Percutaneous nonviral delivery of hepatocyte growth factor in an osteotomy gap promotes bone repair in rabbits: a preliminary study.

Percutaneous Nonviral Delivery of Hepatocyte Growth Factor in a Fracture Gap Promotes Bone Repair in Rabbits: A Preliminary Study

Running title: HGF on Fracture Healing

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Abstract

Hepatocyte growth factor (HGF) was initially identified in cultured hepatocytes and subsequently reported to induce angiogenic mitogen, morphogen, and antiapoptotic activity in various tissues. These properties suggest a potential influence of HGF on bone healing. We asked if gene transfer of human HGF (hHGF) into a fracture gap with a hemagglutinating virus of Japan-envelope (HVJ-E) vector promotes bone healing in rabbits.

HVJ-E that contained either hHGF or control plasmid was percutaneously injected into the fracture gap of rabbit tibias on Day 14. The fracture gap was evaluated by radiography, pQCT, mechanical tests, and histology at Week 8. The expression of hHGF was evaluated by RT-PCR and immunohistochemistry at Week 3. Radiography, pQCT, and histology suggested the hHGF group had faster fracture healing. Mechanical tests demonstrated the hHGF group had greater mechanical strength. The injected tissues at 3 weeks expressed hHGF mRNA by RT-PCR. hHGF-positive immunohistochemical staining was observed in various cells at the fracture gap at Week 3. The data suggests delivery of hHGF plasmid into fracture gap promotes fracture repair, and HGF could become a novel agent for the treatment of bone fractures.

Introduction

The treatment of bone fractures has advanced rapidly in recent years. Various implants for treating fractures have been developed.[3,20] However, complex fractures remain a challenge and often require prolonged fixation. External fixation is preferred by many surgeons method to stabilize such fractures. However, external fixators are still associated with nonunions, pin track infections, and contracture of adjacent joints, some of which relate to the length of the required immobilization.

More rapid bone repair within the fracture gap would avoid the complications that result from prolonged immobilization. Moreover, Einhorn[16] concluded enhancement of the fracture repair process would ensure rapid restoration of function. The ability of injured patients to return earlier to daily life would not only have a substantial economic impact on society, but would also improve the overall physical and mental wellbeing of the patients. Therefore, a compelling need still exists for a safe and effective way to enhance bone repair.

Many studies report enhancement of fracture healing with hormones or growth factors, such as IGF-I,[30] BMP,[17, 29, 51] FGF-2,[4, 10, 42] VEGF,[13] and PTH.[2] Recently, another growth factor called hepatocyte growth factor (HGF) functions as a powerful and versatile factor with angiogenesis, mitogen, morphogen, and antiapoptotic activity. HGF was originally identified from plasma and serum as a molecule that simulated DNA synthesis in rat and human hepatocytes in 1989.[36, 37] Multiple studies subsequently confirmed the role of HGF in enhancing hepatocyte function.[21, 25, 27, 28, 39, 45, 47, 49, 50, 52] However, little is known about the influence of HGF on bone healing, especially *in vivo*. [24] Given the fact that

many growth factors influence various tissues, the question arises as to whether HGF influences bone healing.

We therefore hypothesized gene transfer of hHGF directly into fracture gaps using the HVJ-E vector would promote tibia fracture healing in radiograph, computer tomograph, mechanical test ,and histology in rabbits. Furthermore, we tested the mRNA and protein expression of hHGF to prove the effect of the injected gene.

MATERIALS AND METHODS

In a preliminary study we delivered high human HGF (hHGF) concentrations percutaneously and therefore less invasively than an open method to rabbit tibia fracture gaps. We utilized a novel, nonviral vector, the hemagglutinating virus of Japan envelope (HVJ-E), to deliver hHGF genes to fracture gaps. The HVJ-E vector is effective for gene transfer both in vitro and in vivo.[26] Fracture models were prepared in 50 mature female Japanese white rabbits weighing 2.5 to 3 kg. The rabbits were equally divided into two groups of 25 rabbits each, the hHGF group and the control vector group. Human HGF cDNA (2.2 kb) was inserted between the EcoRI and NotI gaps of the pUC-Sr expression vector plasmid to produce an hHGF expression vector. A pcDNA 3.1(-) plasmid DNA vector (Invitrogen, San Diego, CA) with the same structure, but lacking the hHGF cDNA, was used as a control vector. On Day 7 after injection of HVJ-E, three rabbits in each group were assessed for hHGF mRNA. At 3 and 8 weeks postoperatively, two animals from each group were sacrificed for immunohistochemistry. At 8 weeks, the remaining 40 animals were euthanized and equal numbers used for histological and mechanical

testing. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of Kanazawa University.

HVJ (also known as Sendai virus) envelope vector was prepared as described previously.[26] Briefly, the virus was purified by centrifugation and inactivated by UV irradiation, which disabled the replication capacity of the virus completely without affecting the cell membrane fusing capability of the envelope. HVJ envelope (5 AU) was mixed with 50 μg of either HGF or pcDNA3.1(-) plasmid DNA and 0.3% Triton-X 100. The suspension was then washed with balanced salt solution (BSS - 137 mM NaCl, 5.4 mM KCl, 10 mM Tris-HCl; pH 7.6) and centrifuged (10,000 g, 10 min) at 4°C, and the pellet was resuspended in a final volume of 100 μl BSS for subcutaneous injection. The suspension was stored at 4°C until use. HVJ-E vector is commercially available from Ishihara Sangyo Co. Ltd. (Osaka, Japan).

The rabbits were anesthetized with a subcutaneous injection of ketamine hydrochloride (35 mg/kg body weight; Sankyo Pharmaceutical, Tokyo, Japan), xylazine (5 mg/kg body weight; Bayer, Tokyo, Japan), and an intravenous injection of pentobarbital sodium (40-50 mg/kg body weight; Abbott Laboratories, North Chicago, IL). A longitudinal skin incision was made on the anteromedial aspect of the right tibia, and the periosteum was carefully stripped of the surrounding soft tissue and fascia. Four half pins 2 mm in diameter (Stryker, Geneva, Switzerland) were inserted into the medial aspect of the tibia, perpendicular to its axis, and a unilateral external fixator of our design was applied. To simulate a fracture of the rabbit tibia,[33, 40, 41] we created a 3-mm gap between the bone fragments with a bone saw (Stryker, Geneva, Switzerland). This gap was not critical gap.

Althogh this model was not a fracture in essence, it was an osteotomy, healing process of both models were same.[33, 40,41]

At postoperative Week 2, after induction of anesthesia with ketamine and xylazine, HVJ-E (100 μL) containing either hHGF or pcDNA 3.1(-) plasmid DNA (n = 25 for each group) was percutaneously injected into the fracture gap with a 29-gauge needle (Terumo, Atsugi, Japan) under an image intensifier. The reason we chose postoperative Week 2 was that there were many cells to introduce the gene at the fracture gap.

On Day 7 after percutaneous injection of HVJ-E containing either hHGF or pcDNA 3.1 (-) plasmid DNA (n = 3 for each group), the rabbits were euthanized and the tissue at the fracture gap and the surrounding muscle was harvested and prepared for reverse transcription-polymerase chain reaction (RT-PCR) to detect hHGF mRNA. The reason why we chose 7 days after delivery was that the maximum amount of mRNA from introduced gene was peaked at 5-7 days after the injection.Total RNA was isolated using acid guanidinium thiocyanate-phenol-chloroform and ethanol precipitation. RT-PCR was performed using an amplification reagent kit (TaqMan EZRT-PCR kit; Applied Biosystems, Alameda, CA) with primers specific for hHGF and rabbit GAPDH. The primer pairs for hHGF (sense primer, 5'-ACCCAAGCTGGCTAGCGT-3'; anti-sense primer, 5'-AGTGCTGGATCTATTTTGATTAGG-3') and rabbit GAPDH (sense primer, 5'-GCGCCTGGTCACCAGGGCTGCTT-3'; anti-sense primer, 5'-TGCCGAAGTGGTCGTGGATGACCT-3') [44] were used to amplify hHGF and rabbit GAPDH. PCR reactions of 40 cycles with annealing temperature of 62°C for 1 minute for hHGF, and 26 cycles with annealing temperature 63°C for 1 minute for rabbit GAPDH

were performed. The PCR products (hHGF, 260 bp; GAPDH, 465 bp) were separated by electrophoresis in a 3% agarose gel and stained with ethidium bromide.

At postoperative weeks 3 (for the confirmation of the protein expression) and 8 (for the period of the protein expression), two tibias, respectively, from each group were used for immunohistochemistry. Paraffin sections were treated with anti-hHGF monoclonal antibody (R&D Systems Inc., Minneapolis, MN; dilution 1:100), and peroxidase-conjugated goat antimouse immunoglobulin (EnVision, DAKO, Carpinteria, CA) was used as the secondary antibody. To develop the color, a DAB kit (EnVision, DAKO) was used. The sections were counterstained with Mayer's hematoxylin.

To monitor bone formation, the fracture gaps of the hHGF group were compared to those of the control vector group by radiography at each postoperative time point (Fig 1A-B). The fracture gap was evaluated by comparing bone density of the hHGF and control groups (n = 20 for each group) on anteroposterior and medial-lateral radiographs with an aluminum step wedge (10 steps, 1 mm/step) on the same film. Radiographs were obtained weekly under anesthesia for 8 weeks after the operation. We (HT) evaluated the quantity of callus over the entire 3-mm gap between the proximal and distal native bone using Scion Image Beta-3b software for Windows (Scion Corporation, Frederick, MD). Briefly, the bone density of the gap was measured by interpreting the entire image in units of thickness of the aluminum plate by comparing it with the gradient of luminosity obtained from the aluminum wedge. The bone density is reported in units of aluminum thickness (mm Al).

For the quantitative evaluation of the healing process, 10 rabbits in each group were euthanized by an intravenous dose of sodium pentobarbital 8 weeks after surgery. After the soft tissues were dissected from the tibia and the external fixator was removed. The tibias were stored in gauze soaked in 0.9% saline solution at -20°C and thawed at room temperature before pQCT and mechanical analysis. A quantitative determination of callus development was performed with pQCT (XCT-Research SA+, Stratec, Pforzheim, Germany). A 3-mm gap bone section was analyzed with three consecutive transverse pQCT scans 0.77 mm thick, 2.5 mm apart, and with a pixel size of 0.1 × 0.1 mm. The XCT Series software package (Rev. 6.00B; Stratec, Pforzheim, Germany) was used to calculate the mineral content (mg/mm), mineralized callus area (mm^2), and bone mineral density (mg/cm^3) at the gap level. In order to assess progression of remodeling, areas of higher bone density (> 690 mg/cm^3) within the callus were measured and separated from areas of low-mineral density. The mineral content and the area of this high-mineral-density callus were calculated. The threshold of 690 mg/cm^3 was selected because it corresponds to the lower level of cortical bone. Furthermore, electronic sections through the long axis of each tibia were created on 3-D reconstructed images.

Both tibiae of each rabbit were mechanically tested using an electromechanical testing machine (model MZ-500D; Maruto Machine, Inc., Tokyo, Japan). A three-point bending test was performed at a rate of 2.5 mm/min with 100 kgf of weight axial load. The central loading point was adjusted toward the fracture gap. The lower loading points were separated 30 mm from each other. Failure load values and load-displacement curves were obtained for all samples. The stiffness of each unilateral tibia was calculated as the slope of the linear segment on the load displacement curve. Mechanical data ratios of the

mechanical data of the fractured unilateral tibia to the intact tibia (percent failure load and percent stiffness) were calculated.

Ten rabbits in each group were euthanized 8 weeks after surgery, and the histology of the fracture gap was studied. Heparinized physiologic saline was perfused through both femoral arteries followed by perfusion with 4% paraformaldehyde solution in a phosphate buffer (pH 7.4). The tibias were fixed for 24 hours in the same solution. The tibias were then decalcified with 10% EDTA solution and embedded in paraffin. The specimens were sectioned at a 5-μm thickness parallel to the bone axis and stained with hematoxylin and eosin.

Differences in the bone density, mineral contents, mineralized area, bone mineral density, failure load, and stiffness between control vector group and HGF group were determined by a student's t test.

RESULTS

The administration of HVJ-E/hHGF or HVJ-E/pcDNA to the rabbits produced no obvious adverse effects such as sudden death or abnormal weight loss during the 8 weeks of the experiment.

