Immunohistochemical Analysis Revealed the Expression of Bone Morphogenetic Proteins-4, 6, 7, and 9 in Human Induced Membrane Samples Treated With the Masquelet Technique

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Research Article

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Abstract

Background

Induced membrane (IM) is the key component of Masquelet reconstruction surgery for the treatment of bone defects. It is formed around the cement spacer and is known to secrete growth factors and osteoinductive factors. However, information on the presence of osteoinductive factors in IM is not enough in the literature. The purpose of this study was to investigate the existence of bone morphogenetic proteins (BMPs) in the IM harvested from patients during the treatment of bone defects using the Masquelet technique.

Methods

We included six patients whose bone defects were treated using the Masquelet technique. The affected bone was the femur in three patients and the tibia in three patients. During the second-stage surgery, 1-cm^2 pieces of IM were harvested. Histological sections of IM were immunostained with anti-BMP-4, 6, 7, and 9 antibodies. Human bone tissue served as the positive control.

Results

The existence of BMP-4, 6, 7, and 9 was observed in all IM samples. Further, immunolocalization of BMP-4, 6, 7, and 9 was observed in blood vessels and fibroblasts of all IM samples. Immunolocalization of BMP-4, 6, 7, and 9 was also observed in bone tissue within the IM in one sample, in which osteogenesis inside the IM was observed.

Conclusions

This study revealed that osteoinductive factors BMP-4, 6, 7, and 9 were present in the IM harvested from patients. This helps explain how the Masquelet technique effectively contributes to the healing of large bone defects. It may thus be possible for surgeons to omit the addition of BMPs to bone grafts give the endogenous secretion of BMPs from the IM.

Trial registration

Not applicable.

Background

Reconstruction of a large bone defect caused by trauma, infection, or tumors is a challenging problem in orthopedic surgery. Distraction osteogenesis using the Ilizarov method [1, 2] and vascularized fibula grafts [3, 4] have remained limited treatment options for a long time. The Masquelet technique, also known as the induced membrane (IM) technique, is a third option for the reconstruction of large bone defects [5–7]. It comprises a two-staged surgery. In the first stage, bone defects caused by debridement are filled with a polymethyl methacrylate cement spacer. Subsequently, IM, a bioactive membrane, is formed around the cement spacer. After the IM is formed, the cement is removed, and autologous bone grafting is performed in the space of the bone defect surrounded by IM. The latter serves as a conduit for cells and provides a favorable environment for bone graft osseointegration. IM formed around the cement spacer is a key component of Masquelet reconstruction surgery [5–8]. Previous studies have shown that IM possesses osteogenic and osteoinductive functions, and is richly vascular [9–16].

Bone morphogenetic proteins (BMPs) are representative osteoinductive factors. They cause the proliferation and differentiation of mesenchymal stem cells or chondro-/osteo-progenitor cells involved in endochondral or intramembranous ossification [17, 18]. Furthermore, the expression of BMPs during fracture repair has also been reported [19–21]. Endogenous BMPs are important for bone regeneration and repair. Exogenous BMPs such as BMP-2 and 7 have been applied to promote bone regeneration and repair in open fractures and non-union [22, 23]. Recently, BMPs have been utilized as osteoinductive factors as per the ‘diamond concept’ when using the Masquelet technique [24–27].

The expression of BMP-2 protein has been detected by immunohistochemistry, enzyme-linked immunosorbent assay, and western blotting in human [15, 28] and animal [9–11, 29] IM samples. The gene expression of BMP-2, 3b, 6, 7, 10, and 14 has also been detected in human IM samples [28, 30]. However, the existence of BMP proteins other than BMP-2 has not been demonstrated by immunohistochemistry in IM samples as yet. Hence, this study aimed to investigate the existence of BMPs (BMP-4, 6, 7, and 9) using histological samples of human IM.

Methods

Ethical approval

This study was performed in accordance with the ethical standards laid down by the 1964 Helsinki Declaration and its later amendments, and was approved by the ethics committee of our university. The requirement for informed consent was waived because of the retrospective nature of the study.

Patient inclusion

We included six patients whose bone defects were treated using the Masquelet technique in our department. The affected bone was the femur in three patients and the tibia in three patients.

