

**Figure 1.** MSC-CD44 antibody-magnetic bead complex. A: Electron microscopic view of a magnetic bead, Ferri Sphere 100C<sup>®</sup>. Magnification,  $\times 100,000$ . The scale bar indicates 50 nm. B: Expression of CD44 antigens in rat MSCs. Rat MSCs at passage four were stained immunohistochemically, which showed that the CD44 antigens were exhibited on the surface of rat MSCs up to at least passage four. Magnification,  $\times 400$ . The scale bar indicates 5  $\mu\text{m}$ . C: Electron microscopic views of MSC-CD44 antibody-magnetic bead complex. (a) Magnification  $\times 2500$ . The scale bar indicates 2  $\mu\text{m}$ . (b) Magnification,  $\times 5000$ . The scale bar indicates 1  $\mu\text{m}$ . The arrows indicate CD44 antibody-immobilized magnetic beads attached to the surface of an MSC. D: Light microscopic views of MSC-CD44 antibody-magnetic bead complex (a) and normal non-labeled rat MSCs (b). Magnification,  $\times 400$ . The scale bar indicates 5  $\mu\text{m}$ . The data shown are typical of two independent experiments ( $n = 3$ ). [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

### Assembly of MSC-magnetic beads complex

Two types of MSC-magnetic bead complex were composed using MSCs and a mediator: CD44 antibodies (Chemicon) or RGDS peptides (Peptide institute, Osaka, Japan) with mono-sized and carboxyl group-combined magnetic beads. To assemble the MSC-magnetic bead complex, first a mediator was immobilized to the magnetic beads, and then the prepared magnetic beads were combined with expanded MSCs.

### Carbodiimide-mediated immobilization of CD44 antibody or RGDS peptide to the magnetic beads by amide bond formation

We used uniform, mono-sized magnetic beads composed of styrene-acryl polymers as a core, coated with magnetic ferrite thin film [Fig. 1(A)] (diameter: 310 nm; density: 1.8 g/cm<sup>3</sup>; the amount of magnetization: 27 emu/g; carboxyl groups are introduced on the surface: 0.01–0.04  $\mu\text{mol}/\text{mg}$ ;

Ferri Sphere 100C<sup>®</sup>, Nippon Paint, Tokyo, Japan). The coupling procedure involved the formation of an amide bond between a primary amino group of the CD44 antibody or RGDS peptide and the carboxyl groups on the surface of the magnetic beads, mediated by carbodiimide activation. Because the intermediate product of the reaction between the carboxylic acid and the carbodiimide is very labile and would hydrolyze quickly, a less labile intermediate (NHS: N-hydroxy succinimide) was used. Briefly, after 3 mg of magnetic beads were washed twice with 500  $\mu\text{L}$  0.01 N NaOH for 10 min with thorough mixing, they were washed three times with de-ionised water in the same manner to remove excess liquid. Then, 50  $\mu\text{L}$  of EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) solution (50 mg/mL in 25 mM MES (2-[N-morpholino]ethane sulfonic acid), pH 5) and 50  $\mu\text{L}$  NHS solution (50 mg/mL in 25 mM MES, pH 5) were added, mixed well, and incubated with slow tilt rotation at room temperature for 30 min. After incubation, the tube containing the magnetic beads was placed on a magnet for 4 min and the supernatant was removed. The beads were washed twice with 25 mM MES, pH 5. Twenty micrograms of CD44 antibody or RGDS pep-

tide dissolved in 25 mM MES-buffer, pH 5, were added to the activated beads, making a total volume of 500  $\mu$ L. The mixture was vortexed and then incubated for 3 h at 25°C with slow tilt rotation. After incubation, the tubes were placed on a magnet for 4 min and the supernatant was removed. 0.05 M ethanolamine in PBS(-), pH 8, was added and the magnetic beads were incubated for 1 h at room temperature with slow tilt rotation to quench non-reacted groups. Finally, the beads were washed four times with 0.5% BSA in PBS(-) and suspended in 0.5% BSA in PBS(-) at the concentration of 3 mg beads/mL.

### Complexing of prepared magnetic beads to MSCs

Forty five microliters of CD44 antibody- or RGDS peptide-immobilized magnetic beads (225  $\mu$ g beads) and  $1 \times 10^6$  MSCs were mixed in 355  $\mu$ L of 0.5% BSA in PBS(-) with slow tilting and rotation for 1 h at 4°C or at 37°C, respectively. The tubes were then placed on a magnet for 4 min to collect the complexes. The assembled MSC-CD44 antibody-bead complexes and the MSC-RGDS peptide-bead complexes were washed 4 times with 0.5% BSA in PBS(-), respectively, and resuspended in culture medium at a concentration of  $5 \times 10^6$  cells/mL.

### Preparation of specimens of MSC-magnetic bead complexes for electron microscopy

MSC-magnetic bead complexes were centrifuged and supernatant was discarded. Precipitated complexes were fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C, then post-fixed with 1% OsO<sub>4</sub> in 0.1 M phosphate buffer (pH 7.4) for 2 h at 4°C. Specimens were then dehydrated in an ascending series of ethanols, replaced with propylene oxide, and embedded in epoxy resin. Semi-thin sections ( $\sim 1 \mu$ m) were cut and stained with 0.5% toluidine blue in 0.5% borate for light microscopy. Thin sections (0.1  $\mu$ m) were cut, stained with 3% uranyl acetate and lead citrate, and observed with a transmission electron microscope, JEM-1200 (JEOL, Tokyo, Japan).

### Cell proliferation assay

Cell proliferation was assayed by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-8) procedures basically as previously described.<sup>12</sup> The assay was performed using a commercial kit, Cell Counting Kit-8 (Dojindo laboratories, Kumamoto, Japan). About  $1 \times 10^4$  cells of the MSC-magnetic bead complex or control rat MSCs were seeded into each well of a 24-well plate. At various times after seeding (0, 1, 3, 5, and 7 days of culture), the working solution containing WST-8 and 1-Methoxy PMS (5 and 0.2 mM, respectively, as the final concentration) was added to each well at 1:10 volume of culture medium. After incubation for 4 h, the absorbance of each well was measured at 450 nm, with a

microplate spectrophotometer, SpectraMax 190 (Molecular Devices, Sunnyvale, CA).

### Distribution of prepared MSC-magnetic bead complexes under the influence of an external magnetic force, and monolayer culture for generation of chondrogenic matrix by the complexes

Before seeding MSC-bead complexes, magnetic flux density in the vertical and horizontal directions of the neodymium magnet (diameter: 5 mm; height: 5 mm; magnetic flux density: 0.43 Tesla (T)) was measured using a gauss meter, HGM-8200 (Probe: FS-3, ADS Co., Tokyo, Japan). To evaluate whether MSC-magnetic bead complexes could be gathered in a specific area by an external magnetic force, distribution of prepared MSC-magnetic bead complexes under the influence of an external magnetic force was observed. The cell distribution method used was modified from Hirao's method.<sup>13</sup> Briefly, 40  $\mu$ L of MSC-bead complexes (containing  $2 \times 10^5$  cells) were seeded onto the center of 24-well culture plates, which contained 300  $\mu$ L of chondrogenic differentiation medium: High-glucose DMEM supplemented with 10 ng/mL transforming growth factor (TGF)- $\beta$ 3 (Sigma),  $10^{-8}$  M dexamethasone (Sigma), 50  $\mu$ g/mL ascorbic acid-2-phosphate (Sigma), 40  $\mu$ g/mL L-proline (Nacalai tesque, Kyoto, Japan), ITS-A supplement (Invitrogen, 10  $\mu$ g/mL insulin, 6.7 ng/mL sodium selenite, 5.5  $\mu$ g/mL transferrin, 110  $\mu$ g/mL sodium pyruvate), and 1.25 mg/mL BSA (Sigma). In the experimental group (magnet group), a neodymium magnet was placed beneath the center of the plate (magnetic flux density was 0.20 T at a distance of 2 mm from the plate base). No magnet was used in the control group. After incubation for 1–2 h at 37°C to allow for cell attachment, 700  $\mu$ L of chondrogenic differentiation medium was added to each well. Sixteen hours after adding the differentiation medium, the magnet was removed from underneath the plate. Cell number was counted in the center of the plate (area M) and in four peripheral areas, each 3 mm in diameter [areas C1–C4; Fig. 5(A)]. The cell number in each area in six different plates was counted using a light microscope (100-fold) and the mean was calculated.

After culturing the MSC-bead complexes for 21 days with chondrogenic medium, the plates were washed three times and stained with toluidine blue. The plates were then assessed macroscopically and histologically.

### Pellet culture for chondrogenesis of MSC-magnetic bead complexes

To assess the chondrogenic potential of the MSC-magnetic bead complexes or of control rat MSCs in standard three-dimensional culture conditions, a modification of Johnstone's pellet culture system<sup>14</sup> was used. About  $2 \times 10^5$  MSCs, alone or as an MSC-magnetic bead complex, suspended in the earlier mentioned chondrogenic differentiation medium, were placed in a 15-mL polypropylene tube

(Greiner Bio One, Frickenhausen, Germany), and centrifuged to form a pellet. The pellet was cultured for 21 days at 37°C with 5% CO<sub>2</sub> and 95% air in 1 mL of the chondrogenic differentiation medium. In the control group, culture medium without chondrogenic differentiation factors (TGF- $\beta$ 3 and dexamethasone) was used.

