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Fibroblast growth factor 2 enhances the kinetics of mesenchymal stem cell chondrogenesis

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ABSTRACT

Treatment of mesenchymal stem cells (MSCs) with fibroblast growth factor 2 (FGF-2) during monolayer expansion leads to increased expression of cartilage-related molecules during subsequent pellet chondrogenesis. This may be due to faster differentiation and/or a durable change in phenotype. In order to evaluate changes over time, we assessed chondrogenesis of human MSCs at early and late time points during pellet culture using real-time PCR, measurement of glycosaminoglycan accumulation, and histology. Marked enhancement of chondrogenesis was seen early compared to controls. However, the differences from controls in gene expression dramatically diminished over time. Depending on conditions, increases in glycosaminoglycan accumulation were maintained. These results suggest that FGF-2 can enhance the kinetics of MSC chondrogenesis, leading to early differentiation, possibly by a priming mechanism.

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1. Introduction

Bone marrow-derived mesenchymal stem cells (MSCs) have the potential to differentiate into multiple cell types. Treatment with transforming growth factor beta (TGF-β) superfamily members under three-dimensional conditions (such as pellet culture) stimulates MSCs to form cartilaginous tissues [1]. The ability of MSCs to undergo chondrogenesis is variable, and is affected by multiple factors. For example, differentiation depends on the duration of *in vitro* cellular expansion. Early passage cells are able to undergo chondrogenesis, however late passage MSCs undergo senescence and lose the ability to form cartilage [2]. In addition, the culture conditions used to proliferate MSCs, including the characteristics of the fetal calf serum used [3], strongly affect subsequent chondrogenesis.

Treatment of MSCs with fibroblast growth factor 2 (FGF-2) during monolayer expansion enhances their ability to undergo chondrogenesis. Such cells are able to differentiate into cartilage after more passages in monolayer than control cells [2,4,5]. FGF-2 exposure during expansion also stimulates subsequent MSC chondrogenesis, leading to (1) improved histologic appearance, with increased toluidine blue staining and numbers of chondrocytes in lacunae [2,5,6], (2) increased glycosaminoglycan (GAG) accumulation [2,5,7–9], and (3) increased transcription of cartilage-associated genes, including aggrecan, Sox9, and Col II [5,7,9–11].

FGF-2 may influence at least two aspects of MSC chondrogenesis. First, the growth factor may speed the sequential, time-dependent pattern of gene expression that occurs during cartilaginous differentiation [12,13], leading to earlier production of cartilage-associated molecules. Secondly, FGF-2 treatment may lead to an altered phenotypic state that more closely resembles native cartilage. Current methods for MSC chondrogenesis lead to tissues that do not match the biochemical and biomechanical properties of native cartilage [7,14,15]. Bioactive molecules can influence both kinetics and phenotype during differentiation. For instance, as indicated, TGF-β strongly influences MSC phenotype, leading to chondrogenesis rather than other programs of differentiation. Growth factors can also affect the kinetics of differentiation. For example, bone morphogenetic protein-6 (BMP-6) accelerates the differentiation of limb-bud mesenchymal cells into hypertrophic chondrocytes *in vitro* [16]. Growth differentiation factor 7 (GDF-7) deficiency in mice leads to a shorter hypertrophic phase duration compared to controls during cartilaginous maturation in the growth plate [17]. In contrast, GDF-5 deficiency leads to a longer hypertrophic phase duration [18].

Abbreviations: ACAN, aggrecan; B2M, β2-microglobulin; Col I, type I collagen; Col II, type II collagen; Col X, type X collagen; DMMB, dimethyl-methylene blue; FGF-2, fibroblast growth factor 2; GAG, glycosaminoglycan; GDF, growth differentiation factor; MSC, mesenchymal stem cell; qPCR, quantitative polymerase chain reaction; SOX9, SRY (sex determining region Y)-box 9; TGF-β, transforming growth factor beta.