Histological specimens

During the second surgery (removal of the cement and bone grafting), 1-cm^2 pieces of the IM that were in contact with the cement spacer were harvested and immersed in 10% neutral buffered formalin. Samples were embedded in paraffin, and histological sections (4 μm in thickness) were made.

Histological analyses

Histological sections were stained with hematoxylin and eosin and analyzed by two clinical pathologists. The number of blood vessels per 1 mm^2 within the IM was counted in locations where the capillary density was the highest. Histological findings of inflammation, foreign body reaction, and fibrosis were assessed using a semi-quantified grading scale of 0–3, where grade 3 indicated the highest degree of inflammation, foreign body reaction, and fibrosis, while grade 0 indicated absence of such findings.

Immunohistochemistry

After deparaffinization, the sections were incubated overnight at 4°C with anti-BMP4 primary antibody (1:100 dilution, GTX100875, GeneTex Inc., Hsinchu City, Taiwan), anti-BMP6 primary antibody (1:100 dilution, ab155963, Abcam, Cambridge, MA, USA), anti-BMP7 primary antibody (1:100 dilution, ab84684, Abcam), or anti-BMP9 primary antibody (1:100 dilution, ab35088, Abcam) and subsequently treated with peroxidase-labeled anti-rabbit immunoglobulin (Histofine® Simple Stain MAX PO, Nichirei Bioscience, Tokyo, Japan) at room temperature for 60 min. The signal was observed as the development of a brown reaction product with the peroxidase substrate 3,3’-diaminobenzidine (Histofine® Simple Stain 3,3-Diaminobenzidine Solution, Nichirei Bioscience). The sections were counterstained with hematoxylin and examined using a BZ-X700 confocal microscope (Keyence Corporation, Osaka, Japan). Phosphate-buffered saline (PBS) was used instead of primary antibodies to stain the negative control samples. Formalin-fixed paraffin-embedded human tissue sections (catalog number CS812148, case ID CU0000012835, 63-year-old, male, bone, distal femur) were obtained from OriGene Technologies (Rockville, MD, USA), and were used as the positive control.

Clinical data

The patients’ sex, age, morbidity accounting for the bone defect, free flap application to the affected limb, affected site (bone), impregnation of antibiotics to the cement spacer, duration of cement placement, smoking habit, and comorbidity of diabetes mellitus (DM) or peripheral artery disease (PAD) were investigated using medical charts. The duration of cement placement was defined as the number of days from the first-stage surgery in which the cement spacer was placed, to the second stage surgery in which the cement spacer was removed and bone grafting was performed. Bony union in the enrolled patients was assessed radiologically and clinically. The time point of bony union assessment was set at six months after the second stage of surgery (autologous bone grafting). Radiological bony union was defined as corticalization of the grafted bone and absence of a gap between the grafted and original bone, observed in three or four cortices using orthogonal (anteroposterior and mediolateral) radiographs. Clinically, bony union was defined as the absence of pain on full weight-bearing. Bony union was defined as the achievement of both radiological and clinical bony union. Three experienced orthopedic trauma surgeons individually assessed bony union. Bony union was said to have been achieved when at least two surgeons agreed upon its presence.

Results

Patient characteristics

Five men and one woman were included in the study (Table 1). The mean age was 53.0 ± 11.1 years (range, 42–72). The morbidities accounting for the bone defects were osteomyelitis (3 patients), infected nonunion (1 patient), non-infected nonunion (1 patient), and severely comminuted open fracture (1 patient). The affected sites were the femur (3 patients) and tibia (3 patients). The mean cement placement duration was 77.5 ± 31.3 days (range, 46–126). Three patients underwent free flap surgery. Five patients received antibiotic

impregnation into the cement. Three patients had a smoking habit, and no patient had comorbidities of DM or PAD. All patients achieved a bony union.

Histological findings

IM formation was confirmed histologically in all patients; the histological findings are summarized in Table 2. Blood vessel formation was noted in all patients. The mean number of blood vessels per 1 mm^2 was 35.0 ± 20.4 (range, 15–70). Inflammation, foreign body reaction, and fibrosis were observed in all patients. The histological grading of inflammation, fibrosis, and foreign body reaction is shown in Table 2. Osteogenesis inside the IM was noted in one patient. A two-layered structure was noted in all patients. A synovial-like structure at the surface that was in contact with the cement was identified in three patients, while fibrin deposition was observed in four patients.