### Toluidine blue staining and expression of type II collagen in MSC-magnetic bead complexes

After culturing the pelleted MSC-magnetic bead complexes or control rat MSCs for 21 days, they were fixed with 4% paraformaldehyde and embedded in paraffin. After deparaffinization, sections (5  $\mu$ m) were stained with toluidine blue solution. To assess expression of type II collagen, the sections were permeabilized with proteinase K (Dako, Japan) in 0.1% Tween-20 in PBS(-) for 5 min at room temperature. The expression of type II collagen was assessed using the same immunohistochemical method as used for CD44 expression described earlier, with anti-rat type II collagen goat polyclonal antibodies (Santa Cruz Biotechnology Inc., CA) at a final concentration of 1  $\mu$ g/mL.

### RNA preparation and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA was prepared from the pellets of MSC-magnetic bead complexes or rat MSCs using the RNeasy micro kit (Qiagen, Tokyo, Japan). Prepared RNA was converted to cDNA using the Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's protocol. PCR was performed in a Minicycler (PTC-150, Bio-Rad, Hercules, CA). PCR amplification conditions for rat integrin  $\alpha$ v, aggrecan, type II collagen, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: 94°C for 2 min followed by 35 cycles of 94°C for 15 s, 56°C (or for GAPDH: 58°C) for 30 s, and 68°C for 1 min. The reaction products were resolved by electrophoresis on a 2% agarose gel and visualized with ethidium bromide. The GAPDH primers were designed ourselves, the integrin  $\alpha$ v primers were as described<sup>17</sup> and the aggrecan and type II collagen primers were as described.<sup>15</sup> The primers were as follows:

integrin  $\alpha$ v (forward): 5'-CCTGCTTCTTCAGGACGGC-AC-3',

integrin  $\alpha$ v (reverse): 5'-CCCATCGGAACCTCGGTC-CATGA-3',

aggrecan (forward): 5'-TAGAGAAGAAGAGGGTTAGG-3',

aggrecan (reverse): 5'-AGCAGTAGGAGCCAGGGTTAT-3',

type II collagen (forward): 5'-GAAGCACATCTGGTTTGAG-3',

type II collagen (reverse): 5'-TTGGGGTTGAGGGTTTT-ACA-3',

GAPDH (forward): 5'-GCCAAAAGGGTCATCATCTC-3',

GAPDH (reverse): 5'-GCCTGCTTACCACCTTCTT-3'.

### Statistical analysis

To compare the cell number between each area (area M and areas C1-C4) in the magnet or control group, one-way

analysis of variance was used. If a statistical difference existed, Scheffe's post-hoc test was used. The Mann-Whitney *U*-test was used to compare the cell number between the magnetic group and control group in each area (M and C1-C4). A *P* value of less than 0.05 was regarded as statistically significant.

## RESULTS

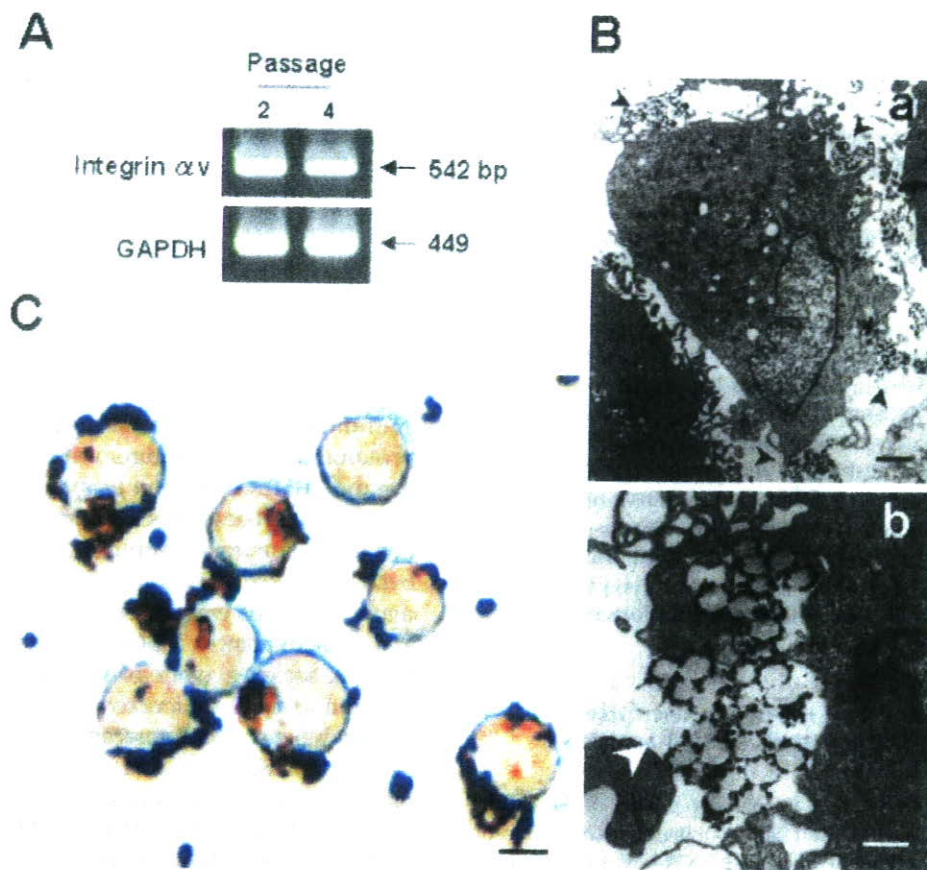
### Assembly of MSC-magnetic bead complexes

Immunohistochemical staining for CD44 antigens revealed that CD44 antigens are expressed in ~90% of expanded MSCs up to at least passage four [Fig. 1(B)]. We decided to use MSCs which were expanded up to passage four for assembling MSC-CD44 magnetic bead complexes. Next, CD44 antibody-immobilized magnetic beads were combined with expanded MSCs, and subsequently, MSC-CD44 antibody-magnetic bead complexes were observed morphologically by electron and light microscopy. This showed that some massive bead conglomerates were attached on the surface of MSCs [Fig. 1(Ca,Cb,Da)]; in contrast no massive bead conglomerates were attached to non-labeled rat MSCs [Fig. 1(Db)]. MSCs could be combined with small-sized magnetic beads through rat CD44 antibodies [Fig. 1(Ea)]. For MSC-RGDS peptide-bead complexes assembled using MSCs expanded up to passage four, the same result was obtained by interaction through the RGDS peptide [Fig. 2(A, Ba,Bb,C)]. Moreover, cell counts performed with a light scope showed that the assembly ratio of complex was ~80–90% after antigen-antibody reactions or interaction between integrins on MSCs and RGDS peptide. Thus, MSCs could be coupled with small-sized magnetic beads through CD44 antibodies or RGDS peptides.

### Proliferation of MSC-magnetic beads complexes

MSC-CD44 antibody-bead complexes did not proliferate up to 3 days of culture although they were attached to the bottom of the well. However, we then observed microscopically that CD44 antibody-immobilized magnetic beads became separated from the surface of MSCs in the well, and the cells then proliferated. After 7 days of culture, cell proliferation of MSC-CD44 antibody-bead complexes was ~50% that of rat MSCs (Fig. 3). MSC-RGDS peptide-bead complexes also attached to the bottom of the well, and gradually proliferated in culture. After 2 days in culture, RGDS peptide-immobilized magnetic beads became separated from the surface of MSCs, and cell





**Figure 2.** MSC-arginine (R)-glycine (G)-aspartic acid (D)-serine (S), RGDS peptide-magnetic bead complex. A: Expression of integrin  $\alpha v$  and GAPDH mRNA in rat MSCs at passage 2 and 4. B: Electron microscopic views. (a) Magnification  $\times 2000$ . The scale bar indicates 2  $\mu m$ . (b) Magnification  $\times 12,000$ . The scale bar indicates 500 nm. The arrows indicate RGDS peptide-immobilized magnetic beads attached to the surface of an MSC. C: Light microscopic view. Magnification,  $\times 400$ . The scale bar indicates 5  $\mu m$ . The data shown are typical of two independent experiments ( $n = 3$ ). [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]

proliferation of MSC-RGDS peptide-bead complexes was then approximately 70% that of rat MSCs at 7 days of culture (Fig. 3). We found that our assembled MSC-magnetic bead complexes could proliferate by separating from the mediator-immobilized magnetic beads, although the proliferation of the complexes was slower during the early period of culture than that of non-labeled rat MSCs.

