

of the augmented grafts to the host bed be evidenced by increased callus formation around the grafts and by evidence of early bone turnover of the devitalized bone leading to the appearance of new bone in the augmented grafts?; and (3) Would the augmented femoral, intercalated, devitalized bone segments acquire more sufficient mechanical bone strength than the unaugmented segments?

MATERIALS AND METHODS

Fifty inbred Sprague-Dawley male rats (7 weeks old, approximately 300 g body weight; Japan SLC Co, Shizuoka, Japan) were randomly divided into three groups (30 for the experimental group, 20 for two control groups) (Table 1).

Each animal was anesthetized by an intramuscular injection of ketamine (30 mg/kg body weight) and xylazine (10 mg/kg body weight). After removal of hair with an electric hair cutter and sterilization of the right thigh skin with 10% povidone iodine and 80% ethanol, the middiaphysis of the femur was exposed through a lateral longitudinal skin incision. An 8-mm-long bone segment with the periosteum attached was removed with a cutting saw. After removing the soft tissue from the bone segment, the bone segment then was autoclaved at 135°C for 15 minutes and reimplanted into the femoral defect. In the experimental group, the cortical surface of the reimplanted bone segment was coated with rhBMP-2-retaining paste (60 mg, 30 mg, or 15 mg in weight) prepared as described below. The cortical surfaces of the replanted segments from animals from the control groups were coated with either 60 mg paste without rhBMP-2 or no paste (CP and C groups, respectively). After reimplantation, all segments were fixed using an 18-gauge injection needle inserted as an intramedullary pin. The wound was closed with 4-0 nylon sutures.

The rhBMP-2-retaining paste consisted of rhBMP-2 (Genetics Institute, Boston, MA); polylactic acid/polyethylene glycol block copolymer (PLA-PEG), a synthetic biodegradable polymer with a molecular weight of 9200 (Taki Chemicals Co Ltd, Kakogawa, Japan); and beta-tricalcium phosphate (β -TCP) powder with a particle size less than 100 μ m in diameter (Olympus Optics Co, Tokyo, Japan). The physicochemical characteristics and efficacy of the block copolymer as carrier material for rhBMP-2 has been reported.^{21,23} To generate the rhBMP-2-retaining paste, we warmed 300 mg PLA-PEG polymer to 50°C, added 100 μ g rhBMP-2 and 300 mg β -TCP powder, mixed well,

and cooled to yield a doughlike paste. Another batch that lacked rhBMP-2 was made and used as a control. Both batches were stored in a deep freezer at -70°C until use.

At surgery, the animals from the experimental group were subdivided into three groups (10 per respective group) and 60 mg, 30 mg, or 15 mg of the rhBMP-2-retaining paste was put on the cortical surface of the autoclaved bone segments. These groups were designated BP60, BP30, and BP15, respectively. During the postoperative period, all rats were fed normal diets. All animals were housed and fed in cages without any limitation of motion and with free access to food and water.

The effect of the application of the rhBMP-2-retaining paste on the incorporation of the reimplanted autograft segments into the femoral defect was evaluated by radiographic, histologic, and biomechanical tests. Plain radiographs of the hind limbs were taken under anesthesia to check for new bone formation in and around the femoral defect sites. The appearance of callus was assessed radiographically and mean radiographic scores were calculated for each group according to the modified scoring system proposed by Yuehwei³⁷ (Table 2). Loss of length of the right femurs resulting from collapse of the devitalized bone segments was measured with calipers and was expressed as a percentage of the length of the contralateral, intact femur. The femurs obtained at each respective interval (2, 4, 8, and 12 weeks after surgery) from each group were processed for routine histologic examination to identify newly formed cartilage, bone, and marrow tissue. Before histologic processing, the intramedullary fixation pins were removed from the femurs. The specific areas of interest for the radiographic and histologic examinations were the junctions of the devitalized bone with the vital cut ends and the circumference of the reimplanted devitalized bone segments. The samples were fixed in neutral-buffered 10% formalin solution, decalcified in 5% formic acid at 4°C, dehydrated in a gradient of ethanol solutions, embedded in paraffin, sectioned at the mid-sagittal plane at 5- μ m thickness, and stained with hematoxylin and eosin. Tartrate-resistant acid phosphatase (TRAP), a marker enzyme specific to osteoclasts, was used to identify these cells in contact with the devitalized bone segments. Briefly, deparaffinized sections were placed in the TRAP staining solution consisting of acetate buffer (pH 5.0) containing 50 mmol/L sodium tartrate, 25 mg/mL naphthol AS-MX phosphate (Sigma Chemical Co, St Louis, MO), and 0.5 mg/mL fast red violet salt (Sigma). The specimens were incubated with the solution at 37°C for 20 minutes. After the solution was removed by washing, the specimens were counterstained with hematoxylin. All of the prepared histologic sections were observed under a light

TABLE 1. Implant Assignment

Group	rhBMP-2 (μ g)	PLA-PEG (mg)	β -TCP (mg)	Concentration of rhBMP-2 (% weight)	Number of Animals
BP60	10	30	30	0.0166	10
BP30	5	15	15	0.0166	10
BP15	2.5	7.5	7.5	0.0166	10
CP60	0	30	30	0	10

PLA-PEG = polylactic acid/polyethylene glycol; β -TCP = beta-tricalcium phosphate

TABLE 2. Radiographic Scoring System

Category	Score
New bone formation in the defect	
Full (across the defect)	3
Moderate (> 50%)	2
Mild (< 50%)	1
None	0
Proximal osteotomy union	
Union	3
Moderate bridge (> 50%)	2
Mild bridge (< 50%)	1
Nonunion	0
Distal osteotomy union	
Union	3
Moderate bridge (> 50%)	2
Mild bridge (< 50%)	1
Nonunion	0
Graft-host bone junction	
Cortex to cortex (both side)	3
Cortex to cortex (one side)	2
Cortex to trabecula	1
No connection	0
Remodeling	
Full remodeling cortex	2
Intramedullary canal	1
No remodeling	0
Maximum total score	14

microscope. At 12 weeks postoperatively, five animals from each group were sacrificed and the femurs of both sides were harvested. After being radiographed, the harvested femurs were subjected to mechanical testing. The mechanical strength of the femurs with autoclaved bone segments was tested in two steps, ie, manual bending and three-point bending strength tests using a mechanical apparatus (EZ Graph; Shimazu Co, Kyoto, Japan) equipped with a computer for data acquisition. When the reim-

planted site of the femur was unstable and flexed easily with mild manual bending, repair of the defect was judged to have failed. When the defect site was solid, the femur was judged to be consolidated and repaired. For quantitative evaluation of the consolidation, the samples were subjected to the three-point bending machine test to evaluate maximum bending strength of the reimplanted femur. Contralateral femurs also were tested in the same manner to obtain normal standard values. The femurs were placed horizontally on two rounded supporting bars located at a distance of 20 mm. The bone was loaded at the midpoint of the diaphysis at the osteogenesis area, including the transplantation site in the anteroposterior plane, by lowering the third bar at a rate of 1 mm per minute until fracture occurred and the load displacement curve was recorded. The ultimate load, and the force causing fracture, was determined from the load displacement curve.

Intergroup comparisons of the radiographic scores, differences in femoral length, and results of the biomechanical testing of the femurs were performed using the Kruskal-Wallis test. Significance was defined as *p* values less than 0.01. When appropriate, differences between experimental groups were evaluated with the post hoc Bonferroni/Dunn test. All sections and radiographic images were viewed by two individuals (TN, KT) who were blinded to the results.

RESULTS

When assessed by gross appearance at 12 weeks after surgery, the reimplanted femurs harvested from the BP15 group were found to be completely repaired as opposed to none of those in the control groups CP60 and C (Fig 1). The average lengths of the reimplanted femurs expressed as a percentage of the contralateral intact femur after 12 weeks were 92.3% in the C group and 97.8% in the CP60 group. No reduction in the femoral length was seen in the

Fig 1A-C. The gross appearance of femurs harvested 12 weeks after reimplantation of 8-mm-long autoclaved segments into intercalated defects is shown. (A) The devitalized bone segment was coated with 15 mg rhBMP-2-retaining paste (containing 2.5 µg rhBMP-2) at surgery. (B) The segment was coated with 30 mg paste without rhBMP-2. (C) The segment was not coated with the paste. The reimplanted bone segment (arrow) is completely repaired in (A) but not in (B) and (C).

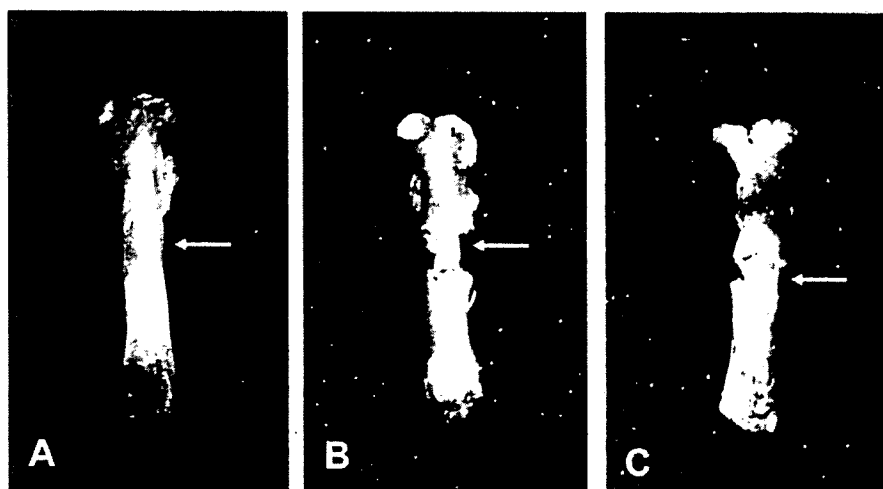
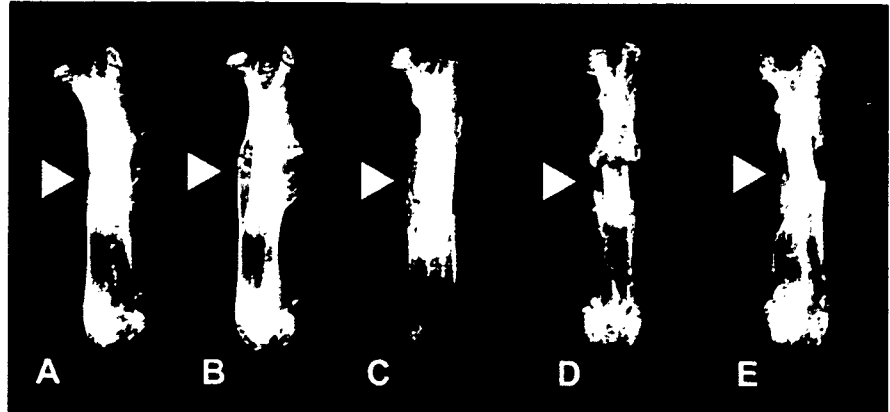


Fig 2A–E. Radiographs of femurs reimplanted with autoclaved bone segments with or without rhBMP-2-retaining paste and harvested 4 weeks after surgery are shown. (A) In the BP60 group, autoclaved bone was coated with 60 mg rhBMP-2-retaining paste (containing 10 μ g rhBMP-2). (B) In the BP30 group, autoclaved bone was coated with 30 mg rhBMP-2-retaining paste (containing 5 μ g rhBMP-2). (C) In the BP15 group, autoclaved bone was coated with 15 mg rhBMP-2-retaining paste (containing 2.5 μ g rhBMP-2). (D) In the CP60 group, autoclaved bone was coated with 60 mg paste without rhBMP-2. (E) In the C group, autoclaved bone was left uncoated. Arrowheads indicate the original defect sites reimplanted with autoclaved bone segments. The callus is more evident bridging the cut ends of femur defects in the BP groups. In control animals (the CP60 and C groups), callus formation was less obvious than in the BP groups.



