAG 18% ( $P = 0.0018$ ), cell-associated GAG 17% ( $P = 0.0091$ ). B: Sets 1 versus 2: NS. Sets 1 versus 3: 3.63-fold ( $P < 0.0001$ ). Sets 3 versus 4: 43% ( $P < 0.0001$ ). Sets 1 versus 5: 47% ( $P < 0.0001$ ). Sets 5 versus 6: NS.



**Fig. 7.** Effect of *sox9* knockdown on collagen production (A) and collagen distribution between the medium and the cells (B). Chondrocytes were transfected by *sox9* siRNA (-) or by non-targeting siRNA (+) and treated with no growth factor (None), FGF-2 or the combination of IGF-I, BMP-2, and BMP-7 proteins. Culture medium was harvested at the designated time points and the cells harvested at 6 days. Collagen in the medium (released collagen) and cell layer (cell-associated collagen) were measured separately. Data are expressed as changes in released collagen and cell-associated collagen (A), and change in the ratio of cell-associated collagen to released collagen (B) for the treatment group compared to the respective control (collagen distribution ratio)  $\pm$  SD for three independent experiments. Comparison groups are illustrated as data sets. A: Sets 1 versus 2: released collagen 1.08-fold ( $P=0.0270$ ), cell-associated collagen 18% ( $P<0.0001$ ). Sets 1 versus 3: released collagen 2.69-fold ( $P<0.0001$ ), cell associated collagen 10.31-fold ( $P<0.0001$ ). Sets 3 versus 4: released collagen 1.19-fold ( $P<0.0001$ ), cell associated collagen 31% ( $P<0.0001$ ). Sets 1 versus 5: released collagen 33%, cell-associated collagen 49% (both  $P<0.0001$ ). Sets 5 versus 6: released collagen 9% ( $P=0.0157$ ), cell associated collagen 19% ( $P<0.0001$ ). B: Sets 1 versus 2: 24% ( $P<0.0001$ ). Sets 1 versus 3: 3.83-fold ( $P<0.0001$ ). Sets 3 versus 4: 42% ( $P<0.0001$ ). Sets 1 versus 5: 23% ( $P<0.0001$ ). Sets 5 versus 6: 11% ( $P=0.0167$ ).

production. The differential effects on chondrocyte function contrast with the similar effects of these treatments on *sox9* and suggest that *sox9* plays different roles in the actions of these growth factors.

Prior studies reported a relation between *sox9* and aggrecan in a cartilage-derived cell line, TC6 [Sekiya et al., 2000], and a correlation



**Fig. 8.** Effect of *sox9* knockdown on cell proliferation. Chondrocytes were transfected by *sox9* siRNA (+) or by non-targeting siRNA (-) and treated with no growth factor (None), FGF-2 or the combination of IGF-I, BMP-2, and BMP-7 proteins, then cultured for 6 days. DNA content was measured in the cell layer. Changes in DNA content serve as an index of cell proliferation. Data are expressed as DNA change compared to the control  $\pm$  SD for three independent experiments. Comparison groups are illustrated as data sets. Sets 1 versus 2: NS. Sets 1 versus 3: 4.37-fold ( $P<0.0001$ ). Sets 3 versus 4: NS. Sets 1 versus 5: 2.81-fold ( $P<0.0001$ ). Sets 5 versus 6: 29% ( $P=0.0003$ ).

between human *SOX9* and *ACAN* expression in FGF-2 stimulated human ear chondrocytes [Mandl et al., 2004]. In the present studies, FGF-2 stimulated both *acan* and *sox9* expression in bovine articular chondrocytes. However, *sox9* knockdown failed to influence FGF-2 stimulated *acan* expression. For this reason, the correlation between *sox9* and *acan* expression in our studies may not be causal. The combination [IGF-I + BMP-2 + BMP-7] also stimulated both *acan* and *sox9* expression, but, unlike its effect on FGF-2 regulation, *sox9* knockdown substantially mitigated the increase in *acan* expression. *Sox9* knockdown also inhibited *acan* expression in the absence of growth factor(s). Taken together, these data suggest that FGF-2, [IGF-I + BMP-2 + BMP-7] and constitutive *sox9* each promote *acan* expression. The data further suggest that [IGF-I + BMP-2 + BMP-7] regulation of *acan* appears to be mediated, at least in part, through *sox9* upregulation, while that of FGF-2 is predominantly through *sox9*-independent mechanisms.