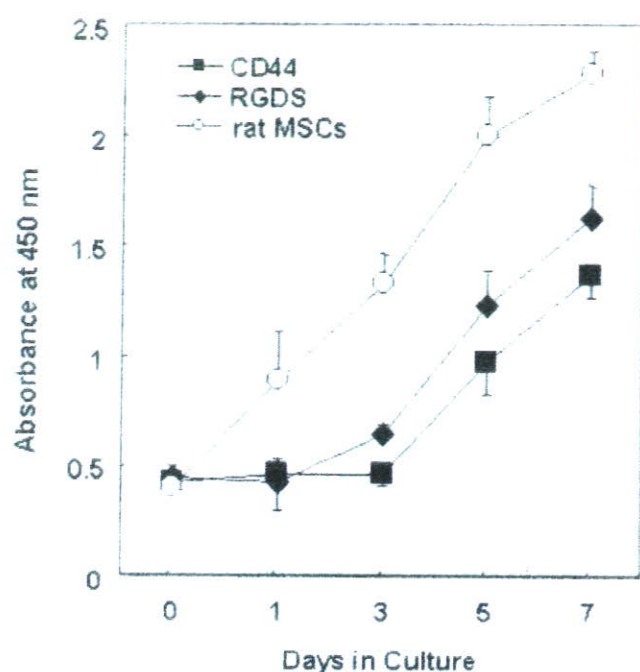
#### Distribution of prepared MSC-magnetic bead complexes under the influence of an external magnetic force, and monolayer culture for generation of chondrogenic matrix by the complexes

In a vertical direction from the magnet, magnetic flux density was 0.44 T at a distance of 0.3 mm from the magnet, and reduced as the height from the magnet increased [Fig. 4(A)]. In a horizontal direction at 1.7 mm above the magnet, magnetic flux density was 0.25 T at 0 mm from the magnet, and reduced as the

distance from the center of the magnet increased [Fig. 4(B)]. To evaluate whether MSC-magnetic bead complexes could be gathered in a specific area by an external magnetic force, the complexes were seeded with or without the influence of a magnetic force. In the magnet group (a neodymium magnet was placed beneath the plate at the center: area M), seeded MSC-bead complexes were attached and gathered mainly in area M [Fig. 5(A,B), M+]. By statistical analysis, there was a significant difference in cell number between area M and areas C1–C4 [Fig. 5(B), M+]. In contrast, in the control group (no magnet), the complexes were scattered mainly in the peripheral area of the plate [Fig. 5(B), M–] and the cell number in area M was statistically lower than those in other areas [Fig. 5(B), M–]. The distributional effects of the magnet were the same for both the two complexes we designed.

Subsequently, 21 days after seeding the MSC-CD44 antibody-bead complexes, in the magnet group the complexes had formed clusters of cells in the center of the plate, and the chondrogenic matrix was strongly stained by toluidine blue [Fig. 5(C), M+]. In the con-





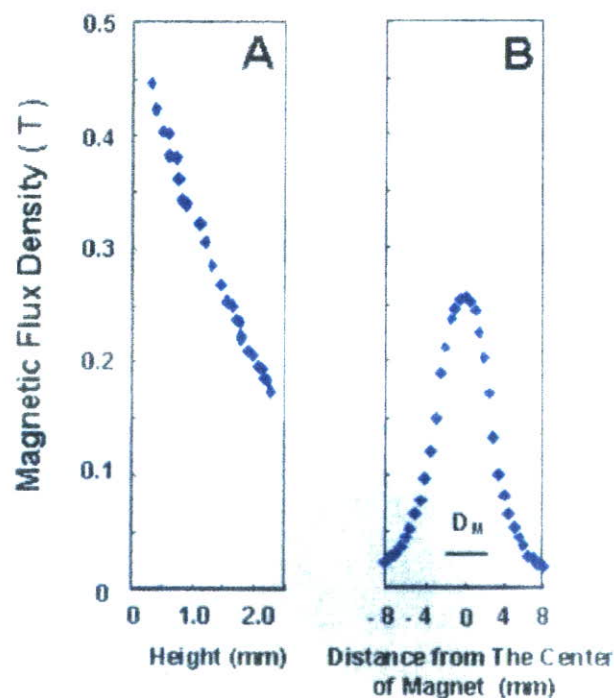
**Figure 3.** Proliferation of MSC-magnetic bead complexes.  $1 \times 10^4$  cells, as MSC-magnetic bead complexes or control rat MSCs were seeded onto each well of a 24-well plate. At various times after seeding (0, 1, 3, 5, and 7 days), cell proliferation of the complexes or rat MSCs was measured by the WST-8 method. The data shown are typical of three independent experiments ( $n = 6$ ). The bar represents the SD of each group.

control group, chondrogenic matrix was observed in the peripheral area, and the cells stained strongly for toluidine blue [Fig. 5(C), M-]. Thus, MSC-CD44 antibody-bead complexes, gathered in a specific area by an external magnetic force, could generate chondrogenic matrix in monolayer culture when supplemented with chondrogenic differentiation medium.

#### Chondrogenesis of MSC-magnetic bead complexes in three-dimensional culture system

After 21 days in culture, MSC-CD44 antibody-bead complex-derived-MSCs were found to be surrounded by cartilage-characteristic proteoglycans that stained metachromatically with toluidine blue [Fig. 6(Aa), D+], while in the pellets cultured without chondrogenic differentiation factors, the area stained with toluidine blue was weak and sparse [Fig. 6(Ac), D-]. Immunohistochemical staining for type II collagen also revealed an increase in type II collagen expression in the pellets cultured with chondrogenic differentiation medium [Fig. 6(Ab), D+] compared with pellets cultured without chondrogenic differentiation factors [Fig. 6(Ad), D-]. This chondrogenic phenotype of MSCs cultured in chondrogenic differentiation me-

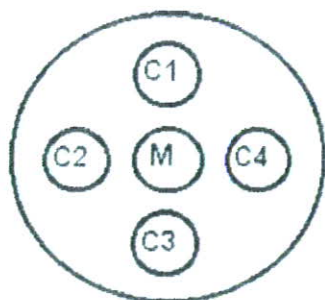
dium was observed in ~50–60% of the total area in MSC-CD44 antibody-bead complex sections, in contrast to 80% in non-labeled MSCs sections (data not shown). In addition, almost the same results were obtained histologically and immunohistochemically in the pellet culture system using the MSC-RGDS peptide-bead complex-derived MSCs (data not shown). Moreover, RT-PCR analysis of the pellet cultures demonstrated that type II collagen and aggrecan mRNA expression could be detected in the MSC-CD44 antibody-bead complex-derived, the MSC-RGDS peptide-bead complex-derived, and control rat MSC (non-magnetically labeled) -derived pellets cultured with chondrogenic differentiation medium [Fig. 6(B), D+], while in the pellets cultured without TGF- $\beta$ 3 and dexamethasone, mRNA expression of those markers was not detected [Fig. 6(B), D-]. Thus, MSC-magnetic bead complexes could also differentiate into chondrocytes in three-dimensional culture systems when supplemented with chondrogenic differentiation factors and the chondrogenic potential of the complexes shows only a slight reduction compared to that of normal rat MSCs.



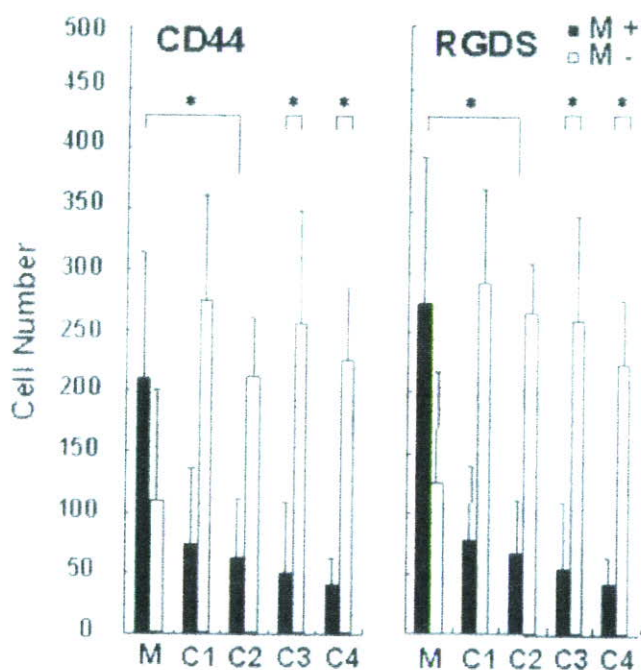
**Figure 4.** Magnetic flux density of the neodymium magnet (diameter: 5 mm, height: 5 mm, magnetic flux density: 0.43 Tesla [T]). A: Magnetic flux density in the vertical direction. B: Magnetic flux density in the horizontal direction at 1.7 mm above the magnet.  $D_M$ : width of magnet. Magnetic flux density was measured using a gauss meter, HGM-8200 (Probe: FS-3, ADS Co.). [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]