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It is unclear whether FGF-2 affects MSC phenotype, the kinetics of MSC differentiation, or both factors. Most previous studies have evaluated differentiation at single time points without addressing temporal changes. The studies that have assessed multiple time points have shown large differences in gene expression and GAG content early, but have suggested that differences may diminish later [8,9,11]. In this study, we sought to determine the effects of FGF-2 treatment on the temporal pattern of MSC chondrogenesis by evaluating early (2 weeks in pellet culture) and late (4 weeks in pellet culture) differentiation. The second time point is later than prior reports, and is also when the biochemical and mechanical properties of MSC-based tissue constructs plateau [15]. MSC chondrogenesis is often evaluated after 3 weeks of pellet culture [1]. We hypothesized that the stimulatory effects of FGF-2 would persist over time, reflecting an improvement in the ultimate phenotype of the cells. We evaluated two FGF-2 treatment regimens, assessing chondrogenesis with quantitative PCR (qPCR), quantification of glycosaminoglycan content, and histology.

2. Materials and methods

2.1. Unless specified, all reagents were obtained from Sigma–Aldrich (St. Louis, MO)

2.1.1. Cell culture

Bone marrow-derived human mesenchymal stem cells (hMSCs; Lonza, Walkersville, MD) were cultured in monolayer with an initial plating density of 5000 cells/cm^2 using mesenchymal stem cell basal medium (MSCBM, Lonza) with supplements (SingleQuot Kit, Lonza). Two FGF-2 treatment regimens were evaluated. First, hMSCs were grown for three passages (six population doublings) in monolayer prior to pellet culture. Cells were treated with 0 ng/mL, 1 ng/mL, or 10 ng/mL human FGF-2 (R&D Systems, Minneapolis, MN) during the second and third passages. In the second regimen, hMSCs were grown for four passages (eight population doublings) in monolayer prior to pelleting. Cells were treated with 0 ng/mL, 1 ng/mL or 10 ng/mL human FGF-2 during the fourth passage.

2.1.2. Pellet chondrogenesis

Upon reaching 80–90% confluence, MSCs were released with trypsin and resuspended in chondrogenesis media [Dulbecco's Modified Eagle Medium with high glucose (Hyclone, Logan, UT) containing ITS+ Premix (BD Falcon, Franklin Lakes, NJ), 40 μg/mL L-proline, 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA), 50 μg/mL ascorbic acid (Stem Cell Technologies, Tukwila, WA), antibiotics (Hyclone), and 10 mM HEPES (Invitrogen)]. Five hundred thousand cells were pelleted in each well of a V-bottom 96 well polypropylene plate. After incubation for 24 h, the resultant cell pellets were transferred to 48 well plates. Pellets were cultured with chondrogenesis media (see above) supplemented with 100 nM dexamethasone (Stem Cell Technologies) and 10 ng/mL TGF-β1 (R&D Systems) for two or four weeks. Media was changed twice a week.

2.1.3. Quantitative PCR

Pellets for gene expression analysis were processed using the Cells-to-Ct kit (Applied Biosystems, Foster City, CA). Samples were lysed, RNA was extracted, and reverse transcription was performed to generate cDNA according to the manufacturer's protocol. Individual qPCR reactions were then performed using TaqMan gene expression assays (Applied Biosystems) for Collagen I (Col I; Hs01076777_m1), Collagen II (Col II; Hs01060345_m1), Collagen X (Col X; Hs00166657_m1), aggrecan (ACAN; Hs00153936_m1),

