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Due to a production error, Figures 2–5 were printed in black and white. The complete article, with color figures, is presented below.

Temporal and Spatial Expression of Bone Morphogenetic Protein-2, -4, and -7 During Distraction Osteogenesis in Rabbits

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The Ilizarov method of limb lengthening makes use of the fact that osteogenesis is induced at an osteotomy site when distraction is applied. It is unknown at present how the mechanical forces created by distraction are translated into biological signals. Because bone morphogenetic proteins (BMPs) are potent inducers of osteogenesis in many experimental systems, they are obvious candidates for playing a role in this process. In this study, we investigated the temporal and spatial expression of BMP-2, -4, and -7 proteins during distraction osteogenesis using immunohistochemistry. An osteotomy was performed on the right tibiae of white New Zealand rabbits. After a delay of 7 days, distraction was started at a rate of 0.25 mm/12 h for 3 weeks, followed by a 3 week consolidation phase. Each week after osteotomy one rabbit was killed for immunohistochemical studies. Staining for BMP-2, -4, and -7 was evident before distraction was applied and was mainly localized to mesenchymal cells and osteoblastic cells in the periosteal region. After distraction was started, the typical fibrous interzone developed between the osteotomy fragments, where both intramembranous and endochondral ossification were noted. In this area, cells resembling fibroblasts and chondrocytes, but not mature osteoblasts, showed intense staining for all three BMPs. This high level of expression was maintained during the entire distraction phase and then gradually disappeared during the consolidation phase. These results are compatible with the hypothesis that BMPs play an important role in the signaling pathways that link the mechanical forces created by distraction to biological responses. (Bone 27:453–459; 2000)

Key Words: Bone morphogenetic protein; Distraction osteogenesis; Immunohistochemistry; Limb lengthening; Rabbits.

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Introduction

Distraction osteogenesis is a well-established technique for bone lengthening that has widespread clinical applications in the treatment of limb length discrepancies, bone defects, limb deformities, and fracture nonunion. The principles of this method were developed by Gavriel Ilizarov in the early 1950s.[7,8] An osteotomy is performed, followed by fixation with an external fixator. After a latency period of about a week, the osteotomy is subjected to controlled distraction. Thereby, osteogenesis is induced and the bone continues to grow in length as long as the distraction is maintained at an adequate rate. When distraction is stopped, bone lengthening ceases and the newly formed bone in the distracted zone gradually consolidates. Although this technique has revolutionized the treatment of many orthopedic disorders, the main problem is the long period during which external fixation is required (approximately 1–2 months for every centimeter lengthened).

The histological features of distraction osteogenesis have been extensively studied by Ilizarov and others.[7,9,13,21,22] At the site of the osteotomy, a fracture callus forms according to the usual pattern of fracture healing.[14] Distraction alters the mechanical environment within the fracture callus from predominance of compressive to mainly tensile forces.[3,5,7,20] Ilizarov[7,8] called the biological consequences of these mechanical changes the "tension-stress effect." A fibrous interzone develops in the gap between the bone fragments, where fibroblasts secrete collagen fibers in the orientation of the distraction force. Further toward the bone fragments, this fibrous tissue is transformed into bone tissue. This occurs either directly by intramembranous ossification or indirectly through a cartilaginous intermediary.[7,22] Formation of new tissue continues as long as distraction is applied, and therefore, a constant supply of specialized cell types is required during the distraction period. This is achieved by high proliferation rates of periosteal and primitive mesenchymal cells, migration of cells to their site of action, and rapid differentiation into the appropriate cell type.[1,12]

It is unknown at present how the mechanical forces created by distraction are translated into biological signals which induce osteogenesis in such a highly coordinated manner. Bone morphogenetic proteins (BMPs) are obvious candidates for playing an important role in these events. They have proliferative effects

