Journal of Cellular Biochemistry 90:777–791 (2003)

Effects of Osteogenic Protein-1 (OP-1, BMP-7) on Gene Expression in Cultured Medial Collateral Ligament Cells

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Abstract Osteogenic protein-1 (OP-1, also called BMP-7), a member of the BMP family and the TGF-β superfamily, induces formation of new bone and cartilage, but also regulates a wide array of processes. In the present study, the expression of several characteristic biochemical markers of ligaments, such as Six1, Scleraxis, aggrecan, and type I collagen in primary cultures of adult rat medial collateral ligament (MCL) cells was determined. The effects of OP-1 on cell proliferation and on gene expression were subsequently examined. OP-1 stimulated cell proliferation, alkaline phosphatase (AP) activity, and the steady-state mRNA levels of the transcription factor Runx2/Cbfa1 in a dose- and time-dependent manner. The mRNA levels of type I collagen only increased slightly, but the activity of the cloned collagen promoter increased by 2-fold in transiently transfected MCL cells. OP-1 also stimulated aggrecan mRNA expression. The mRNA levels of Six1 and Scleraxis were not detectably altered by OP-1. In control cultures, the steady-state mRNA levels of ActR-I, BMPR-IA, BMPR-IB, and BMPR-II increased as a function of time in culture. The mRNA levels of BMP-1 and -4 increased significantly after 12 days, but those of BMP-2 and -6 did not change. The GDF-1, -3, -5, -6, and -8 mRNA levels in the control cultures also increased as a function of time. OP-1 treatment stimulated mRNA expression of BMPR-IA and BMPR-II, but had little effect on ActR-I and BMPR-IB mRNA expression. OP-1 lowered the BMP-1, -2, and -6 mRNA levels without changing the BMP-4 mRNA level. OP-1 treatment also reduced the mRNA levels of GDFs detected. In summary, the present study demonstrated that OP-1 stimulated cell proliferation and mRNA expression of several biochemical markers in this ligament cell culture model and established the spatial and temporal appearance of several members of the TGF-β superfamily. J. Cell. Biochem. 90: 777–791, 2003.

Key words: BMP; OP-1; BMP receptors; ligament; MCL; Runx2/Cbfa1; Six1; Scleraxis; gene expression

The anterior cruciate ligament (ACL) and the medial collateral ligament (MCL) are commonly injured areas of the knee joint. Healing of the ligament is a complex process involving cellular proliferation and migration, as well as synthesis and deposition of ligament cellular components. In vitro and in vivo studies showed that several growth factors stimulate synthesis of extracellular protein molecules and cell proliferation of the ligamentous cells [Woo et al., 1999; Benjamin and Ralphs, 2000]. The growth factors studied thus far include basic fibroblast growth factor (bFGF) [Schmidt et al., 1995; Kobayashi et al., 1997; Marui et al., 1997; Woo et al., 1998], platelet-derived growth factor-B (PDGF-B) [Schmidt et al., 1995; Spindler et al., 1996; Marui et al., 1997; Scherping et al., 1997; Woo et al., 1998], insulin-like growth factor-I and -II (IGF-I and -II) [Abrahamsson, 1997; Murphy and Nixon, 1997], and transforming growth factor-β (TGF-β) [Marui et al., 1997; Natsu-ume et al., 1997; Woo et al., 1998; Spindler et al., 2002]. That these factors are present in the early stage of repairing ACL, MCL, and patellar tendon supports their involvement in the healing process of these tissues [Natsu-ume et al., 1997; Kuroda et al., 2000].

Bone morphogenetic proteins (BMPs) and growth differentiation factors (GDFs) are members of the TGF-β superfamily [Ozkaynak et al., 1990; Sampath et al., 1992; Kingsley, 1994; Hogan, 1996; Reddi, 2000; Wozney, 2002]. Three GDFs, viz., GDF-5 (BMP-14), GDF-6 (BMP-13), and GDF-7 (BMP-12), have been shown to induce neotendon/ligament formation

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Received 10 June 2003; Accepted 4 August 2003

DOI 10.1002/jcb.10666

when implanted at ectopic sites in vivo [Wolfman et al., 1997; Aspenberg and Forslund, 1999; Tashiro et al., 1999; Forslund and Aspenberg, 2001]. BMP-2 has been reported to increase bone ingrowth into a tendon graft that has been implanted in a bone tunnel [Rodeo et al., 1999]. Osteogenic protein-1 (OP-1, BMP-7) not only induces new bone and cartilage formation and repair in vivo [Sampath et al., 1992; Klein-Nulend et al., 1998; Sakou, 1998; Cook, 1999; Koepp et al., 1999], but also plays a key role in normal skeletal development and numerous other biological processes [Dudley et al., 1995; Luo et al., 1995].

BMP transduces its effects through the binding to a complex of trans-membrane serine/threonine kinase receptors: type I and type II receptors [Massague, 1998; ten Dijke et al., 2000]. Both the type I and the type II receptors can bind ligands independently but with different affinities. 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 OP-1. The mRNA expression of these receptors can be altered by OP-1 in vitro in several cell types, e.g., osteoblastic cells [Yeh et al., 2000], the mouse MC615 chondrocyte cells [Yeh et al., 2002a], and the pluripotent mesenchymal cell line C2C12 [Yeh et al., 2002b].

In view of the critical role of OP-1 in bone and cartilage formation and repair as well as in other skeletal development, we hypothesized that OP-1 might be involved in post-natal ligament differentiation and regeneration. To test the hypothesis, we determined whether OP-1 stimulates proliferation and differentiation of MCL cells in vitro. Moreover, the effects of OP-1 on the expression of the other *BMP* genes and the BMP receptors in these cells were studied. This is of particular interest, since previous studies showed that several other BMPs could induce neotendon/ligament formation when implanted in vivo. Information produced should aid in our understanding of the potential functional relationship among the various BMPs. The scientific data should also increase our knowledge on the cellular and molecular mechanism of ligament growth and repair.

MATERIALS AND METHODS

Materials

All reagents were of molecular biology grade. All buffers were prepared with diethylpyrocarbonate-treated water. SeaKem ME and GTG agarose were purchased from FMC BioProducts (Rockland, ME). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Recombinant human OP-1 was provided by Stryker Biotech (Hopkinton, MA) and was dissolved in 47.5% ethanol/0.01% trifluoroacetic acid. Radioisotopes were purchased from ICN (Irvine, CA). Fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), serum-free DMEM, F-12, penicillin/streptomycin, gentamicin, and trypsin–EDTA, were purchased from Life Technologies (Grand Island, NY). TRI Reagent and collagenase were from Sigma (St. Louis, MO).

Primary Cell Culture of MCL Cells

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. MCLs were surgically excised from the surrounding connective tissues at the knee joints, rinsed with HBSS, cut into small pieces, and cultured in DMEM/F12 (1:1) medium with 10% FBS supplemented with 30 μg/ml of gentamicin at 37°C with 5% CO_2. Cells began to emerge from the tissue pieces after 3–4 days in culture and began to attach to the surface of the culture dishes. After 6–7 days, the tissue pieces were removed and the attached cells were cultured in fresh media until confluent. Cells were subcultured until confluent and frozen in liquid N_2. For experimentation, cells were revived from the frozen stock in 100- or 150-mm dishes until confluent and subcultured at a cell density of 4×10^4 cells/ml.