In radiographs, bony callus appeared on the lateral side at postoperative Week 2 in the control vector group and bridged the fracture gap at postoperative Week 3. After 3 weeks, the size of the bridging callus was reduced and the callus became gradually calcified at the fracture gap. At postoperative Week 8, however, corticalization was not sufficient to complete remodeling. The hHGF group demonstrated a similar course to the control

vector group until postoperative Week 3. After that time point, the callus at the fracture gap became calcified to a greater extent and more rapidly than that of the control vector group. Furthermore, remodeling, especially corticalization and formation of the medullary canal, progressed in the hHGF group. At postoperative Week 8, remodeling in hHGF group was complete, and the fracture gap looked homogeneous when compared to the host bone.

The bone density in the hHGF group was greatercompared to the control vector group at postoperative weeks 4 to 8 ($p= 0.3675$; week 2, $p= 0.0099$; week 4, $p= 0.0002$; week 6, $p= 0.0093$; week 8) (Table 1) (Fig 2). Mineral content in the hHGF group was greater ($p = 0.006$) at postoperative Week 8 compared to the control vector group (control vector group, 18.7 ± 4.7 mg/mm; hHGF group, 25.7 ± 5.4 mg/mm;) (Fig 3A). Mineralized callus area in the hHGF group was also greater ($p = 0.015$) at postoperative Week 8 compared to the control vector group (control vector group, 19.4 ± 4.9 mm^2; hHGF group, 26.2 ± 6.4 mm^2) (Fig 3B). In addition, bone mineral density in the hHGF group tended to be greater at postoperative Week 8 compared to the control vector group, however, there was no difference ($p = 0.059$) between the two groups (control vector group, 933.5 ± 67.3 mg/cm^3; hHGF group, 987.7 ± 52.7 mg/cm^3) (Fig 3C). 3D-CT reconstructed images and axial images of specimens obtained 8 weeks after surgery were created (Fig 4A-B). The fracture gaps in the control vector specimen were not bridged completely with a partial defect of the cortical bone. However, no gap was observed in the fracture gap of the hHGF group (Fig 4A). In the axial view, the fracture gaps in the hHGF group had a circular, thick cortical bone. In contrast, the cortex in the control vector group had a partial defect of circular cortical bone (Fig 4B).

The mean ratio of failure loads of the hHGF group was greater ($p = 0.037$) than that of the control vector group (89.4 ± 17.5% versus 76.3 ± 9.1%, respectively) (Fig 5A). The hHGF-treated tibiae were relatively stiffer ($p = 0.001$) than those of the control group when both were compared to their contralateral controls (69.7 ± 6.5% versus 57.5 ± 6.3%) (Fig 5B).

At postoperative Week 8 in the hHGF group, the fracture gaps were completely remodeled with a firm cortex and a reconstructed medullary canal almost identical to that of normal bone. In contrast, a medullary canal was not observed in the control vector group. In place of a medullary canal, mature cartilage and new trabecular bone were present in the middle of the fracture gap, which was not remodeled sufficiently (Fig 6). In RT-PCR, expression of hHGF mRNA was detected only in the callus of hHGF group and in the surrounding muscle of neither group (Fig 7). Thus HVJ-E/hHGF was transfected locally into the fracture gap sufficiently to express mRNA. By immunohistochemistry, We observed expression of hHGF in immature cells, fibroblasts, osteoblasts, and osteocytes (Fig 8A). No immunohistochemical staining was observed in the surrounding muscle of the hHGF group. No hHGF-positive cells were observed in specimens from the control vector group. This indicates the HVJ-E/hHGF was transfected locally into the fracture gap at the level of protein. Human HGF expression was still observed at this time point, however, when expression at 8 weeks was compared to that at 3 weeks, hHGF protein decreased by the endpoint of treatment (Fig 8B).

DISCUSSION

214 HGF was originally identified from rat and human hepatocyte. Multiple studies
215 subsequently confirmed the role of HGF in enhancing hepatocyte function. After that,
216 HGF was recognized as a powerful and versatile factor with angiogenesis, mitogen,
217 morphogen, and antiapoptotic activity in various tissues. The question arose as to whether
218 HGF influences bone healing. We therefore hypothesized gene transfer of hHGF directly
219 into fracture gaps would promote bone healing in rabbit. This study had two major
220 limitations. First, we followed up only eight weeks. Although this time period was chosen
221 because the control model showed bone union and sufficient corticalization in radiograph
222 at postoperatibe Week 8, our mechanical test data did not reach intact level. Different
223 results may occur at later stages of healing. However, this study demonstrates bone
224 healing differences resulting from HGF, especially at early stage. Second, we did not
225 confirm the duration of hHGF gene expression. Because, on clinical setteing, gene
226 therapy always accompanies the problem of its safety, we should ascertain its safety until
227 at least the end of expression. We gave the priority to economic and practically reasons.

228 Our data suggest percutaneous injection of HVJ-E/hHGF into tibial fracture gaps
229 effectively promotes bone repair. Consequently, the treatment time for fractures could be
230 shortened when the hHGF gene is administered during the early stages of fracture repair.
231 These data indicate the high potential of hHGF gene therapy using the HVJ-E vectors for
232 treatment of bone fractures.

233 Some osteogenetic factors have been used as therapeutic molecules for fracture healing
234 and promote fracture repair. HGF, however, has not been used for this purpose to date.
235 Many papers have been published describing therapeutic uses of HGF for various
236 diseases such as limb ischemia,[28, 49, 50] myocardial ischemia,[5] brain ischemia,[47, 52] hearing

237 impairment,[39] nerve injury,[27] and spinal cord injury.[45] Notably, HGF plasmid delivery for
238 peripheral limb ischemia is now in clinical trials.[34]

239 Recently, it was demonstrated that HGF along with vitamin D promoted growth and
240 differentiation of human mesenchymal cells into osteogenic cells.[12] Later, it was reported
241 HGF enhanced osteoblast differentiation in vitro.[22] It has also been reported HGF
242 contributes to fracture repair by inducing the expression of BMP receptors during the
243 early phase of fracture repair.[24] Our data support the previous results that the use of HGF
244 for fracture repair will induce the expression of BMPR, which will differentiate
245 mesenchymal cells into osteoblasts and osteoblasts into ossification. Based on these
246 observations, the use of HGF together with other factors such as BMP or vitamin D could
247 enhance fracture healing more rapidly. This possibility should be investigated in future
248 studies.

249 Gene delivery to bone has been accomplished by several vectors, including adenovirus,
250 retrovirus, adeno-associated virus (AAV), lentivirus, and herpes simplex virus (HSV). In
251 this study, we employed the HVJ-E vector system as the delivery method for bone
252 fracture gaps. HVJ-E is a novel vector system that converts inactivated HVJ into a gene
253 transfer vector by introducing plasmid DNA directly into inactivated HVJ particles after
254 treatment with a mild detergent and centrifugation in the presence of plasmid DNA.[26]
255 Previous studies demonstrated the successful delivery of DNA to cultured cells and
256 animal tissues such as the inner ear,[39] liver, skin, uterus, lung, eye, tumor tissues,[26] and
257 brain.[46] HVJ-E is a nonviral vector that is generally less efficient than other viral delivery
258 vehicles. However, it is inexpensive, safe, nonimmunogenic, and easy to handle.[19]

In principle, gene delivery is performed in two ways, in vivo or ex vivo.[8, 9, 14] In-vivo gene delivery is a direct approach, where the vector is injected directly into the specified target tissue. Ex vivo is an indirect approach, where the therapeutic gene is delivered outside the body to various cells grown in culture prior to implantation into the body. In-vivo gene delivery involves directly delivering the gene into a specific anatomic gap. The advantages of this method are that it is a simple technique that favors its transfer into a clinical application, and that it has the potential for lower costs. The disadvantages are the difficulties in targeting specific cells for transduction and in achieving high transduction efficiency.[14] Ex-vivo gene transfer is considered safer because transfected cells are introduced into the body, and safety tests are possible before introduction. However, it is technically more complex and more expensive.

We used gene delivery rather than protein delivery. Gene therapy has potential advantages over protein delivery due to: (1) long-term expression of the protein from the delivered gene, (2) high local concentration of protein expression, (3) low cost of manufacture, (4) reduced systemic effects, and (5) longer shelf life and easier storage of vectors.[31, 32, 38, 48] Ido[23] reported recombinant hHGF administered intravenously was rapidly decreased in serum with a short half-life of 2.4 min. We detected expression of hHGF 6 weeks after the injection of plasmid. Therefore, gene transfer of HGF plasmid is expected to generate much longer expression of HGF than direct administration of HGF protein. Yoshimura[52] also demonstrated gene transfer of HGF plasmid markedly increased cerebral blood flow in the ischemic brain, whereas a single injection of recombinant HGF failed to do so.

There are multiple studies that relate to gene therapy for bone defects and fractures.[6, 7, 8, 15, 18, 43] However, therapeutic gene therapy for bone regeneration with a nonviral vector has never been demonstrated. Egermann et al[15] reported inflammation due to an immune reaction to adenovirus vectors caused severe retardation of bone formation. Similar results occurred with injection of Ad-BMP-2 into muscles of immunocompetent rats, causing poor bone formation and an inflammatory response at the injection gap.[1]

Furthermore, a small number of studies address direct percutaneous injection of a gene to a fracture gap.[7, 8, 43] Gene transfer by direct percutaneous injection leading to endogenous bioactive protein expression offers the potential advantage of simple direct delivery without the requirement for a carrier or surgery and could be used to treat closed fractures in clinical cases. Rundle[43] also performed percutaneous injection of the BMP-4 gene with a retroviral vector into the subperiosteum, which required the deposit of all vector within the periosteum while avoiding the muscle. Therefore it was technically difficult and not practical. Percutaneous injections of gene to muscle are widely used to promote bone repair due to the high efficiency and ease of transfection and longer duration of gene expression.[1, 11, 35] With intramuscular injections, however, the area of gene expression is uncertain, which raises questions about the effect of gene expression at the fracture gap. Furthermore, the immune response differs between intraosseous and intramuscular gaps, as the muscle has a stronger immunologic reaction.[6]

Our data suggests HGF promotes fracture healing in rabbit tibia and in-vivo gene therapy using HVJ-E/hHGF effectively enhances bone formation in fracture healing. Utilization of these methods could shorten treatment time, resulting in improved physical and mental

303 wellbeing of patients. In the future, the safety evaluation of this gene therapy technique
304 and the mechanisms whereby HGF promotes bone healing should be assessed further.

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References

1. Alden TD, Pittman DD, Hankins GR, Beres EJ, Engh JA, Das S, Hudson SB, Kerns KM, Kallmes DF, Helm GA. In vivo endochondral bone formation using a bone morphogenetic protein 2 adenoviral vector. *Hum Gene Ther.* 1999;10:2245-2253.

2. Alkhiary YM, Gerstenfeld LC, Krall E, Westmore M, Sato M, Mitlak BH, Einhorn TA. Enhancement of experimental fracture-healing by systemic administration of recombinant human parathyroid hormone (PTH 1-34). *J Bone Joint Surg Am.* 2005;87:731-741.

3. Al-Sayyad MJ. Taylor Spatial Frame in the treatment of pediatric and adolescent tibial shaft fractures. *J Pediatr Orthop.* 2006;26:164-170.

4. Aoyama I, Anzai J, Nakamura T, Hiyama Y, Tamura M. Acceleration of fracture healing in nonhuman primates by fibroblast growth factor-2.Kawaguchi H, Nakamura K, Tabata Y, Ikada Y. *J Clin Endocrinol Metab.* 2001;86:875-880.

5. Azuma J, Taniyama Y, Takeya Y, Iekushi K, Aoki M, Dosaka N, Matsumoto K, Nakamura T, Ogihara T, Morishita R. Angiogenic and antifibrotic actions of hepatocyte growth factor improve cardiac dysfunction in porcine ischemic cardiomyopathy. *Gene Ther.* 2006;13:1206-1213.

6. Baltzer AW, Lattermann C, Whalen JD, Ghivizzani S, Wooley P, Krauspe R, Robbins PD, Evans CH. Potential role of direct adenoviral gene transfer in enhancing fracture repair. *Clin Orthop Relat Res.* 2000;379:S120-125.

7. Bertone AL, Pittman DD, Bouxsein ML, Li J, Clancy B, Seeherman HJ. Adenoviral-mediated transfer of human BMP-6 gene accelerates healing in a rabbit ulnar osteotomy model. *J Orthop Res.* 2004;22:1261-1270.