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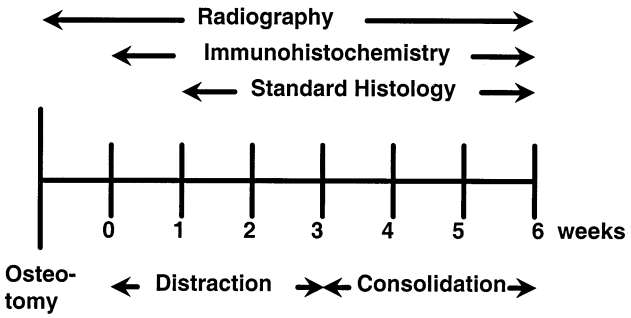

Figure 1. Schematic representation of the study protocol. Time points are indicated in weeks after the start of distraction.

on various cell types, exhibit chemotactic properties, and can induce the differentiation of noncommitted mesenchymal cells into cells of osteoblastic and chondroblastic lineage.[17] Most importantly, BMPs are potent inducers of osteogenesis both during embryological bone formation and in fracture repair.[6,18] Among the members of the large BMP family, BMP-2, -4, and -7 have been shown to be especially important for osteogenesis.[6,18]

Although the known activity profile of BMPs appears to match the requirements exactly for signaling molecules during distraction osteogenesis, there is only scant information on their role during this process. A few studies have examined mRNA expression patterns of various BMPs,[4,11,19] but no data are available on BMP expression at the protein level. Using a rabbit model, we investigated the temporal and spatial expression of BMP-2, -4, and -7 proteins at different time points during distraction osteogenesis. Such data will be important to devise a rational strategy for the use of BMPs in future treatment studies which aim to accelerate the limb-lengthening process.

Materials and Methods

Thirteen skeletally mature (9-month-old) male white New Zealand rabbits, weighing 3.5–4.5 kg, were used. The housing, care, and experimental protocol were approved by the McGill University Animal Care and Ethics Committee.

Operative Protocol

The rabbits were anesthetized by intramuscular administration of ketamine and xylazine. Anesthesia was maintained with halothane, oxygen, and nitric oxide after intubation. A modified Orthofix uniplanar M-100 fixator (Orthofix, Inc., Verona, Italy) was applied to the right tibia under sterile conditions. Four half-pins were inserted, two above and two below the osteotomy site. The tibia was exposed subperiosteally, and the osteotomy was performed with an oscillating saw just below the fusion site between tibia and fibula. The periosteum was reapproximated and the wound closed. Unrestricted weight bearing and activity were allowed postoperatively. After a delay of 7 days, distraction was started at a rate of 0.25 mm/12 h for 3 weeks (**Figure 1**). This was followed by a period of 3 weeks, during which the external fixator was held in place with no distraction (consolidation phase).

The rabbits were examined daily for signs of infection, weight loss, and pain. None of the animals had these manifestations. Antero-posterior and lateral X-ray views of the lengthened tibiae were taken weekly. Each week after osteotomy, one (at time 0) (Figure 1) or two (at 1–6 weeks) rabbits were killed by intravenous injections of Euthanyl (MTC Pharmaceuticals, Cambridge, Ontario). At each time point, material from one animal was used for immunohistochemistry. The samples from the other rabbits were used for standard histology.

Sample Preparation

After the rabbits were killed, the external fixator was removed and the right tibia was resected. One nonoperated left tibia was used as a control. Specimens from rabbits assigned to histology of undecalcified sections were embedded in methylmethacrylate. Undecalcified 6 μm sections were stained with Goldner Trichrome. Specimen harvested for immunohistochemical analysis were fixed in 4% paraformaldehyde overnight, decalcified in 20% ethylenediamine tetraacetic acid for 3 weeks, and embedded in paraffin, and 7 μm sections were cut. Parallel sections were taken so that both the temporal and the spatial expressions of BMP-2, -4, and -7 were evaluated and compared with each other.

Immunohistochemistry

After deparaffinization and hydration, endogenous peroxidase was blocked with 1% hydrogen peroxide for 10 min. Nonspecific binding was blocked by incubation in phosphate-buffered saline containing 1% blocking reagent (Boehringer Manheim Canada, Laval, Quebec, Canada) and 0.1% Triton for 30 min. For immunostaining, commercially available polyclonal goat anti-BMP-2, -4 and -7 antibodies were used (SantaCruz Biotechnologies, Santa Cruz, CA). Sections were incubated with these primary antibodies (25 μg/mL in phosphate-buffered saline with 1% blocking reagent and 0.1% Triton) overnight at 4°C in a humidified chamber. For negative controls, the primary antibody was omitted. A biotinylated antigoat antibody was used as secondary antibody. Sections were stained using the avidin–biotin complex method (Vector Labs, Burlingame, CA) and 3,3′-diaminobenzidine tetrachloride. Finally, the sections were counterstained with hematoxylin and mounted.