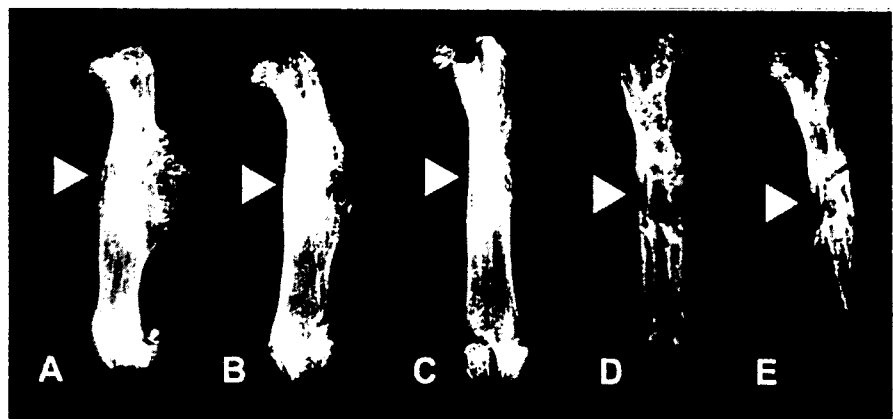
BP60 (100.7%), BP30 (100.7%), and BP15 (100.5%) groups. The control femurs (CP60 and C) were shortened ($p < 0.01$) compared with those in the BP groups.

At 4 weeks after reimplantation, encapsulating callus formation was observed in all BP groups. Shell-shaped calcified shadows bridging the cut ends of the femur and encasing the reimplanted devitalized bone were seen. In the controls (CP60 and C), small areas of callus were seen, but these did not bridge the cut ends (Fig 2). At 12 weeks, the callus containing the reimplanted devitalized bone in the BP60 and BP30 group animals was remodeled to form cortical bone, with the unresorbed, reimplanted bone visible inside the new cortical bone. In the BP15 group animals, the bone defects were repaired with normal anatomic appearance. The femurs from the controls showed no obvious new bone or callus formation around the devitalized

bone segments (Fig 3). The radiographic scores were higher ($p < 0.01$) in all BP groups compared with those of the controls at 4, 6, and 12 weeks after reimplantation (Fig 4).

Histologic sections prepared from all BP groups revealed qualitatively similar reactions over the experimental period. At 4 weeks, the newly formed bone was partially remodeled to form cortical bone connecting the cut ends of the femur, and new living bone and bone marrow were present in the marrow space. In the controls, no new bone was observed and the dead bone segments were not united to the cut ends of the femur (Fig 5). At 12 weeks, the diameter of the repaired bone in the BP groups remained larger than that of normal femurs, and part of the cortical bone of the reimplanted segment appeared to be incorporated to the host bone (Fig 6).

Fig 3A–E. Radiographs of femurs reimplanted with autoclaved bone segments with or without rhBMP-2-retaining paste and harvested 12 weeks after surgery are shown. (A) BP60, (B) BP30, (C) BP15, (D) CP60, and (E) C groups are described in Figure 2. Arrowheads indicate the original defect sites reimplanted with autoclaved bone segments. Newly formed bone mass size was proportional to the amount of paste implant material in the BP groups. The femurs from the control animals (the CP60 and C groups) showed no obvious new bone or callus formation around the devitalized bone segments.



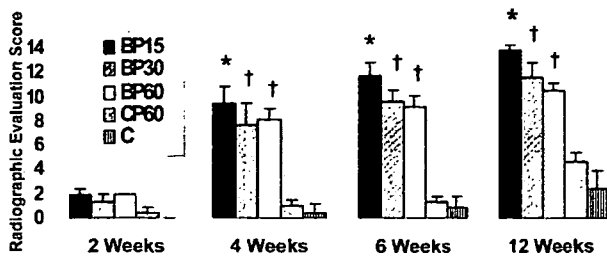


Fig 4. Radiographic evaluation score was measured at each period after surgery. Ten animals at 2 weeks, eight at 4 weeks, seven at 6 weeks, and five at 12 weeks were assigned to each group postoperatively. The bar represents mean \pm standard deviation. * = $p < 0.01$ (compared with all other groups); † = $p < 0.01$ (compared with the C and CP60 groups). The scores in all BP groups were substantially higher than those of the control animals (the CP60 and C groups) at 4, 6, and 12 weeks.

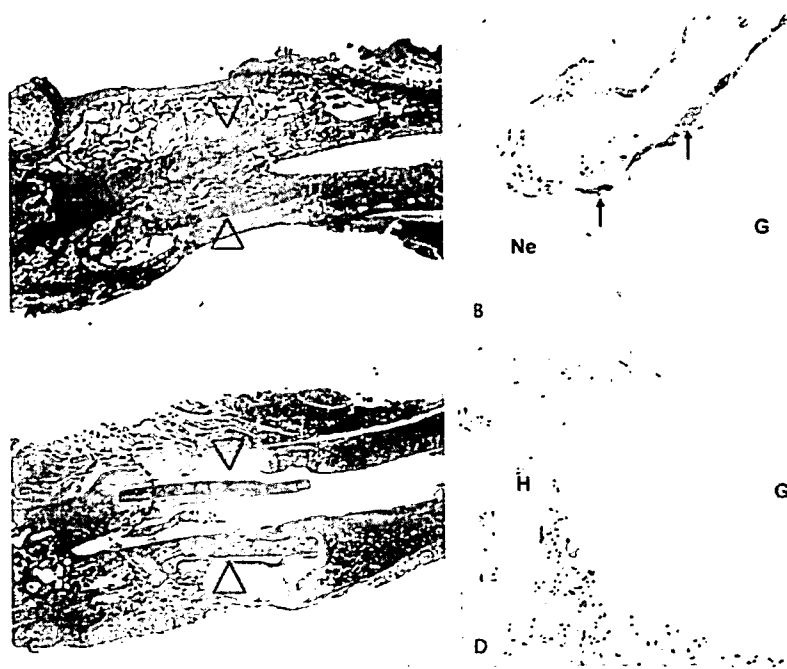
As assessed using manual testing of the samples harvested at 12 weeks, all of the femurs from the three BP groups were estimated to have successful consolidation, whereas those from the control animals had failed consolidation (Table 3). The mean values of maximum strength of the reimplanted femurs as assessed on the three-point bending tests indicated that the three BP groups were stronger ($p < 0.01$) than those from the control groups (Fig 7). Among the three BP groups, the maximum strength was higher ($p < 0.01$) in the BP15 group despite less callus formation than in the other two groups.

DISCUSSION

We assessed a method of reconstructing rat femoral defects using reimplantation of the devitalized, resected segments coated with rhBMP-2 in a biodegradable carrier. The results suggest reimplantation of extracorporeally devitalized autogenous bone segments predictably repair critical-sized rat femoral defect when combined with a rhBMP-2-containing resorbable paste. This method potentially could be used to reconstruct defects frequently encountered during limb salvage surgery for treatment of musculoskeletal malignancies. When considering the practical application of this repair method in conjunction with the devitalized tumor-bearing bone, the dose of rhBMP-2 should be reconsidered because the minimal essential dose of rhBMP-2 is different depending on the recipient species.³⁴ In highly evolved animals or humans, the responsiveness to rhBMP-2 is much lower than in rodents. The dose of rhBMP-2 per square centimeter of devitalized bone surface was 1.4 μg in our study. If the responsiveness of humans is 20 times lower than that of rats, the essential dose in humans may be closer to 28 $\mu\text{g}/\text{cm}^2$. Also, as a previous study indicated,¹⁷ responsiveness to rhBMP-2 is age-dependent. Therefore, additional preclinical experiments in a large animal model and/or in primates and, ultimately, clinical trials will be required to optimize the dose of rhBMP-2.

The reimplantation of a devitalized, autogenous bone segment was first reported by Galle in 1918 with use of

Fig 5A–D. Histologic sections prepared from (A, B) the BP15 group and (C, D) the C group at 4 weeks after surgery are shown (A, C: stain, hematoxylin and eosin; original magnification, $\times 40$; B, D: higher-magnified views from the circled areas; stain, TRAP; original magnification, $\times 200$). Ne = newly formed bone; G = reimplanted bone; H = host bone. Arrowheads indicate the implanted autoclaved bone segment and arrows indicate TRAP-positive cells or osteoclasts on the surface of the autoclaved bone. The reimplanted bone segment was encased in the newly formed bone with marrow and the autoclaved bone was incorporated into the repaired bone in the BP15 group. The reimplanted bone was not repaired by new bone formation in the C group.



