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Bone Morphogenetic Protein-7 Regulates Differentially the mRNA Expression of Bone Morphogenetic Proteins and Their Receptors in Rat Achilles and Patellar Tendon Cell Cultures

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Abstract Previous animal studies have suggested that certain bone morphogenetic proteins (BMPs) may be useful therapeutically in treating tendon healing. To better understand the relationship among the different BMPs in the healing process, we initiated the present study to examine the effects of a member of the BMP family, BMP-7 (also called Osteogenic Protein-1) on the temporal and spatial expression patterns of other BMPs and the BMP receptors in cell cultures of adult rat Achilles and Patellar tendons. Cultures from both tendon types expressed detectable but variable levels of biochemical markers characteristics of tendons. RNAs coding for type II collagen and transcription factors *Six1*, *Scleraxis*, and *Tendin* were detected in both types of cultures. Distinct patterns of expression of several BMP members and their receptors were observed in these cultured cells and BMP-7 exerted differential effects on their expression. The findings may have implications in the treatment of different tendon injuries with BMPs. J. Cell. Biochem. 104: 2107–2122, 2008.

Key words: bone morphogenetic proteins; BMP-7; osteogenic protein-1; BMP receptors; achilles; patellar; tendon cultures; gene expression

Tendons are composed of dense fibrous connective tissues linking muscle to bone. Recent studies have shown that the incidence of tendon injuries has increased in the US, especially in the aging population. Understanding the repair process of tendon and ligament is of paramount importance. Several studies have shown that tendons respond to treatment with several members of the bone morphogenetic proteins (BMP) family.

BMPs consist of a group of structurally related proteins and belong to the transforming growth factor-β (TGF-β) superfamily. Cartilage-derived morphogenetic proteins (CDMPs) and growth and differentiation factors (GDFs) are subfamilies of the BMPs [Ozkaynak et al., 1990; Sampath et al., 1990; Kingsley, 1994; Wozney and Rosen, 1998; Reddi, 2000]. BMPs are multifunctional proteins that are involved in numerous developmental and repair processes [Dudley et al., 1995; Luo et al., 1995; Hogan, 1996]. BMPs transduce their effects via the binding to a complex of type I and type II transmembrane serine/threonine kinase receptors [ten Dijke et al., 1994; Miyazono, 2000]. A number of type II receptors (e.g., BMPR-II and ActR-II) and type I receptors (e.g., BMPR-IA, BMPR-IB, and ActR-I) have been reported to bind BMP-7 [ten Dijke et al., 1994]. These receptors play a role in regulating BMP action.

Several BMPs have been shown to exert different effects on tendons. For example, GDF deficient mice showed altered tendon structure [Gruneberg and Lee, 1973] and function [Mikic et al., 2001], delayed Achilles tendon healing [Chhabra et al., 2003], and disrupted tail tendon formation and function [Clark et al., 2001]. GDF-5, -6, and -7 induce a tendon-like tissue

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when ectopically injected into rat muscles [Wolfman et al., 1997]. CDMP-1 (GDF-5), -2 (GDF-6), and -3 improved tendon repair in rats [Aspenberg and Forslund, 1999; Rickert et al., 2001; Forslund and Aspenberg, 2003; Forslund et al., 2003]. CDMP-3 and CDMP-2 gene transfer studies showed similar results [Helm et al., 2001; Lou et al., 2001; Jayankura et al., 2003]. BMP-2 enhanced the healing process when a tendon graft is transplanted into a bone tunnel [Rodeo et al., 1999]. BMP-2 gene transfer studies also showed enhanced tendon-bone integration in bone tunnels [Martinek et al., 2002]. BMP-7 (OP-1) induces bone and cartilage formation in vivo and is implicated in numerous other biological processes [Lyons et al., 1990; Dudley et al., 1995; Luo et al., 1995]. Animal studies have revealed that BMP-7 promotes tendon repair and healing in rats [Forslund and Aspenberg, 1998; Aspenberg and Forslund, 2000] and integration of tendon graft in anterior cruciate ligament reconstruction in sheep [Mihelic et al., 2004]. A high level of BMP-7 expression has been detected in developing flexor tendons [Marcias et al., 1997].

These studies strongly suggest that these BMPs may be useful therapeutically in treating tendon repair. However, little is known about their inter-relationship and their mechanisms of action in tendons at the molecular level. The specific purpose of the present study was to examine the effects of BMP-7 on the temporal and spatial expression patterns of BMPs and their receptors in cultured rat tendon cells. Two types of tendons (Achilles and Patellar) which have been shown to exhibit distinct mechanical, physiological, and structural properties [Benjamin and Ralphs, 2000] were selected for the study.

MATERIALS AND METHODS

Reagent

DMEM, HBSS, penicillin/streptomycin, trypsin/EDTA were purchased from Gibco/Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Gemini Bio-Products (Woodland, CA). Recombinant human BMP-7 was provided by Stryker Biotech (Hopkinton, MA) and dissolved in 47.5% ethanol/0.01% trifluoroacetic acid.

Cell Culture

Young adult male Long Evans rats were purchased from Charles River (Indianapolis, IN), handled and euthanized according to the procedures approved by the Institutional Animal Care and Use Committee of The University of Texas Health Science Center at San Antonio. Achilles and Patellar tendons (AT and PT) were surgically excised, rinsed with HBSS, cut into small pieces, and incubated in DMEM containing 10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin sulfate at 37°C in 5% CO_2. Cells began to exude from the tissues for 10–15 days and became attached to the culture dish. Media were changed every 3 days. The tendon pieces were removed and the attached cells continued to grow. At confluence, cells were trypsinized and frozen as passage 2. Cells were revived from the frozen stock and cultured in DMEM +10% FBS for experimentation.

Cell Morphology and Cell Proliferation

Morphology of the cultured cells was monitored with an Olympus CK2 inverted microscope equipped with a CCD camera. Images were captured using phase contrast with 100× magnifications. Cell proliferation was measured by a tetrazolium colorimetric assay (CellTiter96AQ Cell Proliferation Assay, Promega, Madison, WI) following the manufacturer's instruction. Briefly, cells were cultured in 96-well plates until confluent and subsequently treated with different concentrations of BMP-7 in serum-free DMEM. After 24 h, cultures were rinsed with sterile PBS and 100 μl of media containing 1% BSA and 20 μl of the 96AQ reagent were added to each well. The color developed after 4 h at 37°C was measured at 490 nm using a MRX microplate reader (Dynex Technologies, Chantilly, VA).

RNA Isolation and Northern Blot Analysis

Cells were cultured in D-100 culture dish until confluent and subsequently treated with different concentrations of BMP-7 in DMEM containing 5% FBS. Total RNA was isolated using TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's recommendation. Northern analyses with cDNA probes were conducted as described previously [Yeh et al., 2000]. All cDNA probes were labeled with ^{32}P-dATP using the Strip-EZ kit from Ambion (Austin, TX). Positive bands were detected using the PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and analyzed using the ImageQuant software. Blots

were stripped with the Strip-EZ probe degradation buffer according to the manufacturer and checked to ensure that the level of radioactivity was reduced to background prior to probing with another probe. The blots were also probed with an 18S rRNA oligonucleotide to correct for loading variations.