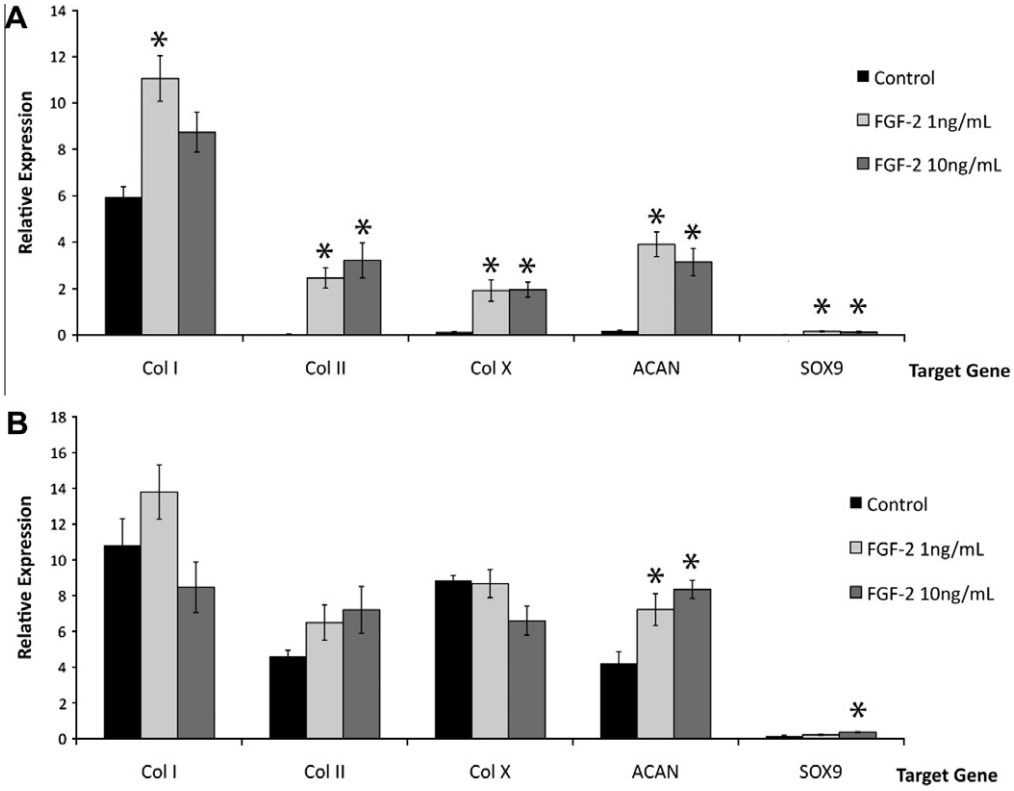

Fig. 1. Gene expression of pellets from third passage hMSCs. (A) qPCR of cartilage-associated genes after 2 weeks of pellet culture. (B) qPCR of cartilage-associated genes after 4 weeks of pellet culture. Gene expression relative to β2-microglobulin is shown ± SD. Asterisks indicate conditions with significantly greater levels of expression ($p < 0.05$) than controls.

and SOX9 (Hs00165814_m1). β2-microglobulin (B2M; Hs99999907_m1) was used as the reference gene. The amplification program consisted of an initial activation step (10 min at 95 °C) followed by 40 cycles of 95 °C for 15 s to denature the template, and an annealing and extension step at 60 °C for 1 min. The threshold cycle (CT) value for each gene was measured and expression levels relative to B2M were calculated (ΔCT Method).

2.1.4. Glycosaminoglycan (GAG) and DNA content

Samples for biochemical analysis were digested at 60 °C overnight with papain (125 mg/mL in 5 mM L-cysteine, 100 mM Na_2HPO_4, and 5 mM EDTA), and then clarified with centrifugation. Aliquots of the supernatant were assayed separately for GAG and DNA content. Sulfated GAG content was estimated using the dimethyl-methylene blue (DMMB) binding assay. The standard curve for the analysis was generated using a chondroitin-4-sulfate standard. Two hundred and fifty microliters of DMMB reagent (16 mg of dye in 1 L of water containing 3.04 g glycine, 2.37 g NaCl, and 95 mL 0.1 M HCl) was added to 40 μL of papain digest and the absorbance at 525 nm was immediately measured. DNA content was determined using the Quant-iT PicoGreen dsDNA Reagent (Invitrogen) according to the manufacture's protocol.

2.1.5. Histology

Samples for histology were fixed in Z-fix (Anatech, Battle Creek, MI) for 24 h, dehydrated with ethanol, washed with xylene, and embedded in paraffin. Thin sections (7 μm thick) were cut from the paraffin blocks, deparaffinized, stained with safranin-O and Fast Green, and examined under light microscopy.

2.1.6. Immunohistochemistry for type I collagen

Immunohistochemistry was performed as previously described [15] with a mouse monoclonal antibody against type I collagen (MAB3391, Millipore, Billerica, MA). Briefly, antigen retrieval was performed on deparaffinized sections by heating to 99 °C for 25 min in citrate buffer (10 mM citric acid, pH 6.0 with 0.05% Tween 20), cooling to room temperature for 20 min, and then incubating with 300 μg/mL hyaluronidase in PBS for 1 h at 37 °C. Samples were incubated with primary antibody, which was detected with the MACH 4 Universal HRP Polymer Detection kit (Biocare Medical, Concord, CA).