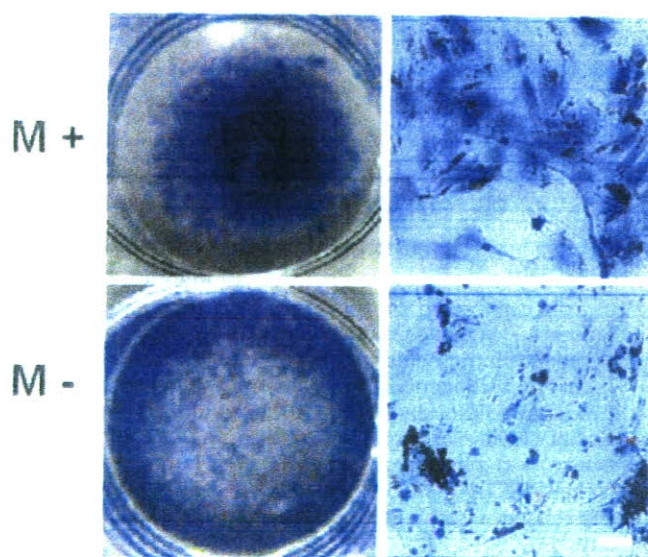
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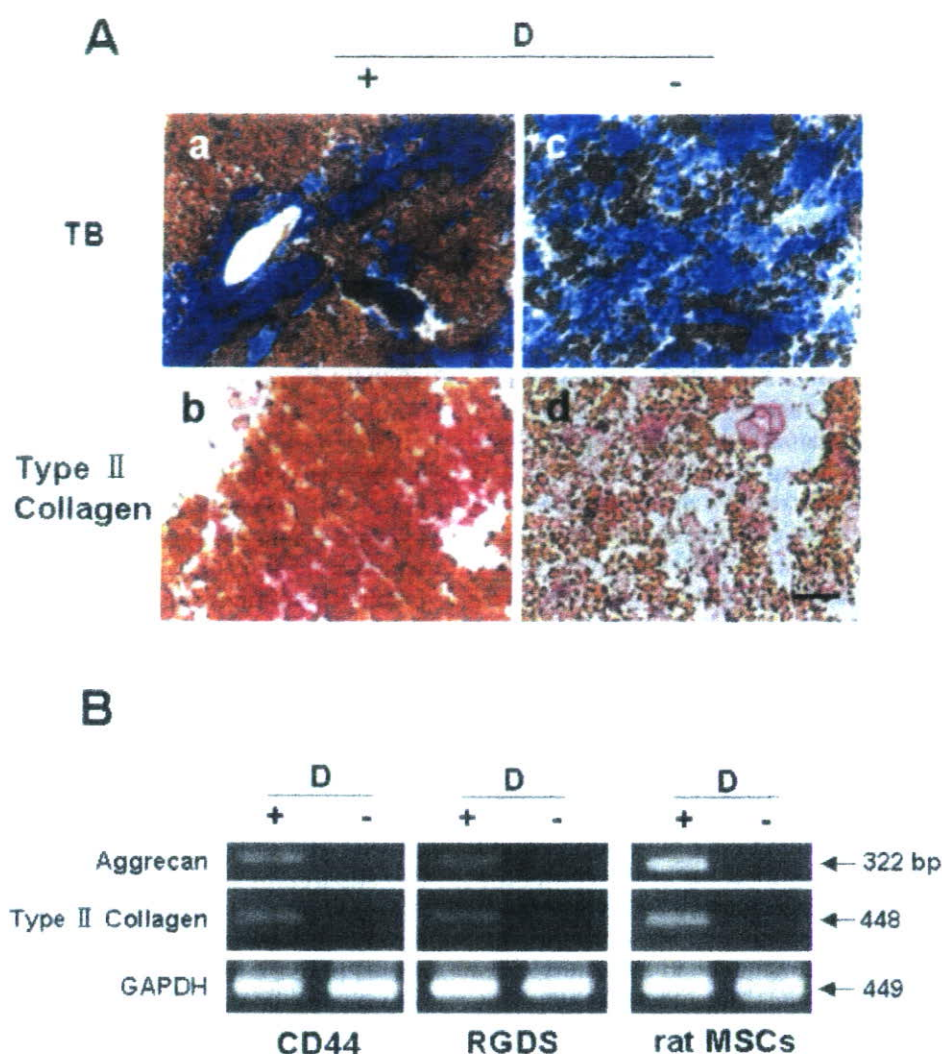
## DISCUSSION

The present study clearly demonstrates that MSCs coupled to magnetic beads can proliferate, after separating from mediator-immobilized magnetic beads, and can be attached and gathered effectively in a desired area by the influence of an external magnet *in vitro*. They can also generate chondrogenic matrix effectively with the addition of chondrogenic differentiation factors after cell accumulation in the monolayer culture system. Moreover, in a three-dimensional culture, they can differentiate into the chondrogenic lineage, as proved by the production of a dense chondrogenic matrix and the expression of type II collagen and aggrecan mRNA, although the chondrogenic potential of the complexes is a little reduced compared to that of normal rat MSCs.

The MSC-magnetic bead complexes were assembled by antigen-antibody reactions between CD44 antigens on MSCs and CD44 antibody-immobilized magnetic beads or by interactions between integrins on MSCs and RGDS peptides. As far as can be ascertained by light and electron microscopy, the assembly of the complexes was not 100% efficient. Before using our MSC delivery system with an external magnetic force *in vivo*, optimization of the number of magnetic beads and the cell number, and modifications of the reaction method such as reaction time and temperature, are

**Figure 5.** Distribution of MSC-magnetic bead complexes under the influence of magnetic force and generation of chondrogenic matrix by the MSC-CD44 antibody-magnetic bead complexes in a monolayer culture system. A, B: In the experimental group (magnet group, M+), a neodymium magnet was placed beneath the center of the 24-well plate (area M). The MSC-magnetic bead complexes were seeded under the influence of the magnetic force (magnetic flux density was 0.20 T at 2 mm from plate base, [Fig. 3(Ba)]). In the control group (M-), a magnet was not used. After allowing the cells to attach, the magnet was removed from underneath the plate and cell numbers were counted in area M and in four peripheral areas (areas C1-C4) (each area was 3 mm in diameter). There was a statistically significant difference in cell number between area M and areas C1-C4 in the magnet group ( $n = 6$ , each area,  $P < 0.05$ ). The data shown are typical of three independent experiments. The bar represents SD of each group. C: Generation of chondrogenic matrix by the MSC-CD44 antibody-magnetic bead complexes in a monolayer culture system. Images on the left show macroscopic views of the plates after 21 days culture with chondrogenic differentiation medium. In the M+ group, the MSC-CD44 antibody-bead complexes were attached and localized in area M, and the chondrogenic matrix stained by toluidine blue was observed macroscopically. In contrast, in the M- group, chondrogenic matrix was observed in the peripheral area of the plate. Images on the right show microscopic views of MSC-CD44 antibody-bead complexes in area M of each group. Magnification,  $\times 200$ . The scale bar indicates 50  $\mu\text{m}$ . [Color figure can be viewed in the online issue, which is available at [www.interscience.wiley.com](http://www.interscience.wiley.com).]





necessary. In addition, morphological observation of the complexes by electron and light microscopy showed that some massive bead conglomerates were attached on the surface of MSCs [Figs. 1(C,Da) and 2(B,C)]. The complexes were able to separate from the mediator-immobilized magnetic beads as the culture period increased, and the cells could proliferate up to at least 7 days of culture (Fig. 3). It has been demonstrated by other researchers that micro-sized magnetic beads are phagocytosed by some cells such as active dendritic cells<sup>16</sup> or CD8 positive lymphocytes.<sup>17</sup> These data and our results suggest that it is possible that our assembled complexes have the potential to cover an injured site, such as a cartilage defect, by proliferation of the complexes, and that mediator-immobilized magnetic beads, once released from the cell surface,

may be taken up by cells such as macrophages when the complexes are applied *in vivo*. Moreover, our designed complexes could be gathered effectively under the influence of an external magnetic force [Fig. 5(B)], and were able to generate chondrogenic matrix in monolayer culture [Fig. 5(C)]. They could also differentiate into the chondrogenic lineage in a three-dimensional culture system when supplemented with chondrogenic differentiation factors [Fig. 6(A,B)], although the chondrogenic potential of the complexes was a little less than that of normal rat MSCs. To assess more clearly the difference in chondrogenic potential between the complexes and normal rat MSCs, quantitative gene expression analysis by real time PCR is required. Taken together, our results show that the coupling of CD44 antibody- or RGDS peptide-immobilized

bilized magnetic beads with expanded MSCs have scarcely any effects on cell proliferation and growth or on the potential of expanded MSCs to differentiate into chondrocytes, compared to non labeled-rat MSCs, but that our system allows their effective delivery to the desired location. When comparing the component of magnetic beads, it is suggested that RGDS peptide-immobilized beads would be more useful for clinical applications than CD44 antibody-beads, because the RGDS peptide is a biodegradable material.

In previous studies, magnetic beads have been applied as a very useful tool for cell separation in biotechnology<sup>18–21</sup> since Kronick et al. successfully reported cancer cell separation using magnetic particles with surface markers, using the ganglioside GM1 in 1978.<sup>22</sup> Recently the application of magnetic beads has been expected as a magnetic system for the therapy of diseases such as liver tumors.<sup>23</sup> We have previously successfully demonstrated the efficacy of a drug delivery system (DDS) using magnetic liposomes containing magnetite:  $\text{Fe}_3\text{O}_4$  (mean diameter: 10 nm) and cytokines (recombinant human bone morphogenetic protein-2, rhBMP-2 or TGF- $\beta$ 1) and the implantation of a permanent magnet into the target site of a bone or cartilage defect in a rabbit model.<sup>24,25</sup> Treatment with magnetic liposomes containing TGF $\beta$ 1 together with a permanent magnet implanted near the osteochondral bone defect resulted in an increase in the concentration of TGF $\beta$ 1 in the osteochondral defect and effectively promoted chondrogenesis.<sup>25</sup> However, in this technique, the permanent magnet had to be surgically implanted at the injured site. Thus, the next step was to use an external magnetic force, which is less invasive and more convenient than surgical implantation of the magnet because the application time and site can be changed based on clinical demands. Recently, we have successfully used >0.3 T (maximum: 0.4 T) of an external magnetic force for systemic chemotherapy with magnetic liposomes containing doxorubicin in osteosarcoma-bearing hamsters.<sup>26</sup> As a modification of this magnetic DDS, we considered using this magnetic system to deliver pluripotent stem cells after combining the cells with magnetic beads, and thus, we created a novel magnetic stem cell delivery system.<sup>4</sup> Before our assembled MSC-magnetic bead complexes can be applied to an *in vivo* study, we think it is important to obtain further experimental results as follows: (1) the external magnetic force needs to be appropriate for all kinds of injury site and carefully shaped to fit the target organ; (2) to attract MSC-bead complexes to the injured site, we have to determine the appropriate time of applying magnetic force; (3) we have to investigate whether MSC-bead complexes induce an immune response in a host, and (4) investigate any potential side effects on transplanted MSCs or host cells when the appropriate magnetic force is applied.