8. Betz OB, Betz VM, Nazarian A, Pilapil CG, Vrahas MS, Bouxsein ML, Gerstenfeld LC, Einhorn TA, Evans CH. Direct Percutaneous Gene Delivery to Enhance Healing of Segmental bone defect. *J Bone Joint Surg. Am*. 2006; 88:355-365.

9. Blanquaert F, Delany AM, Canalis E. Fibroblast growth factor-2 induces hepatocyte growth factor/scatter factor expression in osteoblasts. *Endocrinology*. 1999;140: 1069-1074.

10. Chen WJ, Jingushi S, Aoyama I, Anzai J, Hirata G, Tamura M, Iwamoto Y. Effects of FGF-2 on metaphyseal fracture repair in rabbit tibiae. *J Bone Miner Metab*. 2004;22:303-309.

11. Chen Y, Cheung KM, Kung HF, Leong JC, Lu WW, Luk KD. In vivo new bone formation by direct transfer of adenoviral-mediated bone morphogenetic protein-4 gene. *Biochem Biophys Res Commun.* 2002;298:121-127.

12. .D'Ippolito G, Schiller PC, Perez-stable C, Balkan W, Roos BA, Howard GA. Cooperative actions of hepatocyte growth factor and 1,25-dihydroxyvitamin D3 in osteoblastic differentiation of human vertebral bone marrow stromal cell. *Bone*. 2002;31:269-275.

13. Eckardt H, Ding M, Lind M, Hansen ES, Christensen KS, Hvid I. Recombinant human vascular endothelial growth factor enhances bone healing in an experimental nonunion model. *J Bone Joint Surg Br.* 2005;87:1434-1438.

14. Egermann M, Goldhahn J, Schneider E. Animal models for fracture treatment in osteoporosis. *Osteoporos Int.* 2005;16:S129-138. Review.

15. Egermann M, Lill CA, Griesbeck K, Evans CH, Robbins PD, Schneider E, Baltzer AW. Effect of BMP-2 gene transfer on bone healing in sheep. *Gene Therapy.* 2006;13:1290–1299.

16. Einhorn TA. Enhancement of fracture-healing. *J Bone Joint Surg Am.* 1995;77:940-956. Review.

17. Einhorn TA, Majeska RJ, Mohaideen A, Kagel EM, Bouxsein ML, Turek TJ, Wozney JM. A single percutaneous injection of recombinant human bone morphogenetic protein-2 accelerates fracture repair. *J Bone Joint Surg Am.* 2003;85:1425-1435.

18. Franceschi RT, Yang S, Rutherford RB, Krebsbach PH, Zhao M, Wang D. Gene therapy approaches for bone regeneration. *Cells Tissues Organs.* 2004;176:95-108.

19. Giannoudis PV, Tzioupis CC, Tsiridis E. Gene therapy in orthopaedics. *Injury*. 2006;37:S30-40. Review.

20. Greiwe RM, Archdeacon MT. Locking plate technology: current concepts. *J Knee Surg.* 2007;20:50-55.

21. Hirano S, Bless DM, Rousseau B, Welham N, Montequin D, Chan RW, Ford CN. Prevention of vocal fold scarring by topical injection of hepatocyte growth factor in a rabbit model. *Laryngoscope*. 2004;114:548-556.

22. Hossain M, Irwin R, Baumann MJ, McCabe LR. Hepatocyte growth factor (HGF) adsorption kinetics and enhancement of osteoblast differentiation on hydroxyapatite surfaces. *Biomaterials*. 2005;26:2595-2602.

23. Ido A, Moriuchi A, Kim I, Numata M, Nagata Y, Hasuike S, Uto H, Tsubouchi H. Pharmacokinetic study of recombinant human hepatocyte growth factor administered in a bolus intravenously or via portal vein. *Hepatology Research.* 2004; 30:175–181.

24. Imai Y, Terai H, Nomura-Furuwatari C, Mizuno S, Matsumoto K, Nakamura T, Takaoka K. Hepatocyte growth factor contributes to fracture repair by upregulating the expression of BMP receptors. *J Bone Miner Res*. 2005;20:1723-1730.

25. Jin M, Chen Y, He S, Ryan SJ, Hinton DR. Hepatocyte growth factor and its role in the pathogenesis of retinal detachment. *Invest Ophthalmol Vis Sci.* 2004;45:323-329.

26. Kaneda Y, Nakajima T, Nishikawa T, Yamamoto S, Ikegami H, Suzuki N, Nakamura H, Morishita R, Kotani H. Hemagglutinating virus of Japan (HVJ) envelope vector as a versatile gene delivery system. *Mol Ther.* 2002;6:219-226.

27. Kato N, Nemoto K, Nakanishi K, Morishita R, Kaneda Y, Uenoyama M, Ikeda T, Fujikawa K. Nonviral HVJ (hemagglutinating virus of Japan) liposome-mediated retrograde gene transfer of human hepatocyte growth factor into rat nervous system promotes functional and histological recovery of the crushed nerve. *Neurosci Res.* 2005;52:299-310.

28. Koike H, Morishita R, Iguchi S, Aoki M, Matsumoto K, Nakamura T, Yokoyama C, Tanabe T, Ogihara T, Kaneda Y. Enhanced angiogenesis and improvement of neuropathy by cotransfection of human hepatocyte growth factor and prostacyclin synthase gene. *FASEB J.* 2003;17:779-781.

29. Lane JM. Bone morphogenic protein science and studies. *J Orthop Trauma*. 2005;19:S17-22.

30. Lieberman JR, Daluiski A, Einhorn TA. The role of growth factors in the repair of bone. Biology and clinical applications. *J Bone Joint Surg Am.* 2002;84:1032-1044.

31. Lieberman JR, Daluiski A, Stevenson S, Wu L, McAllister P, Lee YP, Kabo JM, Finerman GA, Berk AJ, Witte ON. The effect of regional gene therapy with bone

morphogenetic protein-2-producing bone-marrow cells on the repair of segmental femoral defects in rats. *J Bone Joint Surg Am.* 1999;81:905-917.

32. Luo J, Sun MH, Kang Q, Peng Y, Jiang W, Luu HH, Luo Q, Park JY, Li Y, Haydon RC, He TC. Gene therapy for bone regeneration. *Curr Gene Ther.* 2005;5:167-179. Review.

33. Matsushita T, Kurokawa T.Comparison of cyclic compression, cyclic distraction and rigid fixation. Bone healing in rabbits. *Acta Orthop Scand.* 1998;69:95-98.

34. Morishita R, Aoki M, Hashiya N, Makino H, Yamasaki K, Azuma J, Sawa Y, Matsuda H, Kaneda Y, Ogihara T. Safety evaluation of clinical gene therapy using hepatocyte growth factor to treat peripheral arterial disease. *Hypertension*. 2004;44:203-209.

35. Musgrave DS, Bosch P, Ghivizzani S, Robbins PD, Evans CH, Huard J. Adenovirus-mediated direct gene therapy with bone morphogenetic protein-2 produces bone. *Bone*. 1999;24:541-547.

36. Nakamura T, Nawa K, Ichihara A, Kaise N, Nishino T. Purification and subunit structure of hepatocyte growth factor from rat platelets. *FEBS Lett*. 1987;224:311-316.

37. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S. Molecular cloning and expression of human hepatocyte growth factor. *Nature*. 1989;342:440-443.

38. Niyibizi C, Baltzer A, Lattermann C, Oyama M, Whalen JD, Robbins PD, Evans CH. Potential role for gene therapy in the enhancement of fracture healing. *Clin Orthop Relat Res.* 1998;355:S148-153.

39. Oshima K, Shimamura M, Mizuno S, Tamai K, Doi K, Morishita R, Nakamura T, Kubo T, Kaneda Y. Intrathecal injection of HVJ-E containing HGF gene to cerebrospinal fluid can prevent and ameliorate hearing impairment in rats. *FASEB J*. 2004;18:212-214.

40. Park SH, O'Connor K, Sung R, McKellop H, Sarmiento. Comparison of healing process in open osteotomy model and closed fracture model. *J Orthop Trauma.* 1999;13:114-120.

41. Park SH, Silva M. Effect of intermittent pneumatic soft-tissue compression on fracture-healing in an animal model. *J Bone Joint Surg Am.* 2003;85:1446-1453.

42. Radomsky ML, Aufdemorte TB, Swain LD, Fox WC, Spiro RC, Poser JW. Novel formulation of fibroblast growth factor-2 in a hyaluronan gel accelerates fracture healing in nonhuman primates. *J Orthop Res.* 1999;17:607-614.

43. Rundle CH, Miyakoshi N, Kasukawa Y, Chen ST, Sheng MH, Wergedal JE, Lau KH, Baylink DJ. In vivo bone formation in fracture repair induced by direct retroviral-based gene therapy with bone morphogenetic protein-4. *Bone*. 2003;32:591-601.

44. Sekalska B, Ciechanowicz A, Dolegowska B, Naruszewicz M. Optimized RT-PCR method for assaying expression of monocyte chemotactic protein type 1 (MCP-1) in rabbit aorta. *Biochem Genet.* 2006;44:133-143.

45. Shi E, Jiang X, Kazui T, Washiyama N, Yamashita K, Terada H, Bashar AH. Nonviral gene transfer of hepatocyte growth factor attenuates neurologic injury after spinal cord ischemia in rabbits. *J Thorac Cardiovasc Surg.* 2006;132:941-947.

46. Shimamura M, Morishita R, Endoh M, Oshima K, Aoki M, Waguri S, Uchiyama Y, Kaneda Y. HVJ-envelope vector for gene transfer into central nervous system. *Biochem Biophys Res Commun.*2003;300:464-471.

47. Shimamura M, Sato N, Oshima K, Aoki M, Kurinami H, Waguri S, Uchiyama Y, Ogihara T, Kaneda Y, Morishita R. Novel therapeutic strategy to treat brain ischemia: overexpression of hepatocyte growth factor gene reduced ischemic injury without cerebral edema in rat model. *Circulation*. 2004;109:424-431.

48. Southwood LL, Frisbie DD, Kawcak CE, McIlwraith CW. Delivery of growth factors using gene therapy to enhance bone healing. *Vet Surg.* 2004;33:565-578.

49. Taniyama Y, Morishita R, Aoki M, Nakagami H, Yamamoto K, Yamazaki K, Matsumoto K, Nakamura T, Kaneda Y, Ogihara T. Therapeutic angiogenesis induced by human hepatocyte growth factor gene in rat and rabbit hindlimb ischemia models: preclinical study for treatment of peripheral arterial disease. *Gene Ther.* 2001;8:181-189.

50. Taniyama Y, Morishita R, Hiraoka K, Aoki M, Nakagami H, Yamasaki K, Matsumoto K, Nakamura T, Kaneda Y, Ogihara T. Therapeutic angiogenesis induced by human hepatocyte growth factor gene in rat diabetic hind limb ischemia model: molecular mechanisms of delayed angiogenesis in diabetes. *Circulation*. 2001;104:2344-2350.

51. Welch RD, Jones AL, Bucholz RW, Reinert CM, Tjia JS, Pierce WA, Wozney JM, Li XJ. Effect of recombinant human bone morphogenetic protein-2 on fracture healing in a goat tibial fracture model. *J Bone Miner Res*. 1998;13:1483-1490.

52. Yoshimura S, Morishita R, Hayashi K, Kokuzawa J, Aoki M, Matsumoto K, Nakamura T, Ogihara T, Sakai N, Kaneda Y. Gene transfer of hepatocyte growth factor to subarachnoid space in cerebral hypoperfusion model. *Hypertension*. 2002;39:1028-1034.

Legends

Fig 1A-B. (A) A series of anteroposterior radiographs taken of the hHGF group (H) and control groups (C) from each single animal show faster callus formation at the 3-mm gap in the hHGF group. **(B)** A series of mediolateral radiographs taken of the hHGF group (H) and control groups (C) from each single animal show faster remodeling at the 3-mm gap in the hHGF group.

Fig 2. The effect of hHGF plasmid on bone healing was determined by bone density of the 3-mm gap. The results of the bone density are given in units of aluminum thickness

Fig 3A-C. (A) At postoperative Week 8 the mineral content (mean ± SD, $p = 0.0061$) was higher in the hHGF group (H) than in the control vector group (C). **(B)** Mineralized callus area at postoperative Week 8 (mean ± SD, $p = 0.015$) was higher in the hHGF group (H) than in the control vector group (C). **(C)** Bone mineral density 8 weeks after the operation (mean ± SD) in the hHGF group tended to be greater compared to the control vector group (C). There was no difference ($p = 0.059$) between the two groups.