According to data provided by the manufacturer, the primary antibodies used in the present study are known to recognize mouse, rat, and human BMPs. Therefore, we tested whether these antibodies also recognize rabbit BMPs to verify whether the observed staining pattern represented BMP specific signal. According to the manufacturer's instructions, 100 μL of goat BMP-blocking peptide at a concentration of 200 μg/mL was inserted in a speedvac (Savant, Farmingdale, NY) to obtain the blocking peptide in a powder form. This was mixed with 20 μL of primary antibody (concentration 200 μg/mL) and preincubated overnight at 4°C. Then, the same protocol as for the sections without the blocking peptides was used (same incubation time of 1 h). When sections were treated as described above, no staining was evident. Thus, the antibodies used in the present study recognized rabbit BMPs.

Quantitation

The number of cells expressing BMP-2, -4, and -7 protein was assessed by cell counting. Chondrocytes, osteoblastic, and fibroblastic cells were identified morphologically. These analyses were performed separately for the callus region and the central region containing the fibrous interzone.

Results

Radiography and Standard Histology

Two weeks after the start of distraction, new mineralized bone became apparent radiologically in the distraction zone (not

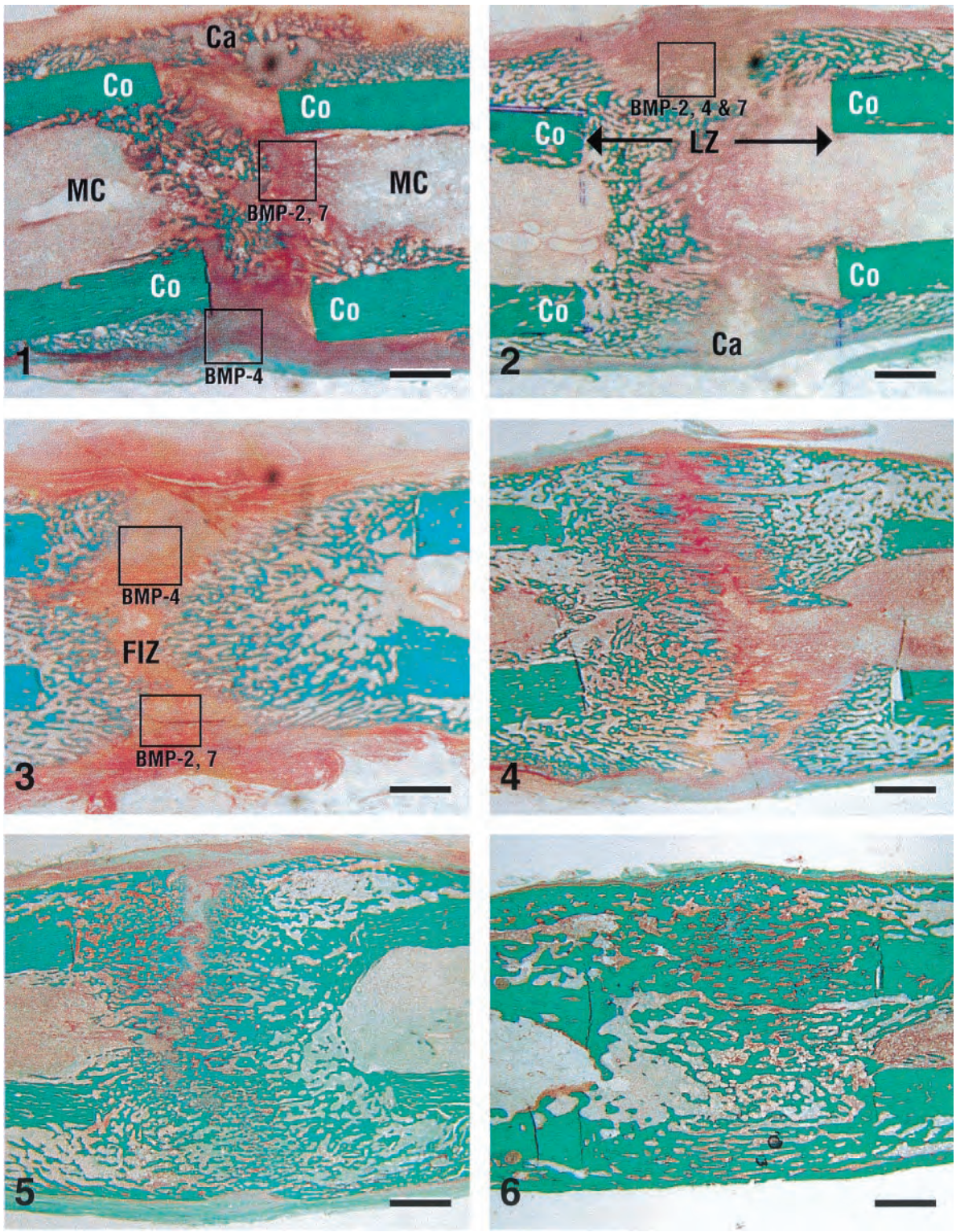