Immunohistochemical findings

The immunohistochemical findings are summarized in Table 3. The existence of BMP-4, 6, 7, and 9 was observed in all IM samples, and immunolocalization of BMP-4, 6, 7, and 9 was observed in blood vessels and fibroblasts of all IM samples. Representative immunohistochemical images are shown in Fig. 1 (Case 3, femur) and Fig. 2 (Case 4, tibia). Immunolocalization of BMP-4, 6, 7, and 9 was also observed in the bone within the IM in one sample in which osteogenesis inside the IM was observed (Fig. 2). Finally, immunostaining of human bone tissue as a positive control demonstrated positive immunoreactivity for BMP-4, 6, 7, and 9 (Fig. 3).

Discussion

This study confirmed the existence of BMP-4, 6, 7, and 9 in IMs using human samples, and demonstrated that these BMPs serve as osteoinductive factors in the treatment of patients with bone defects using the Masquelet technique. This finding helps understand the mechanism by which IM promotes bone regeneration and repair in the treatment of bone defects using the Masquelet technique. Therefore, the presence of these BMPs as osteoinductive factors in IM may lead surgeons to omit the addition of exogenous BMPs to bone grafts. The application of BMPs to surgery is expensive, and use of IM in the Masquelet technique can be considered as an alternative approach to supply BMPs at a low cost.

BMPs constitute the largest subdivision of the transforming growth factor-β (TGF-β) family of ligands, with nearly 30 distinct human proteins bearing the name [17, 18]. Among the various BMPs, we selected four for this study, the reasons for which are enumerated below.

BMP-4 belongs to the same subgroup of bone-inducing BMPs as BMP-2, based on the homology of their amino acid sequences. BMP-2 is known to be the most representative osteoinductive factor and is widely used clinically to treat bone fracture or nonunion [22, 31, 32]. BMP-4 has been detected along with BMP-2 in the area of endochondral ossification, particularly in the matrix between the newly formed osteoid in human fracture callus [19]. The expression of BMP-4 and Noggin, a major BMP antagonist in tissues, is highlighted in the newly formed callus tissue, thereby confirming the central role of BMP signaling in bone fracture repair [20].

BMP-7 has been clinically applied to treat nonunion of fractures. In a randomized controlled trial, the efficacy of recombinant human BMP-7 (rhBMP-7) in tibial nonunion of 124 patients who received either autologous bone grafting or a device containing rhBMP-7 was tested [23]. The bone healing rate was found to be inferior in the rhBMP-7 treated group, albeit not statistically significant, and the bone healing capacity of rhBMP-7 was assessed to be comparable to that of autologous bone grafting. The Food and Drug Administration (FDA) and European Medicines Agency (EMA) approved rhBMP-7 as a “humanitarian use device” for tibial nonunion. In addition, rhBMP-7 has been used off-label for various indications, including nonunion of the scaphoid, humerus, and clavicle [33–36].

BMP-6 is a paralog of BMP-7 with 87% similarity in their amino acid sequences. BMP-6 is more potent in promoting osteoblast differentiation in vitro and inducing bone regeneration in vivo when compared with its closely related BMP-7 paralog. This is explained by the reversible binding of BMP-6 to Noggin, and unlike BMP-7, BMP-6 may dissociate from Noggin and escape Noggin inhibition [37]. A novel rhBMP-6 containing osteogenic device aimed to accelerate bone regeneration has been developed and is being tested in clinical trials [18, 38, 39].

BMP-9 is a recent discovery in the BMP family. BMP-9 is resistant to Noggin, thus facilitating a more robust cellular differentiation of osteoprogenitor cells into preosteoblasts and osteoblasts [40]. It was reported that BMP-9 stimulated callus formation in osteoporotic rats during fracture healing [41]. Besides its osteogenic activity, BMP-9 exerts a broad range of biological functions, including stem cell differentiation, angiogenesis, neurogenesis, tumorigenesis, and metabolism [42]. It is expected that BMP-9 will be a promising alternative to clinically available BMPs.