Moreover, for future directions, our novel approach could be used to deliver stem cells to an injury site but also could be used to deliver appropriate cytokines or signaling molecules, if stem cells are capable of coupling with small-sized magnetic liposomes containing such growth factors for enhancement of tissue repair. Tissue regeneration would be promoted with this novel simultaneous stem cell and growth factor delivery system using a magnetic force. Additionally, we believe that our novel system would also be effective in the treatment of brain or spinal cord injury and for malignant tumors, using natural killer cells instead of autologous bone marrow-derived MSCs.

Currently, to track migration of transplanted cells such as stem and progenitor cells by magnetic resonance (MR) imaging, these cells have been used after labeling magnetically for detection in myocardium<sup>27,28</sup> and brain.<sup>29,30</sup> To our knowledge, magnetic targeting therapy involving cell delivery for repair of cartilage defects has not been reported. In the present study, the door of a novel magnetic targeting therapy using magnetic force and MSC-magnetic bead complexes has been opened. Basic research has started to elucidate whether magnetically labeled MSCs are able to differentiate into several lineages. There has been controversy over whether or not magnetic labeling of MSCs inhibits chondrogenesis. Arbab et al. demonstrated that superparamagnetic iron oxide (SPIO, ferumoxide, ~80–150 nm in size)-labeled MSCs were formed through the mediation of a polycationic transfection agent, poly-L-lysine (PLL),<sup>31</sup> and could differentiate into several lineages including the chondrogenic lineage.<sup>32</sup> On the other hand, most recently, Kostura et al. reported that SPIO-labeled MSCs inhibited chondrogenesis, and that the blocking of chondrogenic activity was mediated by the SPIO, rather than by PLL.<sup>33</sup> This blocking of chondrogenic activity by SPIO may be due to the effect of 'intracellular' magnetic labeling of SPIO-coated PLL, because SPIO-coated PLL acts through electrostatic interactions and binds to the cell membrane, inducing membrane bending, following which the magnetic label is endocytosed.<sup>33</sup> In the present study, Ferri Sphere 100C® (310 nm in size), which is approximately twice the size of SPIO, was used for 'extracellular' magnetic labeling through cell surface molecules, and we found that our magnetically labeled MSCs could differentiate into the chondrogenic lineage, although the chondrogenic potential of the complexes was somewhat reduced compared to that of normal rat MSCs. In fact, as far as could be observed by electron microscopy, magnetic beads could not be observed intracellularly within MSCs [Figs. 1(C) and 2(B)]. However, before clinical application of our magnetic cell delivery system can be considered, further studies by Western blot or real time PCR analysis to rule out any inhibitory effects of magnetic beads on signal transduction molecules such



as the transcription factor, Cbfa1 or Runx 2, which could affect chondrogenic differentiation will be required.

In conclusion, our design of two types of MSC-magnetic bead complexes were able to proliferate, effectively gather at a desired location under the influence of an external magnetic field, and had chondrogenic potential. Because of the fact that MSC-RGDS peptide-bead complexes are composed with an easily biodegradable material, RGDS peptides as a mediator, the RGDS peptide-bead complex is likely to prove more useful for minimally invasive clinical applications of our designed magnetic MSC delivery system than CD44 antibody-bead complexes. Further studies are necessary to evaluate the effectiveness of our stem cell delivery system for cartilage repair *in vivo*, but we have clearly demonstrated the potential of this system *in vitro*.

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MINI REVIEW

Fumitaka Kugimiya\* · Shinsuke Ohba · Kozo Nakamura  
Hiroshi Kawaguchi · Ung-il Chung

## Physiological role of bone morphogenetic proteins in osteogenesis

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**Key words** bone morphogenetic protein · hypertrophic chondrocyte · bone formation · osteoblast

### Introduction

Bone morphogenetic proteins (BMPs) are members of secreted signaling proteins that belong to the transforming growth factor- $\beta$  superfamily. BMPs were originally identified as molecules that induced ectopic bone formation when implanted into rodent muscles [1,2]. In this process, mesenchymal precursor cells condense and differentiate into two distinct tissues, namely the cartilage template and the surrounding perichondrium. Subsequently, chondrocytes in the cartilage template undergo a program of proliferation, cell cycle arrest, hypertrophy, calcification, and ultimately cell death. Ossification of the calcified matrix around the hypertrophic layer occurs upon vascularization and the recruitment of osteoblasts to the site [3]. In accordance with such in vivo effects, BMPs have been shown to regulate osteoblast differentiation in vitro [4].

BMPs bind to a characteristic pair of type I and II transmembrane serine/threonine kinase receptors (Fig. 1). They first bind to the type II receptor, which phosphorylates the GS domain of the type I receptor. The activated type I receptor subsequently recruits and phosphorylates the transcription factors Smad1, Smad5, and Smad8 (R-Smads) through the GS domain. R-Smads then physically associate with Smad4 (Co-Smad), translocate into the nucleus, and

activate the target genes in concert with other coactivators. Smad6 blocks BMP signaling by inhibiting the phosphorylation of the BMP-dependent R-Smads by the BMP type I receptors [5,6].

### Skeletal phenotypes of BMP knockout mice

Because BMP2, 3, 4, 5, 6, 7, and GDF5 are expressed in the developing skeletal elements [7], these BMPs may be involved in skeletogenesis. To clarify the physiological roles of BMPs, a number of BMP knockout mice were generated (Table 1). Briefly, homozygous *Bmp2*-deficient (*Bmp2*<sup>-/-</sup>) or *Bmp4*-deficient (*Bmp4*<sup>-/-</sup>) mice are embryonically lethal before the onset of skeletogenesis [8,9]. Twelve percent of heterozygous *Bmp4*<sup>+/-</sup> mice exhibit preaxial polydactyly. *Bmp3*<sup>-/-</sup> mice exhibit increased bone density [10]. *Short ear* mice, which are caused by a naturally occurring mutation in the *Bmp5* gene, have many anatomical abnormalities: reduced ear size, reduction in body size, reduction in the number of ribs, misshapen xiphoid appendix, hydronephrosis, and lung abnormalities [11]. *Bmp6*<sup>-/-</sup> mice have no skeletal patterning defects except a mild delay in sternum ossification that can be traced to the formation of mesenchymal condensation [12]. *Bmp7*<sup>-/-</sup> mice have patterning abnormalities in the hind limbs. However, histological analysis failed to detect any other abnormalities in osteoblast differentiation [13]. Brachypodism is a spontaneously occurring phenotype caused by a mutation in the *Gdf5* gene, characterized by a reduction in the length of the long bones and the replacement of two bones in most digits by a single skeletal element [14]. A generation of mice harboring mutations in two different BMP genes adds little to the knowledge of physiological bone formation (see Table 1) [15–21]. Thus, all naturally occurring or genetically engineered mice deficient in BMPs reported to date are either normal, exhibit abnormalities in skeletal patterning, or die during early embryonic development, and thus provide no information on the physiological role of endogenous BMPs in bone formation [7].

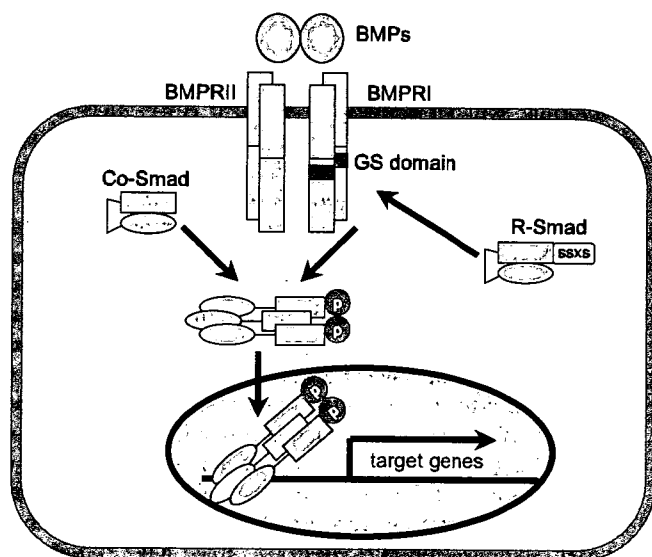
F. Kugimiya · S. Ohba · U. Chung (✉)  
Division of Tissue Engineering, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan  
Tel. +81-3-3815-5411 (ext. 37014); Fax +81-3-3818-4082  
e-mail: uichung-tyk@umin.ac.jp

F. Kugimiya · K. Nakamura · H. Kawaguchi  
Division of Sensory and Motor System Medicine, University of Tokyo, Tokyo, Japan