Figure 2. Goldner-stained sections of rabbit tibiae during distraction and consolidation. Mineralized bone stained green. The numbers indicate the number of weeks after distraction was started. 1–3 distraction phase; 4–6 consolidation phase. The locations of the sections used for immunohistochemistry (see Figures 3–5) are indicated. Co, cortex; LZ, lengthened zone; Ca, callus; FIZ, fibrous interzone. Bar scale = 2 mm.

shown). A radiolucent area remained visible until 1 week after the end of distraction. At 3 weeks into the consolidation phase, the distracted zone was completely bridged with new bone, but the cortices were not yet demarcated.

During the distraction phase, the distance between osteotomy ends increased and a fibrous interzone developed (**Figure 2**). In the middle of this zone, elongated cells produced large amounts of fibrillar matrix. Next to this fibrillar region there were numer-

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Table 1. Bone morphogenetic protein (BMP) expression during distraction osteogenesis: quantitative analysis

		Center			Callus		
Protein	Week	Osteoblastic cells (preosteoblasts)	Chondrocytes	Fibroblastic cells	Osteoblastic cells (preosteoblasts)	Chondrocytes	Fibroblastic cells
BMP-2	1	+	++	+	+	++	+
	2	+	+++	++	+	+++	++
	3	+	++	++	+	++	++
	4	+	+++	++	+	+++	+
	5	+	+	−	+	+	−
	6	−	−	−	−	++	−
BMP-4	1	+	+	+	+	++	+
	2	+	+	+	+	+	+
	3	+	+	+	+	+	−
	4	+	+	+	+	+	+
	5	−	+	−	+	+	−
	6	−	−	−	−	++	−
BMP-7	1	+	+	+	+	+	+
	2	+	++	+	+	++	++
	3	+	+	+	+	++	+
	4	+	+	++	+	+	+
	5	+	−	−	+	+	−
	6	−	−	−	−	++	−

KEY: −, no positive staining; +, less than one third of cells positive; ++, one third to two thirds of cells positive; +++, more than two thirds of cells positive. Weeks 1–3: distraction phase; weeks 4–6: consolidation phase.