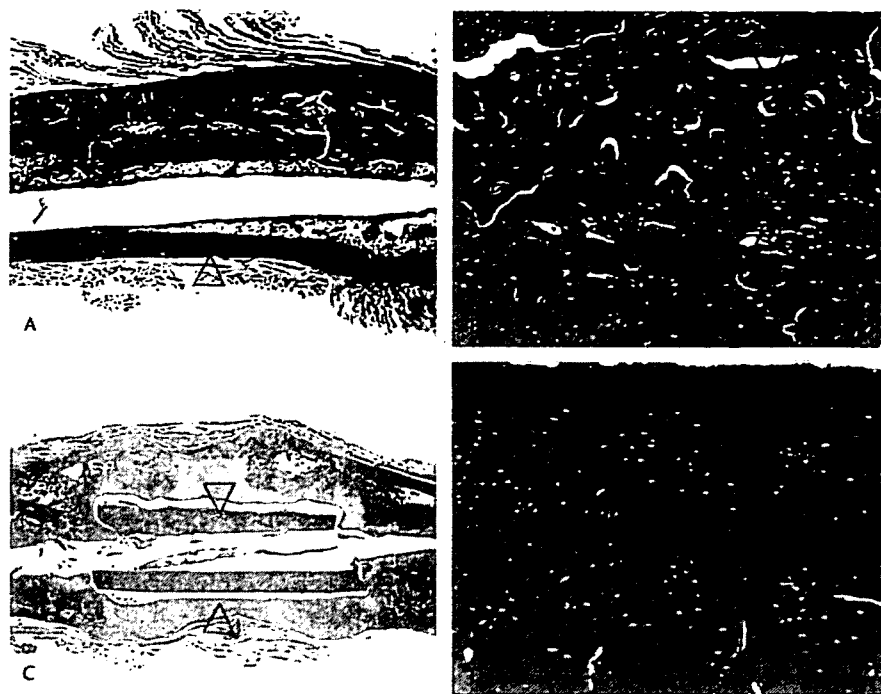


Fig 6A–D. Histologic sections prepared from (A, B) the BP15 group and (C, D) the C group at 12 weeks after surgery are shown (A, C: stain, hematoxylin and eosin; original magnification, $\times 40$; B, D: longitudinal sections of cortical bone of autoclaved segments; stain, hematoxylin and eosin; original magnification, $\times 200$). The reimplanted bone segment is partially resorbed and replaced with new bone (arrowheads) in the BP15 group. The reimplanted bone was embedded in fibrous tissue and remodeling activity was not seen in the C group.

boiled tumor-bearing bone mass. Since then, numerous similar reports have been published, and the reimplantation of devitalized tumor-bearing bone segments have been an option for reconstruction of bone defects generated by wide resection of malignant bone tumors, especially when the resected bone has enough strength to support the mechanical loads applied to the affected bone. Although the reimplanted bone tissue offers advantages in terms of being able to match the size of the defect, a low risk for local tumor recurrence, avoidance of immune reactions, and low cost, the clinical results have been disappointing as a result of delayed or nonunion of the reimplanted bone and gradual resorption of the reimplanted graft leading to mechanical weakness and fracture. These disadvantages appear to result from the lack of the osteogenic potential of the reimplanted bone devitalized by au-

toclaving, pasteurization, or radiation.^{3,30} Restoration of the osteogenic potential of the reimplanted bone should improve the clinical results without loss of the advantages described. This prompted us to look at the possibility of overcoming these problems by combining rhBMP-2 and its delivery system with the devitalized resected bone.

In the rat bone defect model used in our study, rhBMP-2 was used because of its known biologic efficacy

TABLE 3. Union Rates Assessed by Radiography and Manual Palpation

Group	Number of Unions by Radiography	Number of Unions by Manual Palpation
BP60	5/5	5/5
BP30	5/5	5/5
BP15	5/5	5/5
CP60	2/5	0/5
C	0/5	0/5

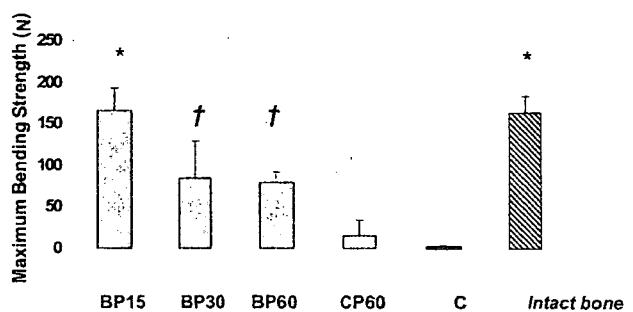


Fig 7. Reimplanted femurs harvested 12 weeks postoperatively were subjected to a three-point bending test. The bar represents mean \pm standard deviation ($n = 5$). * = $p < 0.01$ (compared with the BP30, BP60, CP60, and C groups); † = $p < 0.01$ (compared with the CP60 and C groups). Among the three BP groups, the maximum strength was substantially higher in the BP15 group.

in nonhuman species.^{13,22,25} Clinical use of rhBMP-2 has been approved by the FDA and often is used in combination with a biodegradable bovine collagen carrier for spinal fusion surgery in the United States.^{4,8} In our study, a synthetic biodegradable block copolymer (PLA-PEG) was developed to serve as an alternative biodegradable carrier for rhBMP-2.^{10,26,27} This synthetic biodegradable polymer and β -TCP powder were used instead of collagen.¹⁸ Adding the β -TCP powder in an amount equal to the polymer modifies the sticky gel-like nature of the PLA-PEG at room temperature to create a less sticky dough that was coated on the surface of the devitalized bone segments. As expected, the dough or paste implant was resorbed and new bone was formed, probably through the sustained release of the rhBMP-2 from the degrading paste.

Although we noted a correlation between the amount of rhBMP-2-retaining paste and newly formed bone mass around the reimplanted bone segments on radiographs at times longer than 4 weeks after reimplantation, the mechanical strength of the repaired femurs at 12 weeks did not correlate with the new bone mass around the devitalized bone segments. Unexpectedly, the femur that received 15 mg of paste and yielded the smallest amount of callus most effectively restored the strength of the intact femur at 12 weeks. Devitalized bone segments that received larger amounts of the rhBMP-2-containing dough (BP60 and BP30 femurs) did not show a more rapid recovery in bone strength. Thus, the optimal amount of dough in our model was 15 mg, representing an optimal rhBMP-2 dose of approximately 2.5 μ g. A possible explanation might be that the higher doses of rhBMP-2 led to a dose-dependent larger callus with a mechanically weaker structure at the periphery, resulting in comparative weakness on the three-point bending test. Radiographic and histologic features in the BP15 group indicated a more complete remodeling of the callus resulting in a thicker cortex with greater strength. These features might be the consequence of an accelerated remodeling of the callus at this optimal rhBMP-2 dose. Despite larger calluses in BP30 and BP60 samples, a thinner cortical shell resulting from inadequate remodeling might have resulted in the weakness, suggesting further time for remodeling to achieve comparable mechanical strength is needed at higher rhBMP-2 doses. Another possible explanation is that this is the result of an increased rate of bone resorption in the presence of higher doses of rhBMP-2, as described in previous reports.¹⁹ In fact, histologic examination at 12 weeks showed there was less trabecular bone inside the shell-like calluses in the BP60 groups.

The findings of this experimental study suggest this reimplantation method with the use of rhBMP-2 represents a new approach for reconstruction of bone defects resulting from wide resection of tumors in the extremities or

trunk and offers an alternative to implantation of a prosthesis or the use of vascularized or nonvascularized bone grafting.

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Regenerative Repair of Long Intercalated Rib Defects Using Porous Cylinders of β -Tricalcium Phosphate: An Experimental Study in a Canine Model

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Background: In maxillofacial, spinal, and orthopedic surgery, bony ribs have been used as a source of donor bone. The resultant defects are not usually repaired, despite the pain or cosmetic morbidity experienced by the patient. The authors evaluated the efficacy of an osteoconductive β -tricalcium phosphate and the contribution of the periosteum in rib bone regeneration.

Methods: Two 8-cm-long intercalated rib defects were generated in each of 30 beagle dogs. In the first group ($n = 15$), one defect was implanted with 16 small, short, porous β -tricalcium phosphate cylinders that were connected with a titanium wire, and the other defect was left untreated. In the remaining 15 dogs, the periosteum was devitalized by ethanol, and then the same surgical procedures were performed. Each group was subdivided into three groups ($n = 5$), and the animals were euthanized at 3, 6, and 12 weeks. Bone regeneration was assessed radiologically, histologically, and mechanically.

Results: In the defect implanted with β -tricalcium phosphate on intact periosteum, newly formed bone was present on and in the β -tricalcium phosphate cylinders and bridged both ends of the resected ribs at 12 weeks, with replacement of β -tricalcium phosphate by new bone. Mechanical testing of these ribs revealed that they had 70 percent of the strength of normal ribs when compared in a bending stress test at 12 weeks after surgery. No regenerative bone bridging the rib defects was seen in the ethanol-devitalized or untreated groups.

Conclusions: Porous β -tricalcium phosphate cylinders placed in tandem on the intact periosteum might be useful for the repair of rib bone donated at surgery, presenting a new and unique method for regenerating rib defects. (*Plast. Reconstr. Surg.* 119: 1431, 2007.)

In maxillofacial, spinal, and orthopedic surgery, bone from ribs, ilium, and fibula has served as donor material to repair bone defects and to promote spinal fusion because of its superior osteogenic potential and acceptable clinical results.^{1,2} However, the defects in the donor ribs are currently left unrepaired despite donor-site pain and cosmetic morbidity.^{3,4} This issue has not been addressed in part because of the limited impact on respiratory function and/or lack of effective methods for regenerating the lost rib. In limited cases, various rib

prostheses have been used to preserve respiratory function.^{5,6} However, concerns regarding infection and limited clinical results remain because of limited biocompatibility and osteogenic potential of the prosthesis to regenerate the rib.

For bone substitutes, ceramic implants such as hydroxyapatite and tricalcium phosphate have been investigated extensively because of their good biocompatibility and osteoconductive properties. These have been used clinically and have shown acceptable surgery-related results.^{7,8} Hydroxyapatite has the advantage of mechanical strength, but has the disadvantage of a long residence time. β -Tricalcium phosphate is degraded with the help of osteoclasts and replaced by new bone when incorporated into osseous tissue.^{9,10} Recently, highly pure porous β -tricalcium phosphate has been manufactured using a mechanochemical method.

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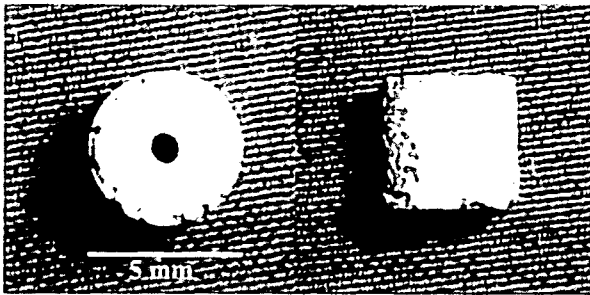


Fig. 1. Macroscopic aspect of the β -tricalcium phosphate cylinder. The cylinder has an outer diameter 5 mm, an inner diameter of 1 mm, and a height of 5 mm.

Porous β -tricalcium phosphate possessing macropores of 100 to 500 μm and micropores of less than 5 μm is now available as a degradable implant. In this material, almost all the macropores are interconnected by means of 100- to 200- μm paths. These structures facilitate the infiltration of osteogenic cells. The effective bioabsorbability and osteoconductivity of this β -tricalcium phosphate have been documented in both animal experiments and clinical studies.¹¹⁻¹⁵

To improve the methods for regenerating ribs or to preserve respiratory function resulting from rib bone loss, we attempted to produce a new implant material using β -tricalcium phosphate. The implants were fabricated into short cylindrical blocks and connected in tandem with titanium wire passed through the cylinders.