Type II collagen is the principal collagen in articular cartilage and a major structural component of cartilage matrix. It is found in few other tissues and is a phenotypic marker of chondrocytes. In prior gene transfer studies, tFGF-2 was shown to markedly inhibit, and [tIGF-I + tBMP-2 + tBMP-7] to markedly stimulate, *col2a1* expression [Shi et al., 2012]. In the present investigation using growth factor proteins, FGF-2 almost completely abrogated *col2a1* expression, while [IGF-I + BMP-2 + BMP-7] increased it to over eightfold control levels. Yet, FGF-2 and [IGF-I + BMP-2 + BMP-7] increased *sox9* expression 3.0-fold and 2.8-fold, respectively, both in a time-dependent fashion. The opposite effects of FGF-2 and [IGF-I + BMP-2 + BMP-7] on *col2a1*, coupled with their similar stimulation of *sox9*

expression suggest that *col2a1* expression is dissociated from *sox9* expression. However, the siRNA data suggest an alternative explanation. In the absence of growth factor(s), *sox9* knockdown repressed *col2a1* expression. Sox9 knockdown also progressively attenuated by 32% to 59% (all  $P < 0.0001$ ) the marked stimulation of *col2a1* expression by [IGF-I + BMP-2 + BMP-7] over time, and further inhibited by 25–41% (all  $P < 0.001$ ) an already profound reduction in *col2a1* expression by FGF-2. Taken together, these results suggest that *sox9* promotes *col2a1* expression under all three treatment conditions tested. However, while [IGF-I + BMP-2 + BMP-7] stimulation of *col2a1* expression is largely *sox9*-dependent, this role of *sox9* is minimal compared to the marked *sox9*-independent inhibition of *col2a1* expression by FGF-2.

The type I collagen genes, *col1a1* and *col1a2* are typically not expressed in adult articular chondrocytes [Eyre, 2002] and are minimally expressed in short term primary chondrocyte culture [von der Mark et al., 1977]. In marked contrast to *col2a1*, *sox9* knockdown increased *col1a1* and *col1a2* expression in the absence of growth factor(s) and in [IGF-I + BMP-2 + BMP-7] treated chondrocytes. In the presence of FGF-2, *sox9* knockdown initially produced a similar increase in *col1a1* and *col1a2* expression but this disappeared with the essentially complete (97%) inhibition by FGF-2 by the end of culture. To our knowledge, this is first study to demonstrate that *sox9* represses *col1a1* and *col1a2* expression in adult articular chondrocytes.

Taken together, these findings indicate that *sox9* plays opposite roles in the regulation of type I and type II collagen gene expression. Sox9 appears to stimulate *col2a1* expression and to suppress *col1a1* and *col1a2* expression. Thus, these data suggest that *sox9* supports the chondrocyte phenotype in these cells. The specific pathways responsible for the differential *sox9* regulation of different collagen genes remain to be elucidated.

The distribution of new GAG and collagen between cell layer (cell-associated matrix molecules) and medium (released matrix molecules) is of considerable importance to articular cartilage repair. GAG and collagen that is retained with the cells contribute to the formation of new cartilage. When lost into the medium, they do not provide structural benefit. The production and retention of these matrix molecules may reflect chondrocyte anabolic activities, while release of the matrix molecules may reflect chondrocyte catabolic activities [Sah et al., 1994; Im et al., 2007; Shi et al., 2013].

The findings that *sox9* knockdown reduced the marked stimulation by [IGF-I + BMP-2 + BMP-7] of total GAG production and of the GAG distribution, but did not affect released GAG, suggest that *sox9* increases the GAG distribution ratio by increasing cell-associated GAG rather than by decreasing released GAG. Although FGF-2 modestly increased total GAG production, it decreased the GAG distribution ratio. Interestingly, *sox9* knockdown did not alter this ratio, even though FGF-2 increased *sox9* expression and *sox9* knockdown reduced this FGF-2 stimulation by as much as 61%. Taken together, these data suggest that *sox9* mediates, at least in part, the regulation of GAG distribution by [IGF-I + BMP-2 + BMP-7], but that *sox9* is not involved in the regulation of GAG distribution by FGF-2.

Treatment with [IGF-I + BMP-2 + BMP-7] increased both total collagen and the collagen distribution ratio. Sox9 knockdown had little effect on total collagen production, but decreased the collagen

distribution ratio 42%. This effect reflects both a further increase in released collagen (2.69- to 3.22-fold), and a repression of the [IGF-I + BMP-2 + BMP-7] stimulation of cell-associated collagen (10.31- to 7.13-fold) by *sox9* knockdown. Taken together, these data suggest that *sox9* plays an important role in directing the newly synthesized collagen to the cell layer. These actions were similar in both direction and magnitude to those on GAG.