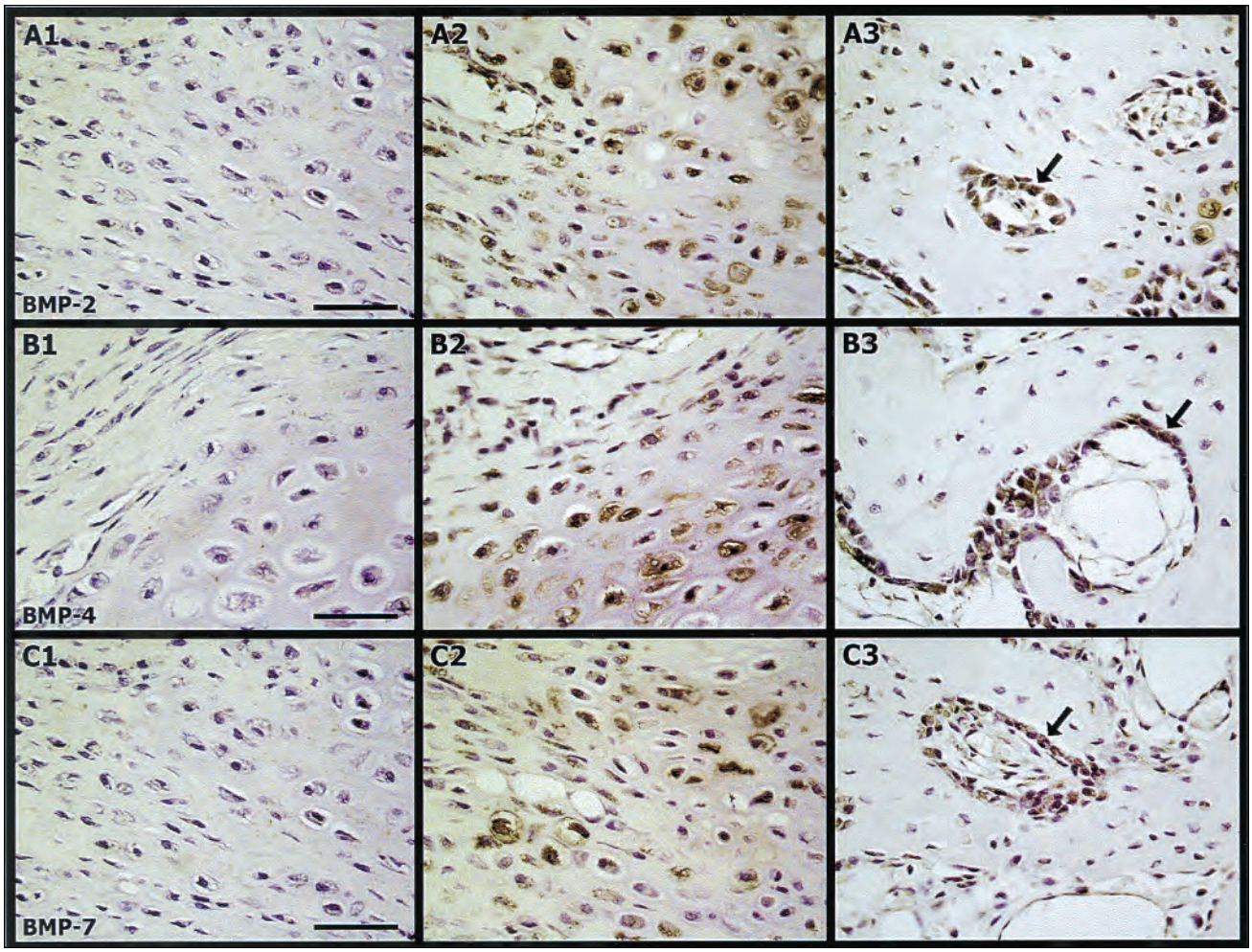

Figure 3. Immunohistochemistry 1 week after the start of distraction. The location of these sections is indicated in Figure 2. Row A: bone morphogenetic protein (BMP)-2; row B: BMP-4; row C: BMP-7. Negative controls are shown in A1, B1, and C1. BMP expression in fibroblastic cells and chondrocytes is shown in A2, B2, and C2. BMP expression in osteoblastic cells (arrows) is shown in A3, B3, and C3. Bar scale = 100 μm.