Cell Proliferation

Cell proliferation was evaluated 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 OP-1 (0, 100, 200, 300, and 400 ng/ml) in serum-free DMEM/F12 (1:1) for 24 h. After the media were removed, cultures were rinsed with sterile PBS. To each well, 100 μl of media containing 1% BSA plus 20 μl of the 96AQ reagent were added. After incubation for 4 h at 37°C, the color developed

was measured at 490 nm using a MRX microplate reader (Dynex Technologies, Chantilly, VA).

Alkaline Phosphatase (AP) Activity Assay

Confluent cells, grown in 48-well plates, were treated in serum-free DMEM/F12 (1:1) for 48 h with varying concentrations of OP-1 as indicated in the figure legend. Control cultures were treated with an equal amount of solvent vehicle. Cells were lysed by sonication in 0.1% Triton X-100 in PBS (100 μl/well) for 5 min at room temperature. Total cellular AP activity was measured using a commercial assay kit (Sigma Chemical Co.) as described previously [Yeh et al., 1996]. Reactions were terminated by the addition of 0.5 N NaOH. Absorbance of the reaction mixture was measured at 405 nm using a MRX microplate reader (Dynex Technologies). Protein was measured according to the method of Bradford [1976] using BSA as a standard. AP activity was expressed as nanomoles of *p*-nitrophenol liberated per micrograms of total cellular protein.

Measurement of the Promoter Activity of the Type I Collagen Gene

A 1.372-kb DNA fragment, comprised of nucleotides from −1,263 bp upstream to +109 bp downstream from the transcription start site (+1) of the rat type I collagen gene, was generated by PCR using genomic DNA isolated from rat liver. The (−1,263/+109) promoter fragment was subcloned into pGL2-Basic vector (Promega Corp.) containing the promoterless luciferase report gene (*Luc*). The clone was confirmed by restriction enzyme mapping and double-stranded DNA sequencing. Sub-confluent MCL cells grown in 6-well plate were transiently transfected using 3 μg of the type I collagen promoter construct and 12 μl FuGENE 6 (Roche Diagnostics Corp., Indianapolis, IN) for 4 h following manufacturer's instruction. The transfected cells were allowed to recover in FBS-containing medium overnight and treated with vehicle or two concentrations of OP-1 (50 and 200 ng/ml) for 6 days. Luciferase activity was then measured using the Dual assay kit (Applied Biosystems, Bedford, MA) and normalized to the β-galactosidase activity.

Northern Blot Assay

Total RNA was isolated using the TRI reagent (Molecular Research Center, Inc., Cincinnati, OH) following the manufacturer's recommendation. The cDNA probes for ActR-I, BMPR-IA, BMPR-IB, and BMPR-II were obtained by digestion of the corresponding plasmids with the appropriate restriction endonucleases as reported previously [Yeh et al., 2000]. Probes for type I collagen, Scleraxis, and Runx2/Cbfa 1 were obtained by PCR. The Six1 probe was purchased from ATCC (clone ID#1152512). All cDNA probes were labeled with ^{32}P-dATP using the Strip-EZ labeling kit from Ambion (Austion, TX).

Northern analyses were conducted as described previously [Yeh et al., 1997]. Briefly, total RNAs (20 g) were denatured and fractionated on 1% GTG agarose gels containing 2.2 M formaldehyde. The fractionated RNA was transferred onto a "Nytran Plus" membrane using a Turboblot apparatus (Schleicher & Schuell, Inc., Keene, NH) and was covalently linked to the membrane using the UV Crosslinker (Stratagene, La Jolla, CA). The membranes were incubated overnight at 42°C with cDNA probes, washed, exposed to a screen for the PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and analyzed using the ImageQuant software. Before probing with another probe, the blots were stripped at 68°C with the Strip-EZ probe degradation buffer (Ambion) according to the protocol of the manufacturer and checked to ensure that the level of radioactivity was reduced to background. The blots were also probed with an 18S rRNA oligonucleotide probe to correct for loading variations.

RNase Protection Assay (RPA)

The antisense RNA for the rat aggrecan was obtained by digestion of the original plasmid in pGEM-3zf(−) with *Hind*III and labeled as described [Yeh et al., 2002a]. The BMP and GDF mRNA levels were measured using the RiboQuant RPA kit with two Mouse Multi-Probe Template Sets from BD PharMingen (San Diego, CA). The mBMP-1 Multi-Probe Template Set permits detection of mRNAs for BMP-1, -2, -3, -4, -5, -6, -7, -8A, and -8B. The protected fragment for BMP-1, -2, -3, -4, -5, -6, -7, -8A, and -8B should be 148, 160, 181, 226, 253, 283, 316, 353, and 133 nucleotides in length, respectively. The mGDF-1 Multi-Probe Template Set allows detection of mRNAs encoding GDF-1, -3, -5, -6, -8, and -9. The protected fragment for GDF-1, -3, -5, -6, -8, and -9 should be 148, 160, 181, 226, 283, and 316 nucleotides in length, respectively.

Both Template Sets also allow detection of mRNAs for ribosomal protein L32 and GAPDH, thus allowing normalization of sampling or technique errors. The anti-sense RNA probes were labeled with ^{32}P-UTP using the RiboQuant in vitro transcription kit from 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).

Statistical Analysis

Data are presented as the mean ± 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

Morphology of MCL Cells in Culture

After several days in DMEM/F12 with 10% FBS, MCL cells diffused out of the ligament pieces into the media and became attached to the tissue culture dish. Figure 1A shows the morphology of control cells as a function of time. Cells exhibited the characteristic elongated shape and spindle-shaped nuclei. The gross morphology of cells from passage 1 and 2 was similar (Fig. 1B).

OP-1 Stimulated Cell Proliferation of Adult Rat MCL Cells

Treatment of MCL cells with varying concentrations of OP-1 in serum-free media resulted in a moderate (~40%), but dose-dependent increase in cell proliferation (Fig. 2A). The finding is consistent with previously published results, indicating that OP-1 is a differentiation factor possessing a low level of mitogenic activity.

Effects of OP-1 on the Expression of Several Biochemical Markers

The effects of OP-1 on the expression of several biochemical markers were examined. The markers were selected mainly based on their reported association with different types of tissues of the skeletal system. Figure 2B shows that the AP activity in MCL cells treated with OP-1 was elevated in a dose-dependent manner, reaching a maximum of about a 70% increase, compared to the untreated control.

Northern blot analysis revealed that control MCL cells expressed Six1 mRNA in a time-dependent manner, with a peak expression occurring at 8 days (Fig. 3A,B). The mRNA expression returned to the day 0 control value afterwards. OP-1 treatment did not change the Six1 mRNA expression pattern.

Northern blot analysis also revealed that MCL cells expressed the *Scleraxis* gene constitutively in a time-dependent manner. The expression level remained unchanged for the initial phase, decreased slightly on day 8, but increased dramatically beginning at about the 12th day (Fig. 3A,C). OP-1 treatment did not change the pattern of expression.

Northern blot analysis revealed that control MCL cells expressed a high level of type I collagen mRNA and the levels remained relatively unchanged. OP-1 treatment increased the levels only slightly (Fig. 4A). However, OP-1 stimulated the promoter activity of type I collagen in a dose-dependent manner in transiently transfected MCL cells (Fig. 4B). OP-1 stimulated the basal luciferase activity of the control vector pGL2-basic by about 15% (data not shown), but stimulated the promoter activity by approximately 1.8- and 2.5-fold at 50 and 200 ng/ml of OP-1, respectively compared to the no treatment control.

mRNA coding for the osteoblast specific transcription factor Runx2/Cbfa1 was also detected in control MCL cells and the level was low throughout the entire 16 days of culture (data not shown). OP-1 treatment decreased the Runx2/Cbfa1 mRNA level (compared to the same day control) for the first 8 days, but thereafter increased gradually, reaching about 1.5-fold (Fig. 5A).