The presence or absence of intact periosteum at the rib defect might influence the regeneration potential because of the osteogenic capacity of the periosteum. In clinical cases such as defects in donor sites after vascularized rib grafting or by excision of malignant or benign tumors originated from ribs or soft tissue of the thoracic cage, the rib defects should be repaired under the condition without intact periosteum. Thus, a rib defect model with periosteum devitalized by contact with ethanol was also made to examine

the contribution of the periosteum to rib defect repair in this study. The purpose of this study was to evaluate the efficacy of small cylinders of an osteoconductive biomaterial, β -tricalcium phosphate, and the contribution of the periosteum in rib bone regeneration.

MATERIALS AND METHODS

Preparation of Porous β -Tricalcium Phosphate

β -Tricalcium phosphate powder was synthesized by wet milling (a mechanochemical method). The preparation of this material involves milling dicalcium phosphate dihydrate and calcium carbonate at a molar ratio of 2:1 with pure water and zirconium beads, followed by drying at 80°C. The resultant crystalline solid is converted to β -tricalcium phosphate by calcinations at 750°C for 1 hour. On sintering of the β -tricalcium phosphate powder at 1050°C for 1 hour, a porous β -tricalcium phosphate block was created, which was then characterized in terms of surface area and pore size distribution. The porosity of the block, as measured by the Archimedes method, was 75 percent. The β -tricalcium phosphate block possesses macropores of 100 to 500 μm and micropores of less than 5 μm . Nearly all macropores are interconnected. The porous β -tricalcium phosphate was cut into cylinders (outer diameter, 5 mm; inner diameter, 1 mm; height, 5 mm) with an average weight of 70 mg, and provided to us as a sterile material by Olympus Biomaterial Co., Ltd. (Tokyo, Japan) for this study (Fig. 1).

Preparation of Composite Implants

To assemble a composite implant, a curved titanium wire 0.8 mm in diameter and 12 cm in length was passed through the 15 or 16 β -tricalcium phosphate cylinders under sterile conditions (Fig. 2).

Experimental Animals

Thirty male beagle dogs, 8 months old, with an average body weight of 10 kg (range, 9.5 to 10.5



Fig. 2. Macroscopic aspect of the β -tricalcium phosphate cylinder. Sixteen cylinders are connected by passing a titanium wire (0.8 mm in diameter) through each cylinder.

kg) were purchased from Oriental Yeast Co. (Tokyo, Japan), and housed in separate cages with free access to food and water.

Surgical Procedures

Each animal was anesthetized by means of an intramuscular injection of ketamine (10 mg/kg) and xylazine (1.2 mg/kg) and maintained under anesthesia with an intravenous injection of pentobarbital (25 mg/kg). Cephazolin (10 mg/kg) was administered subcutaneously as a prophylactic antibiotic. After intratracheal intubation, the animal was placed in the right lateral position on an operating table, and skin hair over the left thoracic cage was removed with a hair cutter. The skin was sterilized with povidone-iodine solution and wrapped using sterile technique. In 15 dogs, a skin incision 10 cm long on and along the left tenth rib was made, and the outer surface of the thoracic cage was exposed. The tenth ribs, 8 cm long, were removed together with periosteum on the outer surfaces of the ribs. The periosteum on the inner wall of the ribs remained on the thoracic cage. The assembled prosthesis was placed into the defects in the tenth rib (tricalcium phosphate group) (Table 1). The implanted prosthesis was fixed to the cut ends of the ribs by intramedullary insertion of the titanium wires. The end of the wires was pushed out of the cortical bone of the rib through a drill hole to facilitate removal at 6 weeks after surgery (Fig. 3). The intercostal muscles were brought into contact with the implants by rough sutures and skin with close interrupted sutures. By the same surgical procedures through an additional skin incision along the fourth rib, the defect in this rib did not receive any implants (defect group). In another group of 15 dogs, the inner periosteum of the tenth rib defects was treated with 99% ethanol to devitalize the osteogenic cells on the cambium layer of the periosteum. Then, the prosthesis was placed (tricalcium

phosphate/ethanol group). In addition, the inner periosteum in the fourth rib defect with no implant received similar alcohol treatment (defect/ethanol group). Table 1 summarizes the surgical procedures for each rib group. After surgery, all the animals were monitored closely and had ad libitum access to food and water for 12 weeks. The experimental protocol was approved by the Institutional Committee for Animal Care and Experiments of Osaka City University Graduate School of Medicine and performed under strict accordance with appropriate guidelines.

Sample Preparation

These two groups were each subdivided into three groups ($n = 5$), and the animals were euthanized by means of an overdose of anesthetics at 3, 6, and 12 weeks. At each of these time points, the fourth and tenth ribs, including defects, were dissected free. The seventh ribs were removed from dogs that had not been operated on for use as normal controls. All dissected samples were radiographed and fixed in 10% neutral buffered formalin. The rib specimens were examined for evidence of new bone formation by the use of radiographic, histologic, and biomechanical methods.

Radiographic Examinations

Plain radiographs of the removed ribs were taken by means of routine procedures. The samples were cut to 3-mm thickness perpendicular to the rib axis at the middle of the original defects and radiographs were obtained. These radiographic images were examined for changes in radiographic densities in and around the implants at the rib defects.

Mechanical Testing

The tricalcium phosphate group and the tricalcium phosphate/ethanol group ribs retrieved from chest walls at 3, 6, and 12 weeks after surgery were subjected to mechanical testing by three-point bending using an apparatus designed for this purpose (Maruto Testing Machine Co., Tokyo, Japan). The stiffness of the defect segment was measured on a stress/deformity curve. The stiffness of normal ribs was measured as normal controls.

Histology

After the radiographic and biomechanical examinations, undecalcified histologic sections of the rib defects were prepared. The rib samples

Table 1. Surgical Procedures for Each Group*

	β -TCP	
	Negative	Positive
ETOH		
Negative	Defect	TCP
Positive	Defect/ETOH	TCP/ETOH

β -TCP, the defect was implanted with 16 small short porous β -tricalcium phosphate cylinders; ETOH, the periosteum of rib defect was devitalized by 99% ethanol.

*These groups were each subdivided into three groups ($n = 5$), and the animals were euthanized at 3, 6, and 12 weeks.

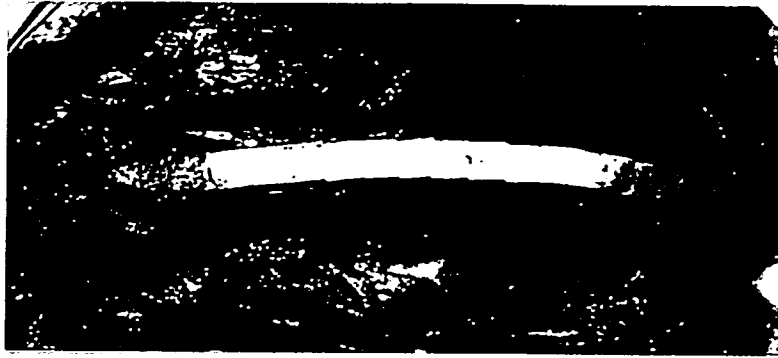


Fig. 3. Intraoperative view of β -tricalcium phosphate cylinders placed in the defect of the rib. The ends of the titanium wire were placed into the medullary canal of both ends of the original rib and passed through the outer cortices of the rib to fix the implant. This also enabled the removal of the titanium wire after repair of the rib defect.

fixed in neutral buffered formalin were dehydrated in gradient ethanol and embedded in polymerized methylmethacrylate and cut to 10- μ m thickness perpendicular to the rib axis at the middle of the original defects and stained by means of the Villanueva bone staining method. In addition, a histochemical method to detect tartrate-resistant acid phosphatase, an enzyme specific to osteoclast, was used. Briefly, the paraffin-removed sections were placed in the tartrate-resistant acid phosphatase staining solution consisting of acetate buffer (pH 5.0) containing 50 mM sodium tartrate, 25 mg/ml naphthol-ASMX phosphate (Sigma Chemical Co., St. Louis, Mo.), and 0.5 mg/ml fast red violet salt (Sigma). The sections were incubated with the solution at 37°C for 20 minutes. After washing, the sections were counterstained with methyl green and observed under a light microscope.

Statistical Analysis

The quantitative values obtained from measurement of stiffness were expressed as means \pm SD and compared using the Kruskal-Wallis test. Differences between experimental groups were evaluated with the post hoc Tukey-Kramer test. Differences at $p < 0.05$ were considered significant.

RESULTS

Gross Inspection and Palpation Test

All 30 dogs underwent surgery successfully and survived without any difficulty during the 12 postoperative weeks. No abnormality relating to surgical-site infection was noted. The respiratory movement of the chest wall with the prosthetic

implants was normal, without paradoxical movement when inspected just before the animals were euthanized. On inspection of the surface of the harvested chest cages at 3 weeks postoperatively, the cylinder implants placed in defects in the tricalcium phosphate group and the tricalcium phosphate/ethanol group ribs were identical and the tandem arrangement of β -tricalcium phosphate cylinders was recognized in the rib defects. In the defect group ribs and the defect/ethanol group ribs, the defects were filled with a fibrous tissue mass. At 6 weeks after surgery, the rib defects in the tricalcium phosphate group were harder than those from other control defects, but there was no significant difference in gross appearance of the defects of other respective group rib samples compared with those harvested 3 weeks after surgery. At 12 weeks after surgery, the rib defects in the entire tricalcium phosphate group were covered with hard tissue that bridged both ends of the rib defects and restored mechanical stiffness on palpation. In the defects in the tricalcium phosphate/ethanol group ribs, no evidence of hard tissue adjacent to the β -tricalcium phosphate cylinder implants or mechanical stiffness was found. In the defect group ribs, a thin layer of hard tissue mass with connection to the rib ends was found in the defects on palpation. In the defect/ethanol group, there was no evidence of palpable hard tissue mass.