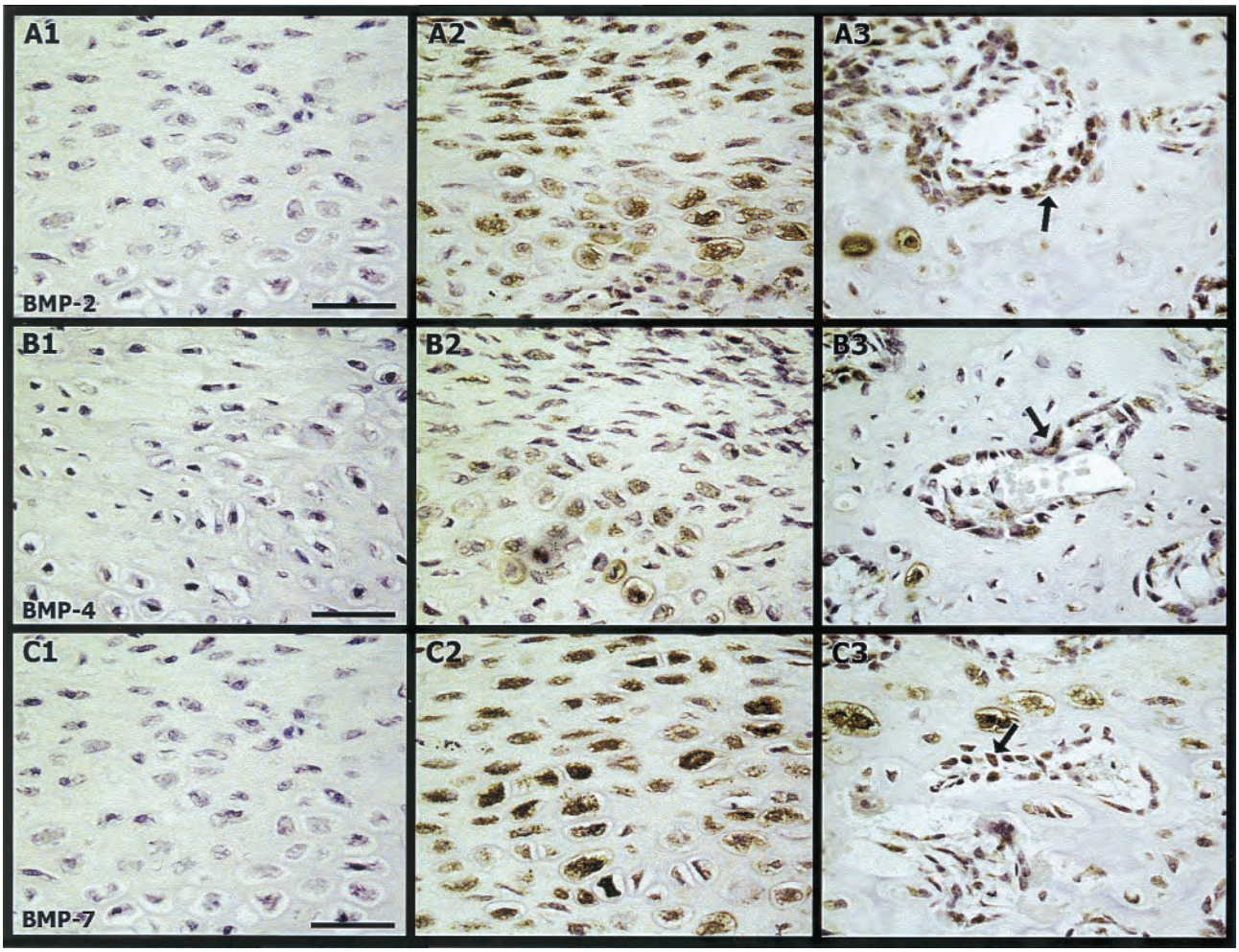

Figure 4. Immunohistochemistry 2 weeks after the start of distraction. The location of these sections is indicated in Figure 2. Row A: BMP-2; row B: BMP-4; row C: BMP-7. Negative controls are shown in A1, B1, and C1. BMP expression in fibroblastic cells and chondrocytes is shown in A2, B2, and C2. BMP expression in osteoblastic cells (arrows) is shown in A3, B3, and C3. Bar scale = 100 μm.

ous cells which morphologically represented a continuum between fibroblasts and chondrocytes. In neighboring locations osteoblasts arose. Thus, there was a mixture of endochondral and intramembranous bone formation. However, endochondral bone formation clearly predominated in the area between the cortical fragments.