\*F. Kugimiya is a recipient of the JSBMR Encouragement Award 2004

**Table 1.** Information from BMP knockout mice

BMP2 <sup>-/-</sup>	Embryonically lethal, small allantois, lack of amnion
BMP3 <sup>-/-</sup>	Increased bone density
BMP4 <sup>-/-</sup>	Preaxial polydactyly of hindlimb, cystic kidney
BMP4 <sup>-/-</sup>	Embryonically lethal, lack of allantois, heart defect
BMP5 <sup>-/-</sup>	Short ear mice, abnormality of rib cage
BMP6 <sup>-/-</sup>	Delayed sternum ossification
BMP7 <sup>-/-</sup>	Abnormality of rib cage, polydactyly
BMP8a <sup>-/-</sup>	Defect in spermatogenesis
BMP8b <sup>-/-</sup>	Defect in spermatogenesis
BMP11 <sup>-/-</sup>	Abnormality of A-P patterning of axial skeleton
BMP12 <sup>-/-</sup>	Hydrocephalic abnormality
BMP15 <sup>-/-</sup>	Defect in oogenesis
GDF5 <sup>-/-</sup>	Brachypodism, shortened limbs, reduced digit bones
GDF8 <sup>-/-</sup>	Skeletal muscle hypertrophy
GDF9 <sup>-/-</sup>	Defect in oogenesis
BMP2 <sup>+/-</sup> ; BMP4 <sup>+/-</sup>	Fewer primordial germ cells than BMP2 <sup>+/-</sup> or BMP4 <sup>+/-</sup>
BMP2 <sup>+/-</sup> ; BMP6 <sup>-/-</sup>	Reduction in bone formation due to osteoblast dysfunction
BMP2 <sup>+/-</sup> ; BMP7 <sup>+/-</sup>	No skeletal abnormality
BMP4 <sup>+/-</sup> ; BMP7 <sup>+/-</sup>	Skeletal defect in rib cage, polydactyly
BMP5 <sup>-/-</sup> ; BMP6 <sup>-/-</sup>	Almost the same as BMP5 <sup>-/-</sup>
BMP5 <sup>+/-</sup> ; BMP7 <sup>+/-</sup>	No skeletal abnormality
BMP5 <sup>-/-</sup> ; BMP7 <sup>-/-</sup>	Embryonically lethal, defect in cardiac cushion and septation
BMP5 <sup>-/-</sup> ; GDF5 <sup>-/-</sup>	Combination of BMP5 <sup>-/-</sup> and GDF5 <sup>-/-</sup>
BMP6 <sup>-/-</sup> ; BMP7 <sup>-/-</sup>	Embryonically lethal, lack of allantois, heart defect
BMP7 <sup>+/-</sup> ; BMP8a <sup>-/-</sup>	More severe defects in spermatogenesis than BMP8a <sup>-/-</sup>
BMP8a <sup>+/-</sup> ; BMP8b <sup>+/-</sup>	Defects in spermatogenesis and epididymis



**Fig. 1.** BMP/Smad signaling pathway. BMPs bind to type I and type II receptors that are membrane-bound serine/threonine kinase receptors. Upon ligand binding, type II receptor phosphorylates the GS domain of the type I receptor, and type I receptor induces phosphorylation of R-Smads. Phosphorylated R-Smads form complexes with Co-Smad, and translocate into the nucleus

### Induction of bone formation by hypertrophic chondrocytes

The vertebrate skeleton is derived from three distinct embryonic lineages: neural crest cells, paraxial mesoderm, and lateral plate mesoderm. Cells of these lineages proliferate and migrate into distinct mesenchymal condensations at the

sites of future elements. Subsequently, they follow two distinct ways of bone formation: intramembranous and endochondral [3]. During intramembranous bone formation, condensed mesenchymal cells differentiate directly into osteoblasts. Most craniofacial bones and clavicles are formed through this process. Most of the bones are formed through the latter process, which is characterized by the replacement of a cartilage mold by bone and bone marrow [22]. In endochondral bone formation, there are several pieces of evidence that show that hypertrophic chondrocytes induce bone formation in adjacent tissues. First, bone formation always occurs in locations adjacent to the hypertrophic chondrocytes [23]. The primary spongiosa and bone collar are formed around the hypertrophic chondrocytes. This rule applies to all endochondral bones, large or small, without exception. Second, in mice lacking the *parathyroid hormone* (*Pth*)-related peptide (*Pthrp*) gene or its receptor, the *Pth/Pthrp* receptor (*Ppr*) gene, the regulation of hypertrophy is disturbed, and ectopic chondrocyte hypertrophy and ectopic bone formation appear [24–26]. Third, both the hypertrophy of chondrocytes and the formation of bone collar and primary spongiosa are suppressed in transgenic mice expressing the constitutively active *Ppr* under the control of the chondrocyte-specific type II collagen promoter (Col II *Ppr* transgenic mice) [27]. Furthermore, by the introduction of the Col II *Ppr* transgene, both the ectopic hypertrophy and the ectopic bone formation seen in *Pthrp*<sup>-/-</sup> mice are reversed [23]. Thus, the chondrocyte-specific manipulation of PTHrP signaling influences the appearance of ectopic bone formation. Fourth, in the growth plate of *Ppr*<sup>-/-</sup>/WT chimeric mice, ectopic bone collars are formed near clusters of ectopic prehypertrophic/hypertrophic chondrocytes [23]. Thus, during endochondral bone formation, hypertrophic chondrocytes in the growth plate link chondrogenesis to



osteogenesis by inducing osteogenesis in adjacent perichondrium and primary spongiosa.

Hypertrophic chondrocytes express a number of growth factors, cytokines, and matrix proteins. Among them, Indian hedgehog (Ihh) has been proven to be indispensable for osteogenesis by hypertrophic chondrocytes [23,28]. However, Ihh alone cannot induce bone formation [29], suggesting that other factors secreted from hypertrophic chondrocytes may also be necessary for osteogenesis. Because some BMPs can induce ectopic bone formation when implanted into rodent muscles and promote osteoblast differentiation in vitro, they are strong candidates for osteogenic factors secreted from hypertrophic chondrocytes.

### **BMP subtypes expressed by hypertrophic chondrocytes**

Because hypertrophic chondrocytes induce bone formation in the primary spongiosa and the perichondrium during endochondral bone development, BMPs that are important for physiological bone formation are likely to be expressed by hypertrophic chondrocytes. BMP2, 3, 4, 5, 6, 7, and GDF5 are known to be expressed in the growth plate [7,12]. BMP2 is expressed in the hypertrophic chondrocytes, the perichondrium, and the developing joints. Expression of BMP3 is detected in the perichondrium along the shaft, but not surrounding the epiphyses. BMP4 transcripts are detected in the cells of the perichondrium and the transition zone adjacent to the mature hypertrophic chondrocytes. BMP5 is expressed in the perichondrium. BMP6 expression is localized to the hypertrophic chondrocytes and joints. Expression of BMP7 is detected in the proliferative chondrocytes and the inner perichondrium. These reports indicate that the main BMP subtypes expressed by the hypertrophic chondrocytes are BMP2 and BMP6 [12,30].

### ***Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice exhibited growth retardation with proportional growth plates**

As is the case with the other BMPs, however, there is no direct evidence that BMP2 and BMP6 are essential for physiological bone formation, because *Bmp2*<sup>-/-</sup> mice die at an early embryonic stage [8], and *Bmp6*<sup>-/-</sup> mice show no skeletal abnormality except for a slight delay in the ossification of the sternum [12]. Because there may be genetic redundancy between BMP2 and BMP6 in the regulation of bone formation, compound knockout mice lacking one allele of the *Bmp2* gene and both alleles of the *Bmp6* gene (*Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup>) were generated and the effect on bone formation was analyzed [30].

Although BMPs are known to play important roles in skeletal patterning during embryonic development, there was no apparent abnormality of skeletal patterning in *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice. In contrast, there was growth

retardation in *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice. The size of the *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> growth plate was smaller than that of the WT, but the proportions of the distinct layers of growth plate chondrocytes (periarticular proliferative, columnar proliferative, and hypertrophic) showed no significant differences. Although several lines of evidence indicate that BMPs promote chondrocyte hypertrophy, *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice did not show a significant difference in chondrocyte differentiation.

### **Impaired bone formation due to osteoblast dysfunction in *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice**

As for bone formation, the trabecular bone volume and cortical thickness were significantly reduced in *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice. Bone histomorphometric analysis showed that bone volume was decreased by 31% compared with that of the WT. Regarding the parameters of bone formation, there was a significant reduction in mineral apposition rate and bone formation rate per bone surface, but little difference in osteoblast number. On the other hand, the parameters of bone resorption were normal. These data suggest that bone loss in *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice was caused by the inhibition of bone formation due to an impairment of osteoblast function. Urinary deoxypyridinoline, a marker for bone resorption, showed little difference between the two groups.

Fracture healing recapitulates some aspects of the intramembranous and endochondral bone development [31], and the abnormalities of such development are sometimes exaggerated in fracture healing. The endochondral, not the intramembranous, bone formation was defective in *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice. In *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice, a massive cartilaginous callus persisted, which contained hypertrophic chondrocytes. The hypertrophic differentiation of chondrocytes was similar between WT and *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice, suggesting that the bone induction ability of the hypertrophic chondrocytes was impaired in *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice.

### **Comparison of *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice and mice deficient in the BMP signaling cascade**

Recently, transgenic mice expressing a dominant-negative form of *Bmp receptor 1b* (*Bmpr-1b*) under the control of the type I collagen promoter were generated, and they closely resembled *Bmp2*<sup>+/-</sup>; *Bmp6*<sup>-/-</sup> mice [32]. They were smaller than the WT mice and showed impairment of postnatal bone formation with the number of osteoblasts and the parameters of bone resorption unchanged, suggesting that osteoblast function was impaired. In addition, transgenic mice lacking the *Bmp receptor 1a* (*Bmpr-1a*) specifically in osteoblasts using the Cre/loxP system under the control of the osteocalcin 2 promoter also exhibited low bone mass due to impaired osteoblast function [33]. Noggin

is a secreted glycoprotein and acts as a BMP antagonist. Noggin binds to BMP2, 4, 5, 6, and 7 and inhibits their binding to the BMP receptors. Transgenic mice expressing *Noggin* under the control of the osteocalcin promoter exhibited a reduction in the trabecular bone volume and the bone formation rate, but not in the number of osteoblasts [34,35]. *Smurf1* is a HECT domain ubiquitin E3 ligase and inhibits BMP signaling by inducing the degradation of R-Smads and BMP receptors. A deficiency of *Smurf1* in mice showed an increase in bone mass due to the enhanced activities of osteoblasts [36], and transgenic mice expressing *Smurf1* under the control of the type I collagen promoter exhibited osteopenia [37]. The bone phenotypes of these genetically manipulated mice, in which the osteoblasts could not transduce normal BMP signaling, were similar to those of *Bmp2*<sup>-/-</sup>; *Bmp6*<sup>-/-</sup> mice. These data support our hypothesis that BMP2 and BMP6 act on osteoblasts to induce normal bone formation.