Ribonuclease Protection Assay (RPA)

The BMP, GDF, and BMPR mRNA levels were measured using the RiboQuant RPA kits with the Mouse Multi-Probe Template Sets from BD PharMingen (San Diego, CA). The mBMP-1 Multi-Probe Template Set detects mRNAs for BMP-1, -2, -3, -4, -5, -6, -7, -8A, and -8B with protected fragments of 148, 160, 181, 226, 253, 283, 316, 353, and 133 nucleotides in length, respectively. The mGDF-1 Multi-Probe Template Set detects mRNAs encoding GDF-1, -3, -5, -6, -8, and -9, with protected fragments of 148, 160, 181, 226, 283, and 316 nucleotides in length, respectively. The mBMPR kit detects ALK-1, ALK-2 (ActR-I), ALK-3 (BMPR-IA), ALK-4, ALK-5, ALK-6 (BMPR-IB), ALK-7, AVR-2 (ActR-II), AVR2B (ActR-IIB), and MIS2R with the protected fragments of 430, 388, 349, 313, 280, 250, 223, 199, 178, and 161 nucleotides in length, respectively. All three kits also detect mRNA for ribosomal protein L32 and GAPDH, whose mRNA levels were used for correcting sampling or technique errors. The anti-sense RNA probes were labeled with ^{32}P-UTP using the RiboQuant in vitro transcription kit (BD PharMingen). The protected fragments were analyzed on 5% polyacrylamide gels containing 8 M urea, detected using the PhosphorImager, and quantified using the ImageQuant Software (Molecular Dynamics, Sunnyvale, CA).

Statistical Analyses

Data are presented as the mean $\pm$ SEM. Statistical differences between means were determined by one-way ANOVA, followed by *post-hoc* Least Significant Difference Multiple Comparisons in the SIMSTAT3 software package (Normand Peladeau, Provalis Research, Montreal, Canada).

RESULTS

Effects of BMP-7 on Cell Morphology and Cell Proliferation

Achilles and Patellar tendon cells differed morphologically as examined under phase contrast microscope. Achilles cells were elongated and fibroblastic-like (Fig. 1A). Patellar cells were less elongated and more polygonal in

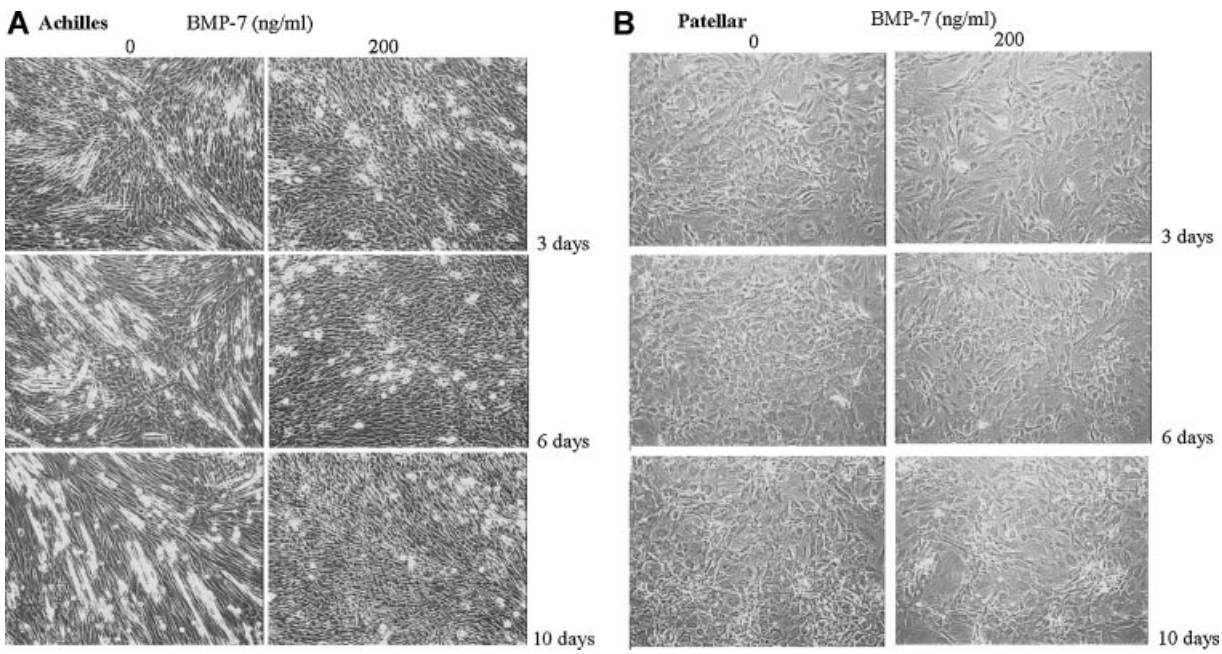

Fig. 1. Cell morphology of (**A**) Achilles and (**B**) Patellar tendon cells in culture as a function of time. Cells from tendons were treated with 0, 50, and 200 ng/ml of BMP-7 in DMEM plus 5% fetal bovine serum. Media were changed every 3–4 days. Images of the cultures were captured with a CCD camera. Representative images (phase contrast with 100× magnification) are presented.

shape (Fig. 1B). The morphology of the Achilles and the Patellar cells was not significantly changed after 10 days of treatment with 200 ng/ml of BMP-7 (Fig. 1A,B).

To determine whether BMP-7 stimulated cell proliferation, cultures of Achilles and Patellar tendons were treated with different concentrations of BMP-7 and cell proliferation was measured. Low concentrations (<100 ng/ml) of BMP-7 stimulated the proliferation of both types of tendons in a dose-dependent manner (Fig. 2A). A maximum stimulation of about 1.4-fold in Achilles at 100 ng/ml of BMP-7 and 1.3-fold in Patellar at 50 ng/ml of BMP-7 was achieved. Higher concentrations of BMP-7 did not stimulate cell proliferation further. Although the extent of stimulation at all protein concentrations appeared to be higher in Achilles than patellar, the differences were not statistically significant.