We revealed the existence of BMP-4, 6, 7, and 9 in all human IM samples. In addition to immunolocalization of BMP-4, 6, 7, and 9 in blood vessels and fibroblasts, they were also observed within the bone inside the IM in one sample, in which osteogenesis inside the IM was observed. This histological finding of osteogenesis inside the IM was found in one sample out of six. Other investigators have also reported this finding in limited samples [14, 28]. We consider BMP-4, 6, 7, and 9 existing inside the IM and contributing to osteoinduction as an important finding.

The Masquelet technique is frequently applied to the lower extremity rather than the upper extremity. The tibia and femur are long bones of the lower extremities and frequently become subject to bone defects. The volume of the surrounding soft tissue is greater in the femur than in the tibia, and can be considered to reflect vascularity, thereby potentially affecting the formation and properties of IM. Therefore, we included both tibia and femur cases in this study. The results of our study revealed that IMs harvested from both tibia and femur cases expressed BMP-4, 6, 7, and 9.

The strength of this study was the use of human IM samples harvested from patients with bone defects treated using the Masquelet technique. In addition, we revealed the existence of BMPs that have not been proven at the protein or the mRNA level. The limitation of this study is that specimens were harvested from a limited number of patients. Moreover, this was a retrospective study conducted at a single institution, and could therefore be susceptible to selection bias and limited generalizability.

Conclusions

Osteoinductive factors, BMP-4, 6, 7, and 9 exist in the IM of patients treated with the Masquelet technique.

Abbreviations

IM: induced membrane

BMP: bone morphogenetic protein

DM: diabetes mellitus

PAD: peripheral artery disease

FDA: Food and Drug Administration

EMA: European Medicines Agency

Declarations

Ethics approval and consent to participate

This retrospective study involving human participants was conducted in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki Declaration and its later amendments. This study was approved by the ethics committee of the Kobe University. The requirement for informed consent was waived because of the retrospective nature of the study.

Consent for publication

Not applicable.

Availability of data and materials

Data are available upon reasonable request by contacting the corresponding author.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

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Authors' contributions

Conceptualization: TN; Methodology: NJ, MK, TI; Formal analysis and investigation: TO, KO, TF; Writing - original draft preparation: TN; Writing - review and editing: TM, SH; Supervision: RK.

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Tables

Table 1
Patient characteristics

Case	Sex	Age	Affected site	Morbidity accounting for the bone defect	Duration of the cement placement (days)	Free flap surgery	Antibiotics within the cement	Smoking	DM	PAD
1	M	58	femur	uninfected nonunion	46	-	-	+	-	-
2	M	51	femur	osteomyelitis	54	-	+	+	-	-
3	F	72	femur	osteomyelitis	56	-	+	-	-	-
4	M	43	tibia	severely comminuted open fracture	83	+	+	+	-	-
5	M	52	tibia	osteomyelitis	126	+	+	-	-	-
6	M	42	tibia	infected nonunion	100	+	+	-	-	-

M: male, F: female, DM: diabetes mellitus, PAD: peripheral artery disease

Table 2
Summary of the histological findings

Case	Sex	Age	Affected site	Blood vessel counts per 1mm^2	Inflammation	Foreign body reaction	Fibrosis	Osteogenesis within the membrane	Two-layer structure	Synovial-like structure at the surface	Fibrin deposition
1	M	58	femur	70	1	2	1	-	+	+	-
2	M	51	femur	20	1	3	2	-	+	-	+
3	F	72	femur	30	2	1	1	-	+	+	+
4	M	43	tibia	55	1	1	2	+	+	+	-
5	M	52	tibia	15	2	2	1	-	+	-	+
6	M	42	tibia	20	2	3	1	-	+	-	+

M: male, F: female. The degree of inflammation, foreign body reaction, and fibrosis was graded from 0 to 3.

Table 3
Summary of the immunohistochemical findings

	Blood vessel	Fibroblast	Bone
BMP-4	6/6	6/6	1/6
BMP-6	6/6	6/6	1/6
BMP-7	6/6	6/6	1/6
BMP-9	6/6	6/6	1/6

BMP: bone morphogenetic protein

6/6 means that immunolocalization of the BMPs was observed in the blood vessels and fibroblasts of all samples, out of a total 6 samples.