Radiographic Examinations

In the tricalcium phosphate group ribs, on plain radiographs of the rib defects harvested at 12 weeks after surgery, a radiopaque shadow or new bone was consistently noted on and between the

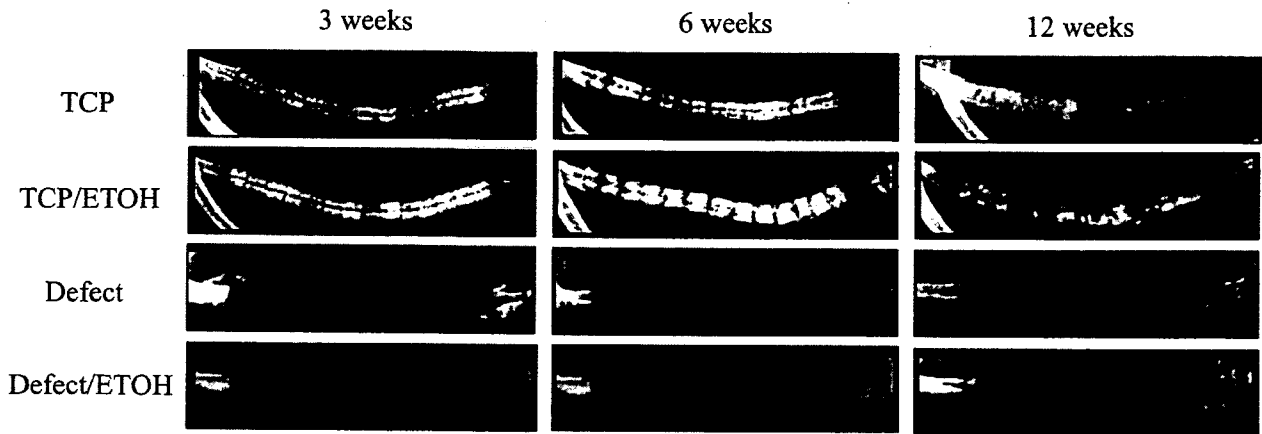


Fig. 4. Representative plain radiographs of the ribs (above) implanted with β -tricalcium phosphate. (Second row) β -Tricalcium phosphate with alcohol treatment of the periosteum; (third row) control defects with no implants; and (below) defects with no implants and alcohol treatment of the periosteum. Sequential radiographs show bone repair at 3, 6, and 12 weeks after implantation in the tricalcium phosphate group.

series of the β -tricalcium phosphate cylinders and connected to both ends of the original rib in four of five samples (Fig. 4). In one of five, new bone was noted over almost the entire defect but not connected completely. The radiopaque shadows were noted predominantly on the inner surface of the β -tricalcium phosphate cylinders at 3 weeks after surgery (Fig. 5). These shadows were not noted in defects in the tricalcium phosphate/ethanol group ribs. The respective β -tricalcium phosphate cylinder encased within the new bone in the tricalcium phosphate group was significantly reduced from its original size over the 12-week test period. The size reduction was also seen in the tricalcium phosphate/ethanol group ribs but to a lesser degree when compared with that in the tricalcium phosphate group. In all defect group ribs, radiopaque shadows in the defects that were connected from cut ends of the ribs were observed. In the defect/ethanol group, these radiopaque shadows were absent over the experimental period of 12 weeks. Table 2 shows the incidence of bone defect repair on radiographic assessments.

Mechanical Testing

The stiffness of the tricalcium phosphate group ribs started to increase 6 weeks after surgery and reached 70 percent of that found in a normal rib at 12 weeks. The tricalcium phosphate/ethanol group ribs had much less stiffness than the tricalcium phosphate group ribs. The stiffness of the tricalcium phosphate and tricalcium phosphate/ethanol group ribs at 3 and 6 weeks and of the tricalcium phosphate/ethanol group ribs at 12

weeks was significantly lower than that of normal ribs at the same time points (Fig. 6).

Histology

On undecalcified cross-sections of the midportion of the defect in the tricalcium phosphate group harvested at 3 weeks, a thin layer of new bone in contact with the inner surface of the cylinder was observed (Fig. 7). At both ends of the defect in the tricalcium phosphate group, the new bone united the preexisting rib bone and the regenerated rib. Multinucleated giant cells were located in the new bone predominantly on the surface of the porous β -tricalcium phosphate implant at the midportion. Those cells revealed an osteoclastic phenotype based on the tartrate-resistant acid phosphatase-positive histochemical staining (Fig. 8). In contrast, in the tricalcium phosphate/ethanol group, no apparent bone formation was noted and fibrous tissue layers were seen on the surface of the implants. A small mass of new bone was seen at the cut ends of the ribs, where tartrate-resistant acid phosphatase-positive multinucleated cells were sparsely distributed on the surface of the β -tricalcium phosphate implant. On sections of the 6-week samples of the tricalcium phosphate group ribs, new bone growth with hematopoietic marrow was seen on and in the porous β -tricalcium phosphate implants. On sections of the defect group ribs, a thin layer of bone mass at the cut ends of the ribs was noted. On cross-sections of the 12-week samples of the tricalcium phosphate group, the β -tricalcium phosphate implant was almost completely resorbed, presumably because of continuous resorption of the implant

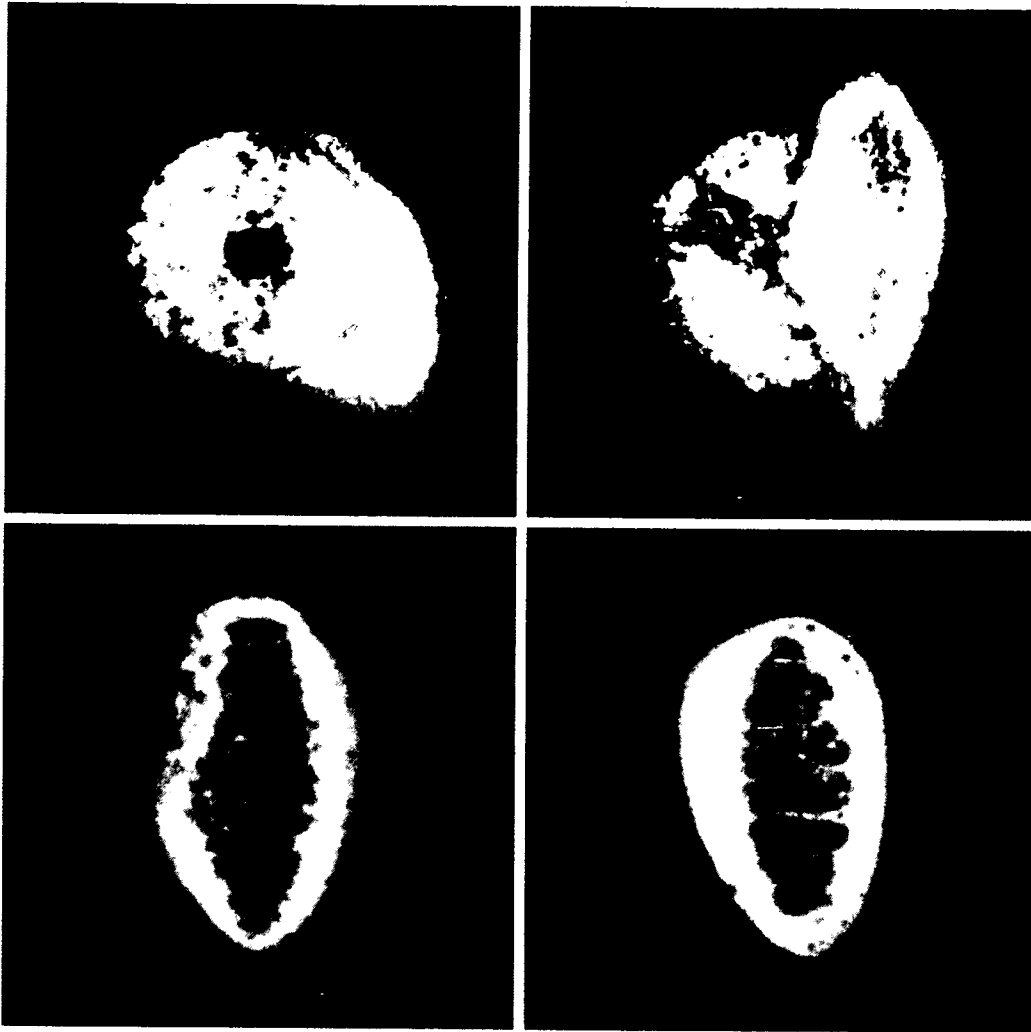


Fig. 5. Representative radiographs of the axial section of the midportion of the defects. Tricalcium phosphate group at 3 weeks (*above, left*), 6 weeks (*above, right*), and 12 weeks (*below, left*). Normal rib (*below, right*). The radiopaque shadows were noted on the inner surface of the β -tricalcium phosphate cylinders. The respective β -tricalcium phosphate cylinder encased within the new bone was significantly reduced from its original size. Regenerative repair with restoration of anatomical configuration was achieved.

by the large population of tartrate-resistant acid phosphatase-positive osteoclastic cells (Fig. 7). In sections from tissues retrieved from animals in the tricalcium phosphate/ethanol group, the implants remained nonresorbed without new bone

formation. In the defect group, small bone masses were formed on the periosteum, but these were of insufficient volume to repair the rib defects or unite with the rib ends. In the defect/ethanol group, no new bone was recognized on histologic examination.

Table 2. Incidence of Rib Defect Repair on Radiographic Assessments

Group of Ribs	3 Weeks	6 Weeks	12 Weeks
TCP	0/5	1/5	4/5
TCP/ETOH	0/5	0/5	0/5
Defect	0/5	0/5	0/5
Defect/ETOH	0/5	0/5	0/5

TCP, tricalcium phosphate; ETOH, ethanol.

DISCUSSION

The results presented in this study show successful and reproducible regenerative repair of intercalated rib defects of critical size (8 cm) through the use of biocompatible, osteoconductive, and degradable porous β -tricalcium phosphate placed on the intact periosteum in dogs.

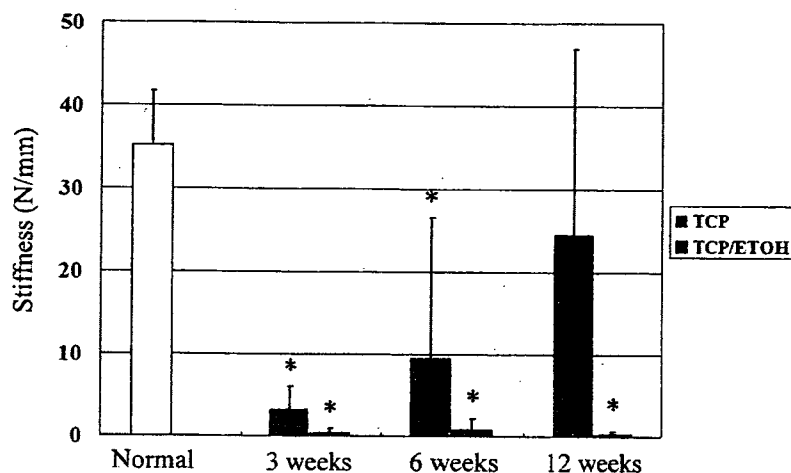


Fig. 6. The results of the three-point bending test of the defects in the tricalcium phosphate group, the tricalcium phosphate/ethanol group, and the normal control ribs at 3, 6, and 12 weeks after surgery are shown. The stiffness of the tricalcium phosphate group at 12 weeks reached a value corresponding to 70 percent of a normal rib. *Statistically significant difference from a normal rib.