Immunohistochemistry

A quantitative evaluation of BMP protein expression is given in **Table 1**. **Figures 3–5** provide representative examples of immunostained sections. Staining for BMP-2, -4, and -7 was evident at 1 week after surgery, even before distraction was started. At that point, the signal was mainly localized to mesenchymal cells and preosteoblasts in the callus region (not shown).

During the distraction period, a marked and continuous increase in BMP expression was noted both in the callus region and in the fibrous interzone, at the center of the distraction zone (Table 1 and Figures 3–5). Both cells resembling fibroblasts and chondrocytes stained intensively for all three BMPs throughout the whole duration of the distraction phase (Table 1 and second column in Figures 3–5). Mature osteoblasts did not express BMPs, but preosteoblasts and early osteoblasts did (Table 1 and third column in Figures 3–5). There was no positive signal in the control nonoperated tibia.

The expression of BMP-2, -4, and -7 started to decrease when distraction stopped (Table 1). Only the chondrocytes in the central fibrous interzone and the periosteal areas continued to show strong positive staining during the early consolidation phase.

Of the three proteins, BMP-2 and -7 exhibited the most intense signal (Table 1). All three BMPs appeared to be located intracellularly, although BMP-7 was also detectable in the extracellular space close to chondrocytes.

Discussion

Distraction osteogenesis can be regarded as a form of fracture repair which occurs under specific mechanical conditions. After an artificial fracture is created by osteotomy, callus formation proceeds similar to other types of fracture repair.[14] It is thus not surprising that our observations on BMP expression in callus tissue before the start of distraction matched the results obtained in other models of fracture repair.[2,10,15,16] After a fracture, expression of BMP-2, -4, and -7 is quickly induced in cells close to the periosteum and appears to be limited mostly to immature

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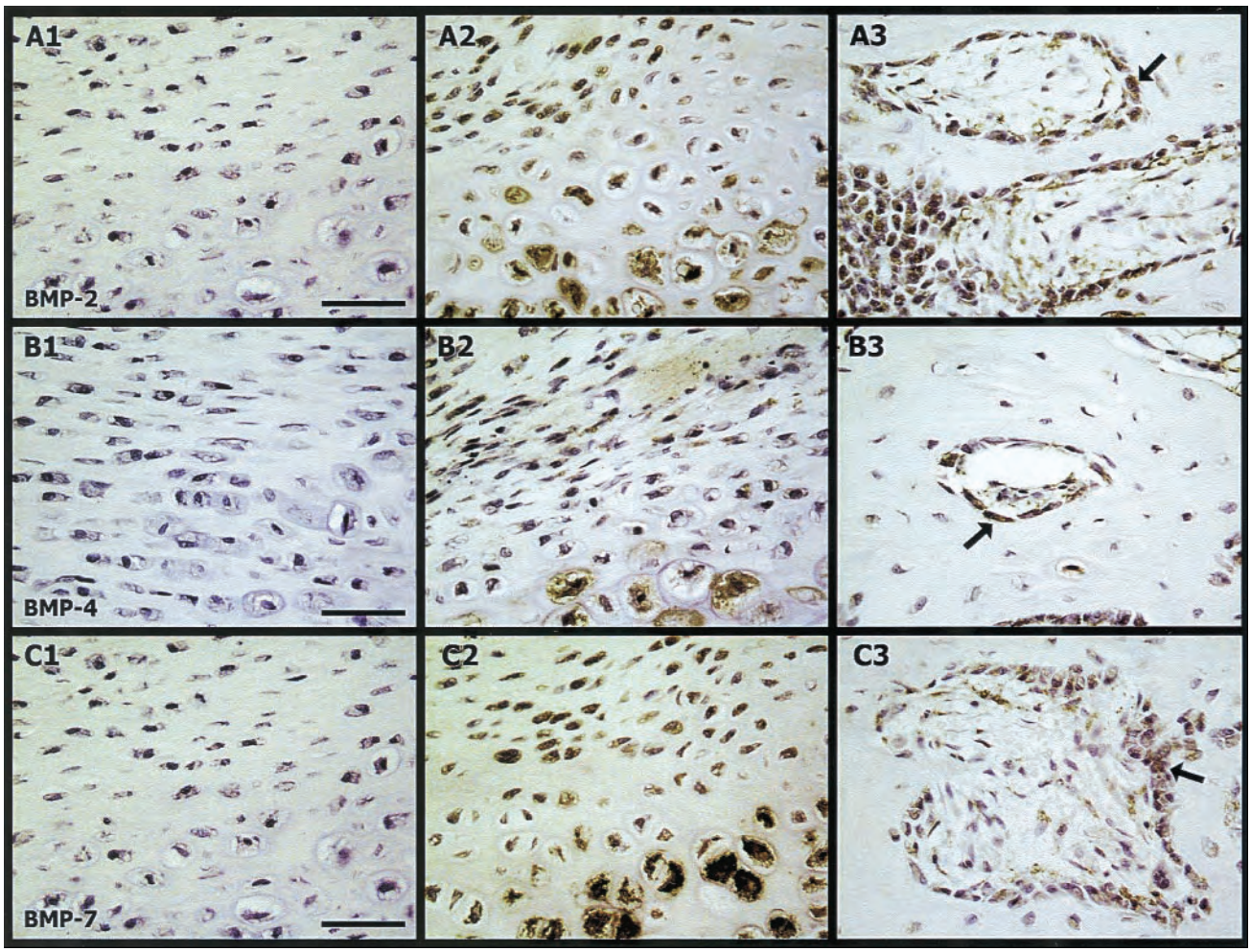