Fig 4A-B. (A) A reconstruction image of whole tibia at postoperative Week 8 shows complete bridging in the hHGF group (H), and not in the control vector group (C) with a partial defect in cortical bone. **(B)** An axial image at the gap level 8 weeks after the operation shows thick, circular cortical bone in the hHGF group (H), and not in the control vector group (C) with a partial defect of cortical bone.

Fig 5A-B. (A) Mean percentage of failure load at postoperative Week 8 (mean ± SD, p = 0.0375) was higher in the hHGF group (H) than in the control vector group (C). **(B)** Mean percentage stiffness at postoperative Week 8 (mean ± SDM,p = 0.0011) was stronger in the hHGF group (H) than in the control vector group (C).

Fig 6. Representative longitudinal histologic sections of the 3-mm gap at postoperative Week 8 are shown. The arrows indicate the original 3-mm defect region (top row). Magnified histology of 3-mm gap (bottom row). The gap in the control vector group (C) had a trabecular bone in the middle of the fracture gap, meanwhile in the hHGF group (H), a firm cortex and a reconstructed medullary canal were observed. (Stain, Hematoxilin-Eosin stain; original magnification, ×1 for left side and ×40 for right side)

Fig. 7. RT-PCR analysis demonstrated the expression of hHGF mRNA in the injected callus and the surrounding muscle of both groups. hHGF mRNA was specifically detected in the callus of the hHGF group (H), but was not detected in the callus and muscle of the control vector group, or in muscle of the hHGF group.

Fig. 8A-B. (A) Immunohistochemistry of hHGF at the fracture gap at postoperative Week 3 (1 week after the injection of plasmid)(Stain, Immunohistochemistry of hHGF ; original magnification, ×400). In the hHGF group (H), hHGF expression was markedly observed in immature cells, fibroblasts, osteoblasts, and osteocytes. No immunohistochemical staining was observed in specimens from the control vector group (C). **(B)** Immunohistochemistry of hHGF of the fracture gap at postoperative Week 3 or 8

(1 and 5 weeks after the injection of plasmid) (Stain, Immunohistochemistry of hHGF ; original magnification, ×400). hHGF expression was still observed at postoperative Week 8; however, it decreased compared to that of postoperative Week 3.

Fig. 1A

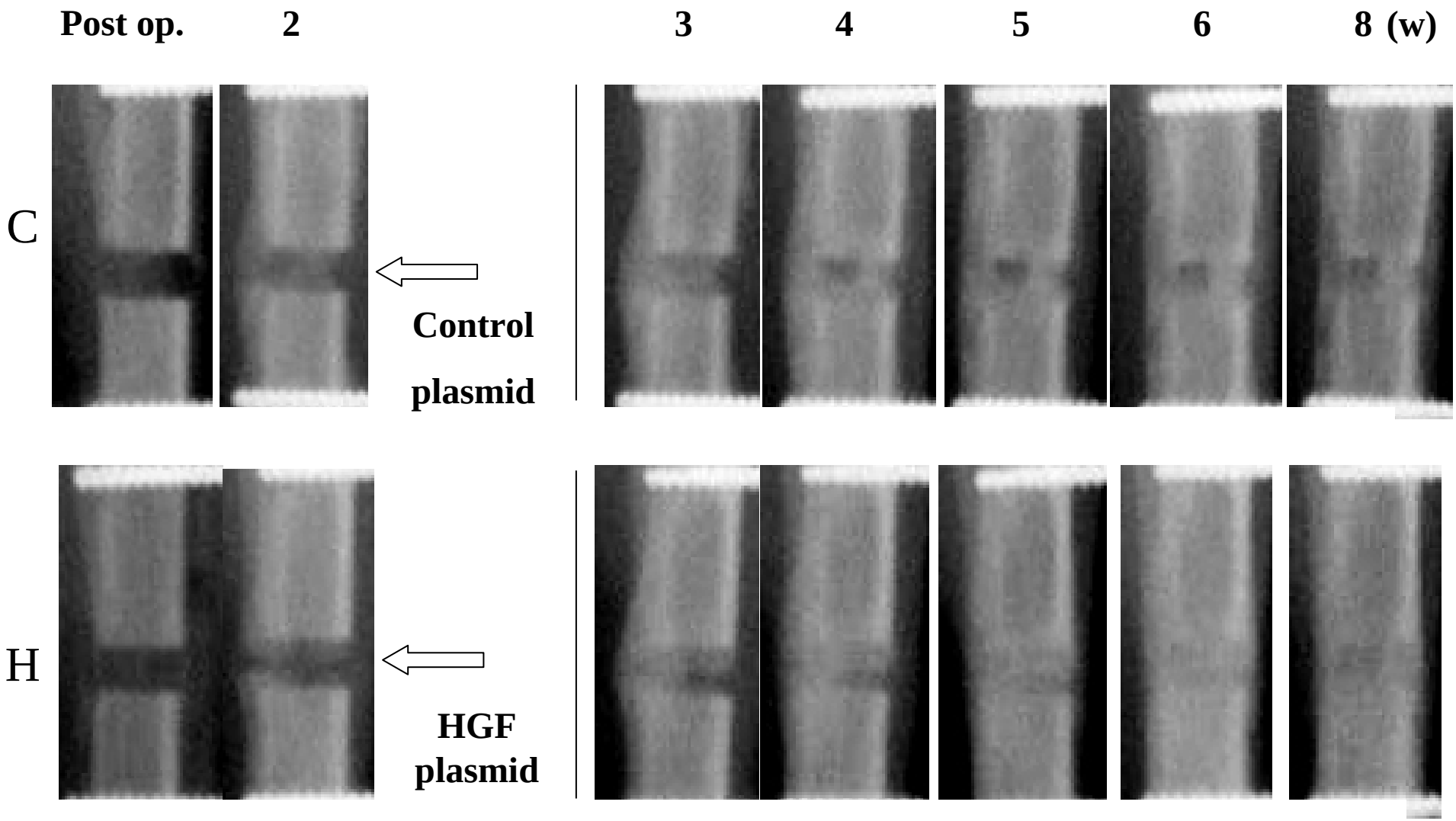


Fig. 1B

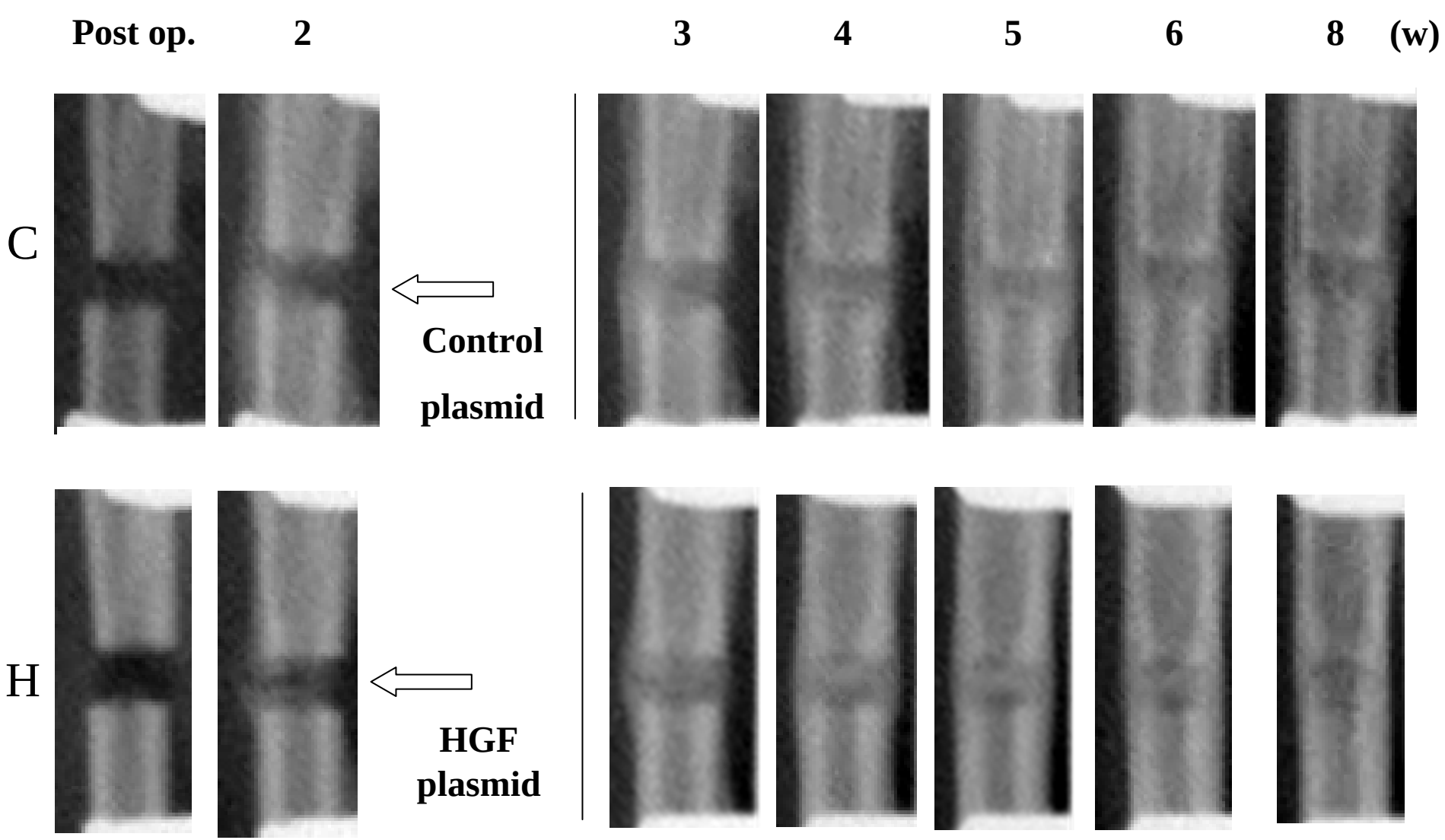
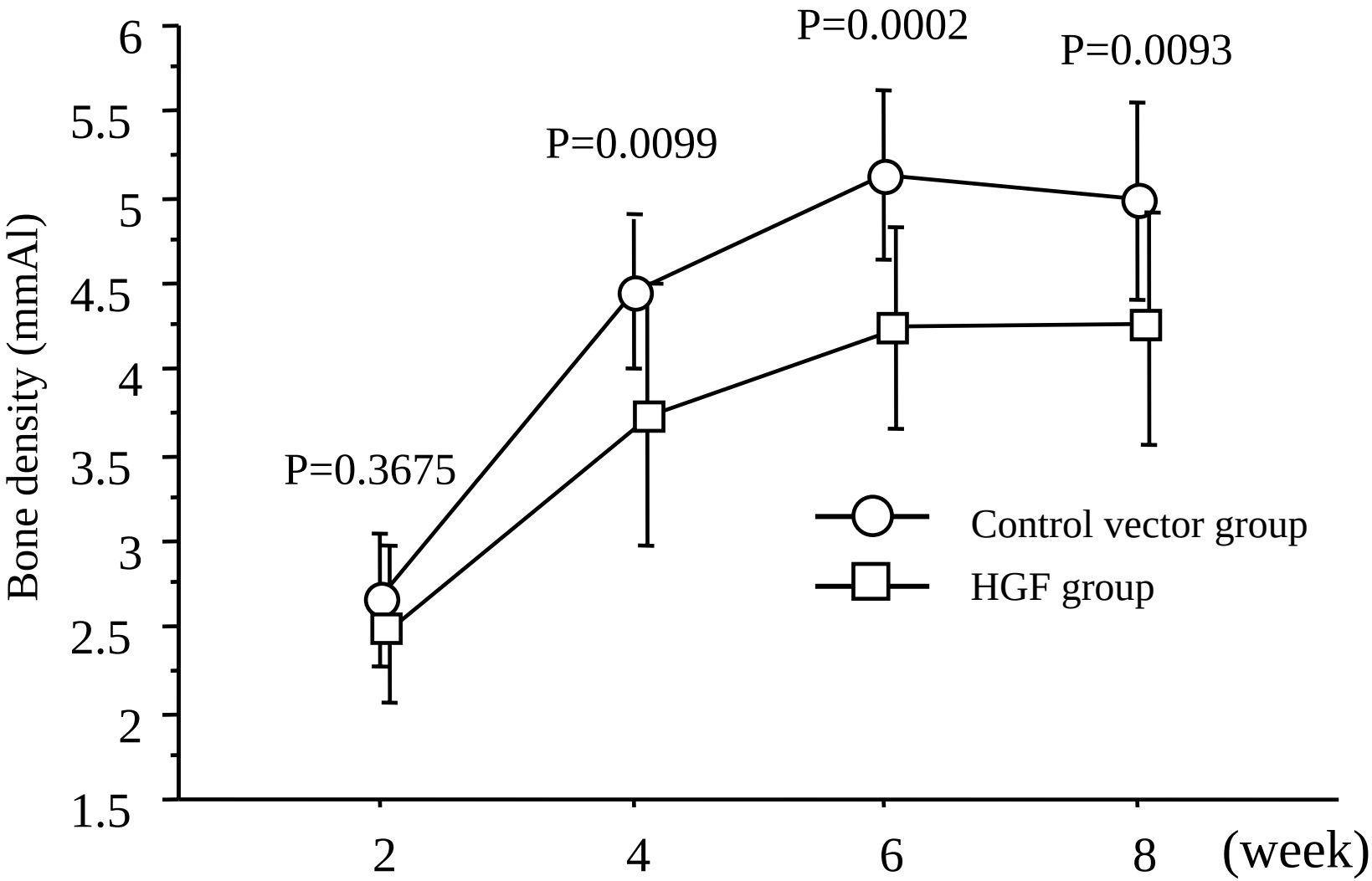