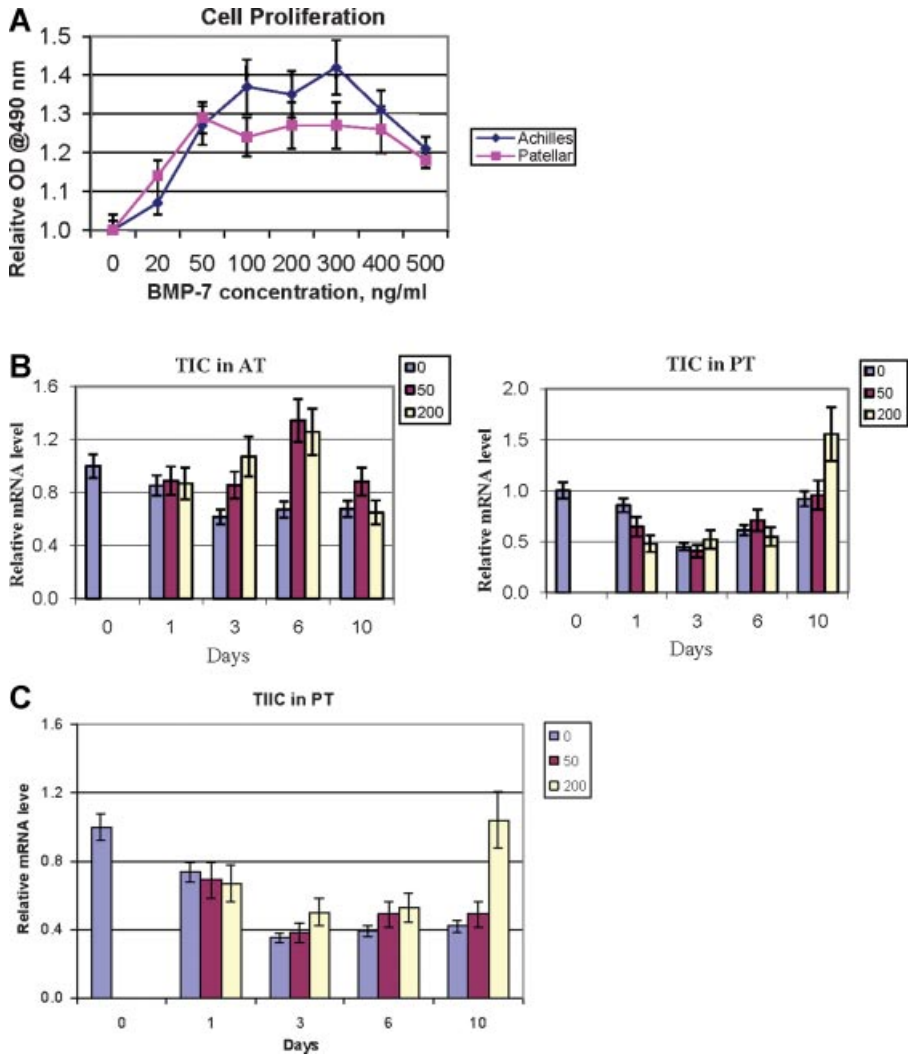

Fig. 2. **A**: Proliferation of Achilles and Patellar tendon cells treated with BMP-7. Confluent cultures were treated with different concentrations (0–500 ng/ml) of BMP-7 for 24 h in serum-free DMEM. Cell proliferation was assayed using a commercial kit as described in ''Materials and Methods'' Section. Values represent mean ± SEM of two independent determinations. **B**: Northern blot analysis of type I (TIC) expression in Achilles and Patellar tendon cells. Confluent tendon cell cultures were treated with 0, 50, and 200 ng/ml BMP-7 for 1, 3, 6, and 10 days. Total RNA was isolated on the designated day, denatured, resolved on 1% agarose gel containing formaldehyde, and transferred onto a Nytran Plus membrane. Blots were hybridized with the indicated ^{32}P-labeled probes. The blots were processed as described in ''Materials and Methods'' Section. The intensity of the hybridized RNA species was analyzed by the ImageQuant software. The mRNA level was normalized to the 18S rRNA level. The normalized mRNA level was then compared to the control value on Day 0 (the day treatment began) as 1. Values represent the mean ± SEM of two measurements using two independent RNA samples. **C**: Northern blot analysis of type II collagen (TIIC) expression in Patellar tendon cells. Conditions are identical to those described in (B). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Effects of BMP-7 on Expression of Tendon/ Ligament Biochemical Markers

The expression of selected tendon/ligament markers was examined by Northern blot analysis. Type I collagen (TIC) mRNA levels in control Achilles cultures decreased as a function of time (Fig. 2B). BMP-7 stimulated TIC expression beginning on day 3 in a protein concentration dependent manner and reached a maximum on Day 6 by about 2.5-fold ($P < 0.05$), compared to the same day control. TIC expression was low in control Patellar cultures, decreased in the early time period but increased towards the later time period, compared to Day 0 control (Fig. 2B). BMP-7 stimulated TIC expression by about 1.5-fold at 200 ng/ml on Day 10, compared to the same day control ($P < 0.05$).

A very low level of type II collagen (TIIC) mRNA was detected in control Achilles cultures and BMP-7 did not stimulate its expression (data not shown). By contrast, type II collagen mRNA was detected in control Patellar cultures and its expression levels decreased by as much as 60% ($P < 0.01$) as a function of culture time (Fig. 2C). BMP-7 did not change the expression pattern of type II collagen, compared to the same day control. However, on Day 10 and at 200 ng/ml of BMP-7, type II collagen mRNA was stimulated by almost threefold ($P < 0.005$), compared to the same day control (Fig. 2C).

In Achilles control cultures, the *Six1* mRNA expression level decreased by about 20% as a function of time and is statistically insignificant. BMP-7 treatment did not change the expression pattern (Fig. 3A). In Patellar control cultures, the *Six1* mRNA expression level did not change as a function of time, except on Day 10, at which the value was about 30% ($P < 0.05$) higher than that of the Day 0 control (Fig. 3A). BMP-7 treatment elevated the *Six1* mRNA level by about 45%, compared to the Day 0 control, but the elevation was not statistically significant, compared to the same day control.

In Achilles, the *Scleraxis* (*scx*) mRNA level decreased by as much as 30–40% compared to Day 0 control. BMP-7 treatment further decreased the *Scx* mRNA level by as much as 60% ($P < 0.01$) on Day 10 (Fig. 3B). In Patellar, the *Scx* mRNA level did not change as a function of time and BMP-7 treatment did not significantly change the expression pattern of *Scx* mRNA level.

The mRNA expression of *Tendin* in control Achilles cultures increased by 25% by Day 10 (Fig. 3C). BMP-7 treatment did not change *Tendin* expression on Days 1 and 3, compared to the same day control. BMP-7 reduced *Tendin* mRNA expression on Days 6 and 10, compared to the same day control in a dose-dependent and statistically significant ($P \leq 0.01$) manner. *Tendin* mRNA was not detected in Patellar tendons under the present experimental conditions and BMP-7 did not change its expression (data not shown).

Effects of BMP-7 on the mRNA Expression of BMPs

BMP mRNA expression in control Achilles cultures. We first established the expression pattern of BMPs in the Achilles and Patellar tendon cell cultures. Effects of exogenous BMP-7 on their expression were then examined. Figure 4A shows representative PhosphorImages showing the protected fragments for five out of the nine BMPs detectable by the commercial kit in control and treated Achilles cultures. The mRNA expression level of each BMP was quantified and normalized to that of the ribosomal protein L32. The relative levels of mRNA for the different BMPs as a function of time are shown in Figure 4B. Based on the intensity of the protected band on the gel, the relative expression level of the detectable BMPs on Day 0 can be arranged in the following order: BMP-1 > BMP-4 > BMP-7 > BMP-6 > BMP-3. The BMP-1 mRNA level did not change significantly over the 10-day culturing period. During culturing, BMP-3, -4, and -6 mRNA levels increased gradually, reaching a twofold change on Day 6, compared to Day 0 ($P < 0.05$) and persisted at the elevated level after 10 days. In contrast, the BMP-7 mRNA level increased more drastically, reaching a 3.4-fold elevation on Day 6, compared to Day 0 ($P < 0.05$) and persisted at the elevated level on Day 10.

BMP mRNA expression in treated Achilles cultures. Effects of continuous treatment with BMP-7 on the mRNA expression of BMPs as a function of culture time were also examined by RPA. These treatments were evaluated via comparison to their respective same-day controls (Fig. 4B). The BMP-1 mRNA levels were stimulated by about 2-fold (compared to the Day 0 control). The BMP-3 mRNA levels were stimulated in a time-dependent manner by 50 ng/ml of BMP-7 to about 2.5-fold ($P < 0.01$) on Day 10. However, at the higher protein concentration (200 ng/ml), the