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SPECIAL REPORT

Jun Hashimoto · Ikko Ohno · Kiyoshi Nakatsuka  
Noriko Yoshimura · Shinjiro Takata · Masaaki Zamma  
Hiroo Yabe · Satoshi Abe · Masaki Terada · Kousei Yoh  
Masao Fukunaga · Cyrus Cooper · Hirotohi Morii  
Hideki Yoshikawa

## Prevalence and clinical features of Paget's disease of bone in Japan

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**Abstract** The present study aimed to evaluate the prevalence and clinical presentation of Paget's disease of bone (PDB) in Japan. As PDB is a very rare disease in Japan, a

The Japanese Committee on Clinical Guidelines of Diagnosis and Treatment of Paget's Disease of Bone of the Japan Osteoporosis Society, Japan

J. Hashimoto (✉) · I. Ohno · H. Yoshikawa  
Department of Orthopaedic Surgery, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Suita 565-0871, Japan  
Tel. +81-6-6879-3552; Fax +81-6-6879-3559  
e-mail: junha@ort.med.osaka-u.ac.jp

K. Nakatsuka  
Department of Endocrinology, Metabolism and Molecular Medicine, Osaka City University Graduate School of Medicine, Osaka, Japan

N. Yoshimura  
Department of Joint Disease Research, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan

S. Takata  
Department of Orthopaedics, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima, Japan

M. Zamma  
Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan

H. Yabe  
Department of Orthopaedic Surgery, School of Medicine, Keio University, Tokyo, Japan

S. Abe  
Department of Orthopaedic Surgery, Teikyo University, School of Medicine, Tokyo, Japan

M. Terada  
Department of Radiology, Wakayama Medical University, Wakayama, Japan

K. Yoh  
Department of Orthopaedic Surgery, Sasayama Hospital, Hyogo College of Medicine, Hyogo, Japan

M. Fukunaga  
Department of Nuclear Medicine, Kawasaki Medical School, Okayama, Japan

C. Cooper  
MRC Environmental Epidemiology Unit, University of Southampton School of Medicine, Southampton General Hospital, Southampton, UK

H. Morii  
Emeritus Professor, Osaka City University, Osaka, Japan

nationwide mail survey was conducted targeting doctors in the specialty most frequently diagnosing and treating PDB patients in Japan. First, the literature for all case reports in Japan published between January 1990 and December 2002 was reviewed to determine who was diagnosing and treating PDB in Japan. This literature review for all case reports in Japan revealed that 72.1% of cases in Japan were reported from departments of orthopaedic surgery. A nationwide two-phase mail survey was conducted for the departments of orthopaedic surgery of 2320 general hospitals accredited by the Japanese Orthopaedic Association. Phase 1 involved determining how many patients with PDB were followed at each hospital. If the answer was one or more, phase 2 of the survey gathered information on the clinical presentation of current patients. The mail survey yielded a final response rate of 75.4% for phase 1 and 87.6% for phase 2. Phase 1 indicated that the prevalence of PDB in Japan is about 2.8 cases per million capita. Phase 2 revealed a slight female predominance, lower frequency of familial clustering, higher frequency of femoral fracture in the affected femur, and a higher ratio of symptomatic PDB in Japan compared with findings in countries displaying a higher prevalence of PDB. The present epidemiological study revealed that the disorder is extremely rare in Japanese individuals, and that some differences exist with regard to the clinical features of PDB between Japanese patients and patients from high-prevalence countries.

**Key words** Paget's disease of bone · prevalence · clinical manifestation · nationwide mail survey · Japanese population

### Introduction

Paget's disease of bone (PDB) is very common in elderly Caucasian populations in Europe, the United States, and Australia, and is well known to display distinct geographical variation. The prevalence of PDB varies from 0.1% to 5% in individuals, depending on the country [1–9]. While PDB

is highly prevalent in countries such as the United Kingdom, France, Germany, New Zealand, and Australia, prevalence is relatively low in Northern Europe [6], and the disease is extremely rare in Asia and Africa, where data are limited to a small number of published case reports [10–14] and no prevalence data are currently available.

Recent studies in Europe, North America, and New Zealand have suggested a decline in the prevalence and an attenuation of the clinical severity of PDB [15–18]. Although a wide range of clinical manifestations is reported among patients with PDB, including bone pain, fracture, secondary osteoarthritis, hearing loss, and secondary sarcoma, the precise clinical manifestations of PDB in Asia and Africa remain unknown. Because epidemiological research in countries with an extremely low prevalence of PDB would provide valuable insights into the etiology of PDB, by comparing patients' clinical manifestations among geographical areas with clearly different prevalences, the present study sought to conduct such research in the Japanese population.

Estimates of the prevalence of PDB have primarily been derived from autopsies, bone scans, or radiographic surveys of subjects investigated in hospitals, and population-based radiological and biochemical surveys [9,19,20]. In a preliminary study, lumbar and pelvic lesions were surveyed, using abdominal radiographs of 1000 Japanese population-based subjects in a limited area, but the study failed to identify any Pagetic lesion. The present study therefore selected a nationwide mail survey to estimate the prevalence of PDB in Japan. However, contacting all Japanese medical doctors was clearly impractical. The nationwide mail survey was therefore targeted at doctors in the specialty most frequently involved in diagnosing and treating PDB patients in Japan. In order to determine which kind of doctor most frequently diagnoses and treats PDB patients in Japan, a search of the literature for case reports in Japan was conducted.

The objectives of this study were to evaluate the prevalence and clinical presentation of PDB in Japan.

## Materials and methods

### Review of published case reports of PDB in Japan

The literature was reviewed for all case reports in Japan published between January 1990 and December 2002, to determine who was diagnosing and treating PDB patients. A systematic search was performed using the key words "Paget's disease in bone" in electronic databases (Japania Centra Revuo Medicina). Duplication of cases was avoided by referring to the key content of each report, and cases were classified by the reporting affiliation as Department of Orthopedic Surgery, Department of Internal Medicine, or Other.

### Mail survey

A mail survey was conducted from September 2002 to May 2003, proceeding in two phases. Phase 1 involved determin-

**Table 1.** Sources of referral (by specialty) of 154 cases of Paget's disease in Japan (1990–2002)

Department	Frequency listed (%)
Orthopedic surgery	111 (72.1%)
Others	43 (27.9%)
Total	154

ing how many patients with PDB were followed at each hospital at the time of the mail survey. If the answer was one or more, phase 2 of the survey gathered the following information on the clinical presentation of current patients: diagnostic procedure; age; sex; familial aggregates; types of clinical symptoms; complications; pattern of bone involvement; serum alkaline phosphatase levels; therapeutic agents used; and prognosis. Surveys were mailed on three occasions to nonresponders in order to maximize the response rate.

## Results

### Review of published case reports of PDB in Japan

Use of the key words "Paget's disease in bone" in the published databases yielded 245 hits, representing 154 cases after meticulous exclusion of duplicated data. These final 154 cases were categorized as 111 cases reported by Departments of Orthopedic Surgery (72.1%) and 43 cases reported by Others (27.9%; Table 1).

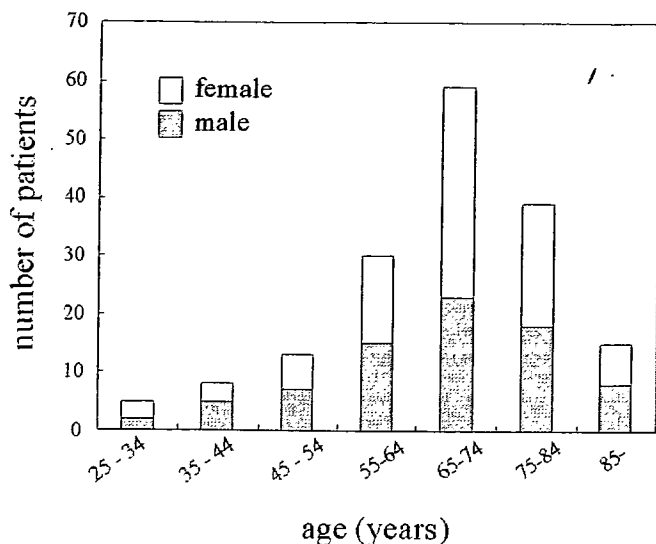
### Mail surveys

Because PDB was diagnosed mainly at departments of orthopedic surgery in Japan, we conducted a mail survey of the departments of orthopedic surgery at 2320 general hospitals accredited by the Japanese Orthopaedic Association. The response rate for phase 1 of the survey was 75.4%, revealing a total of 194 patients, including 1 Indian patient, being followed at departments of orthopedic surgery at 162 general hospitals. The response rate for the phase 2 survey that gathered data on the 194 PDB patients was 87.6%. Of the 194 cases identified in phase 1, phase 2 responses were gained for 170 cases (169 Japanese patients, 1 Indian patient). No duplication of cases was found among the 170 cases according to the obtained clinical data, age, sex, and initials of the name, but duplication could not be excluded from the remaining 24 cases.