1/6 means that immunolocalization of the BMPs was observed in the bone within the induced membrane in one sample, in which osteogenesis inside the induced membrane was observed.

Figures

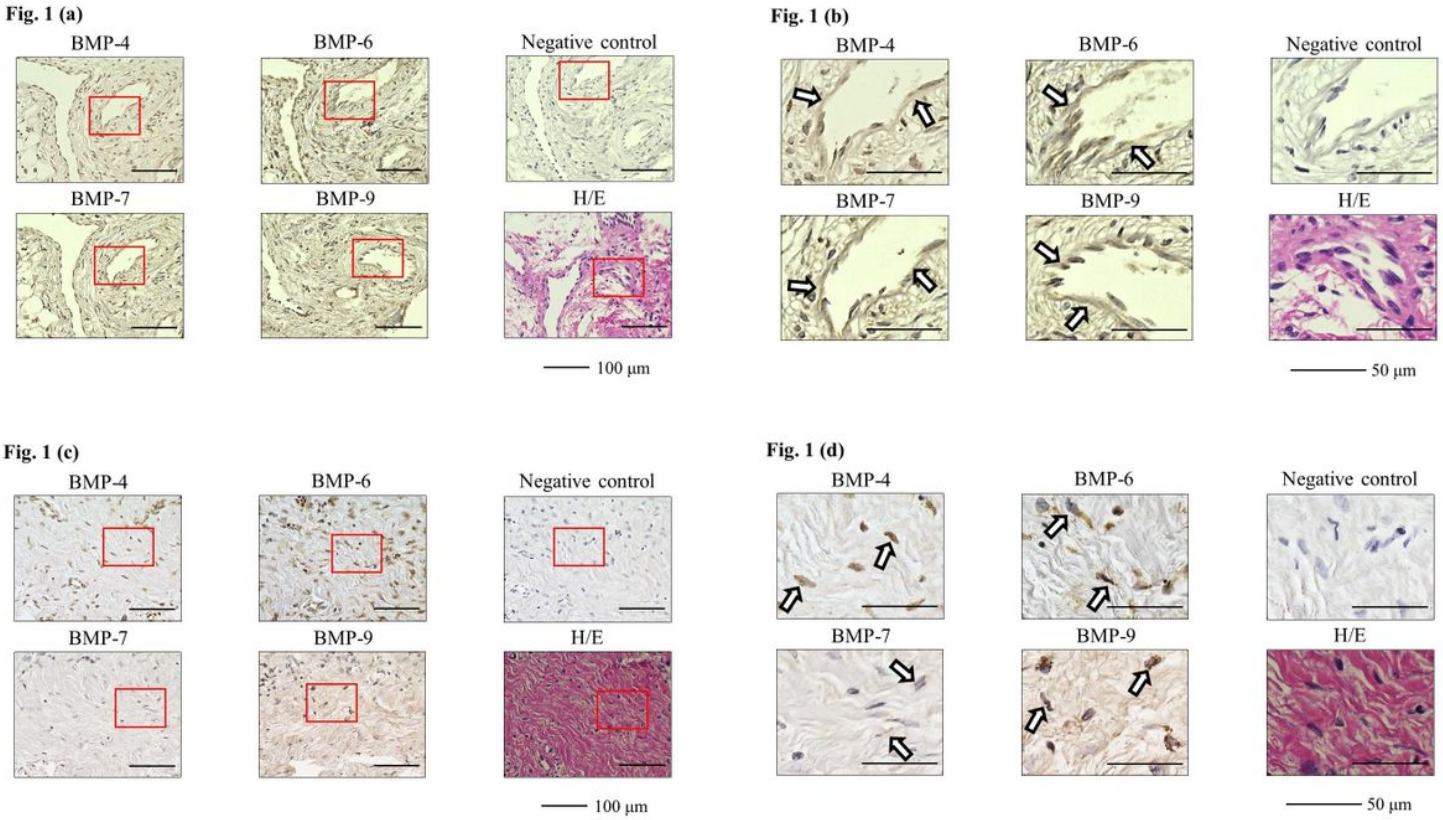

Figure 1

Representative immunohistochemical images of the induced membrane (Case 3, femur) (a) blood vessel, low-power field (b) blood vessel, high-power field (c) fibroblast, low-power field (d) fibroblast, high-power field BMP: bone morphogenetic protein, H/E: hematoxylin and eosin