Fig. 2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P=0.0093
P=0.0099
P=0.3675
Bone density (mmAl)
6
5.5
5
4.5
4
3.5
3
2.5
2
1.5
2
4
6
8
(week)
Control vector group
HGF group

Fig. 3A

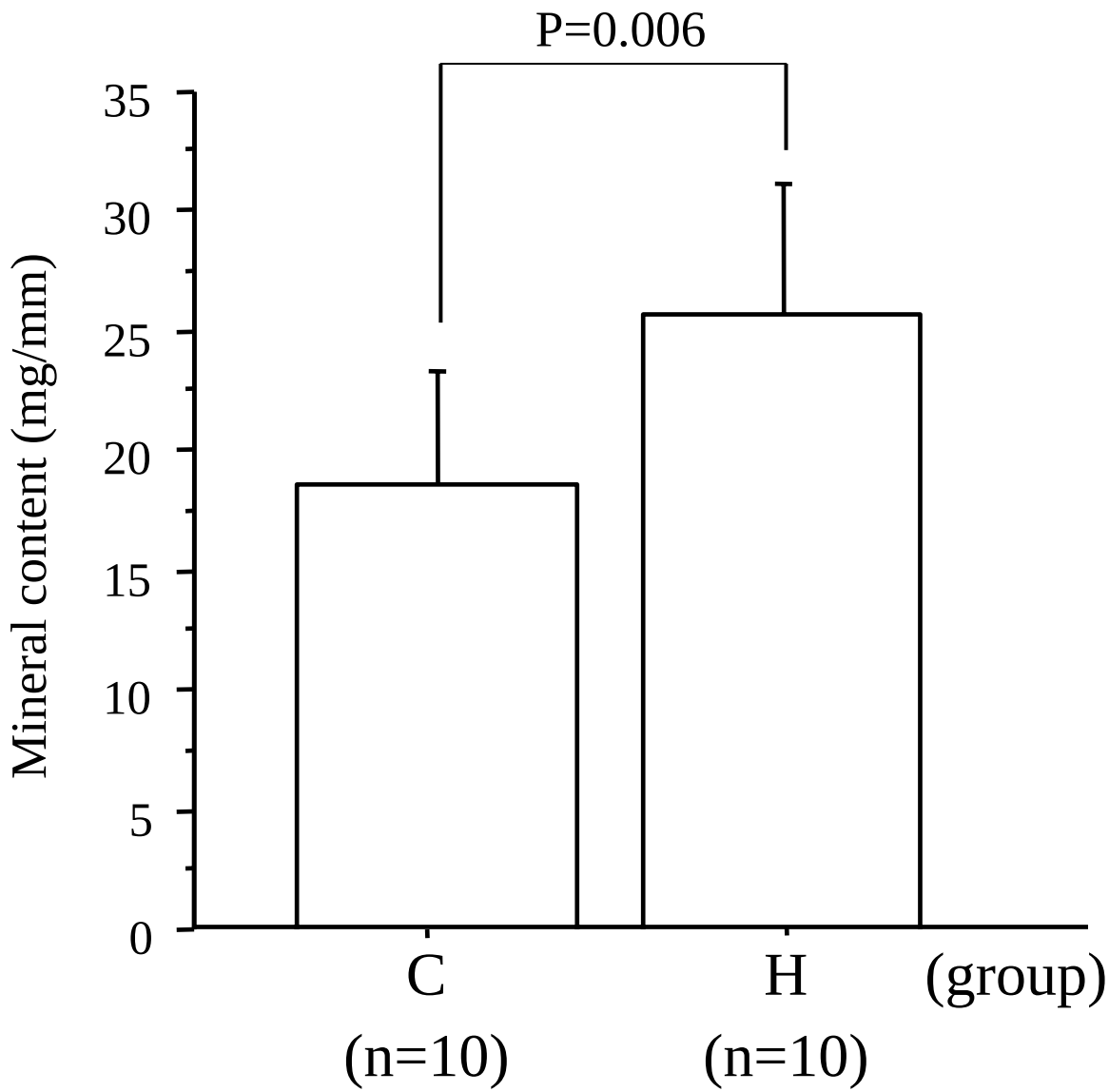

P=0.006
35
30
25
20
15
10
5
0
Mineral content (mg/mm)
C
H
(group)
(n=10)
(n=10)

Fig. 3B

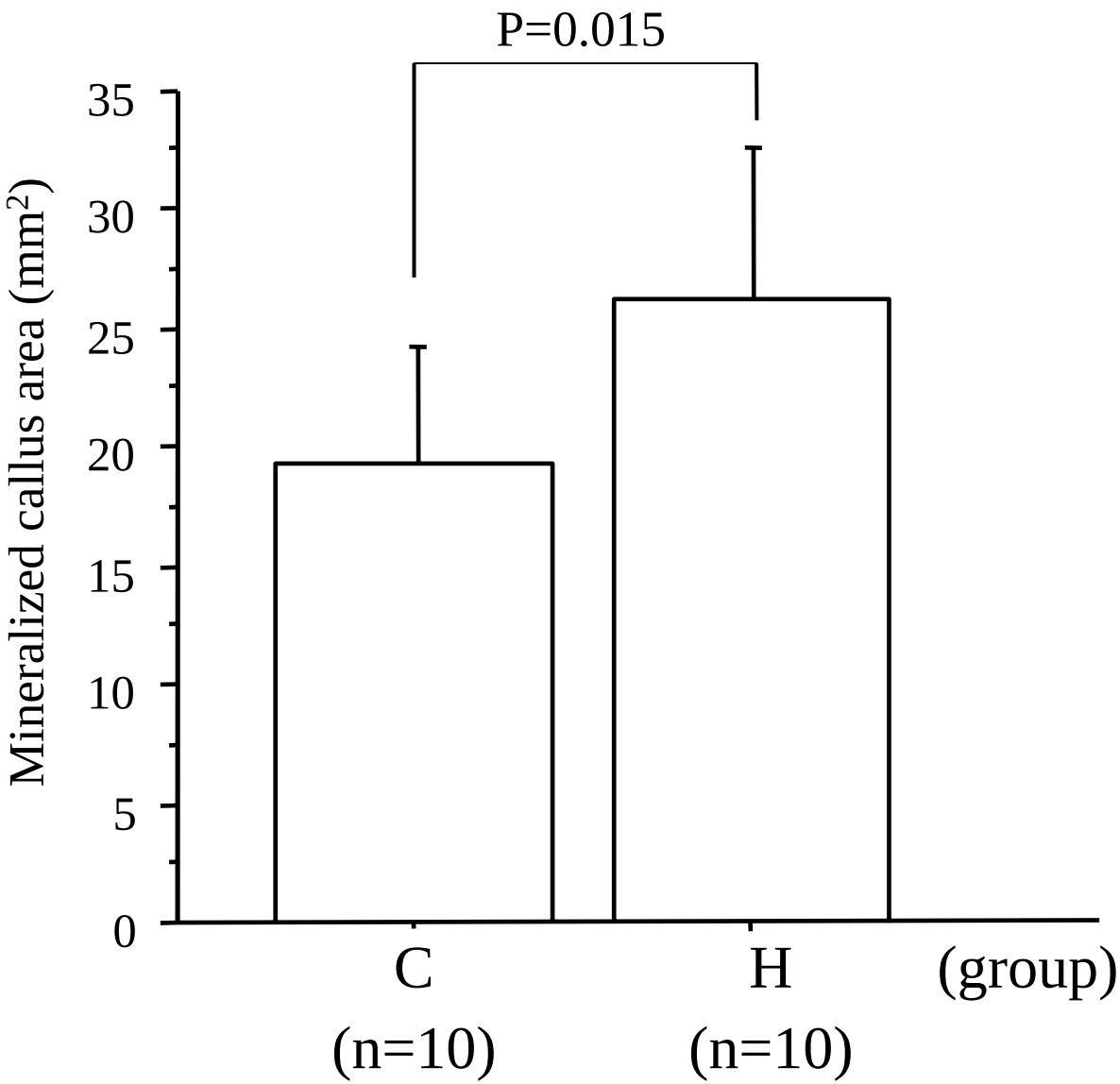

P=0.015
Mineralized callus area (mm²)
35
30
25
20
15
10
5
0
C
H
(group)
(n=10)
(n=10)