2.1.7. Statistical analysis

We used *t*-tests to determine if there were significant differences between results at 2 and 4 weeks within each treatment group. To see if results for any of the three treatments (control, 1 ng/mL FGF-2, or 10 ng/mL FGF-2) were significantly different at each time point, we used the Tukey–Kramer honestly significant difference test for multiple comparisons. A *p*-value of less than 0.05 was taken to be statistically significant for each test. Data analysis was performed with JMP 9.0.0 (SAS Institute Inc., Cary, NC).

3. Results

3.1. Chondrogenesis of third passage hMSCs: gene expression

The commercially-obtained hMSCs used in the current study are commonly expanded for three to six passages *in vitro* prior to experimental manipulation [19–21]. We therefore cultured hMSCs in monolayer for three passages (six population doublings), with FGF-2 added (1 ng/mL or 10 ng/mL) during the second and third passages prior to pellet chondrogenesis. Gene expression was assessed with qPCR after 2 and 4 weeks of pellet culture. At 2 weeks, pellets from cells grown with 1 ng/mL of FGF-2 had significantly ($p < 0.05$) higher levels of Col I (2-fold), Col II (70-fold), Col X (15-fold), ACAN (23-fold) and SOX9 (14-fold) gene expression compared to controls expanded without FGF-2. Pellets from cells grown with 10 ng/mL of FGF-2 had significantly higher levels of Col II (92-fold), Col X (15-fold), ACAN (18-fold), and SOX9 (10-fold) gene expression compared to controls (Fig. 1A).

Compared to pellets harvested after 2 weeks, 4 week pellets had significant ($p < 0.05$) increases in gene expression for all genes within each treatment group except for (1) Col I for the 1 ng/mL FGF-2 and the 10 ng/mL FGF-2 groups and (2) SOX9 for the 1 ng/mL FGF-2 group. However, the differences in gene expression between pellets from MSCs grown with and without FGF-2 were much less pronounced at 4 weeks than at 2 weeks. Compared to controls, a significant increase in expression of only ACAN (2-fold) was found with 1 ng/mL FGF-2. Only ACAN (2-fold) and Sox9 (2-fold) were more highly expressed in pellets from cells treated with 10 ng/mL of FGF-2. The expression levels of other chondrogenic markers were similar in all three groups after 4 weeks of pellet culture (Fig. 1B).