RPA was used to detect presence of aggrecan mRNA, a component of the extracellular matrix. Figure 5B shows the aggrecan mRNA levels were significantly elevated in OP-1 treated MCL cell cultures as a function of culturing time.

Adult Rat MCL Cells Expressed mRNAs Coding for ActR-I, BMPR-IA, BMPR-IB, and BMPR-II and OP-1 Stimulated BMPR-IA and -II Expression

BMPR mRNA expression in control cultures. Figure 6 is a representative Northern blot revealing that MCL cells expressed constitutively the genes coding for the three

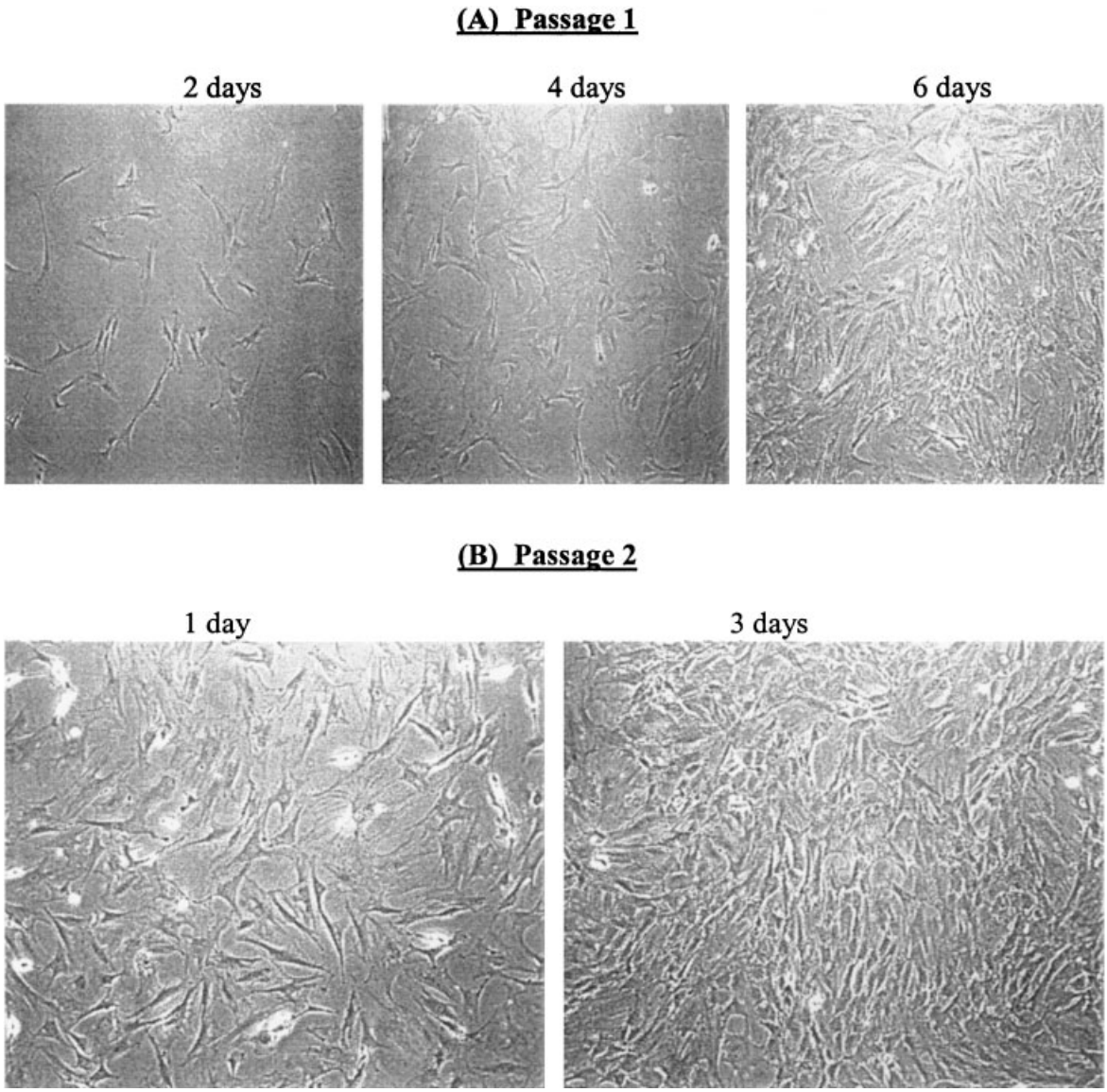

Fig. 1. Morphology of primary cultures of rat medial collateral ligament (MCL) cells. Cells were cultured in DMEM/F12 medium with 10% fetal bovine serum (FBS). Media were changed every 3 days. Cell morphology was monitored as a function of time with an Olympus CK2 inverted microscope equipped with a CCD camera. Representative images (phase contrast with 100× magnification) of cells of passage 1 (**A**) and passage 2 (**B**) are presented.

type I and one type II receptors during the 16 days in culture. In control cultures, the ActR-I mRNA level did not change throughout the culture period (Fig. 6). The BMPR-IA and -IB mRNA levels increased gradually, reaching a maximum of 1.6-fold on day 16. Two positive bands of equal intensity were detected with the BMPR-II probe, although their relationship remained to be elucidated. The BMPR-II mRNA level remained at the basal level during the first 2 days in culture, but increased significantly (by about 1.5-fold, except day 8) thereafter.

BMPR mRNA expression in OP-1 treated cultures. The ActR-I and the BMPR-IB mRNA levels did not change, compared to the same day control. The BMPR-IA mRNA level in OP-1-treated MCL cells increased significantly in a time-dependent manner, reaching a maximum of about 1.6-fold compared to the same day control beginning on day 4 until day 8. The level returned to the control level thereafter. Compared to the other receptors, the BMPR-II mRNA level increased most dramatically, reaching a maximum of about 2-fold compared

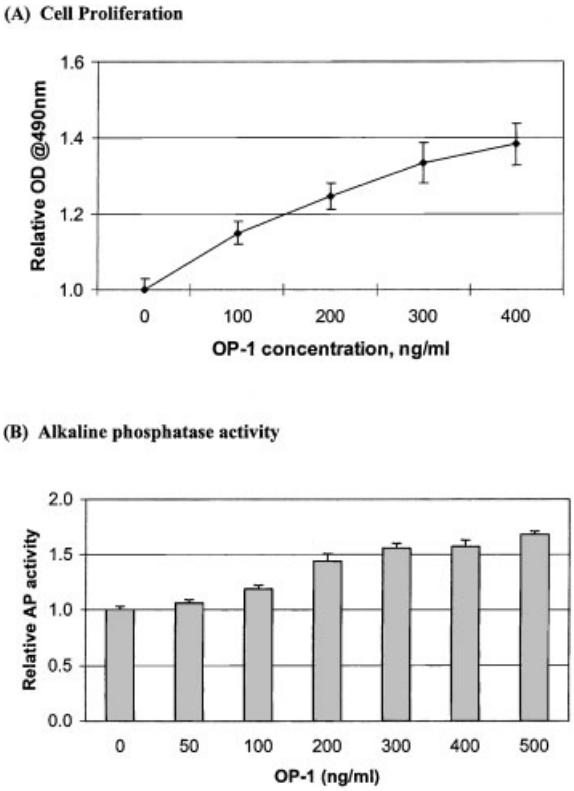

Fig. 2. Effects of osteogenic protein-1 (OP-1) on (**A**) cell proliferation and (**B**) alkaline phosphatase (AP) activity in primary cultures of MCL cells. A: MCL cells were grown to confluency and treated with various amounts of OP-1 for 24 h. Cell proliferation was determined by a colormetric assay as described in ''Materials and Methods.'' Values were normalized to the vehicle control (as 1) to facilitate data comparison and represent the mean ± SEM of seven independent measurements. B: MCL cells were grown to confluency and treated with various concentrations of OP-1. Total AP activity in the cell lysate was measured after 48 h as described in ''Materials and Methods.'' Values were normalized to the solvent control (as 1) and represent the mean ± SEM of three different determinations on two different MCL cell preparations.

to the same day control beginning on day 4 and thereafter (Fig. 6).