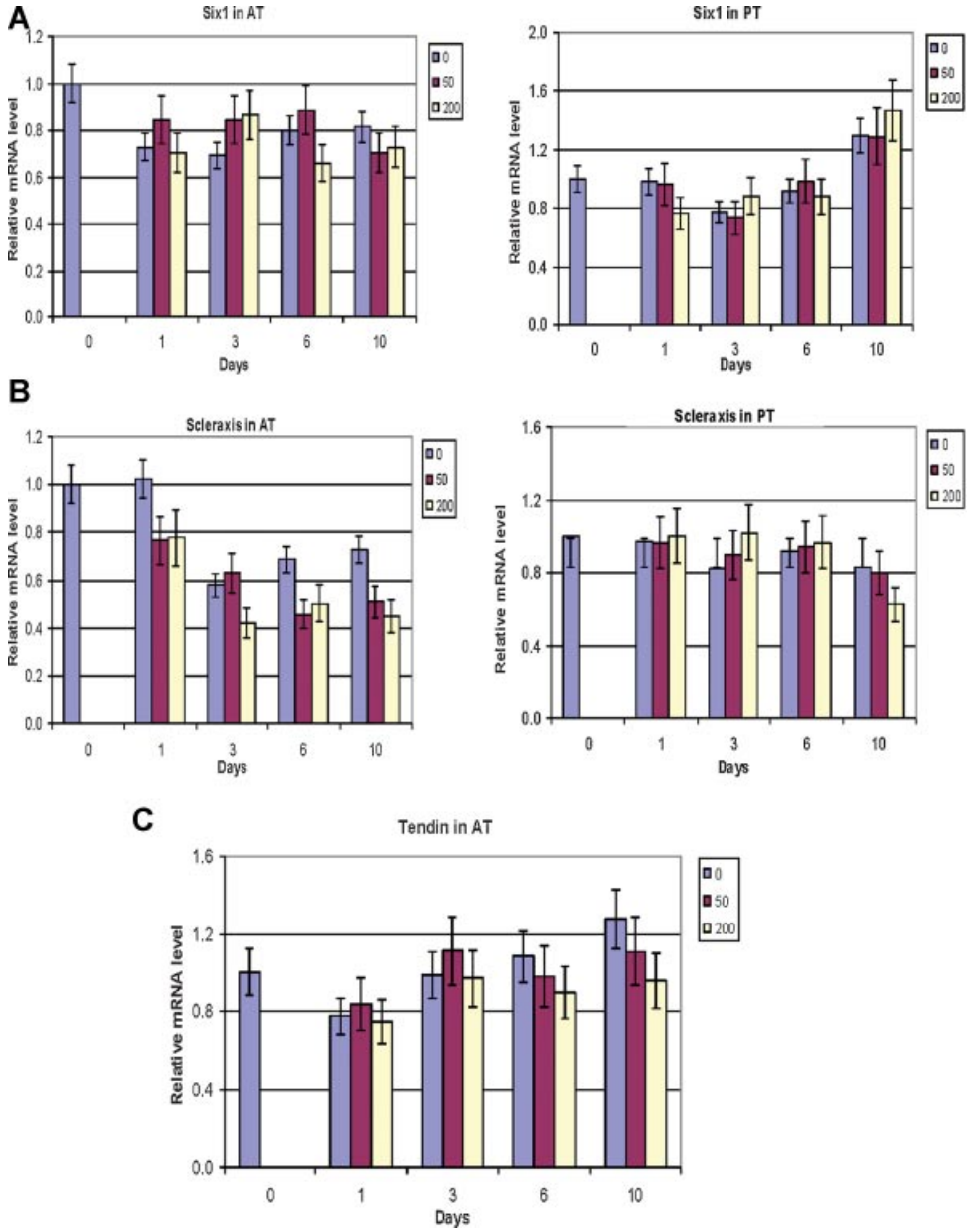

Fig. 3. Quantitative analysis of (**A**) *Six1,* (**B**) *Scleraxis,* and (**C**) *Tendin* expression in Achilles and Patellar tendon cells as determined by Northern blot analysis. Conditions are similar to those described in Figure 2B. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

extent of stimulation was <2-fold ($P < 0.05$). In contrast, the BMP-4, -6, and -7 mRNA levels were down-regulated by approximately 40–50% throughout the entire culture period.

BMP mRNA expression in control Patellar cultures. Figure 5A shows representative PhosphorImages showing the protected fragments for six out of the nine BMPs detectable by the commercial kit in control and treated Patellar cultures. The relative levels of mRNA for the different BMPs as a function of time are shown in Figure 5B. The relative expression level of the detectable BMPs on Day 0 can be arranged in the following order: BMP-1 > BMP-6 > BMP-4 > BMP-2 > BMP-3 ≅ BMP-7. The BMP-1 mRNA levels were slightly elevated over the 10-day period. The BMP-2 mRNA levels were not changed over the 10-day period. The BMP-3 mRNA did not change through the first 6 days of culture but increased by twofold ($P < 0.05$, compared to Day 0) on Day 10. The BMP-6 mRNA decreased gradually through the

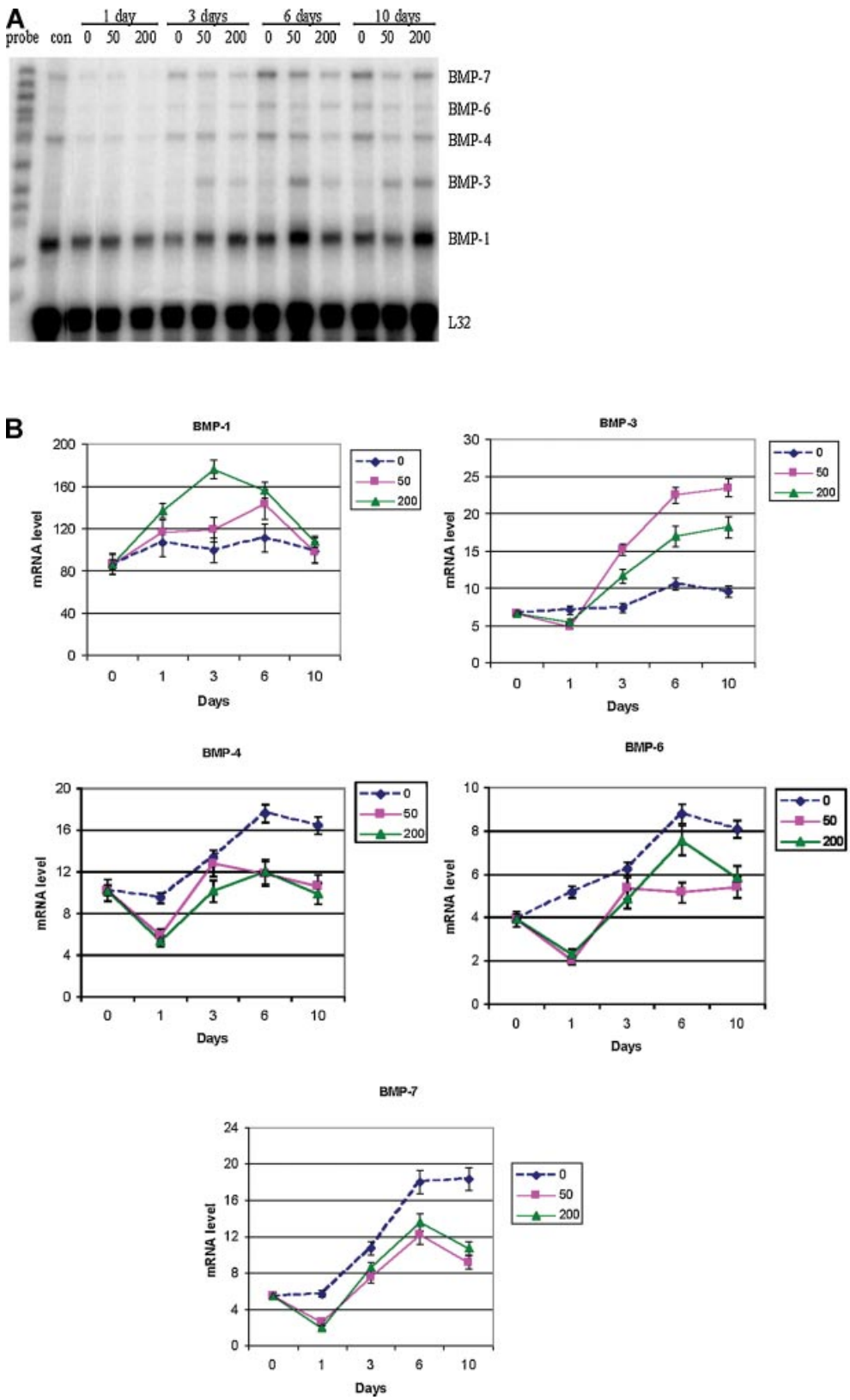

Fig. 4. **A**: RNase protection assay of BMP expression in Achilles tendons. Confluent cells in D-100 tissue culture dishes were treated with 0, 50, or 200 ng/ml BMP-7 for 1, 3, 6, and 10 days. Total RNA was isolated with TRI reagent. Ten micrograms of RNA were hybridized to ^{32}P-labeled mBMP multiple template set overnight at 56°C. The protected fragments were analyzed on a 5% polyacrylamide/8 M urea gel. The signals were detected with the PhosphoImager. **B**: Quantification of BMP expression. The intensity of the hybridized RNA species shown in (A) was analyzed by the ImageQuant software. The mRNA level was normalized to the L32 mRNA level. The normalized mRNA level was then compared to the control value on Day 0 (the day treatment began) as 1. Values represent the mean ± SEM of two measurements using two independent RNA samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