The rib defects that did not receive implants were not repaired in 12 weeks, thus confirming the belief that the defect size adopted in this experimental study had exceeded the natural regenerating potential in dogs.

The successful regeneration of the rib defects might have been caused by the osteogenic potential of the periosteum of the ribs remaining on the chest wall, because alcohol treatment of the periosteal surface and the resultant devitalization of the osteogenic cells eliminated the regenerating reaction.^{16,17} Another possible reason for the successful repair could have been the use of osteoconductive porous β -tricalcium phosphate cylinders in combination with titanium wires to accommodate the anatomical curvature of the rib. The inherent flexibility of this implant was likely permissive to the reciprocating microdeformities caused by respiratory movements of the chest cage. As a result, there was no displacement or breakage of the brittle β -tricalcium phosphate cylinders or fixation to the ribs. In an osteoinductive environment with intact periosteum, β -tricalcium phosphate might be useful for the repair of load-bearing skeletal defects, even in long bones. However, because β -tricalcium phosphate is brittle under mechanical loading, internal or external fixation should be used to avoid collapse of β -tricalcium phosphate blocks in such situation. The porous structure of the implants was beneficial for bone growth into the biomaterial by providing a microenvironment for the differentiation of osteogenic precursor cells to osteoblasts.¹⁰⁻¹⁵ In ad-

dition to the porous structure of the β -tricalcium phosphate implants, the biodegradable character of the β -tricalcium phosphate appeared to be advantageous for the regenerative repair of the rib defects, because the progressive replacement of the β -tricalcium phosphate implants by new bone was seen over the time period of this study.^{13-15,18,19} The rib defects had been repaired to their original anatomy after removal of the titanium wires from the ribs of animals and resorption of the β -tricalcium phosphate implants at 12 weeks. The use of biomaterials such as hydroxyapatite might repair the rib defects, but bioresorption of implants will not occur. The regenerative repair with restoration of anatomical configuration and degradation of β -tricalcium phosphate implants were most likely achieved through enhanced remodeling of the newly formed bone by the large number of osteoclasts recruited to bone in contact with the β -tricalcium phosphate implant. Bone resorption by osteoclasts is coupled with bone formation by osteoblasts.²⁰ In this study, osteoblasts, which were differentiated from precursor or stem cells lining the cambium layer of periosteum, may have been involved in the formation and activation of osteoclasts. The close proximity of the osteoclasts to the β -tricalcium phosphate may in turn have enhanced the bone-remodeling rate, leading to the ultimate replacement of the implant material with bone and marrow. In addition, an ability to restore the original anatomy and mechanical strength of the ribs would make this implant methodology unique and potentially useful when used in clin-

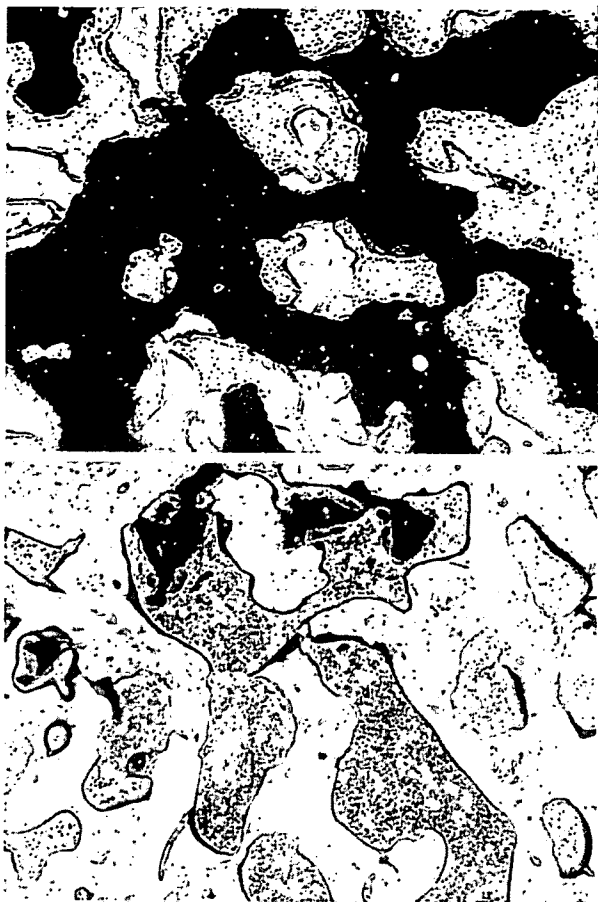


Fig. 7. Representative histologic undecalcified sections in the tricalcium phosphate group (Villaneuva bone stain; original magnification, $\times 100$) at 3 weeks (*above*) and 12 weeks (*below*). At 12 weeks, the β -tricalcium phosphate implant (*black*) was almost completely resorbed, and new bone growth (*pink*) with hematopoietic marrow was seen.

ical practice to regenerate rib defect, only when the periosteum has been left on the chest cage.^{10,21}

Limitations of this osteoconductive implant would be the time taken to achieve repair, the reduced mechanical strength of the repaired rib, and the requirement for periosteum. To overcome these problems, the addition of osteoinductive agents such as bone morphogenetic proteins to enhance bone formation both temporally and spatially would be necessary. Attempts to generate these types of implants are ongoing in our laboratory.

CONCLUSIONS

This study demonstrated successful and reproducible regenerative repair of long intercalated rib defects with small cylinders of the biocompatible, osteoconductive biodegradable biomaterial

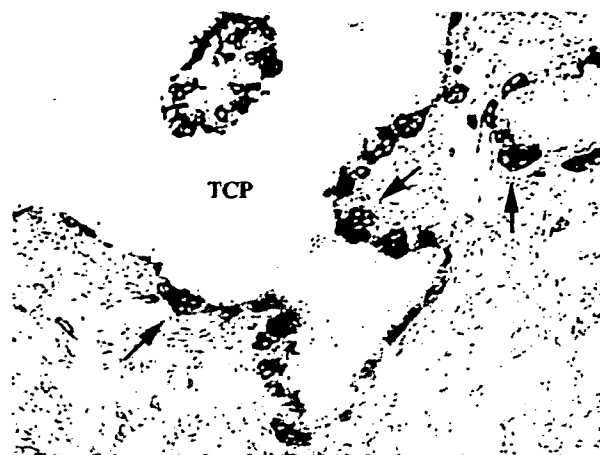


Fig. 8. Representative histologic section at 3 weeks in the tricalcium phosphate group (tartrate-resistant acid phosphatase stain; original magnification, $\times 200$). Multinucleated giant cells were located predominantly on the surface of the porous β -tricalcium phosphate implant (*red, arrows*).

β -tricalcium phosphate, placed in tandem on the periosteum. This type of implant might be useful for the repair of ribs used as a source of bone for reparative or restorative operations.

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DISCLOSURE

None of the authors has a financial interest in any of the products, devices, or drugs mentioned in this article.

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Comparative roles of Twist-1 and Id1 in transcriptional regulation by BMP signaling

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Summary

Basic helix-loop-helix (bHLH) transcription factors are known as key regulators for mesenchymal differentiation. The present study showed that overexpression of Twist-1, a bHLH transcription factor, suppresses bone morphogenetic protein (BMP)-induced osteoblast differentiation, and downregulation of endogenous Twist-1 enhances BMP signaling. Maximal inhibition of BMP signaling was observed when Twist-1 was bound to E47, which markedly enhanced the stability of Twist-1. Co-immunoprecipitation assays revealed that Twist-1 formed a complex with Smad4 and histone deacetylase (HDAC) 1 in MC3T3-E1 cells stably expressing Twist-1. With trichostatin, an HDAC inhibitor, osteogenic factors such as alkaline phosphatase, Runx2 and

osteopontin increased. Those results suggested that Twist-1 inhibited BMP signaling by recruiting HDAC1 to Smad4.

Furthermore, the inhibitory effects of Twist-1 on BMP signaling were overcome by Id1 through induction of Twist-1 degradation. These findings suggest that Twist-1 can act as an inhibitor of BMP signaling, and Id1 can regulate BMP signaling through a positive feedback loop repressing Twist-1 function. These two molecules may therefore regulate differentiation of mesenchymal cells into progeny such as osteoblasts by controlling BMP signaling.

Key words: Twist-1, Id1, BMP, Smad, HDAC

Introduction

Members of the transforming growth factor (TGF)- β superfamily regulate important biological and developmental processes, including cell proliferation, differentiation and migration (Derynck and Zhang, 2003). This is achieved through the ability to induce or repress transcription of diverse gene targets. For example, differentiation of mesenchymal cells into components of bone, cartilage or adipose tissue is regulated by bone morphogenetic proteins (BMPs), which belong to the TGF- β superfamily. BMPs induce not only new bone formation in vivo when implanted into ectopic sites (Urist, 1965), but also osteoblast differentiation of mesenchymal cells in vitro (Katagiri et al., 1994; Thies et al., 1992). TGF- β /BMP signaling is initially mediated by interactions with heterodimeric complexes of type I and type II serine/threonine kinase receptors. Activated receptor kinases phosphorylate receptor-regulated Smads (R-Smads). R-Smads then form activated complexes with common-mediator Smads (C-Smads). These complexes translocate into the nucleus to act as transcriptional regulators (Derynck and Zhang, 2003; Massague, 2000; Wrana, 2000). Studies on the mechanisms by which Smads mediate TGF- β /BMP-regulated gene transcription have led to the discovery of co-activators and co-repressors (Derynck and Zhang, 2003). However, the molecular mechanisms underlying the inhibition of BMP signaling have not been fully elucidated.

Twist-1, originally identified in *Drosophila*, is a member of the basic helix-loop-helix (bHLH) family of proteins (Leptin,

1991; Thisse et al., 1987). Twist-1 is expressed in mesodermal and cranial neural crest cells during embryogenesis (Wolf et al., 1991). In homozygous Twist-1-knockout mice the cranial neural tube fails to close and they die at embryonic day 11.5 (Chen and Behringer, 1995). Twist-1 heterozygous mice present a craniosynostotic phenotype (Bourgeois et al., 1998). Expression of Twist-1 has been implicated in the inhibition of differentiation for multiple mesenchymal cell lineages, including muscle (Hebrok et al., 1997; Spicer et al., 1996) and bone cells (Lee et al., 1999; Rice et al., 2000). The mechanisms of inhibition have been well established in muscle (Hamamori et al., 1999; Hebrok et al., 1997; Spicer et al., 1996), but little is known about the mechanisms behind inhibition of osteoblast differentiation by Twist-1. Very recently, Twist-1 has been reported to interact directly with Runx2, a key transcriptional factor regulating osteogenic gene expression. Direct interaction of Twist-1 with Runx2 causes inhibited DNA binding of Runx2 followed by gene inactivation in osteoblast precursors (Bialek et al., 2004). The fact that neurogenin, another bHLH family member, inhibits glial cell differentiation by sequestering Smad1 of the transcription complex away from glial differentiation genes (Sun et al., 2001), suggests that Twist-1 may likewise affect BMP signaling in the process of mesenchymal cell differentiation into osteoblasts.