BMP mRNA Expression in Control and OP-1-Treated MCL Cell Cultures

BMP mRNA expression in control cultures. The mRNA expression of several BMPs in control and OP-1-treated MCL cultures as a function of time was examined by RPA (Fig. 7). Significant levels of BMP-1, -2, -4, and -6 mRNAs were detected in control MCL cells. The BMP-1 mRNA level increased as a function of time, reaching a maximum of about 3-fold above the day 0 control on day 16 in culture. The BMP-2 mRNA level changed slightly in a time-dependent, cyclical manner in the control cultures during the 16 days of culturing. The BMP-4 mRNA level increased dramatically as a function of time, reaching a maximum of about 7-fold above the day 0 control on day 16. Similar to BMP-2, the BMP-6 mRNA expression changed in a cyclical manner in the control cultures.

BMP mRNA expression in OP-1-treated cultures. The BMP-1 mRNA level in OP-1-treated cells was reduced to approximately that of the day 0 control throughout the entire 16 days. The BMP-2 mRNA level was reduced by 20–40% in the OP-1-treated cells. The BMP-4 mRNA level was not altered significantly by OP-1 treatment. The cyclical nature of the BMP-6 mRNA expression was abolished, and the expression level was significantly reduced (by as much as 50%) at all time points examined.

GDF mRNA Expression in Control and OP-1-Treated MCL Cell Cultures

GDF mRNA expression in control cultures. The mRNA expression of GDFs in MCL cultures was also evaluated by RPA. Figure 8 shows a representative PhosphorImage of the RPA results. Quantitative analysis of these results showed that significant levels of mRNA coding for GDF-1, -3, -5, -6, and -8 were detected in control MCL cultures (Fig. 9). During the 16 days of culturing, the GDF-1, -3, -5, -6, and -8 mRNA levels increased in the control cultures as a function of time, reaching a maximum of about 5-, 7-, 2-, 3-, and 2-fold, respectively, compared to the day 0 control.

GDF mRNA expression in OP-1-treated cultures. The GDF-1 mRNA level increased in the OP-1-treated cultures as a function of time, reaching a maximum of about 3-fold, above the day 0 control. OP-1 treatment lowered the GDF-3, -6, and -8 mRNA levels without abolishing the time-dependent changes such that the maximum achieved on day 16 was about 4-, 2-, and 1.5-fold, respectively, compared to the day 0 control. OP-1 reduced the GDF-5 mRNA level to that in the control cultures.

DISCUSSION

Understanding ligament growth and repair will require knowledge about the molecular and cellular events occuring during these processes. Information on the time sequence of gene expression in a cell culture model should be useful. The present study showed that primary

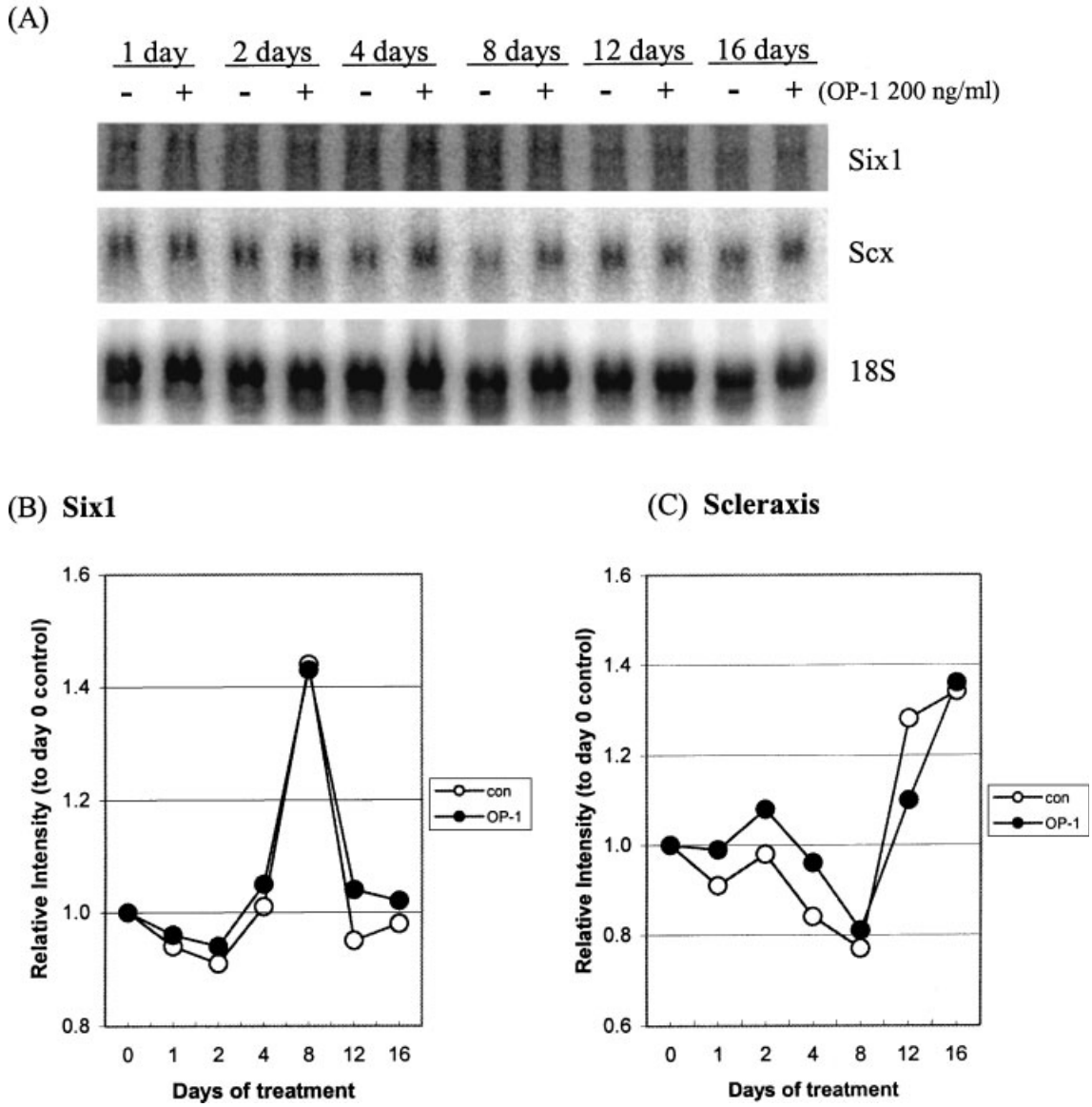

Fig. 3. Effects of OP-1 on Six1 and Scleraxis (Scx) mRNA expression pattern in long-term cultures of MCL cells. **A**: Northern analysis. Confluent MCL cells were treated with solvent vehicle or 200 ng/ml of OP-1 for different durations. Total RNA was isolated on the designated day, denatured, resolved on 1% agarose gel containing formaldehyde, and transferred onto a Nytran Plus membrane. The blots were hybridized with the cDNA probes for Six1, Scx, or the oligonucleotide probe for 18S rRNA. After washing under appropriate conditions, the blots were exposed to a PhosphorImage screen. **B**: Quantitative analysis of the Six1 mRNA level in MCL cells. The intensity of the hybridized RNA species shown in Figure 3A 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. **C**: Quantitative analysis of the Scx mRNA level in MCL cells. Values represent the mean ± SEM of two measurements per RNA sample using two to three separate RNA preparations.