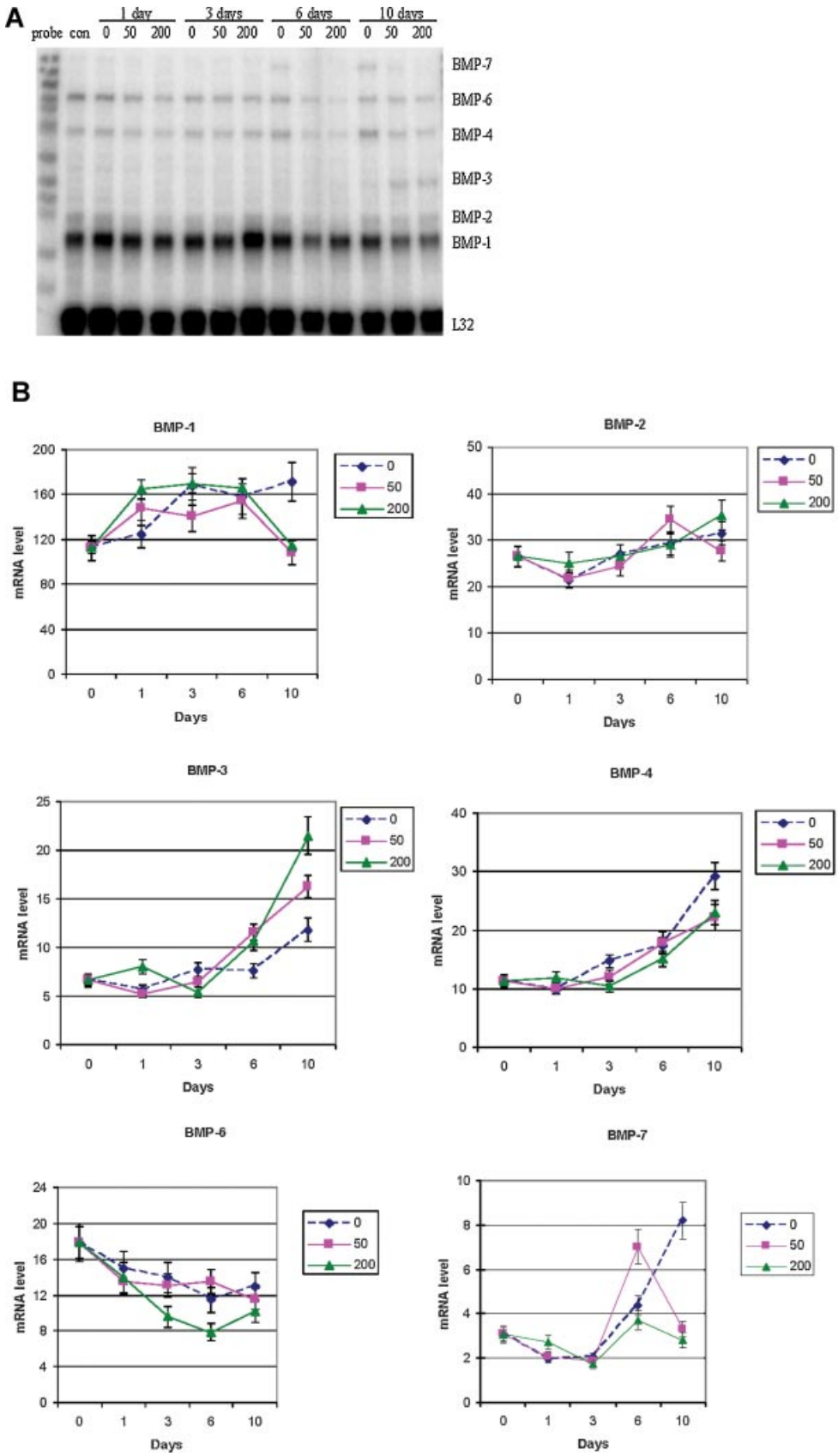

Fig. 5. **A**: RNase protection assay of BMP expression in Patellar tendons. Conditions are similar to those described in Figure 4A. **B**: Quantification of BMP expression. Similar analysis as described in Figure 4B was used. Values represent the mean ± SEM of two measurements using two independent RNA samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

first 6 days of culture but appeared to level off thereafter. The BMP-4 and -7 mRNA levels increased steadily, reaching about threefold elevation on Day 10, compared to the Day 0 control ($P < 0.01$).

BMP mRNA expression in treated Patellar cultures. In BMP-7-treated Patellar cultures, BMP-1 mRNA was not affected, except on Day 10 in which the level dropped by almost 30% (compared to the control). The BMP-2 mRNA level did not change as a function of time or protein concentration (Fig. 5B). The BMP-3 mRNA level increased in a dose-dependent manner only late during culture (Days 6 and 10). In particular, on Day 10, the BMP-3 mRNA level in cultures treated with 50 ng/ml of BMP-7 increased by about 1.5-fold (not statistically significant), but in cultures treated with 200 ng/ml of BMP-7 the level elevated by about twofold ($P < 0.01$), compared to the same day control. In the BMP-7-treated Patellar cultures, the BMP-4 mRNA levels were similar to the untreated controls. The BMP-6 mRNA levels were down-regulated. The BMP-7 mRNA levels were unchanged in the treated cultures, except on Days 6 and 10. On Day 6, the BMP-7 mRNA level in cultures treated with 50 ng/ml of BMP-7 was elevated by about twofold ($P < 0.01$) but the increase was not detected in the cultures treated with 200 ng/ml of BMP-7. On Day 10, the level appeared to be down-regulated by about 2.5-fold in cultures treated with either protein concentrations.

Effects of BMP-7 on the mRNA Expression of GDFs

GDF mRNA expression in control Achilles cultures. Effects of BMP-7 on the temporal and spatial mRNA expression of several GDFs in both tendon types were examined by RPA. Figure 6A shows representative gels revealing the expression pattern of BMPs in Achilles as a function of BMP-7 concentrations and treatment duration. Figure 6B shows the quantitative data derived from the gel data. In control Achilles cultures, only mRNAs encoding GDF-1, -5, -6, -8, and -9 were detected (Fig. 6). The relative expression level of the detectable GDFs can be arranged in the following order: GDF-1 > GDF-5 > GDF-6 ≅ GDF8 ≅ GDF-9. GDF-1 and -5 mRNA levels increased gradually over time such that a maximum increase of 1.5- and 1.9-fold, respectively ($P < 0.02$ and $P < 0.01$, compared to Day 0) was observed on Day 6. In both cases, the level dropped slightly on Day 10. Both GDF-6 and -9 mRNA levels dropped by about twofold on Day 1, ($P < 0.05$, compared to Day 0 control), but returned to the Day 0 level subsequently and remained at that level until Day 10. The GDF-9 mRNA level also dropped on Day 1 by about 2.5-fold ($P < 0.01$, compared to Day 0) and returned to the Day 0 level subsequently.