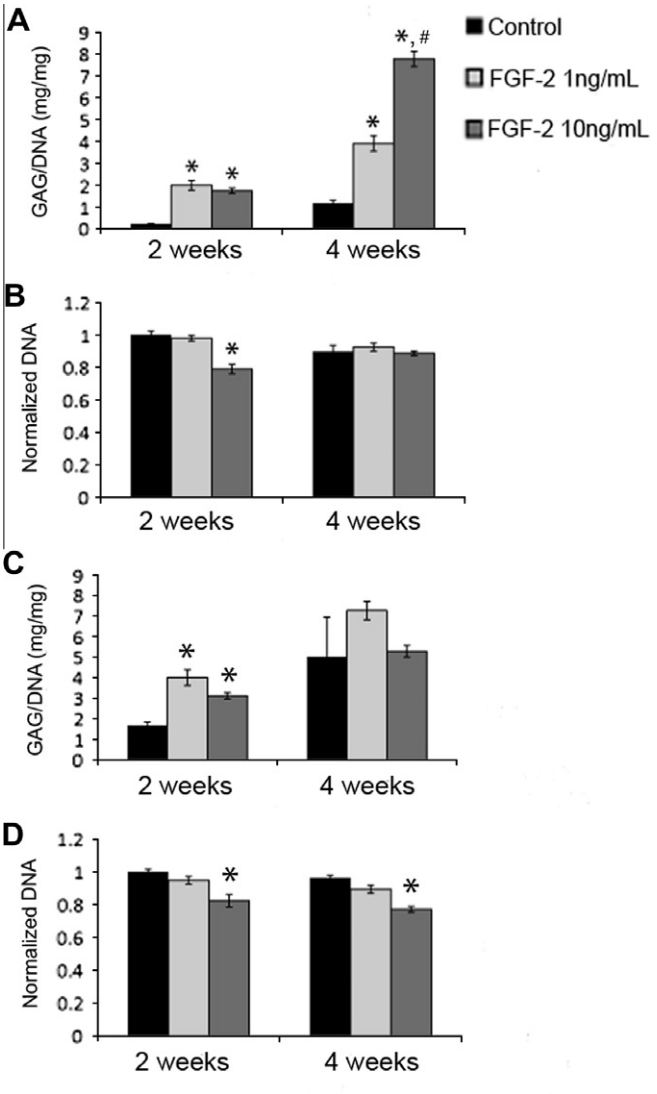

Fig. 2. GAG and DNA content of hMSC pellets. (A) GAG content of pellets from third passage hMSCs normalized to DNA content. (B) DNA content of pellets from third passage hMSCs normalized to DNA content of 2 week control pellets. (C) GAG content of pellets from fourth passage hMSCs normalized to DNA content. (D). DNA content of pellets from fourth passage hMSCs normalized to DNA content of 2 week control pellets. Means ± SEM are shown. Asterisks indicate significant differences ($p < 0.05$) from controls. Hash mark indicates significant difference from 1 ng/mL FGF-2 treatment group.