cultures of rat MCL expressed several characteristic biochemical markers of tendon/ligament. For example, high levels of mRNAs coding for type I collagen mRNA, aggrecan, Six1, and Scleraxis were detected in these cultures. The findings that the current primary culture system is capable of expressing these characteristic markers strongly suggest that this system might serve as a useful model for future in vitro studies on ligaments.

The present study shows that the *Six1* gene was expressed constitutively in long-term MCL cell cultures in a time-dependent manner. *Six1*, a novel murine homeobox-containing gene, has been suggested as a specific molecular marker for the limb tendons and ligaments [Oliver et al.,

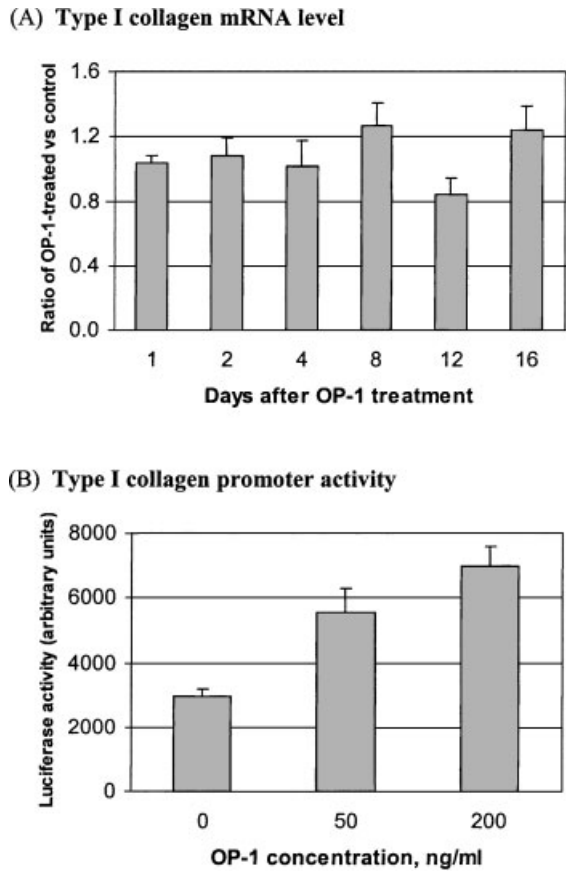

Fig. 4. Effects of OP-1 on (**A**) type I collagen mRNA expression in long-term cultures of MCL cells, and (**B**) the type I collagen promoter activity in transiently transfected MCL cells. Confluent MCL cells were treated as described in Figure 3. A: Blots were probed with the cDNA probe for type I collagen. The data are presented as relative band intensity which is the value normalized to the housekeeping gene and then normalized to the control. Values represent the mean ± SEM of two measurements per RNA sample using two to three separate RNA preparations. B: For promoter activity measurements, primary cultures of rat MCL cells were transfected with the rat type I collagen promoter construct described in ''Materials and Methods'' and treated with solvent, 50 or 200 ng/ml of OP-1 for 6 days. The luciferase activity was then measured and normalized to the β-galactosidase activity using the Dual assay kit (Applied Biosystems). Values represent the mean ± SEM of two independent determinations.

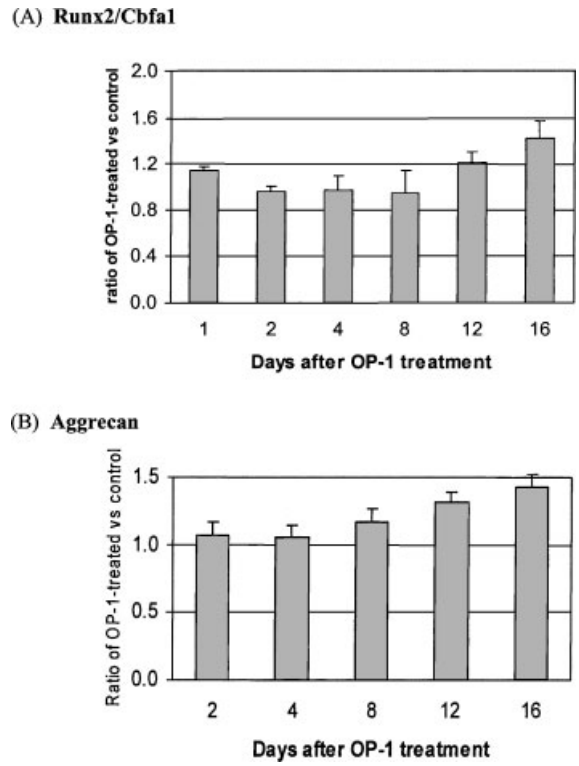

Fig. 5. Effects of OP-1 on (**A**) Runx2/Cbfa1 and (**B**) aggrecan mRNA expression in long-term cultures of MCL cells. Experimental conditions are identical to those described in Figure 4, except that blots were probed with the cDNA probe for Runx2/Cbfa1. The aggrecan mRNA level was measured by RPA as described in the ''Materials and Methods.''

1995]. This suggestion is mainly based on the observation that Six1 mRNA is detected in cells corresponding to those forming connective tissue-derived extensor tendons and ligaments. A recent study also showed that *Six1* gene was expressed in mouse tendon cell clones [Salingcarnboriboon et al., 2003]. We further showed that OP-1 did not change the mRNA expression pattern of Six1.

The current study also showed that the gene coding for Scleraxis, a novel class II helix-loop-helix transcription factor [McLellan et al., 2002], was constitutively expressed in the long-term MCL culture and its expression increased significantly at the late stage of MCL culture. We further showed that OP-1 did not appear to alter its expression pattern. Although the precise function of Scleraxis in ligament remains to be fully understood, Scleraxis was shown to be essential for mesodermal development and was found to be a specific marker for developing tendons and ligaments [Schweitzer et al., 2001]. Scleraxis mRNA was detected in tendons [Salingcarnboriboon et al., 2003] and in mesenchymal progenitors that later form connective tissues [Asou et al., 2002]. Scleraxis appears to act with E47, another bHLH protein, to stimulate transcription of E-box containing promoters [Carlberg et al., 2000]. Thus, the present finding provides a rationale for planning future experiments to understand the role