The best-studied example of dimerization partners for known tissue-specific bHLH transcription factors involves the gene products of the *E2A* gene (Lassar et al., 1991). Through differential splicing, this gene gives rise to two different bHLH

proteins, E12 and E47, the so-called E-proteins (Murre et al., 1989; Sun and Baltimore, 1991). Id is an internal dominant negative form of HLH transcription factor, lacking a basic region. By sequestering E-proteins, Id prevents myogenic transcription factors, such as MyoD, from forming heterodimer complexes (Benezra et al., 1990; Sun et al., 1991). Recent reports have indicated that BMP induces expression of Id1, resulting in degradation of tissue-specific bHLH transcription factors (Vinals et al., 2004; Vinals and Ventura, 2004). Furthermore, Id1 is critical to BMP-induced osteoblast differentiation (Peng et al., 2004). As a result, we hypothesized that Id1 may be an antagonist of Twist-1 in osteoblast differentiation. The present study found that Twist-1 inhibits BMP-induced osteoblast differentiation. Inhibition of BMP signaling by Twist-1 is enhanced by E-protein. Moreover, the inhibitory effect of Twist-1 is overcome by Id1 through the induction of Twist-1 degradation. These findings suggest that Twist-1 and Id1 can regulate differentiation of mesenchymal cell lineages by controlling BMP signaling.

Results

Overexpression of Twist-1 inhibits BMP-induced osteoblast-specific gene expression

To examine the effect of Twist-1 on expression of BMP2-induced osteoblast differentiation marker genes, a cell line (MC3T3-E1-Tw) stably expressing Twist-1 was established. Since MC3T3-E1 cells contained less endogenous Twist-1 (Tamura and Noda, 1999) and Id1 (data not shown) than C3H10T1/2 cells, the cell line was appropriate to minimize the effect of endogenous Twist-1 and Id1. Exogenous Flag-Twist-1 was detected in MC3T3-E1-Tw1 by western blotting of immunoprecipitates using anti-Flag antibody (Fig. 1A).

Next, ALP activity stimulated by BMP2 was examined. BMP2-induced ALP activity was elevated in parental cells (MC3T3-E1-WT; Fig. 1B). By contrast, Twist-1 overexpression significantly suppressed BMP2-enhanced ALP activity. Basal levels of ALP activity in untreated MC3T3-E1-WT and MC3T3-E1-Tw clone 1 (MC3T3-E1-Tw1) cells were low. We analyzed ALP activity under BMP stimulation in other stable transformant clones. ALP activity in all the clones (data not shown) was much lower than in the parental MC3T3-E1-WT. We further examined whether Twist-1 overexpression also suppressed expression of other osteoblast marker genes, such as osteopontin (Hullinger et al., 2001; Shi et al., 1999; Yang et al., 2000) and osteocalcin (Katagiri et al., 1994; Thies et al., 1992). The level of BMP2-induced osteopontin expression in MC3T3-E1-Tw1 cells was lower than in MC3T3-E1-WT cells. BMP2-induced osteocalcin expression also decreased in MC3T3-E1-Tw1 cells (Fig. 1C). These results indicate that Twist-1 could affect BMP2-induced osteoblast differentiation.

Downregulation of endogenous Twist-1 enhances transcriptional activity mediated by BMP signaling

To examine the function of Twist-1 in BMP signaling, we attempted to downregulate endogenous Twist-1 expression in C3H10T1/2, a mesenchymal progenitor cell line with high levels of Twist-1 expression, using RNA interference (RNAi) technology. We selected the most effective Twist-1-specific siRNA (siTwist-691). Transient transfection of siTwist-691 resulted in a 50-60% decreases in mRNA levels (Fig. 2A). Next, we examined the direct effects of downregulating

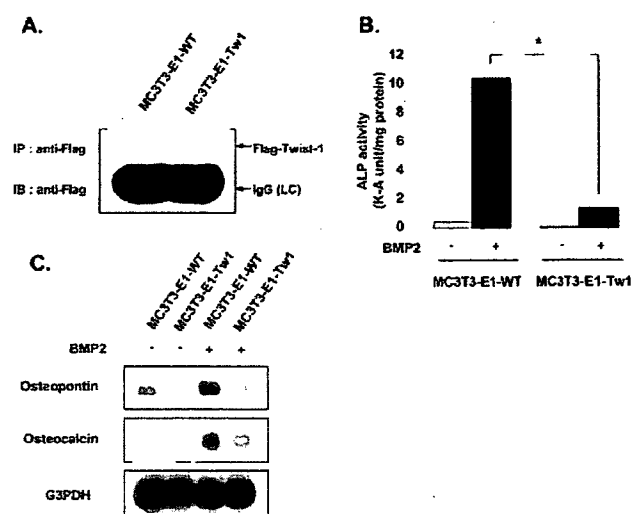


Fig. 1. Twist-1 overexpression suppresses BMP2-induced osteoblast differentiation. (A) Immunoprecipitation of overexpressed Flag-tagged Twist-1 in the nuclear extracts of MC3T3-E1-Tw1 and MC3T3-E1-WT cells. (B) MC3T3-E1-Tw1 and MC3T3-E1-WT cells were grown in the presence or absence of rhBMP2 (300 ng/ml) for 6 days. ALP activity was measured as described in the Materials and Methods (* $P < 0.01$). (C) MC3T3-E1-Tw1 and MC3T3-E1-WT cells were grown in the presence or absence of rhBMP-2 (300 ng/ml) and total RNA was isolated on day 6. Northern blot analysis was performed using osteopontin and osteocalcin cDNA probes.

endogenous Twist-1 on transcriptional activity mediated by Smads using 3GC2-Lux, which contains three tandem repeats of a Smad-binding GC-rich sequence linked with the collagen X core promoter inserted into pGL2-Basic (Ishida et al., 2000). 24 hours after transfection of C3H10T1/2 cells with Twist-1-specific siRNA, cells were transfected with 3GC2-Lux and TK-Renilla luciferase and fed with or without rhBMP2 treatment. Suppression of endogenous Twist-1 expression by Twist-1-specific siRNA resulted in increased BMP-dependent Smad transcriptional activity (Fig. 2B). Additionally, when the siTwist-691 was co-transferred to C3H10T1/2 cells with Twist-1 expression plasmid by lipofection, real-time PCR analysis indicated that endogenous Twist-1 expression was partially recovered (at most 32% compared with Twist-1 level in the cells received the siRNA alone). However, no significant increase of endogenous Twist-1 expression was obtained using control plasmid, pCAGIP, without Twist-1 cDNA (data not shown).

Next, we overexpressed Smad1, Smad4 and BMPR-IB(QD) in C3H10T1/2. Downregulation of endogenous Twist-1 using Twist-1-specific siRNA also enhanced transcriptional activity mediated by overexpressed Smads (Fig. 2C). These results indicate that Twist-1 could inhibit BMP/Smad signaling.

Twist-1 inhibits BMP signaling cooperatively with E-protein

To examine whether overexpressed Twist-1 could inhibit BMP-induced transcriptional activity, co-transfection studies were performed. P19 cells that respond to BMPs and express some of the BMP target genes were transfected using Twist-1,

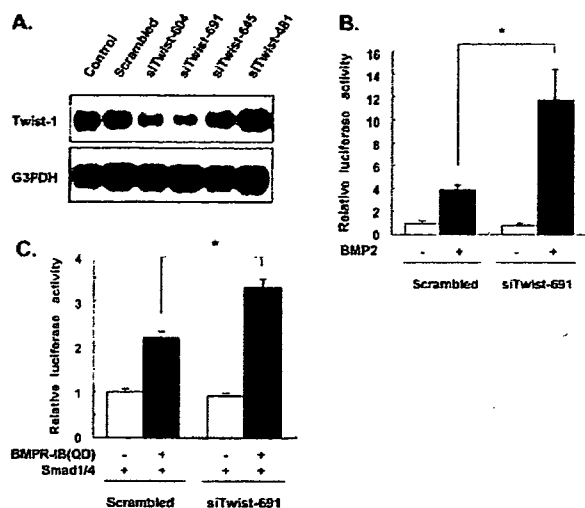


Fig. 2. Downregulation of endogenous Twist-1 using RNAi methods enhances BMP-induced transcriptional activity mediated by Smads. (A) C3H10T1/2 cells were transfected with either Twist-1-specific siRNA (siTwist-604, siTwist-691, siTwist-645, siTwist-481) or control siRNA (Scrambled) as described in the Materials and Methods. Total RNA was extracted 24 hours later, and northern blot analysis was performed using a Twist-1 cDNA probe. (B) C3H10T1/2 cells were transfected with either siTwist-691 or control siRNA (Scrambled). Cells were transfected with 3GC2-Lux and TK-*Renilla* luciferase 24 hours after siRNA transfection. At 12 hours after second transfection, cells were treated, with BMP (300 ng/ml) or left untreated, for 12 hours. Cells were lysed and luciferase activity was assayed ($*P < 0.01$). The mean value of firefly luciferase and *Renilla* luciferase activity in the scrambled sample without BMP was approximately 2,000 and 34,000 RLU (relative light unit), respectively. (C) C3H10T1/2 cells were transiently transfected with either 90 pmol of siTwist-691 or Scrambled in combination with 100 ng of 3GC2-Lux luciferase construct, Smad1, Smad4 and BMPR-1B(QD). Cells were lysed and luciferase activity was assayed 24 hours after transfection ($*P < 0.01$). The mean value of firefly luciferase and *Renilla* luciferase activity in the scrambled sample without BMPR-1B(QD) was approximately 411,000 and 30,000 RLU, respectively.

Smad1 and Smad4 expression constructs and 3GC2-Lux containing a Smad-binding sequence. Co-transfection of BMPR-1B(QD) with Smad1 and Smad4 enhanced transcription of 3GC2-Lux (Fig. 3A). Exogenous Twist-1 inhibited this activity, but inhibitory effects were very weak. Since Twist-1 reportedly inhibits MyoD trans-activation by E-protein sequestration, and the Twist-1-E-protein heterodimer inhibits myocyte enhancer-binding factor 2 (MEF2) trans-activation by direct interaction (Spicer et al., 1996), we hypothesized that the Twist-1-E-protein heterodimer could also act as a repressor in BMP signaling. We examined the effects of E47, an alternatively spliced product of the *E2A* gene (Murre et al., 1989; Sun and Baltimore, 1991), on suppression of Smad signaling by Twist-1. Twist-1 bound to E47 further increased the inhibition of BMP-induced transcription of 3GC2-Lux in a dose-dependent manner (Fig. 3A).