GDF mRNA expression in treated Achilles cultures. Figure 6B shows that In BMP-7-treated Achilles cultures, the GDF-1 expression level was increased by almost twofold ($P < 0.01$), compared to the same day control, but was not changed on the other days. The GDF-5 expression levels were not changed. The expression levels of GDF-6 was elevated by twofold ($P < 0.01$, compared to Day 0) on Day 3 in cultures treated with both protein concentrations. On Days 6 and 10, the magnitude of stimulation was statically insignificant. The GDF-8 mRNA levels were elevated by fourfold ($P < 0.01$) on Day 3 and by about twofold ($P < 0.01$) on subsequent days. The GDF-9 mRNA level was elevated on Day 3 also by twofold ($P < 0.01$) in a concentration-dependent manner, but on subsequent days, the magnitude of stimulation was statically insignificant.

GDF mRNA expression in control and treated Patellar cultures. In contrast to the Achilles Tendons, mRNAs coding for only three GDF family members (GDF-1, -5, and -6) were barely detectable in the Patellar Tendons (data not shown). Since their levels were very low, they were not quantified. BMP-7 treatment did not appear to change their expression patterns.

Effects of BMP-7 on BMP Receptor mRNA Expression

The results described above indicated that the Achilles and Patellar tendon cultures could response to BMP-7. Published reports also showed that tendons could respond to selected BMPs. Since BMP action is known to be regulated at the receptor level, we were interested to determine the mRNA expression pattern of the type I and type II BMP receptors in the Achilles and Patellar tendon cultures as well as the effects of BMP-7 on their expression.

Figure 7A shows representative PhosphorImages revealing the mRNA expression pattern of selective BMP receptors in Achilles cultures and the quantitative data are shown in Figure 7B. In control Achilles cultures, only mRNAs coding for ActR-I (ALK-2), BMPR-IA (ALK-3), and ALK-7 out of the 10 receptors

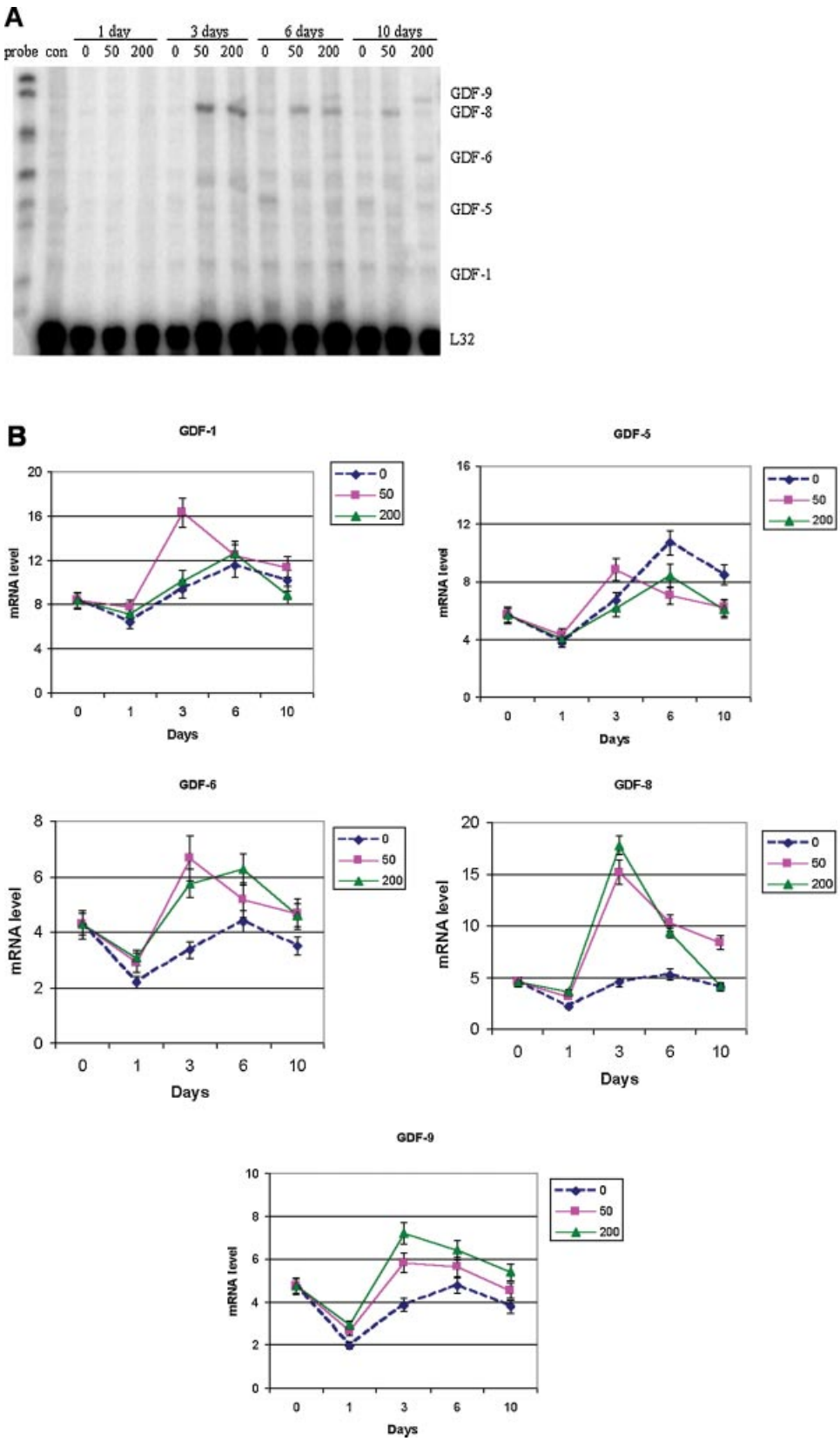

Fig. 6. **A**: RNase protection assay of GDF expression in Achilles tendons. Conditions are similar to those described in Figure 4A, ^{32}P-labeled mGDF multiple template set was used for hybridization. **B**: Quantification of BMP expression. Similar analysis as described in Figure 4B was used. Values represent the mean ± SEM of two measurements using two independent RNA samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