Fig. 3C

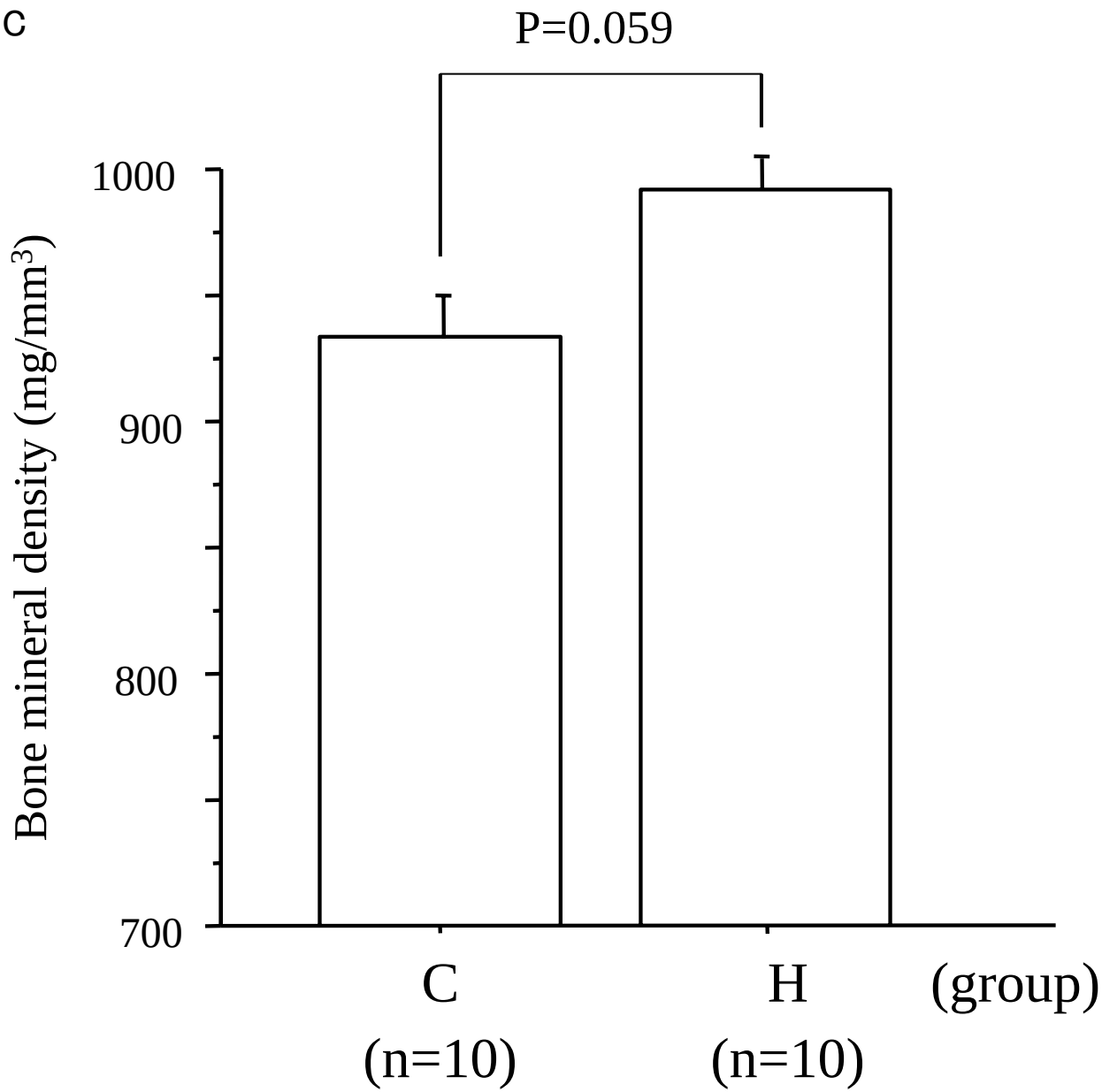
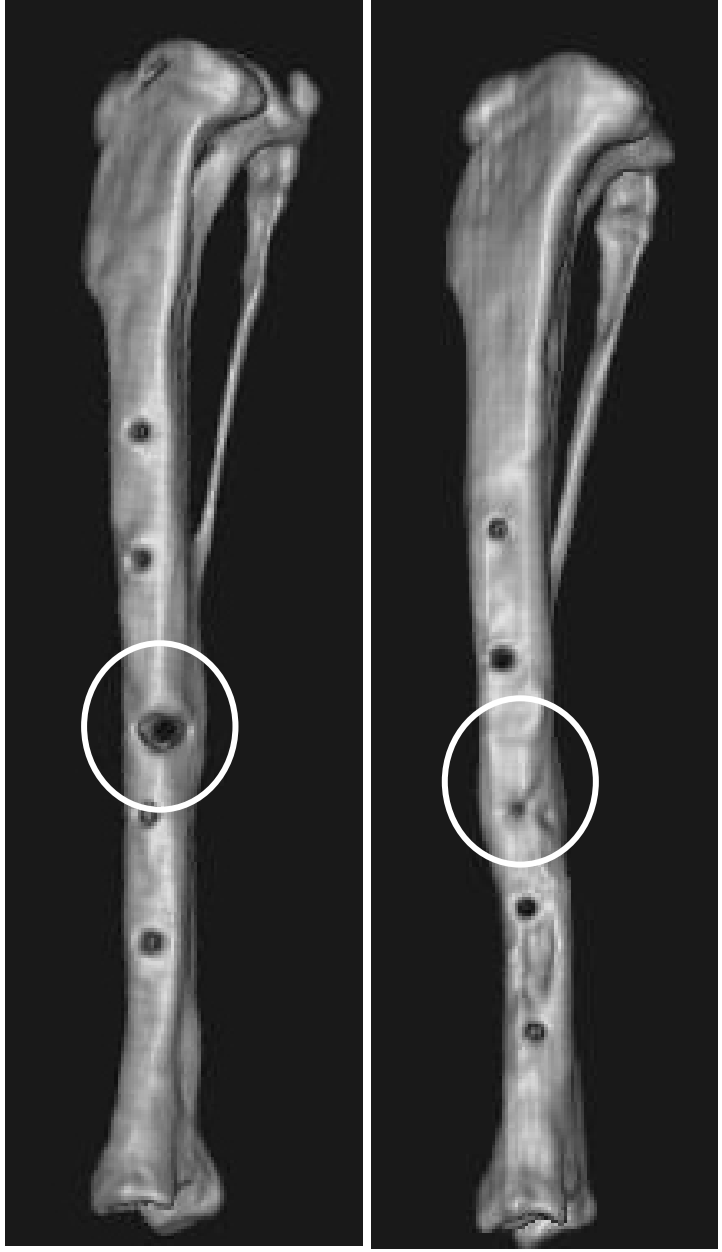
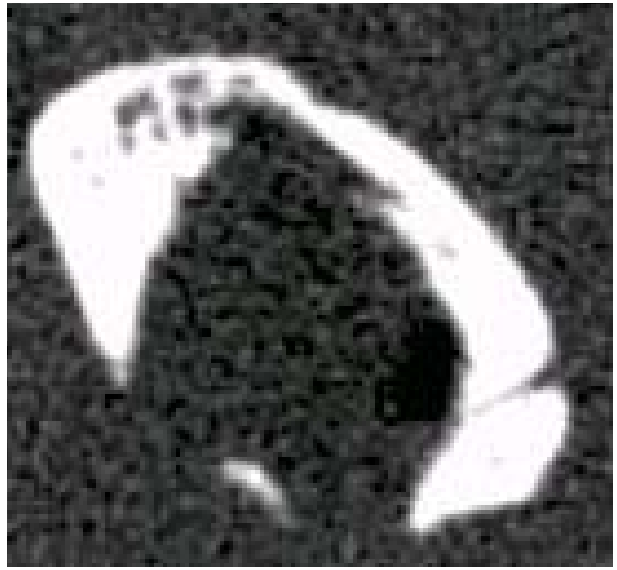

P=0.059
1000
900
800
700
Bone mineral density (mg/mm3)
C
(n=10)
H
(n=10)
(group)