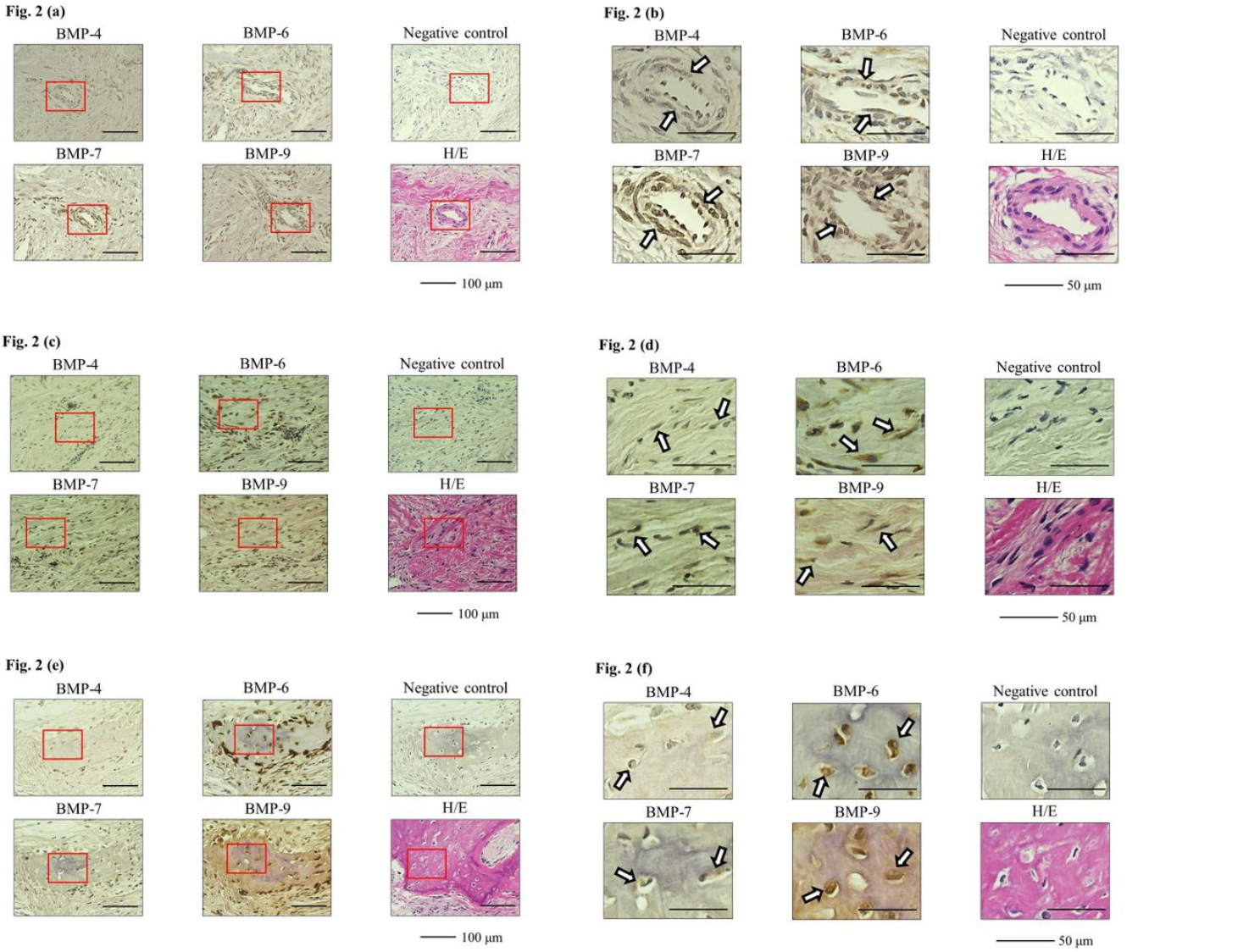

Figure 2

Representative immunohistochemical images of the induced membrane (Case 4, tibia) (a) blood vessel, low-power field (b) blood vessel, high-power field (c) fibroblast, low-power field (d) fibroblast, high-power field, (e) bone inside the induced membrane, low-power field, (f) bone inside the induced membrane, high-power field BMP: bone morphogenetic protein, H/E: hematoxylin and eosin