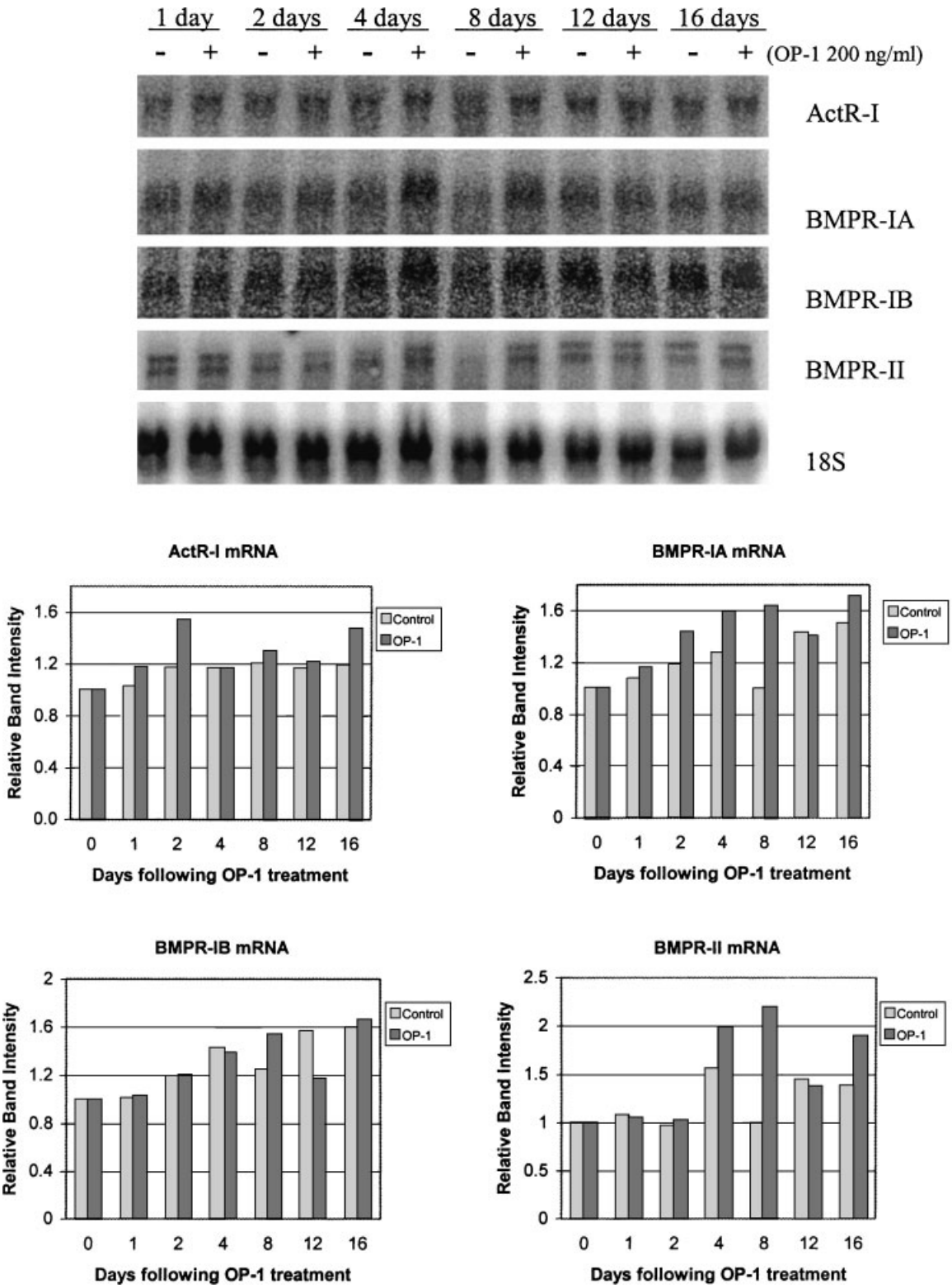

Fig. 6. Effects of OP-1 on BMP receptor mRNA expression in long-term cultures of MCL cells. **A**: Northern blot analysis. MCL cells were treated with OP-1 (200 ng/ml) for the indicated time. Media were refreshed every 3 days. Total RNA was isolated and processed as described in Figure 3. The blots were hybridized with the cDNA probes for ActR-I, BMPR-IA, -IB, and -II, respectively, or the oligonucleotide probe for 18S rRNA. **B**: Quantitative measurements of the steady-state mRNA levels of ActR-I, BMPR-IA, -IB, and -II. The results shown were quantified as described in Figure 3. The data are presented as relative band intensity which is the value normalized to the housekeeping gene and then normalized to the control.