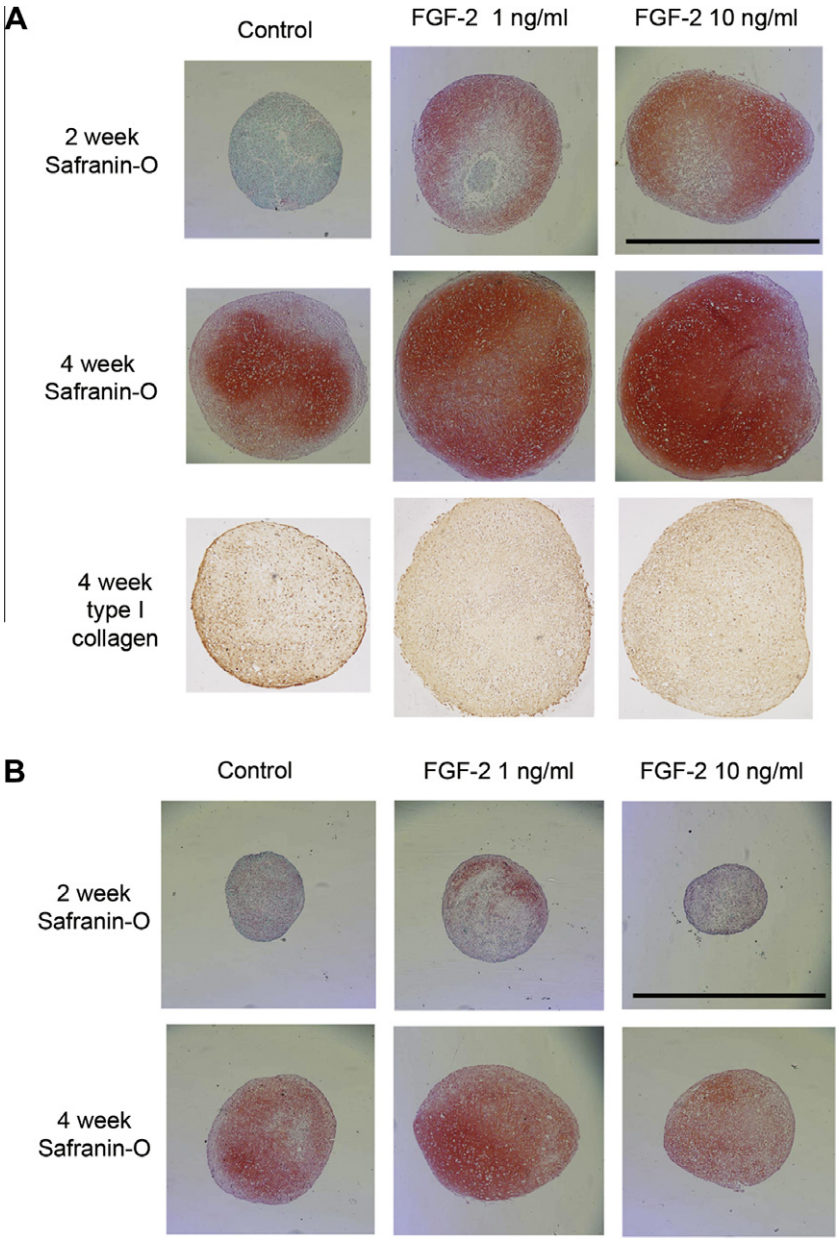

Fig. 3. Histological appearance of hMSC pellets. (A) Histology of pellets from third passage hMSCs. Third row shows immunohistochemistry for type I collagen. (B) Histology of pellets from fourth passage hMSCs. Time in pellet culture and staining are indicated. 1 mm scale bar for all images is shown.

3.1.1. Biochemical analysis

Sulfated proteoglycans are a major component of the extracellular matrix of cartilage. To quantify the degree of chondrogenesis achieved by the MSC pellets, the amounts of sulfated GAGs normalized to cellular (DNA) content at 2 and 4 weeks were determined. At 2 weeks, treatment with 1 ng/mL FGF-2 led to an 8.6-fold increase in GAG production, and treatment with 10 ng/mL FGF-2 led to a 7.5-fold increase ($p < 0.05$) compared to controls. At 4 weeks, significant increases of 3.3- and 6.6-fold were observed with 1 ng/mL and 10 ng/mL of FGF-2, respectively (Fig. 2A). The DNA content of the pellets was similar among all treatment groups at both time points (Fig. 2B).

3.1.2. Histology

Safranin-O staining for sulfated GAGs gave results that were consistent with both the qPCR analysis for ACAN and the biochemical analysis of GAG content. At 2 weeks, both FGF-2 groups showed more staining than controls, indicating higher GAG content. After 4 weeks of chondrogenesis, control pellets showed increased levels of staining compared to controls at 2 weeks, however, pellets produced from FGF-2 treated MSCs still exhibited more intense safranin-O staining than controls (Fig. 3A).

3.1.3. Immunohistochemistry for type I collagen

Previous studies have indicated that continuous treatment of MSCs with FGF-2 prior to pellet chondrogenesis leads to decreased production of type I collagen as assessed by immunofluorescence [2,8]. Since we found increased or similar levels of Col I transcripts from FGF-2 pellets compared to controls, we evaluated the protein levels of type I collagen with immunohistochemistry. Consistent with our qPCR results, immunostaining for type I collagen among the 3 groups was similar throughout most of the pellets at 4 weeks. However, the periphery of the control pellets was more intensely stained than the periphery of the FGF-2 treated pellets (Fig. 3A).