Fig. 4A



C H

Fig. 4B



C

H

Fig. 5A

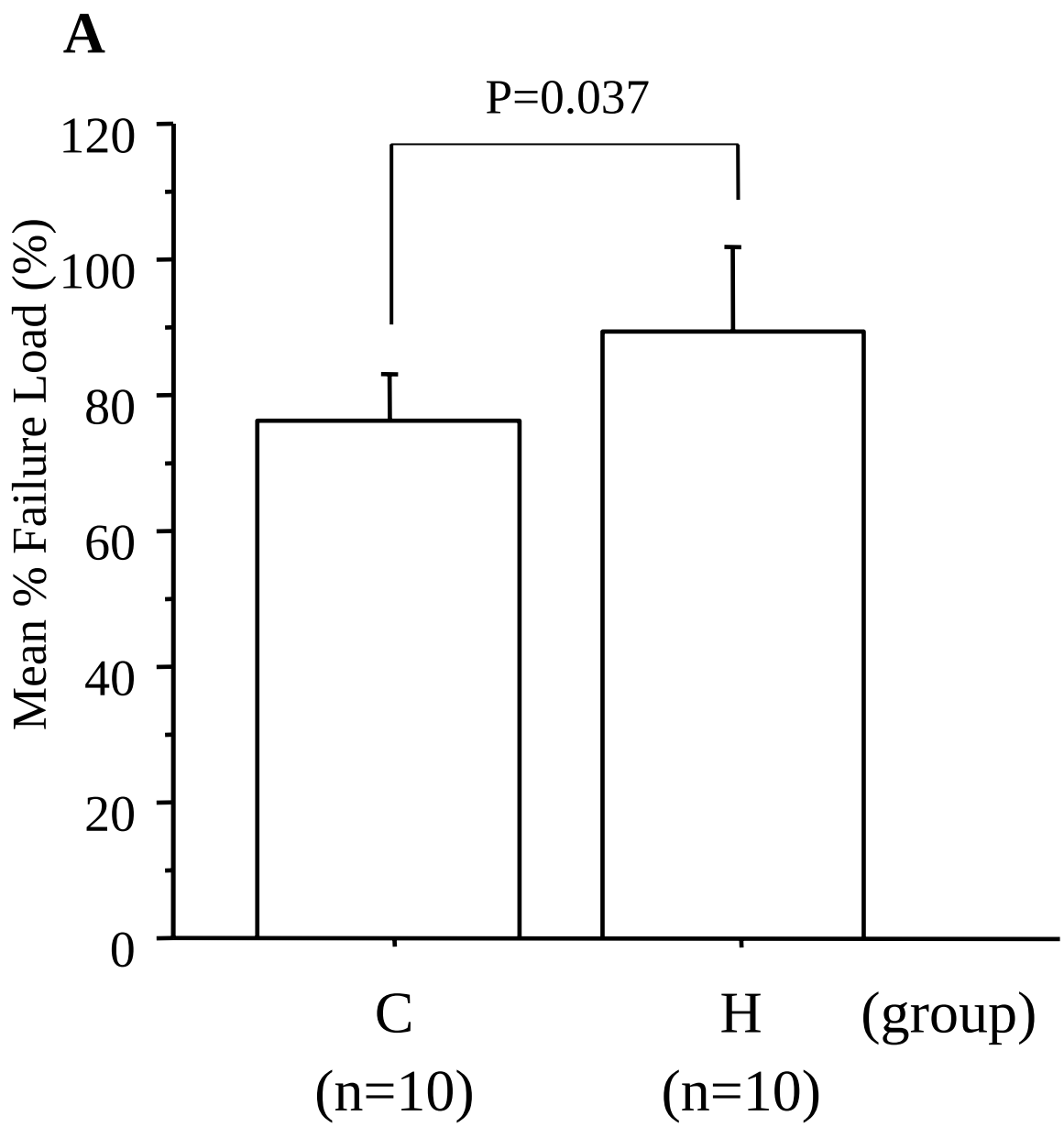


Fig. 5B

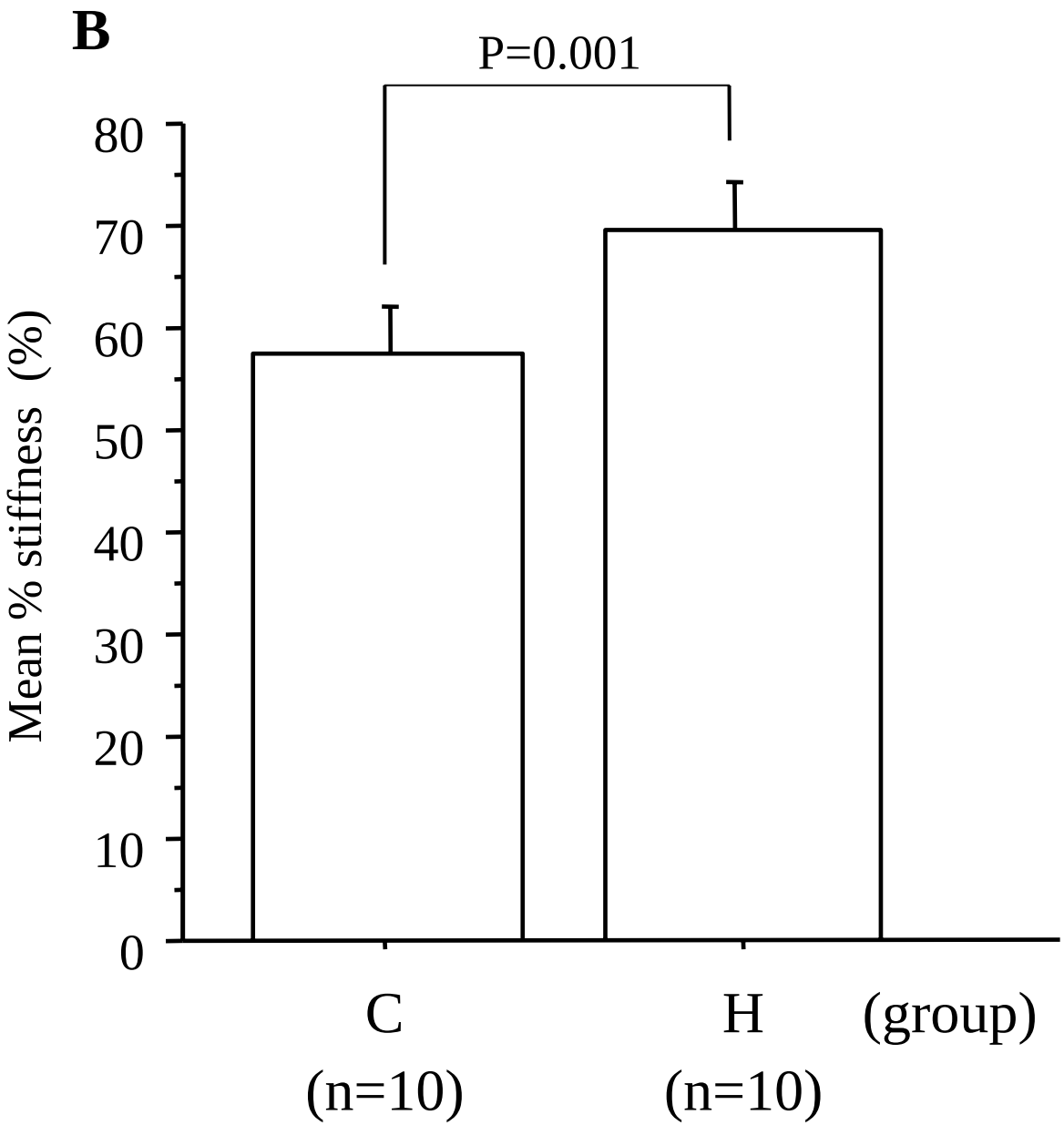
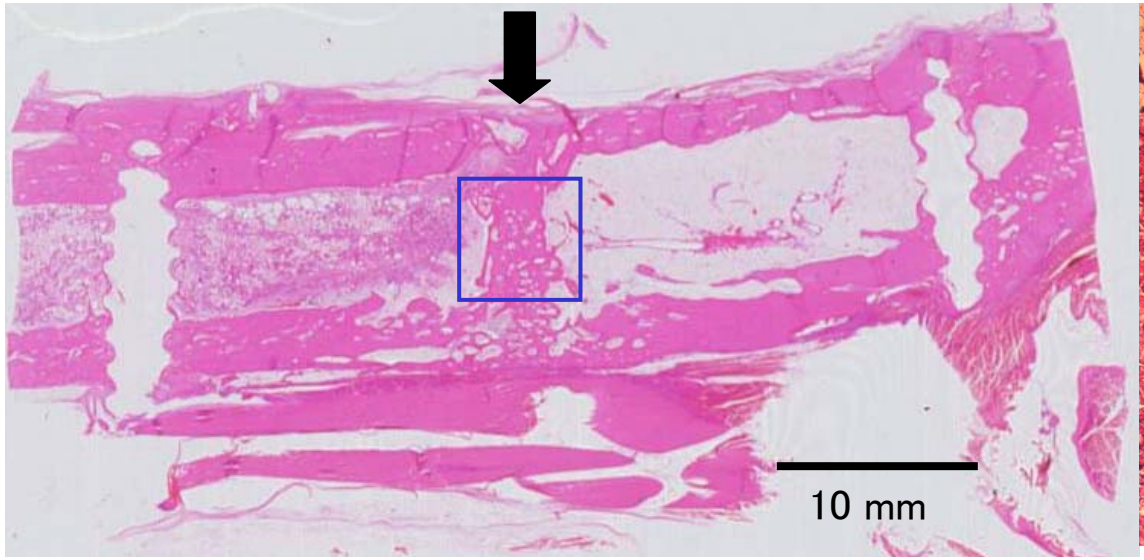
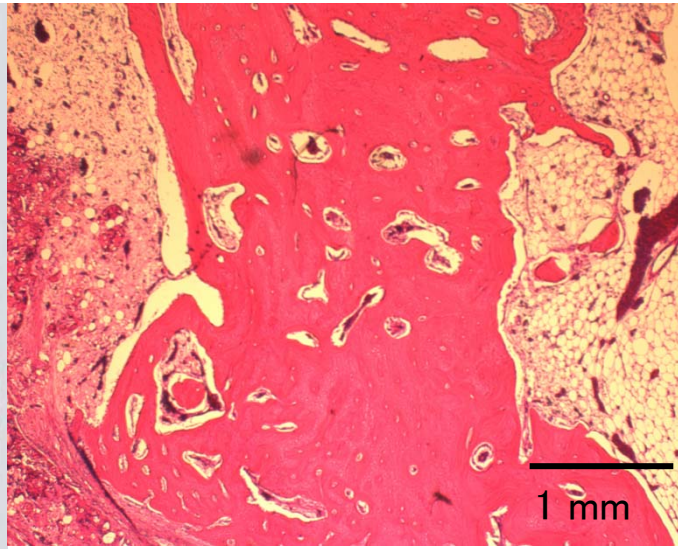
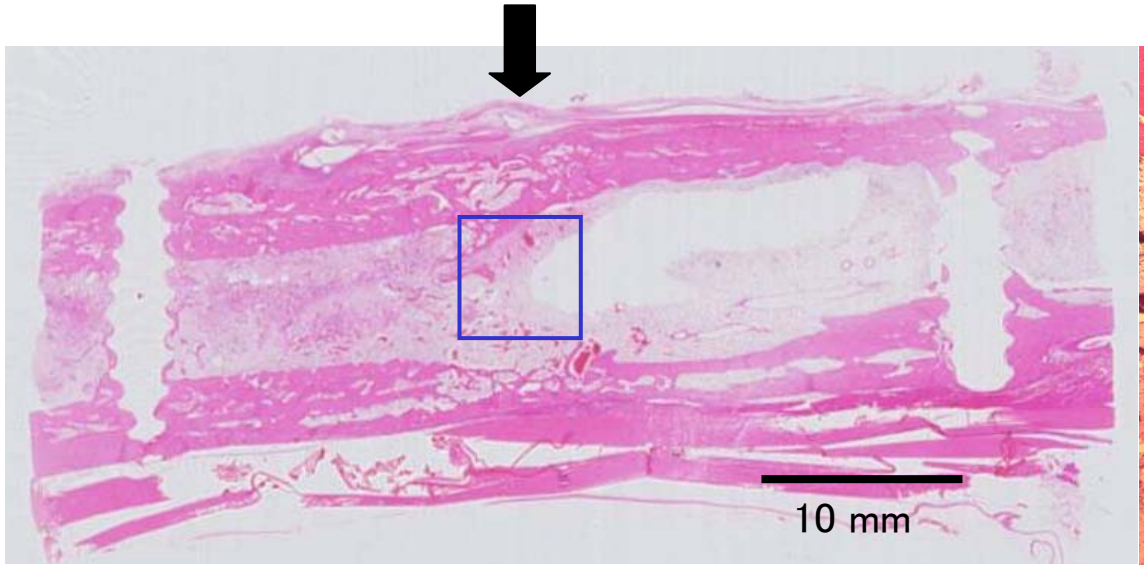