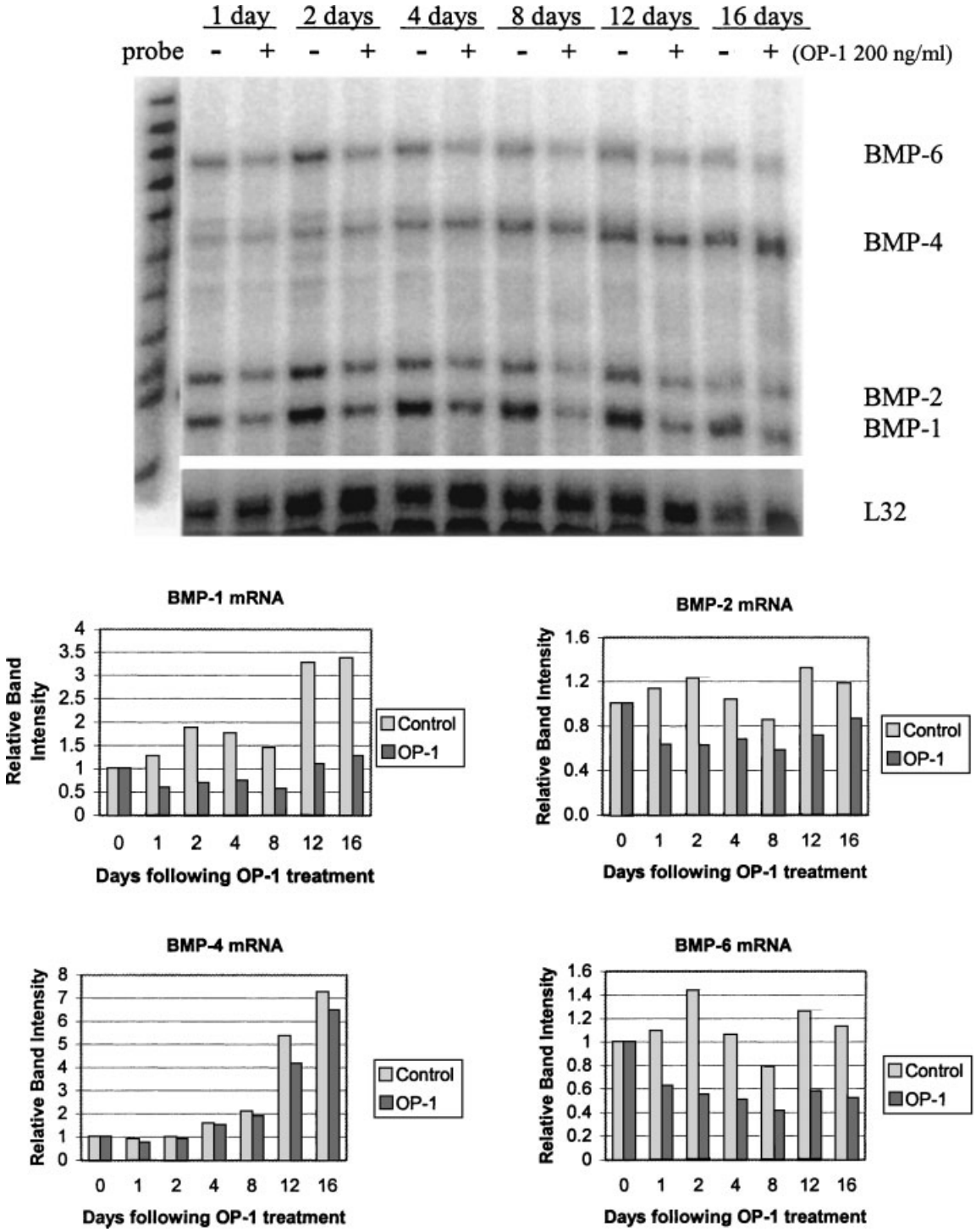

Fig. 7. RNase protection analysis of the effect of OP-1 on BMP mRNA expression in MCL cells. Confluent cultures were treated with vehicle or OP-1 (200 ng/ml) for the designated days. Total RNA was isolated using the TRI reagent. Twenty micrograms of total RNA was used for the measurement of BMP mRNA expression by RNase protection assay (RPA). The protected RNA fragments were fractionated on 5% polyacrylamide gels containing 8 M urea and detected by PhosphorImaging. Positions of labeled probes for the different BMPs and the housekeeping gene control (ribosomal protein L32) are on the left of the image. The protected fragments are indicated on the right. The intensity of the each protected fragment was analyzed and quantified using the ImageQuant software. The data are presented as relative band intensity which is the value normalized to the housekeeping gene and then normalized to the control. Values represent mean ± SEM from two to three different determinations.

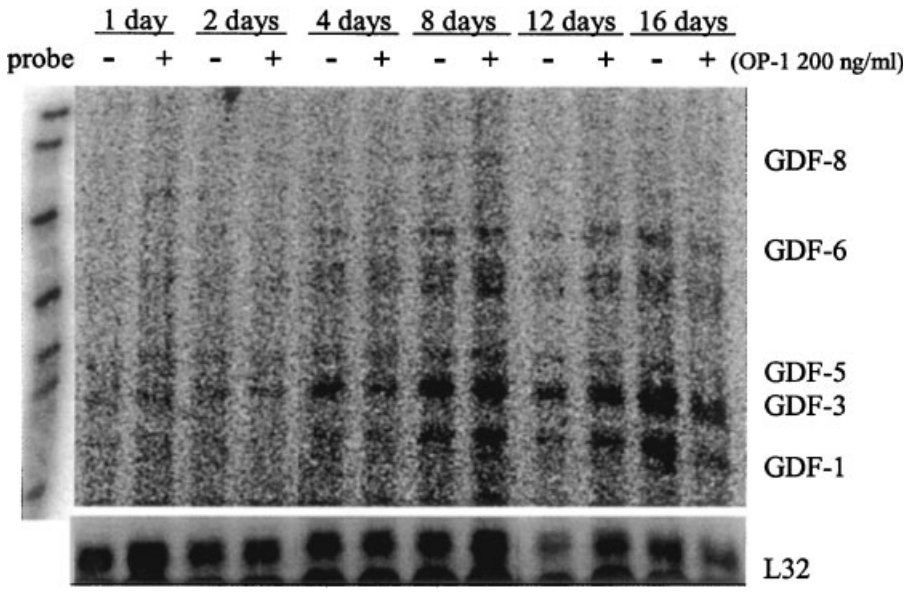

Fig. 8. RNase protection analysis of the effect of OP-1 on GDF mRNA expression in MCL cells. Confluent cultures were treated with vehicle or OP-1 (200 ng/ml) for the designated days. Total RNA was analyzed as described in Figure 7. Positions of labeled probes for the different GDFs and the housekeeping gene control (ribosomal protein L32) are on the **left** of the image. The protected fragments are indicated on the **right**.

of this transcription factor in ligament cell physiology. It has been suggested that Scleraxis may play multiple roles in mesoderm formation and chondrogenesis [Brown et al., 1999; Schweitzer et al., 2001]. In vitro studies showed that Scleraxis mRNA was expressed in C2C12 myoblasts and its expression was decreased by BMP-2 but was increased by Noggin [Liu et al., 1997a]. In a chondrocyte-like cell line TC6, TGF-β elevated but BMP-2 down-regulated Scleraxis expression [Kawa-uchi et al., 1998]. Over-expression of Scleraxis in the ROS17/2.8 osteosarcoma cell line increased expression of the cartilage markers aggrecan and type II collagen and suppressed expression of osteoblast markers, type I collagen and AP [Liu et al., 1997b].

These primary cultures of MCL cells also expressed constitutively high level of type I collagen mRNA. OP-1 stimulated type I collagen gene expression at the transcription level. In this regard, TGF-β has been reported to stimulate type I collagen expression in healing rabbit MCL [Spindler et al., 2002]. Synthesis of type I collagen begins shortly after injury of the rabbit MCL [Sakai et al., 2001] and remains elevated during healing [Boykiw et al., 1998]. Aggrecan mRNA was also detected in MCL cultures and OP-1 elevated its mRNA level. Thus, OP-1 is capable of stimulating expression of components of the extracellular matrix of the MCL cells. Taken together, the current findings are consistent with the concept that OP-1 is capable of stimulating MCL cells to undergo repair processes.

The present study also revealed that OP-1 stimulated two osteoblastic cell markers, AP and Runx2/Cbfa1. A detectable level of Runx2/Cbfa1 mRNA was observed in long-term MCL cultures, and the level was decreased by OP-1 early in culture, but was stimulated significantly later in culture. Effects of BMPs on the expression of the *Runx2/Cbfa1* gene appear to vary widely depending on the BMP and the cell type. For example, treatment of the myogenic C2C12 cells with BMP-2 and TGF-β resulted in an induction of Runx2/Cbfa1 [Lee et al., 1999, 2000]. In particular, BMP-2 induced the expression of the type II/p57 isoform of Runx2/Cbfa1 [Banerjee et al., 2001]. Treatment with a BMP4/7 heterodimer (100 ng/ml) enhanced Runx2/Cbfa1 mRNA expression [Tsuji et al., 1998]. However, these authors also observed that overexpression of Runx2/Cbfa1 resulted in a suppression of collagen and osteocalcin mRNA expression. By comparison, OP-1 treatment did not stimulate Runx2/Cbfa1 mRNA expression in C2C12 cells [Yeh et al., 2002b]. Additional studies are necessary to elucidate the relationship between the expression of this