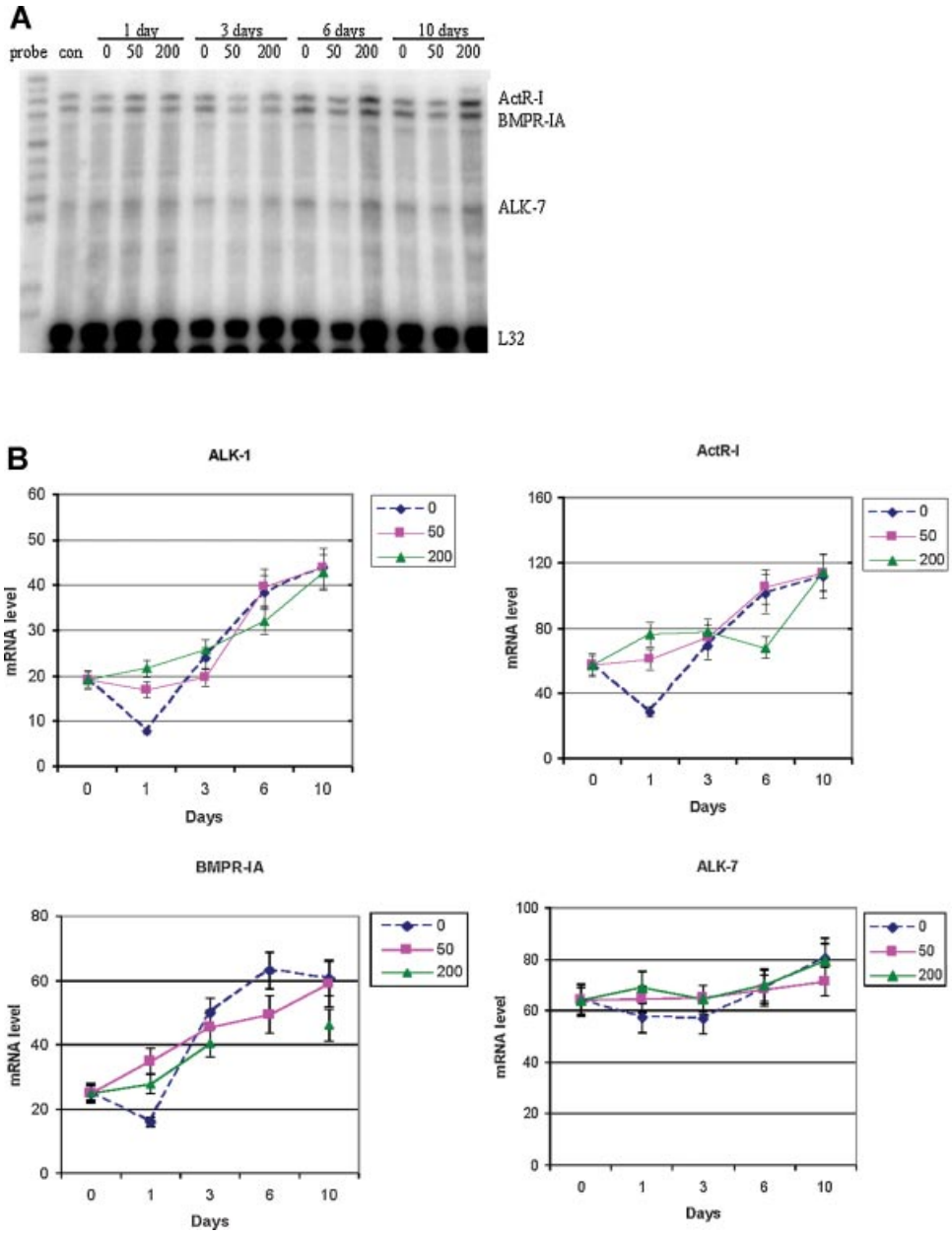

Fig. 7. **A**: RNase protection assay of BMPR expression in Achilles tendons. Conditions are similar to those described in Figure 4A, ^{32}P-labeled BMPR multiple template set was used for hybridization. **B**: Quantification of BMPR expression. Similar analysis as described in Figure 4B was used. Values represent the mean ± SEM of two measurements using two independent RNA samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

measurable by the kit were detected. Their relative expression levels are: ActR-I ≅ BMPR-IA > ALK-7. Their mRNA levels increased by two- to threefold, compared to the Day 0 control over the 10-day culturing period. A very low abundance of BMPR-IB mRNA was also detected but was not further analyzed.

At 50 ng/ml, BMP-7 stimulated the mRNA levels of ActR-I by twofold ($P < 0.01$) on Day 6 but the level returned to that of the same day control on Day 10. In contrast, at 200 ng/ml, BMP-7 stimulated ActR-I mRNA only on Day 10 by twofold ($P < 0.01$). At 50 ng/ml, BMP-7 stimulated BMPR-IA mRNA by twofold ($P < 0.01$) on Day 6 but the level returned to that of the same day control on Day 10. In contrast to ActR-I and BMPR-IA, the mRNA levels of ALK-7 were unchanged in cultures

treated with both concentrations of BMP-7. The slight elevation on Day 3 in cultures treated with 50 ng/ml of BMP-7 was statistically insignificant.

In contrast to the Achilles Tendons, mRNAs for ALK-1, ActR-I, BMPR-IA, and ALK-7 were detected in Patellar tendons (Fig. 8A). A very low level of AvR2B mRNA was observed and was not further quantified. Except for ALK-7, the mRNA levels for ALK-1, ActR-I, and BMPR-IA increased by two- to threefold ($P < 0.01$) as a function of time in culture. The mRNA levels for ALK-7 did not change over the 10-day period (Fig. 8B). BMP-7 did not change the expression pattern of these receptors.

The expression levels of type II receptors were also examined by RPA. Very low levels of mRNAs coding for ActR-IIB and BMPR-II were detected in control Achilles and Patellar cultures. Because of their low abundance, their mRNA levels were not further analyzed. Their

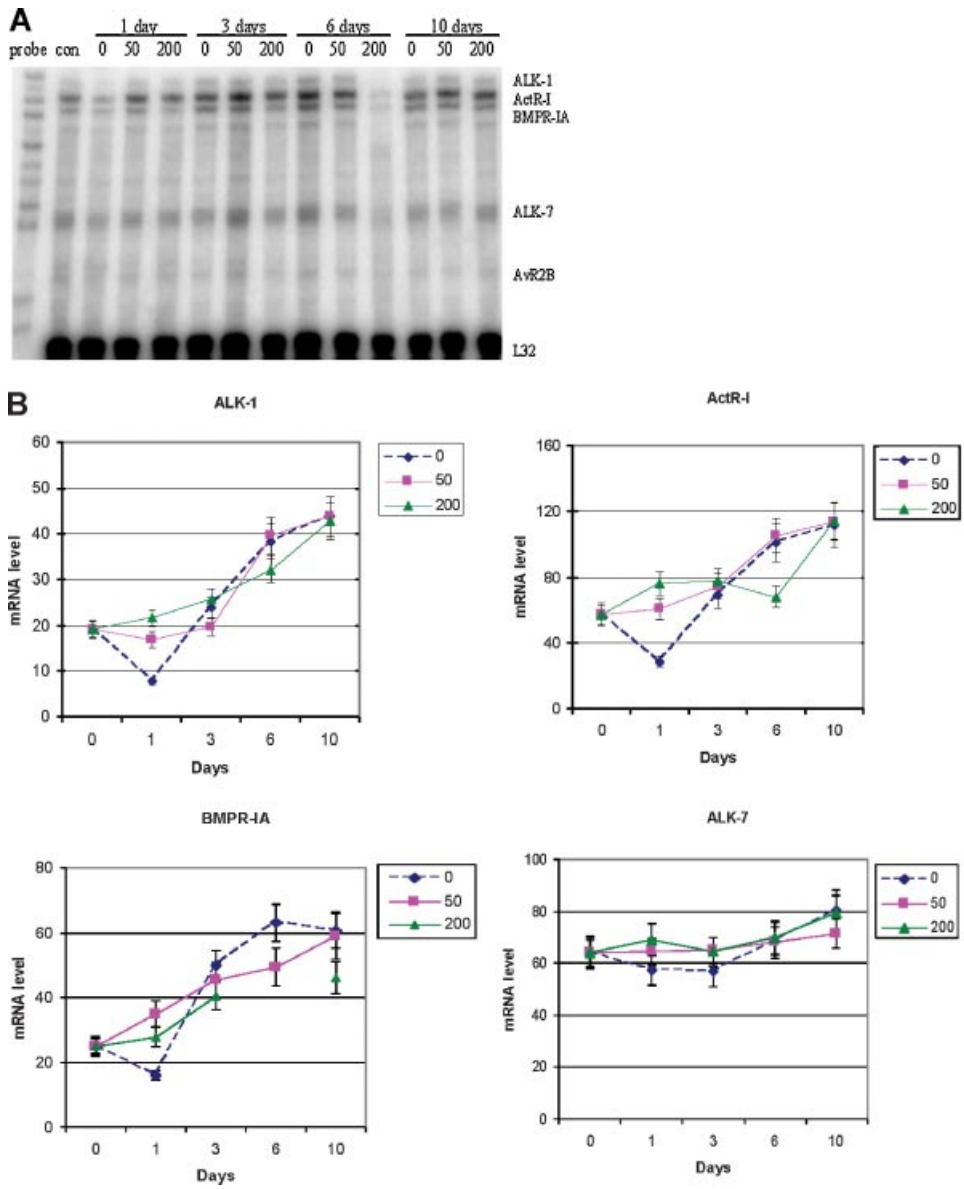

Fig. 8. **A**: RNase protection assay of BMPR expression in Patellar tendons. Conditions are similar to those described in Figure 4A, ^{32}P-labeled BMPR multiple template set was used for hybridization. **B**: Quantification of BMPR expression. Similar analysis as described in Figure 4B was used. Values represent the mean ± SEM of two measurements using two independent RNA samples. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

expression levels were not altered by BMP-7 over the 10-day culture period (data not shown).