Fig. 6







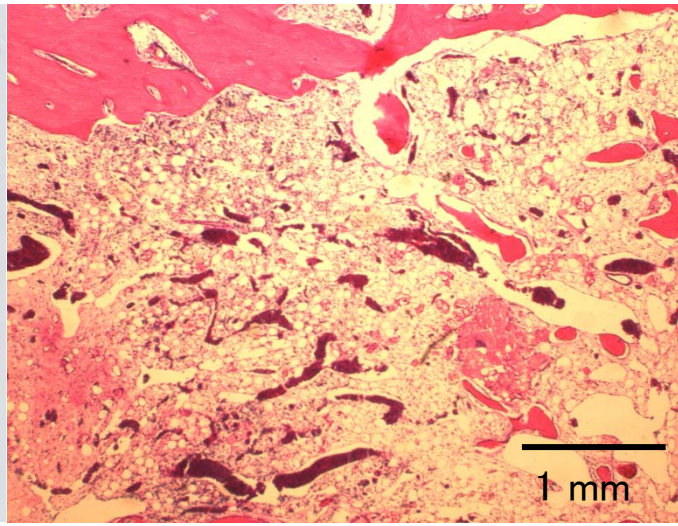


Fig. 7

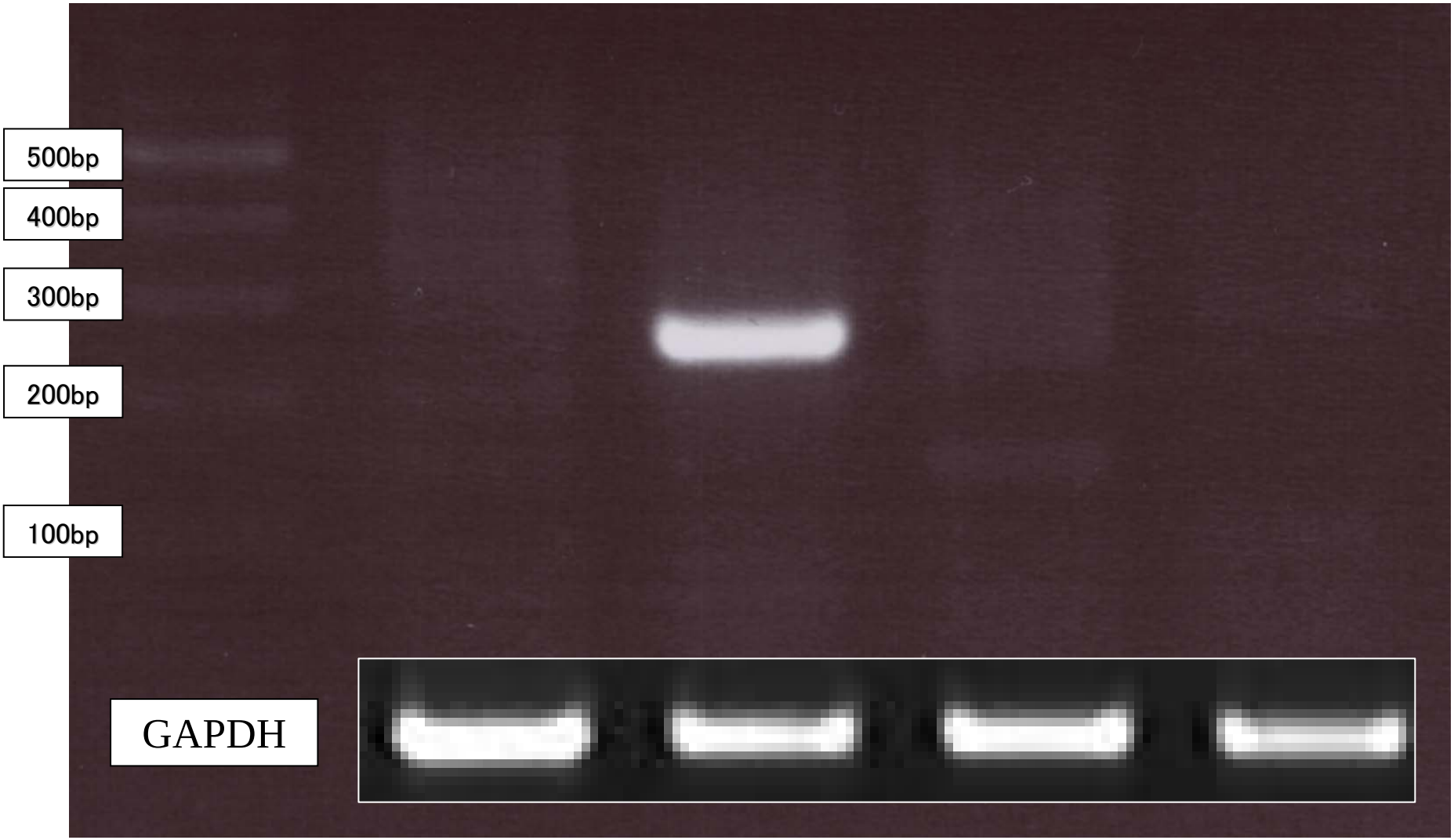
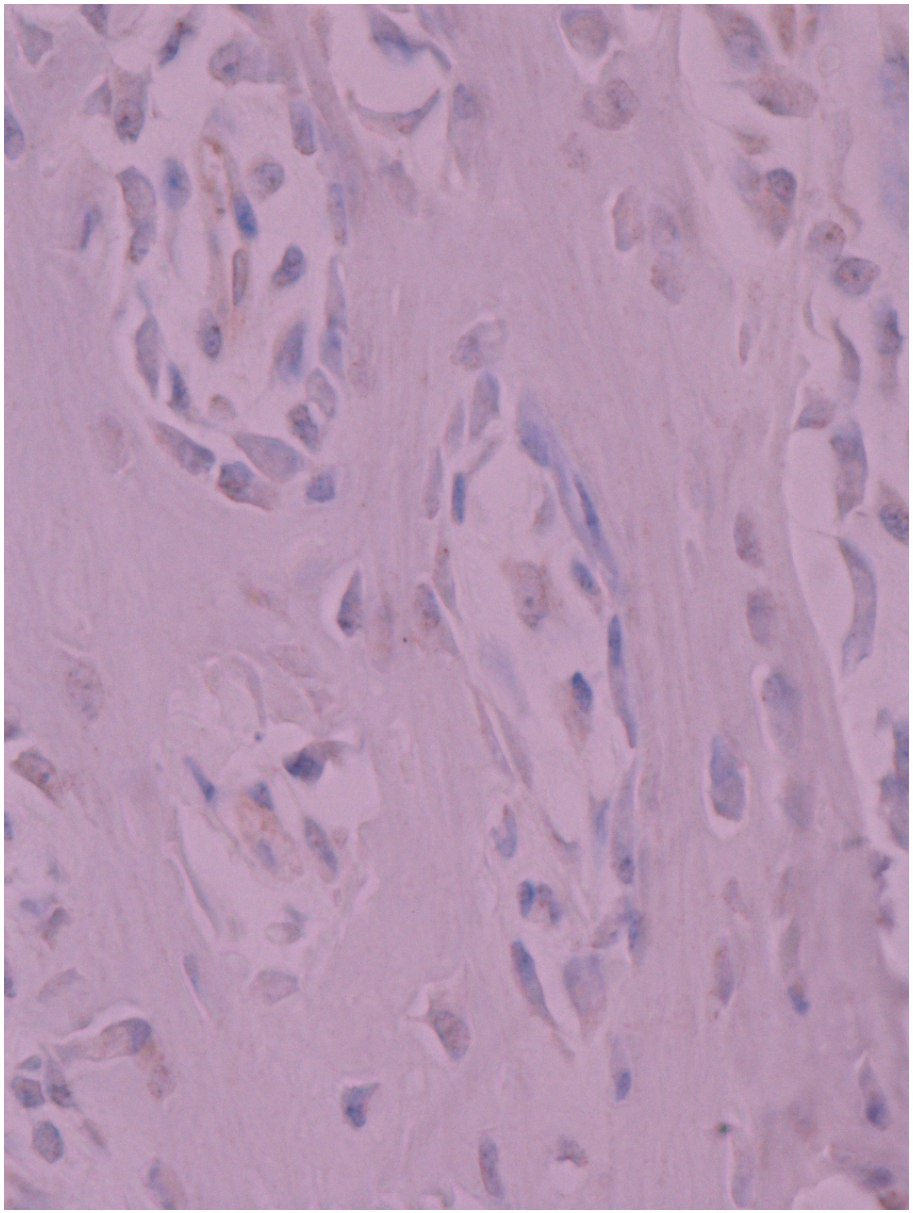
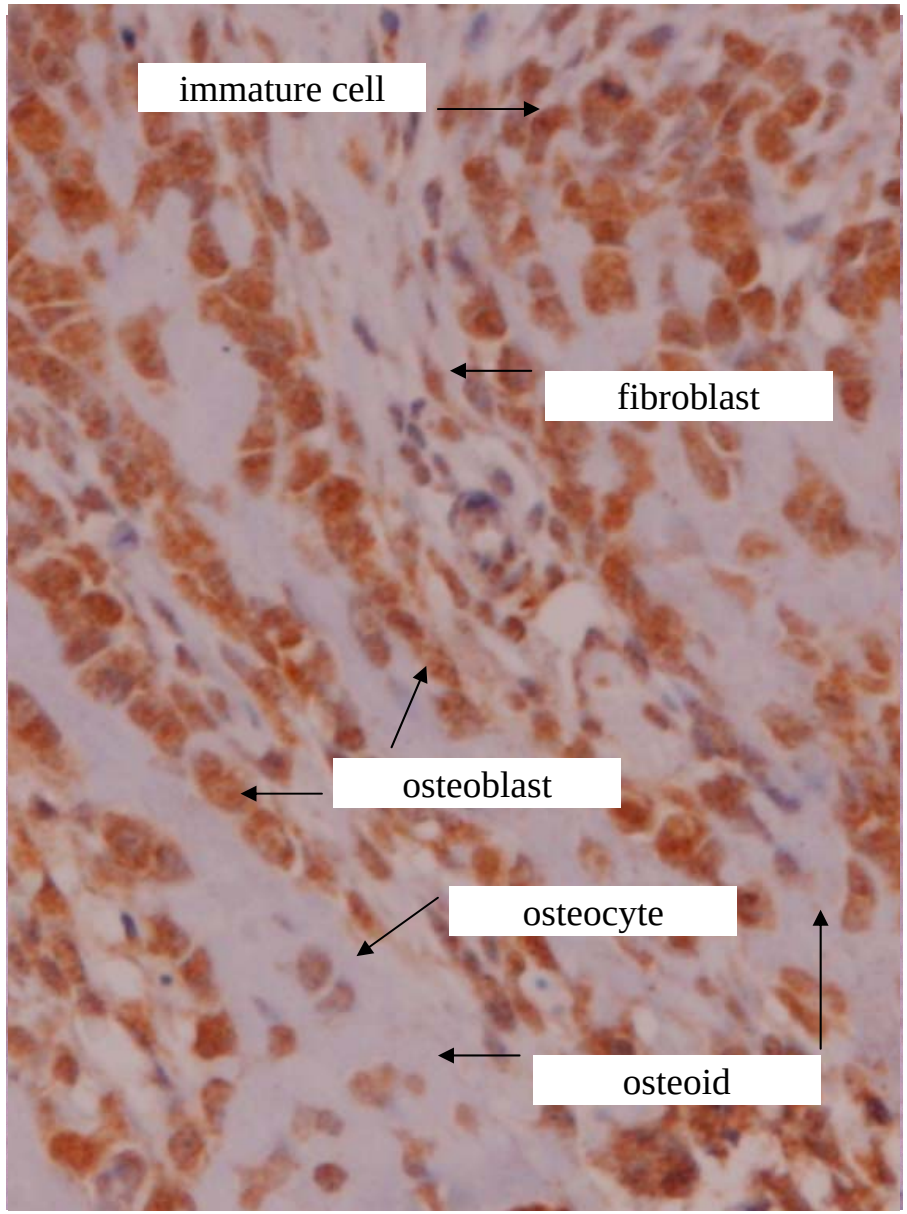

500bp
400bp
300bp
200bp
100bp
GAPDH
C
Callus
H
Callus
C
Muscle
H
Muscle

Fig. 8A

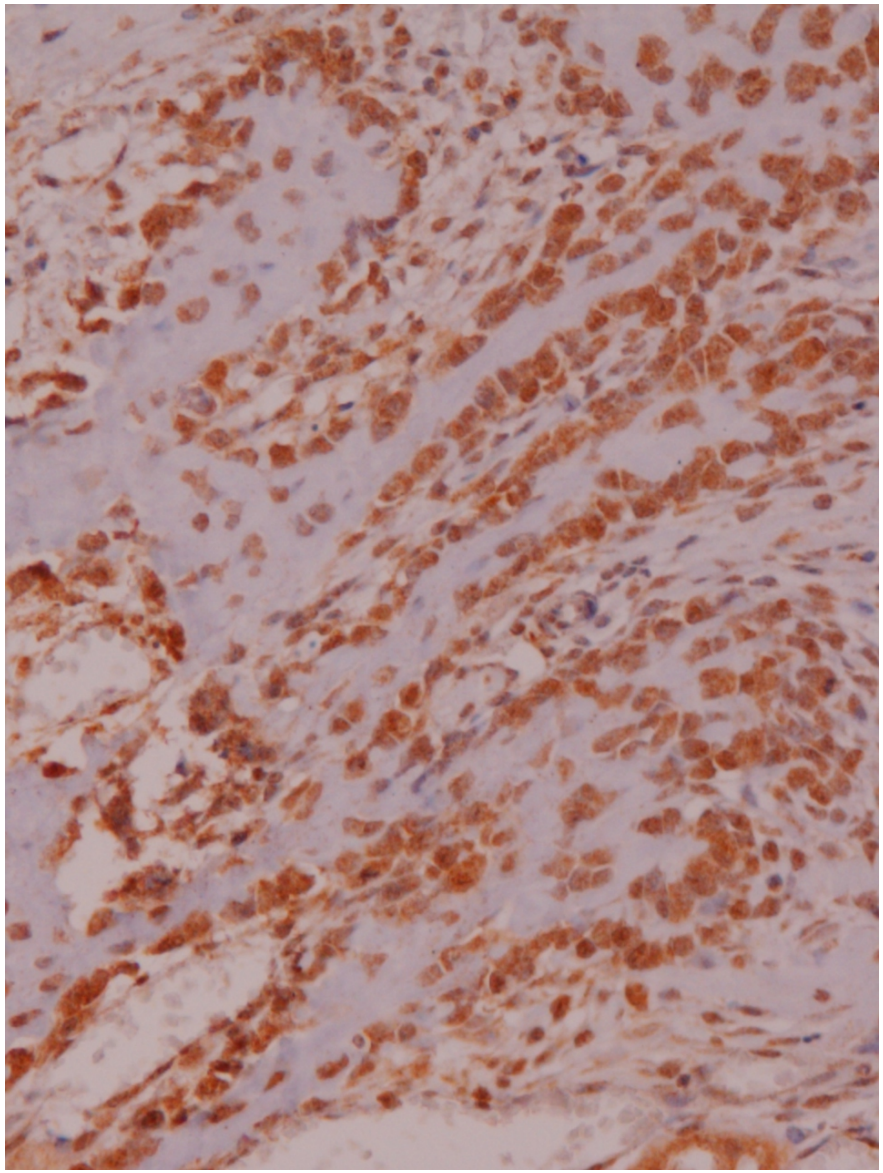


C



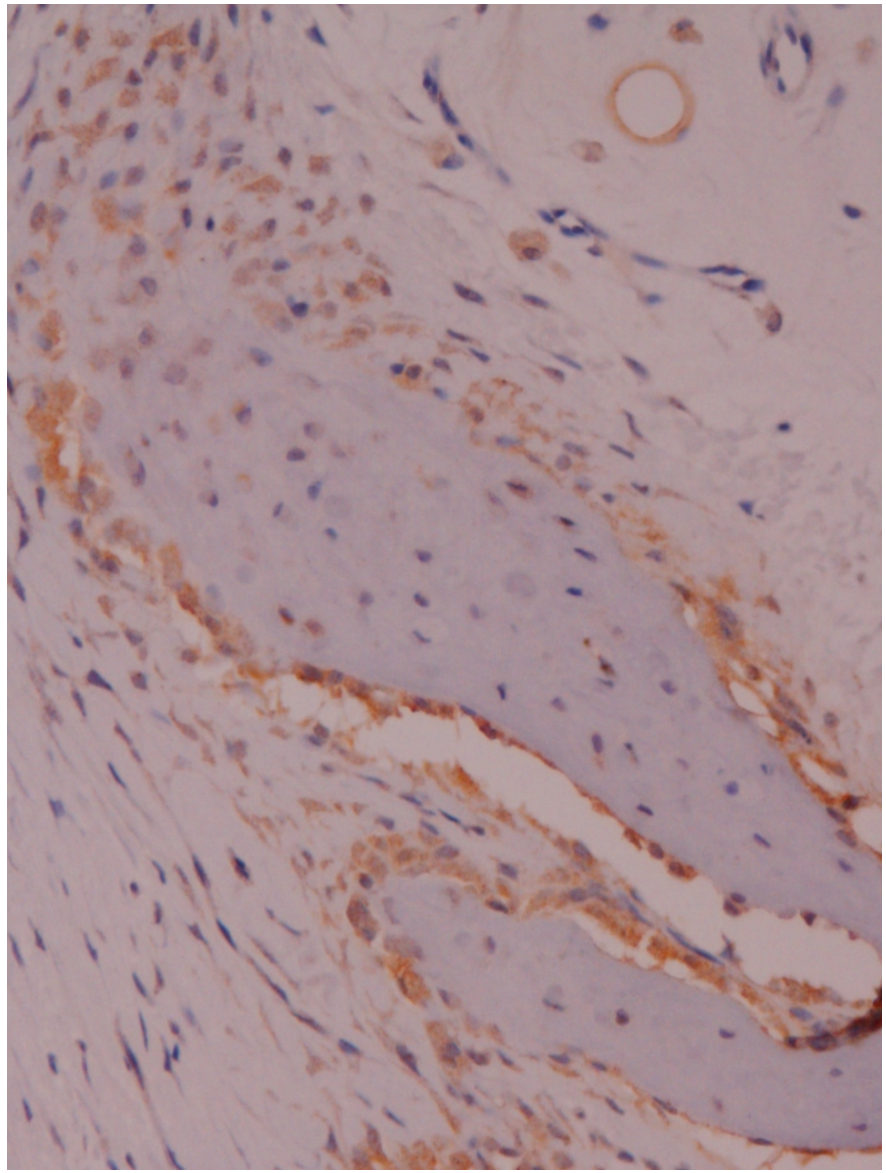
H

Fig. 8B



3w

(1w. after injection)



8w

(5w. after injection)