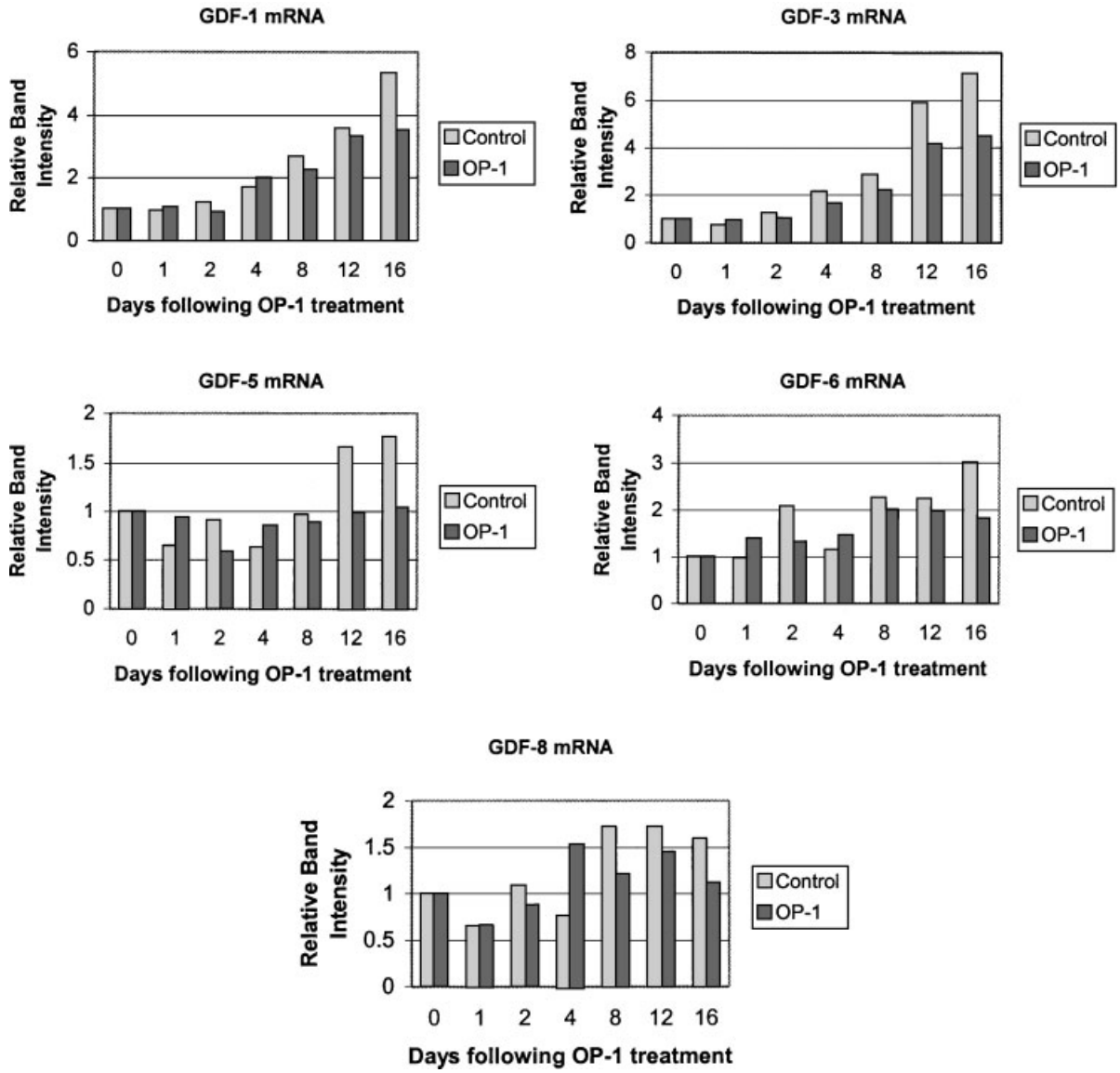

Fig. 9. Quantitative measurements of the steady-state mRNA levels of GDFs in MCL cells. The intensity of the protected fragments as shown in Figure 8 was analyzed and quantified using the ImageQuant software. The data are presented as relative band intensity, which is the value normalized to the housekeeping gene, and then normalized to the control. Values represent the mean ± SEM from two to three different determinations.

transcription factor and the different BMP family members. Nonetheless, the current finding that OP-1 stimulated Runx2/Cbfa1 mRNA expression in a time-dependent manner provides a rational basis for future experimentation into the possible role of this transcription factor in ligament/tendon cell physiology. Moreover, the present findings that the MCL-derived cells expressed genes related to the osteogenic linkage present an intriguing question whether these cells possess mesenchymal stem cell-like properties.

Published animal studies showed that several BMPs could induce ligament formation when implanted in vivo. To aid in furthering our understanding of these in vivo data, we first established the number and identity of BMPs that are expressed in the MCL cells, and then examined their expression pattern in long-term culture. We showed that the MCL cells expressed BMP-1, -2, -4, and -6 mRNA as well as GDF-1, -3, -5, -6, and -8. Although BMP-2 and -4 belong to the same subgroup of BMPs, their spatial and temporal expression patterns are

different. Of the subgroup consisting of BMP-5, -6, -7, and -8, only BMP-6 mRNA was detected. GDF-1 and -3 appear to be the most abundant of the GDFs in MCL cells and their mRNA expression patterns are similar. GDF-5 and -6 belong to the same subgroup and exhibit similar spatial and temporal expression patterns. It is noteworthy to point out that GDF-5 and -6 were shown to induce formation of ligament-like tissues in vivo [Wolfman et al., 1997; Aspenberg and Forslund, 1999; Tashiro et al., 1999; Forslund and Aspenberg, 2001]. GDF-8 appears to be the least abundant of the five detectable GDFs and its level of expression increased as a function of the culture period.

The present study also revealed the effects of OP-1 on the expression of the various BMPs and GDFs. OP-1 suppressed the mRNA expression of all detectable BMPs (except BMP-4) and GDFs to varying extents in MCL cultures. OP-1 suppressed mRNA expression of BMP-1, which cleaves procollagens I and II, and may be involved in the activation of latent TGF-β. This observation is in contrast to the report that TGF-β stimulates type I collagen synthesis in healing rabbit MCL [Spindler et al., 2002]. The mRNA expression levels of BMP-2 and -6 as well as those of all five GDFs detected in MCL cells were suppressed by OP-1. Taken together, the present results provide the first demonstration of the effects of OP-1 on the gene expression of several other BMPs and reveal a complex interplay of these BMPs in MCL cells.

Previous studies suggested that BMPs may regulate their actions by affecting the expression of individual BMP receptors. The present study revealed that three type I BMP receptors (BMPRs) and one type II receptor were expressed in MCL cells. OP-1 stimulated, to varying degrees, mRNA expression of BMPR-IA and BMPR-II, but had little effect on ActR-I and BMPR-IB mRNA expression. Treatment of MCL cells with OP-1 resulted in the most dramatic elevation in BMPR-II mRNA expression. This latter finding is reminiscent of the observation that in primary cultures of fetal rat calvaria (FRC) cells, OP-1 elevated the BMPR-II mRNA most extensively, compared to the other BMP receptors [Yeh et al., 2000]. By comparison, treatment of C2C12 cells with OP-1 resulted in an elevation of the ActR-I mRNA without a significant effect on the mRNA expression of BMPR-IA, -IB, and -II [Yeh et al., 2002b]. Treatment of human SaOS-2 osteosarcoma cells with OP-1 resulted in an increase in the ActR-I mRNA expression and a drop in BMPR-II mRNA level without any changes in the BMPR-IA and -IB mRNA levels. In human TE85 osteosarcoma cells, the mRNA levels for ActR-I, BMPR-IA, BMPR-IB, and BMPR-II were significantly elevated following OP-1 treatment for 24 h. Taken together, it appears that OP-1 not only regulates the expression of individual BMP receptors but also asserts such regulation depending on the tissue types as well as the physiological and differentiation stage of the cell. Whether these observed changes in the steady-state mRNA levels are translated to the surface receptor protein levels requires additional experimentation.

In summary, the current findings establish the temporal appearance of several members of the TGF-β superfamily during MCL cell culturing and by virtue of their expression patterns, suggest specific functional roles. We also showed that OP-1 differentially regulates the mRNA expression of several of these members and their receptors, implying that OP-1 action on ligament growth involves a complex regulation of gene expression of several members of the BMP and the GDF family.

ACKNOWLEDGMENTS

We thank Stryker Biotech for their support.

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