Figure 5. Immunohistochemistry three weeks after the start of distraction. The location of these sections is indicated in Figure 2. Row A: BMP-2; row B: BMP-4; row C: BMP-7. Negative controls are shown in A1, B1, and C1. BMP expression in fibroblastic cells and chondrocytes is shown in A2, B2, and C2. BMP expression in osteoblastic cells (arrows) is shown in A3, B3, and C3. Bar scale = 100 μm.

cells.[2,10,15,16] As confirmed by our data, BMP expression is high before significant new bone formation is evident by either radiography or histology. Thus, the temporal pattern of BMP expression is consistent with a role of BMPs in the regulation of new bone formation.

As soon as distraction is applied, the patterns of BMP expression start to diverge between normal fracture repair and distraction osteogenesis. As the callus tissue matures, BMP expression decreases in the usual fracture repair process.[2,10,15,16] In contrast, as shown in the present study, expression of BMP-2, -4, and -7 protein increases after distraction is started. This confirms earlier reports that examined the expression of BMP-2 and -4 on the mRNA level.[4,11,19] When distraction is discontinued, BMP expression appears to be quickly down-regulated with the exception of chondrocytes. This is in accordance with reports on the pattern of BMP-2 and -4 mRNA expression during the consolidation phase.[19] However, BMP-7 mRNA expression is not induced during distraction osteogenesis in rats,[19] whereas the BMP-7 protein was up-regulated in our rabbit model. Possibly there are species differences that could account for this discrepancy.

The temporal pattern of expression strongly suggests that cellular BMP production is directly or indirectly enhanced by the mechanical stimulus provided by distraction. Our data do not provide insight into the downstream effects of increased BMP expression. However, BMPs are known to stimulate the proliferation of precursor cells[6] and the temporal and spatial expression of BMPs appears to match that of the proliferative activity in the distracted callus.[1] In addition, BMPs can induce the differentiation of mesenchymal cells into both chondrocytes and osteoblast lineages.[18] In fact, we observed both intramembranous and endochondral ossification in our system. Thus, our findings are compatible with the hypothesis that BMPs are implicated in the regulation of both precursor cell proliferation and tissue differentiation during distraction osteogenesis.

In summary, our data suggest that the change in mechanical environment created by distraction leads to increased BMP expression. Thus, BMPs could play an important role in the signaling pathways that link the mechanical forces created by distraction to cellular responses. From a more clinical perspective, our observations indicate that attempts to accelerate the process of distraction osteogenesis by exogenous BMPs should focus on the consolidation phase after endogenous BMP production has stopped.

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