### Estimated prevalence of PDB in Japan

Based on 72.1% of Japanese cases of PDB being diagnosed and the patients treated at departments of orthopedic surgery, the response rate of 75.4% in the phase 1 survey, and data from the Ministry of Public Management that the Japanese population was 126 008 000 in 2002, the prevalence of PDB in Japan was calculated as 2.8 per million capita, using





**Fig. 1.** Age distribution of patients with Paget's disease of bone in Japan

**Table 2.** The clinical features of 169 patients with PDB in Japan

Age (years) mean $\pm$ SD	64.7 $\pm$ 14.5
Sex, male/female (ratio)	78 : 91 (0.86 : 1)
Familial aggregation (n = 158) <sup>a</sup>	10 (6.3%)
Symptom	
Asymptomatic (%)	42 (24.9%)
Symptomatic (%)	127 (75.1%)
Monostotic/polyostotic (ratio)	87 : 82 (1.06 : 1)
Skeletal distribution (%)	
Pelvis	93 (55.0)
Spine	54 (32.0)
Femur	46 (27.2)
Skull	34 (20.1)
Tibia	25 (14.8)
Serum ALP levels (n = 164) <sup>b</sup>	
Normal range	17 (10.4)
$\leq$ 3-fold above ULN	74 (45.1)
3- to 6-fold above ULN	49 (29.9)
$\geq$ 6-folds above ULN	24 (14.6)

ULN, upper limit of normal range

<sup>a</sup>Data not available for 11 patients

<sup>b</sup>Data not available for 5 patients

the following equation:  $193 \text{ cases} \times (100\% / 72.1\%) \times (100\% / 75.4\%) \times (1\,000\,000 / 126\,008\,000)$ .

#### Analysis of clinical characteristics in Japanese PDB patients

Data for the 169 Japanese patients gathered in the phase 2 study were analyzed. Among these 169 patients (age range, 26 to 92 years; mean age  $\pm$  SD, 64.7  $\pm$  14.5 years; Table 2), PDB markedly increased in prevalence with increasing age up to age 74 (Fig. 1). The male/female ratio was 0.86:1, and only 6.3% of patients had familial clustering; 75.1% of patients were symptomatic, and the most frequent symptoms were pain (including lumbago, coxalgia, buttock pain, and gonalgia) followed by skeletal deformity (Tables 2 and 3). Data regarding skeletal distribution revealed that monosto-

**Table 3.** Symptomatology of 127 patients with symptomatic PDB in Japan

Lumbago	30
Coxalgia	29
Skeletal deformity	24
Buttock pain	18
Gonalgia	11
Hearing loss	8
Dental problem	5
Others	9

The total number of patients listed is more than 127 because several patients had more than one symptom

tic and polyostotic disease exhibited almost equal prevalence. Commonly involved skeletal sites were the pelvis, spine, and femur. Serum alkaline phosphatase levels were elevated beyond the upper limit of normal in 89.6% of patients (Table 2).

In addition to the fundamental diagnostic procedures of radiology, bone scans, and biochemical parameters of bone metabolic activity, bone biopsy was performed for diagnosis in 55% of the patients. Medical management was limited almost exclusively to the use of calcitonin and etidronate, because these are the only two agents licensed for the management of PDB in Japan. However, some doctors have used other bisphosphonates, including alendronate, risedronate, and pamidronate, which are approved in countries advanced in the management of PDB. Despite the high frequency of medical management, more than half of the patients remained symptomatic. In regard to complications, 16 patients displayed fractures, 1 developed hip osteoarthritis requiring total hip replacement, 2 developed osteosarcomas, and 1 had malignant fibrohistiocytoma (MFH) in bone. The most frequent sites of fracture were the hip and pelvis (Table 4).

#### Discussion

PDB is known to be a very rare disease in Japan, although its prevalence and clinical features have not been clarified. Thus, the majority of cases of PDB in Japan tend to be reported in the literature as case reports. This permitted us to ascertain the sources of referral in Japanese patients with PDB through a review of published case reports. Between January 1990 and December 2002, 154 cases were reported of which 72.1% were reported from departments of orthopedic surgery in the country. We therefore performed a nationwide mail survey to the departments of orthopedic surgery of 2320 general hospitals accredited by the Japanese Orthopaedic Association; the final response rate in this mail survey was 75.4% for phase one and 87.6% for phase two. These response rates may be sufficient to delineate the prevalence of PDB in Japanese patients.

The study has revealed that the prevalence of PDB in Japan across all ages is 2.8 per million. The prevalence of PDB is reported to range from 0.1% to 5% in high-prevalence countries [1-9]; thus, the prevalence in Japan is

**Table 4.** Treatment, prognosis, and complications of PDB in 169 Japanese patients

	Frequency listed (%)
Diagnostic procedures	
Biopsy	93 (55.0)
Without biopsy	76 (45.0)
Treatment	
No drug	26 (15.4)
NSAID	65 (38.5)
Calcitonin	105 (62.1)
Etidronate	25 (14.8)
Alendronate	15 (8.9)
Risedronate	2 (1.2)
Other bisphosphonates	4 (2.4)
Prognosis ( <i>n</i> = 152)*	
No symptoms	57 (37.5)
Pain decrease	50 (32.9)
Pain persistence	25 (16.4)
Complications	20 (13.2)
Complications	
Fractures	16 (9.5)
Femur	10
Pelvis	2
Lumbar spine	1
Tibia	1
Unreported	2
Total hip arthroplasty	1 (0.6)
Sarcoma	3 (1.8)

\*Data not available for 17 patients

The sum of listed percentages for treatment is more than 100%, because this list includes drugs that were used formerly, as well as currently used drugs

extremely low. PDB increased markedly in prevalence with advancing age, and this age distribution is somewhat similar to that observed in high-prevalence countries. The male/female ratio in the Japanese population is 0.86:1, with slight female predominance, although most studies in high-prevalence countries have reported a slight male predominance, with male/female ratios ranging from 1.2 to 1.8 [15,16,21–22]. The frequency of familial clustering in the Japanese population (6.3%) was lower than that of 15%–40% in high-prevalence countries [23–25].

In the present study, 75.1% of patients were symptomatic, although up to 30% of PDB patients in the United Kingdom and the United States had symptoms related to the disease [16,22]. This study has several limitations. First, the data were analyzed for subjects followed only at departments of orthopedic surgery. Second, these data might not reflect the true proportion of patients with PDB in Japan, through selection bias (missing asymptomatic patients with PDB might result in underestimation of the overall prevalence of asymptomatic PDB). Despite potentially missing asymptomatic PDB cases in this study, we consider any such bias to be insufficient to dispel the findings of an extremely low prevalence of PDB in Japan.

The most frequent clinical symptoms in Japanese PDB patients were musculoskeletal pain and skeletal deformity, findings similar to those in high-prevalence countries. There was a slight difference in the monostotic/polyostotic ratio among Japanese and Caucasian populations, with the prevalence of polyostotic disease in Japan being 48.5%,

contrasting with 66% in high-prevalence countries [26]. The common sites of involvement were pelvis, spine, and femur, and this distribution is similar to that seen in the Caucasian population [27]. Serum alkaline phosphatase levels were elevated beyond the upper limit of normal in 89.6% of Japanese patients, a finding similar to that in high-prevalence countries, where levels are elevated in 85% of patients with untreated Paget's disease [28].

Diagnostic bone biopsy was performed in 55% of patients in Japan. In high-prevalence countries, bone biopsy is not recommended for the diagnosis of Paget's disease [29,30]. This tendency in diagnostic procedures probably reflects the fact that Japanese physicians are unfamiliar with the disease and our principal concern in diagnosis is to exclude possible malignant bone tumor. The present study showed that more than half of patients remain symptomatic in Japan, possibly due to the limited number of agents licensed in Japan for PDB treatment. It remains unclear whether aggressive treatment of PDB to normalize disease activity would result in fewer long-term complications, as the only two licensed drugs, etidronate and calcitonin, did not sufficiently alleviate pain for the majority of PDB patients. However, despite insufficient medical management of PDB in Japan, the frequency of hip osteoarthritis and secondary sarcoma was not high compared with frequencies in high-prevalence countries. The ratio of arthroplasty performed to frequency of hip osteoarthritis in Paget's disease was 1.5% in high-prevalence countries [22], while it was 0.6% in Japan. The frequency of malignant bone tumor in PDB was between 0.1% and 5% in high-prevalence countries [22,31–33], while it was 1.8% in Japan. In contrast to the lower frequency of hip osteoarthritis and secondary sarcoma, that of fracture in the affected femur was much higher in Japan than in high-prevalence countries {21.7% (10 cases/46 cases), compared with 3% [27]}. The higher ratio of symptomatic PDB in Japan, which might be a result of insufficient therapeutic intervention in Japan compared to that in high-prevalence countries, might explain some part of this difference.

In conclusion, the present epidemiological study clarified the prevalence and clinical manifestations of PDB in Japan, revealing that the disorder is extremely rare in Japanese individuals and that there are some differences in the clinical features of PDB between Japanese and patients from high-prevalence countries. Familial aggregation (6.3%) and polyostotic PDB (48.5%) were less common in Japan than in high-prevalence countries. Based on the results of this study, we are currently developing a registration system for PDB patients in Japan in order to facilitate the dissemination of knowledge to physicians of this rare disease among Japanese.

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