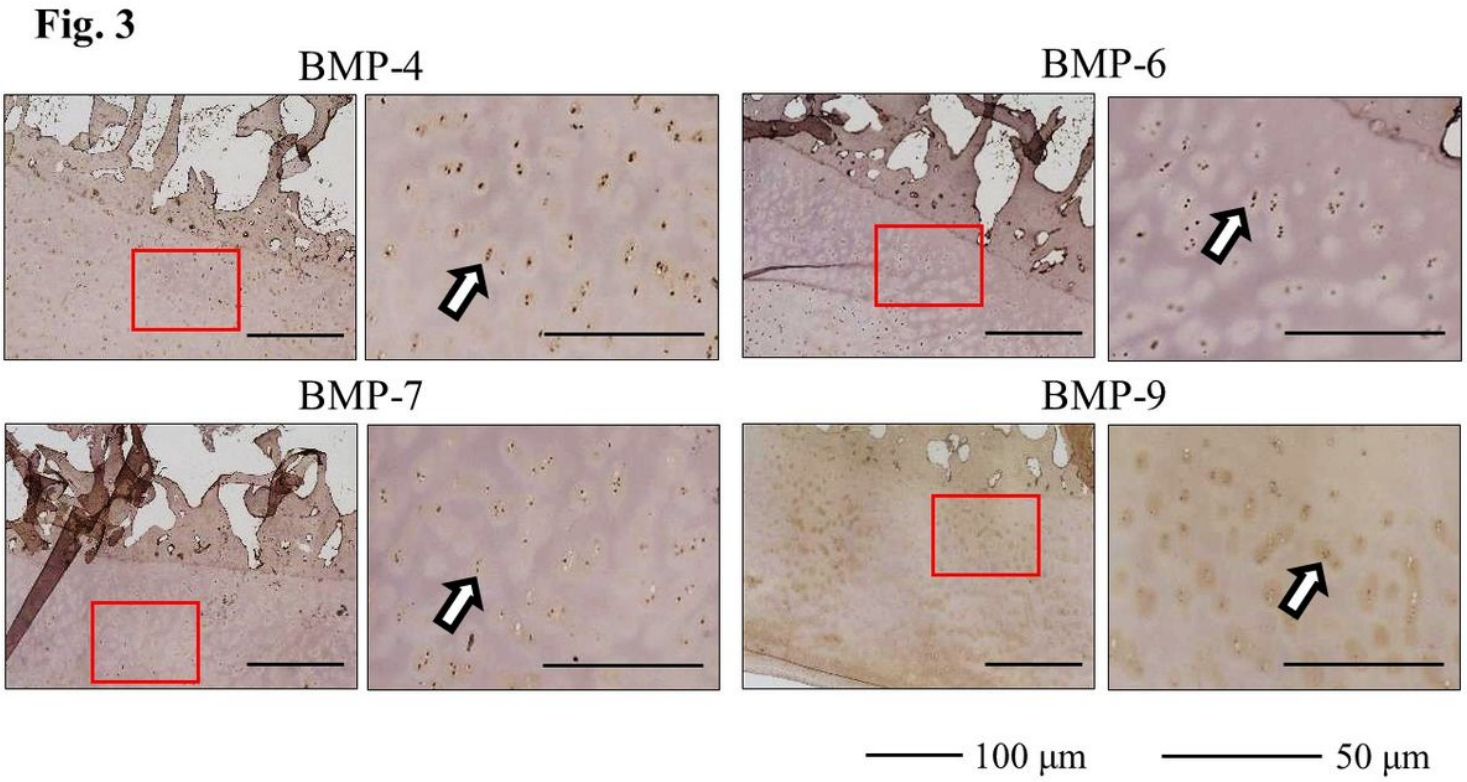

Figure 3

Immunostaining of human bone tissue as a positive control. Left side, low-power field; right side, high-power field BMP: bone morphogenetic protein