In contrast to [IGF-I + BMP-2 + BMP-7], FGF-2 markedly inhibited total collagen production and decreased the collagen distribution ratio. Sox9 knockdown had little effect on this FGF-2 regulation of total collagen production or collagen distribution. Coupled with the finding that FGF-2 stimulated *sox9* expression and *sox9* protein production, and that *sox9* knockdown reduced this stimulation, these results suggest that the marked reduction in collagen production and collagen ratio by FGF-2 are not mediated by *sox9*.

Few studies have investigated a potential role for *sox9* role in the proliferation of adult articular chondrocytes. Although FGF-2 and [IGF-I + BMP-2 + BMP-7] each increased both chondrocyte proliferation and *sox9* expression, *sox9* knockdown reduced the proliferative effect of FGF-2, but did not influence that of [IGF-I + BMP-2 + BMP-7]. These results suggest that the mechanism of FGF-2 stimulation of chondrocyte proliferation involves *sox9*, while this effect of [IGF-I + BMP-2 + BMP-7] is mediated by other pathways.

Taken together, these data suggest that the role of *sox9* differs for the anabolic and the mitogenic functions regulated by these growth factors. Specifically, *sox9* appears to participate in the regulation of matrix production by [IGF-I + BMP-2 + BMP-7], but not by FGF-2, and in the mitogenic activity of FGF-2, but not of [IGF-I + BMP-2 + BMP-7].

Although the importance of *sox9* in mesenchymal cells [Wright et al., 1995] and embryonic chondrocytes in endochondral skeletal development [Lefebvre and Smits, 2005] and chondrocyte-derived dedifferentiated cells [Shakibaei et al., 2006], is well-established, its role in adult articular chondrocytes is controversial. In adult human and osteoarthritic articular chondrocytes, *SOX9* expression did not correlate with *COL2a1* [Aigner et al., 2003; Yagi et al., 2005] or with *ACAN* expression [Yagi et al., 2005]. In adult mouse articular cartilage, *sox9* protein was only faintly detectable in comparison to growth plate chondrocytes [Davies et al., 2002]. On the other hand, *SOX9* overexpression has been shown to restore extracellular matrix production in primary human osteoarthritic chondrocytes [Cucchiari et al., 2007] and to promote the repair of articular cartilage defects in an in vivo rabbit knee cartilage repair model [Cucchiari et al., 2013]. The present studies found a strong correlation between *sox9* expression and both *col2a1* and *acan* expression only in the chondrocytes treated with the combination of IGF-I, BMP-2, and BMP-7. The seeming inconsistency in these reports with respect to type II collagen gene expression may reflect *sox9* binding to different *col2a1* regulatory elements. In primary rabbit articular chondrocytes, low levels of *sox9* overexpression increased *col2a1* expression through its specific intronic enhancer, while high levels of *sox9* overexpression inhibited *col2a1* expression through the -266 bp promoter [Kyriiotou et al., 2003]. The specific pathways responsible for the observed growth factor actions remain to be elucidated.

A limitation of these studies is our finding that the siRNA and growth factor transgenes required different transfection reagents, and



that these failed to achieve satisfactory transfection efficiency when used in concert. We resolved this limitation by employing growth factor proteins to perform the *sox9* knockdown studies. We found that the growth factor transgenes and growth factor proteins generated similar effects on *sox9* regulation and chondrocyte function.

Both FGF-2 and [IGF-I + BMP-2 + BMP-7] increased cell proliferation. For this reason, the observed changes in matrix production may, in part, reflect changes in the number of cells. Because the changes in cell proliferation were similar for both treatments, this effect on the comparisons is likely to be small. An additional limitation is that, as with any knockdown study employing siRNA, blockade of target transcripts is incomplete. The siRNA data is likely to underestimate the result that would be obtained by eliminating *sox9* expression.

Growth factors regulate articular chondrocyte functions through complex signaling networks and, subsequently, by transcription factors. While many of the signaling pathways employed by the tested growth factors have been well-described [Chen et al., 2004; Werner et al., 2008; Ellman et al., 2013], the role of the central chondrogenic transcription factor, *sox9*, in their mechanism of action is less well investigated. The present data suggest that *sox9* mediates, at least in part, the action of certain growth factors, but not of others. The data further indicate that the role of *sox9* also differs with respect to different chondrocyte functions regulated by these growth factors, including pro-anabolic, anti-catabolic, and mitogenic activity. Despite these differences, *sox9* consistently supported the chondrocyte phenotype. Interventions that target *sox9* in the mechanisms of action of these growth factors may help improve the results of articular cartilage repair.

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