DISCUSSION

In the present study, the expression of BMPs and their receptors and effects of BMP-7 on their expression in a primary cell culture system for Achilles and Patellar tendons were studied. Previous studies showed that BMP-7 is involved in developing tendons [Marcias et al., 1997] and promotes tendon repair and healing in vivo [Forslund and Aspenberg, 1998; Aspenberg and Forslund, 2000; Ripamonti et al., 2002; Mihelic et al., 2004].

Published studies showed that the tendon-derived cells stained positive for markers of fibroblasts and mesenchymal progenitor-cells but negative for markers of endothelial cells, smooth muscle cells and pericytes [de Mos et al., 2007]. In the present study, both cultured Achilles and Patellar tendon cells exhibited fibroblastic morphology, but they differed from each other. The former showed more elongated and the latter was more polygonal in shape. These observations were consistent with published in vivo observations. Expression of several characteristic tendon/ligament markers, such as *Six1*, *Scleraxis*, and *Tendin* were also detected. These findings would suggest that the culture system is a useful model for in vitro studies on Achilles and Patellar tendons. Using this system, we demonstrated that Achilles and Patellar tendons respond differently to BMP-7 at the molecular level.

Effects of BMP-7 on Achilles and Patellar Tendon Cell Proliferation

The present study is the first to compare the effects of BMP-7 on Achilles and Patellar; whereas published studies showed effects of a BMP on either Achilles or Patellar. The current results show that BMP-7 stimulated moderately the proliferation of Achilles and Patellar cells. By comparison, a recent study also showed that OP-1 (BMP-7) stimulated cell proliferation, DNA content, synthesis of collagen and proteoglycans in bovine tendon cell cultures [Yamada et al., 2008]. BMP-2, which was previously shown to enhance tendon healing in vivo, did not stimulate proliferation of two clonal Achilles tendon cell lines [Salingcarnboriboon et al., 2003]. TGF-β stimulated modestly cell proliferation of Achilles [Salingcarnboriboon et al., 2003] and Patellar tendons as well as anterior cruciate ligament (ACL) [Spindler et al., 1996]. BMP-12 (also called CDMP-3 and GDF-7), which also has been shown to enhance tendon repair in vivo, stimulated cell proliferation of human Patellar tendon cultures [Fu et al., 2003]. Taken together, these observations would suggest that Achilles and Patellar tendons respond differently to different BMPs.

Effects of BMP-7 on Tendon/Ligament Gene Expression

The present results that BMP-7 stimulated type I collagen mRNA expression is consistent with published results that type I collagen plays a structural and functional role in healing tendons [Monboisse et al., 1990; Gillery et al., 1995, 1996; Rufai et al., 1995]. Additionally, tendons/ligaments expressed several tendon-specific gene markers, such as *Six1*, a murine homeobox-containing transcription factor [Oliver et al., 1995], *Tendin* and *Scleraxis*, a twist-related bHLH transcription factor [Salingcarnboriboon et al., 2003]. Our results show that the *Six1* and *Tendin* genes were expressed constitutively in long-term Achilles tendon cultures but *Scleraxis* transcripts were barely detectable by Northern blot analysis. BMP-7 treatment of the Achilles tendon cell cultures did not result in a significant enhancement of markers expression. On the other hand, *Six1* and *Scleraxis* genes were expressed constitutively in long-term Patellar tendon cultures, and BMP-7 treatment did not increase their expression significantly. By comparison, mRNAs for *Six1*, elastin, decorin, and aggrecan were elevated in GDF-7-treated tendons [Wolfman et al., 1997]. These results indicate that BMP-7 was not as robust in stimulating the expression of tendon gene markers in these cultured tendon cells. In contrast, published animal studies clearly indicated that BMP-7 stimulates tendon repair. One possible explanation for the difference is that the current culture conditions might not be optimal. The culture system used in the present study was grown as monolayer and consisted of mostly fibroblastic. Furthermore, contributions from other cell types both within the tendon and other tissues under in vivo conditions are also likely. For example, published in vivo studies showed that BMP-7 (25 μg) promoted integration of ACL graft into newly formed trabecular bone in sheep [Mihelic et al., 2004]. BMP-7 was capable

of inducing regeneration of the complex periodontal ligament system containing alveolar bone and cementum in the primate *Papio ursinus* [Ripamonti et al., 2002]. BMP-2 (90–260 μg) enhanced ingrowth of the transplanted long digital extensor tendon through new bone formation at the bone-tendon interface in the bone tunnel [Rodeo et al., 1999]. Further research may provide answers to some of these issues.

Effects of BMP-7 on Expression of Other BMPs and BMP Receptors in Tendon Cultures

Numerous studies have shown that the expression of BMPs and their receptors can be altered depending on the physiological state of the tendon. For example, expressions of BMP-2, -4, -7, and CDMP-1 as well as several BMP receptors are changed in pathological conditions of ossified posterior longitudinal ligaments, ossification of the spinal ligament, or around the calcified zone at the insertion of the ligamentum flavum to the bone [Hayashi et al., 1997; Yonemori et al., 1997; Nakase et al., 2001; Tanaka et al., 2001]. The current results provide a demonstration that Achilles and Patellar tendons exhibit a different expression pattern of BMPs and their receptors. The results further reveal a complex interplay of these BMPs in both Achilles and Patellar tendons. Furthermore, the present data on the expression of other BMPs and their receptors in the two tendon types further reveals that BMP-7 not only regulates the expression of individual BMP receptors but also asserts such regulation depending on the tissue types. Whether these observed changes in the steady-state mRNA levels are translated to the surface receptor protein levels will have to wait for additional experimentation.

A current medical treatment of rupture of anterior cruciate ligament typically involves replacement of the ligament with a tendon autograft from another site. It is noteworthy that the effects of BMP-7 on gene expression in medial collateral ligament (MCL) and Achilles tendons (AT) are similar in many cases but are also different is some cases. For example, BMP-7 stimulated cell proliferation and type I collagen mRNA expression and did not alter *Six1* mRNA levels in both MCL [Tsai et al., 2003] and AT (present study). By contrast, BMP-7 down-regulated *Scleraxis* mRNA expression in PT and not in MCL. BMP-7 exerts the mRNA expression patterns of BMPs and their receptors in MCL and AT distinctively.

In summary, BMP-7 exerts differential effects on the spatial and temporal expression of several members of the BMP family in Achilles and Patellar tendons. By virtue of their expression patterns, the findings also imply specific functional roles. Additionally, the complex and differential regulation of the expression of these different BMPs and their receptors by BMP-7 in the two tendon types implies that BMP-7 action on tendon growth involves a complex regulation of gene expression of several members of the BMP and GDF family.

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