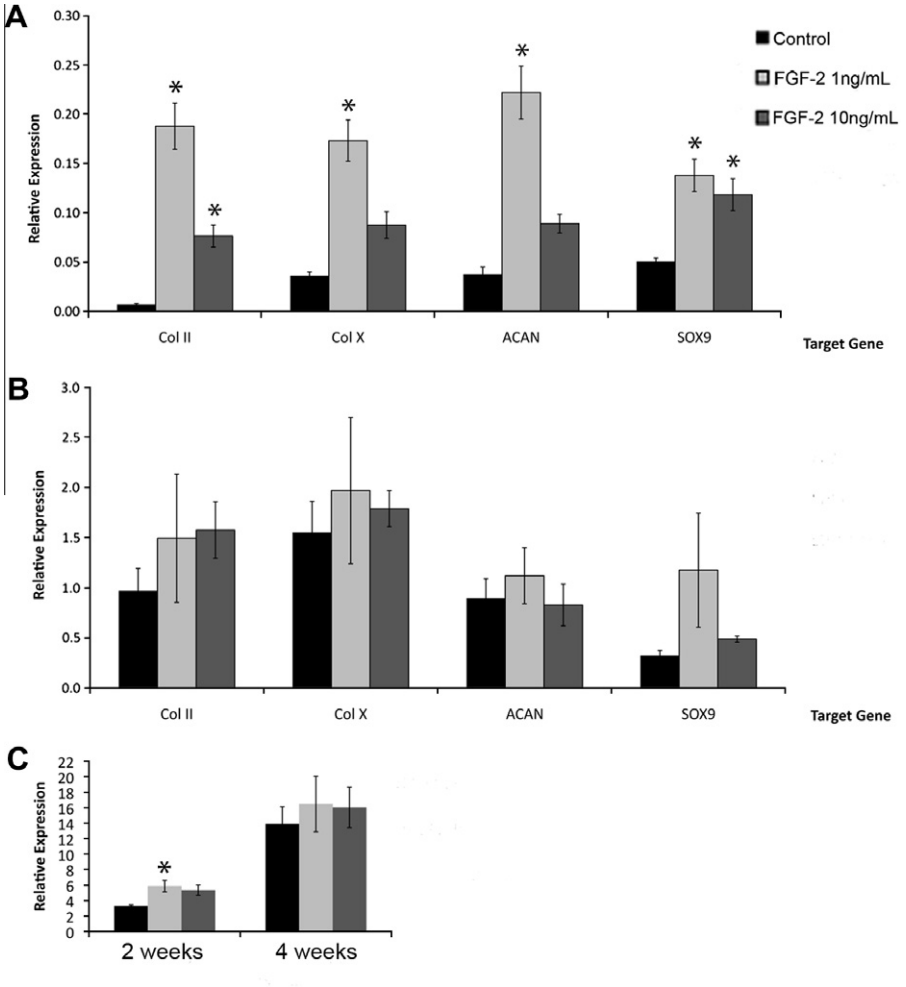

Fig. 4. Gene expression of pellets from fourth passage hMSCs. (A) qPCR of cartilage-associated genes after 2 weeks of pellet culture. (B) qPCR of cartilage-associated genes after 4 weeks of pellet culture. (C) qPCR of type I collagen. Gene expression relative to β2-microglobulin is shown ± SD. Asterisks indicate conditions with significantly greater levels of expression ($p < 0.05$) than controls.

3.2. Chondrogenesis of fourth-passage hMSCs

We next evaluated the effects of limited FGF-2 exposure on later passage cells in order to evaluate the robustness of FGF-2 effects. We expanded hMSCs for four passages (eight population doublings) prior to pelleting, with FGF-2 treatment (1 ng/mL or 10 ng/mL) during the final monolayer passage. FGF-2 had qualitatively similar effects on fourth passage cells as on third passage cells, with strongly enhanced chondrogenesis early, but more modest effects late. Quantitatively, chondrogenesis of fourth passage hMSCs was reduced compared to third passage cells, with lower levels of chondrogenic gene expression and GAG accumulation.

3.2.1. Gene expression

At 2 weeks, treatment with 1 ng/mL FGF-2 led to increased expression of Col I (2-fold), Col II (29-fold), Col X (5-fold), ACAN (6-fold), and SOX9 (3-fold) compared to control pellets ($p < 0.05$). Treatment with 10 ng/mL FGF-2 resulted in significant increases in Col II (12-fold) and SOX9 (2-fold) expression compared to controls (Fig. 4A and C). At 4 weeks, there was no significant difference in expression of any of the marker genes among the three groups (Fig. 4B and C), although SOX9 was most highly expressed in the 1 ng/mL FGF-2 group.

3.2.2. Biochemical analysis

After two weeks of chondrogenesis, biochemical quantification of GAGs showed significant increases of 2.4- and 1.9-fold with FGF-2 treatment at 1 ng/mL and 10 ng/mL, respectively, compared to control pellets. No statistically significant difference was found among the groups at 4 weeks (Fig. 2C and D), although there was a trend toward higher amounts of GAG in pellets from cells treated with 1 ng/mL FGF-2. The DNA content of the pellets was similar among all treatment groups at both time points.

3.2.3. Histology

Pellets stained with safranin-O at 2 weeks showed limited staining, with some peripheral staining observed for the 1 ng/mL FGF-2 group. At 4 weeks, all pellets showed more intense staining for proteoglycans, most noticeably the 1 ng/mL FGF-2 group. However, the differences between the controls and FGF-2 treated groups were not dramatic (Fig. 3B).