Next, the effect of Twist-1 and E47 on TGF- β signaling was analyzed using 3TP-Lux, which was empirically designed to have maximal responsiveness to TGF- β (Wrana et al., 1992).

However, TGF- β signaling was not inhibited by Twist-1 and E47. Id proteins are dominant-negative-type HLH proteins that lack the basic DNA-binding domain. In muscle development, Id1 forms heterodimers with E-protein and prevents myogenic bHLH proteins from forming complexes with E-protein (Benezra et al., 1990; Sun et al., 1991). BMP stimulation also reportedly induces Id1 expression (Katagiri et al., 1994; Nakashima et al., 2001; Ogata et al., 1993), an adverse pattern as compared to Twist-1 expression (Tamura and Noda, 1999). We therefore investigated whether Id1 also cooperated with E-protein to inhibit BMP signaling. In contrast to Twist-1, however, Id1 failed to repress BMP signaling in the presence or absence of E47 (Fig. 3B), suggesting distinctly different roles for Id1 and E47 in BMP signaling. Moreover E47 also failed to repress Smad signaling without Twist-1 (Fig. 3B). To assess whether formation of heterodimer complex with E-protein is critical for the inhibitory effect of Twist-1, we overexpressed a Twist-1 mutant (Twist-NBCT), which lacks the HLH domain (El Ghouzzi et al., 2000; Hamamori et al., 1997; Hebrok et al., 1997; Spicer et al., 1996). This Twist-1 mutant did not interact with E-protein (Fig. 3C). In contrast to with wild-type Twist-1, Twist-NBCT failed to suppress transcriptional activity in the presence or absence of E47 (Fig. 3D). These findings suggest that maximal inhibition of BMP signaling by Twist-1 requires heterodimer formation with E-protein. Additionally, by over expression of Twist-1 and E47, the inhibition of luciferase gene expression by siRNA was abolished (Fig. 3E).

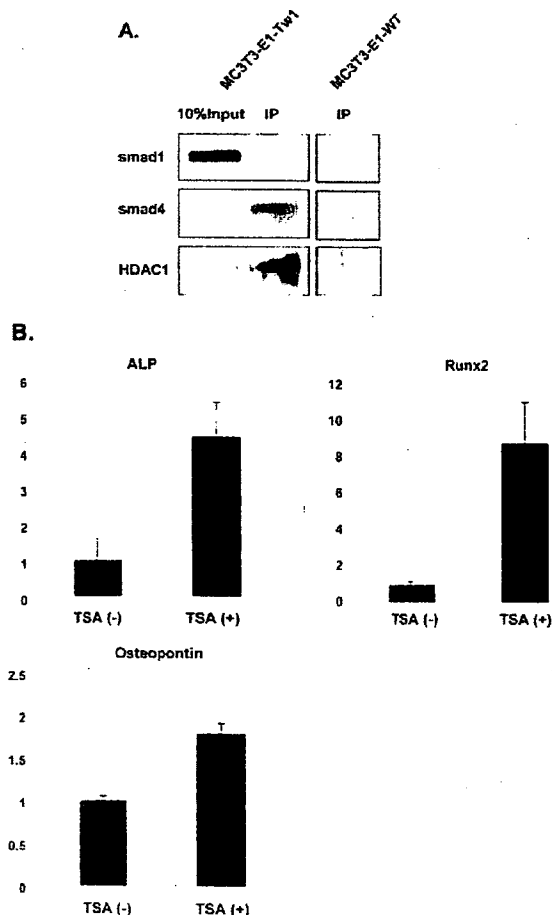
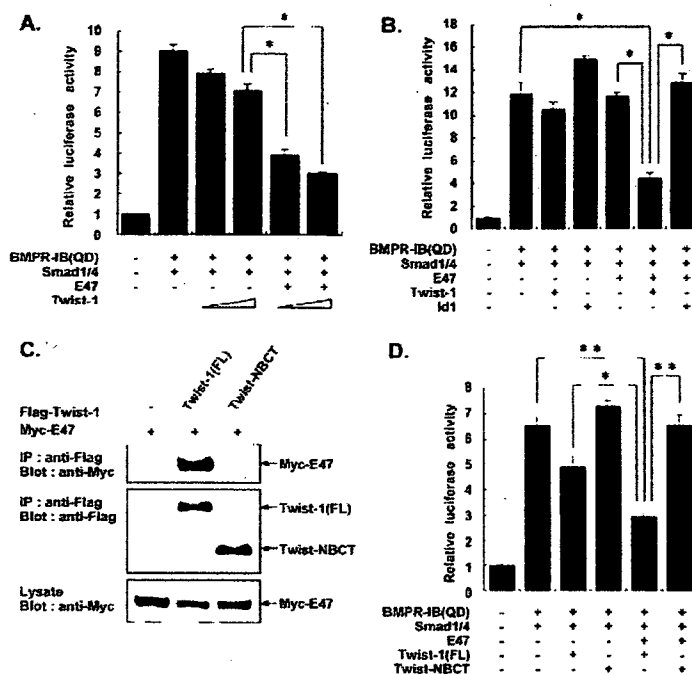
Twist-1 inhibited BMP signaling by recruiting HDAC1 to the Smad1-Smad4 complex

Histone deacetylases (HDACs) are involved in the repression of MyoD and MEF2 by Twist-2, which shares high homology with Twist-1 (Gong and Li, 2002; Li et al., 1995; Tamura and Noda, 1999). To analyze the involvement of HDAC in the inhibition of BMP signaling by Twist-1, Flag-tagged Twist-1 was immunoprecipitated using anti-Flag antibody in MC3T3-E1-Tw1 and the presence of HDAC and Smad was investigated in the precipitate. As shown in Fig. 4A, HDAC1 and Smad4 were detected in the precipitate, but not Smad1. In MC3T3-E1-WT, neither HDAC1 nor Smad4 was precipitated. Next, trichostatin (TSA), an HDAC inhibitor, was used to rescue the inhibition of osteogenic gene expression mediated by Twist-1. As shown in Fig. 4B, TSA treatment significantly increased the expression of ALP, Runx2 and osteopontin in the MC3T3-E1-Tw1 under BMP2 stimulation. These results suggested that Twist-1 could inhibit BMP signaling by recruiting HDAC1 to Smad complex via Smad4.

Twist-1 protein stability is increased by the formation of heterodimer complex with E-protein

Stability of bHLH proteins is reportedly increased by the formation of heterodimer complexes with other bHLH proteins (Deed et al., 1996; Vinals et al., 2004; Vinals and Ventura, 2004). We therefore examined whether co-expression of E47 increases the level of Twist-1 protein. Myc-tagged Twist-1 was expressed with or without Myc-tagged E47 in COS-7 cells, and the levels of both were immunodetected by western blotting. Co-expression of E47 greatly increased Twist-1 protein levels (Fig. 5A). Next, to determine whether enhancement of Twist-1 protein expression is caused by

Fig. 3. Twist-1 inhibits BMP-induced Smad transcriptional activity with E-protein. (A) P19 cells were transiently transfected with 3GC2-Lux luciferase construct in combination with 50 ng of BMPR-IB(QD), Smad1, Smad4, E47 and increasing doses (12.5 or 50 ng) of Twist-1 expression construct. Cells were lysed and luciferase activity was assayed 24 hours after transfection ($*P < 0.01$). The mean value of firefly luciferase and *Renilla* luciferase activity in cells transfected with luciferase expression plasmids was approximately 109,000 and 681,000 RLU, respectively. (B) P19 cells were transiently transfected with 3GC2-Lux luciferase construct in combination with 50 ng of BMPR-IB(QD), Smad1, Smad4, E47, Twist-1 and Id1 construct. Cells were lysed and luciferase activity was assayed 24 hours after transfection ($*P < 0.01$). The mean value of firefly luciferase and *Renilla* luciferase activity in cells transfected with luciferase expression plasmids was approximately 51,000 and 199,000 RLU, respectively. (C) Flag-tagged full-length (FL) Twist-1 or deletion Twist-1 mutant (Twist-NBCT) and Myc-tagged E47 constructs were transfected into COS-7 cells. Lysates were immunoprecipitated using anti-Flag antibody and blotted with anti-Myc antibody. (D) P19 cells were transiently transfected with 3GC2-Lux luciferase construct in combination with 50 ng of BMPR-IB(QD), Smad1 and Smad4, E47, full-length Twist-1 and deletion Twist-1 mutant (Twist-NBCT) expression construct. Cells were lysed and luciferase activity was assayed 24 hours after transfection ($**P < 0.01$, $*P < 0.05$).



avoidance of degradation, we examined Twist-1 protein turnover using a pulse-chase assay. Twist-1 was rapidly degraded in cells, with protein levels at 180 minutes reduced to 17% compared with the baseline at 0 minutes. By contrast, Twist-1 protein stability was greatly enhanced by co-expression with E47 (Fig. 5B). Recent reports have shown that Id genes are rapidly upregulated by BMP stimulation (Nakashima et al., 2001; Ogata et al., 1993; Peng et al., 2004). BMP2 decreases myogenin and Mash1 protein stability through induction of Id1 (Vinals et al., 2004; Vinals and Ventura, 2004). In addition, our previous data showed that Twist-1 could inhibit BMP signaling (Fig. 3A,B,D). These results prompted us to investigate whether Id1 would induce Twist-1 degradation and inhibit Twist-1 function. Twist-1 was degraded by co-transfection of Id1 in the presence of E47 (Fig. 5B).

Id1 inhibits Twist-1 function by interfering with functional Twist-1-E47 heterodimer formation

The finding that Twist-1 stabilization by E47 was partially lost by co-transfection of Id1 suggests that Id1 may sequester E47 from Twist-1, resulting in Twist-1 degradation. We tested this

Fig. 4. Twist-1 can interact with HDAC1 and Smad4. (A) MC3T3-E1-WT and MC3T3-E1-Tw1 cells were treated with BMP2 (300 ng/ml) for 24 hours. Lysates were immunoprecipitated using anti-Flag antibody and blotted with anti-Smad4 and HDAC1 antibody. 10% In, 10% of input; IP, immunoprecipitated fraction. (B) MC3T3-E1-Tw1 cells were treated with BMP alone [TSA (-)] or the mixture of BMP and TSA (82.5 μ M) [TSA (+)]. After 24 hours, total RNA was extracted and the expression of ALP, Runx2 and osteopontin was quantified by real-time PCR. The expression levels were normalized by GAPDH, and the ratio was shown in each sample. Data are presented as mean \pm s.d. of triplicate samples ($*P < 0.05$).