4. Discussion

The goal of this study was to evaluate the time-dependent effects of FGF-2 pretreatment on MSC chondrogenesis, especially late effects. We hypothesized that MSC phenotype would be durably improved, and that significant improvements in chondrogenesis would be maintained after 4 weeks of pellet culture. Instead, the differences between controls and FGF-2 treated groups decreased dramatically from 2 to 4 weeks. In the case of third passage hMSCs, fewer genes exhibited significant differences in expression at the later time, and these differences were also smaller in magnitude.

There were also smaller relative differences in GAG content, although significant differences from controls persisted. For the fourth passage cells, no statistically significant differences were found between experimental pellets and controls in either gene expression or GAG accumulation at 4 weeks. The histological appearances of pellets were also similar among all three treatment groups. We therefore propose that FGF-2 can enhance the kinetics of MSC chondrogenesis, with the exact response to the growth factor depending on the specific treatment conditions.

The early induction of chondrogenesis of fourth passage MSCs by brief FGF-2 exposure is consistent with prior studies of limited growth factor treatment. As little as one day of FGF-2 administration enhances repair of full-thickness articular cartilage injuries in rabbits [22]. Continuous treatment with FGF-2 for one, four, or seven passages in monolayer enhanced MSC chondrogenesis, although the cells grown until passage 7 showed variable differentiation [2]. However, continuous FGF-2 treatment is not required for enhancement of chondrogenesis. Exposure of MSCs to FGF-2 after the second passage in monolayer leads to increased transcription of cartilage-associated molecules during pellet culture [11,23].

In addition to demonstrating an enhancement of differentiation kinetics, these results also shed light on FGF-2's mechanism of action. Two mechanisms have been proposed for the enhancement of MSC chondrogenesis by FGF-2. First, FGF-2 may select for MSCs with inherent chondrogenic potential during monolayer culture (selection mechanism) [4]. Secondly, FGF-2 may generally enhance the chondrogenic potential of MSCs (priming mechanism) [9,24], in part by increasing Sox9 protein levels. Our results support a priming mechanism. For a selection mechanism, pellets derived from FGF-2-treated cells would maintain enhanced chondrogenesis over controls at all times, since the number of cells undergoing chondrogenesis would always be greater in the former case. Chondrogenesis of our control pellets improved from 2 to 4 weeks, eventually approaching the level of cells expanded with growth factor in the case of fourth passage cells. Therefore, under the conditions studied, we believe that FGF-2 primes MSCs to undergo chondrogenesis more quickly by inducing a differentiation-competent state. Although this study supports priming, it does not exclude selection. In contrast to the late FGF-2 treatment in our experiments, FGF-2 may act to select for the survival of mesenchymal chondroprogenitors with long telomeres extremely early during *in vitro* culture, even during the first monolayer passage [4].

Priming may require the switch from a state of high FGF-2 signaling during monolayer proliferation to a state of low FGF-2 signaling during pellet differentiation. While FGF-2 treatment during MSC expansion stimulates subsequent chondrogenesis, FGF-2 treatment during pellet culture inhibits differentiation, with a decrease in proteoglycan production as well as decreased Col II transcription [25]. FGF-2 treatment enhances proliferation and decreases expression of specialized extracellular matrix molecules during monolayer expansion of MSCs [8]. We therefore hypothesize that FGF-2 during expansion supports a proliferative state, whereas the subsequent removal of the factor contributes to the exit from the cell cycle and entry into a differentiation-competent state. Withdrawal of FGF-2 stimulates differentiation in other tissues, including muscle [26] and neuronal [27] cells.

In conclusion, our results indicate that FGF-2 treatment increases the kinetics of MSC chondrogenesis, possibly by priming cells to undergo differentiation. The resultant early differentiation can lead to enhanced extracellular matrix accumulation (as in the third passage MSCs). These kinetic effects can occur even with brief growth factor exposure of late passage MSCs (as in the fourth passage cells). Our results stress the importance of evaluating stem cell differentiation over time, and support a role for bioactive factors in speeding differentiation.

Acknowledgments

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