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Effects of Pleiotrophin (PTN) Over-expression on Mouse Long Bone Development, Fracture Healing and Bone Repair

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Abstract. Pleiotrophin (PTN) was found to have potent effects on regulation of osteoblast recruitment, proliferation and differentiation. The present study examined the long-term effects of targeted PTN over-expression on bone development and repair in a transgenic mouse model. Femurs and tibiae from the PTN transgenic mice and the wild type mice at age 1, 2, 4, 6, 12 and 24 months were collected, and examined by radiography, peripheral quantitative computed tomography (pQCT), histology and mechanical testing. Age-matched PTN and the control mice received a standardized femoral fracture, followed by regular x-rays and sacrificed at day 16 post-fracture for histology examination. A cortical hole was drilled on the tibiae of age-matched PTN and wild type mice, collagen sponge with either saline, 100 ng of rhBMP-2 or rhPTN was implanted in the holes, and animals were sacrificed 10 days later, subject to pQCT and histology examinations. During early stages of bone development, the PTN mice had advanced bone growth in length and maturation, but the difference diminished in later life. The fracture healing was impaired in the PTN mice, and there was delayed callus formation and remodelling. The cortical holes treated with BMP-2 in the PTN mice had significantly less trabecular bone formation. The current study confirmed that the targeted PTN over-expression in mouse bone has moderate enhancing effects on early bone development; but the bones become brittle in later life. Fracture healing was impaired in the adult PTN mice and this may be due to inhibitory effects of PTN over-expression on BMP-2 mediated bone induction.

Key words: Pleiotrophin — Over-expression — Bone development — Fracture repair — rhBMP-2.

Pleiotrophin (PTN) is secreted, lysine-rich peptide associated with extracellular matrix of neuronal cells and bone cells [1]. PTN is also known as a heparin-binding growth-associated molecule (HB-GAM) [2], heparin affin regulator peptide (HARP) [3], or osteoblast-stimulating factor-1 (OSF-1) [4]. It is now realized that all these molecules were actually the same but had been found in different tissues throughout the body.

During embryonic development, the PTN gene is widely expressed in many tissues [5]. In postnatal life, PTN expression is mainly seen in the nerve (brain) and bone tissues [2, 4]. It has diverse functions in stimulating neurogenesis, tumor cells migration and angiogenesis [6]. Under normal conditions PTN is not expressed in adult bone and neither is its receptor, the N-syndecan [7]. However, in a bone injury model, PTN became highly expressed in the osteocytes and their canaliculi at the bone surface and in the periosteum of the injured bones [7]. Recently it has been suggested that PTN has multiple roles in bone formation that are dependent on its concentration, the time of development and the interaction with other factors [8].

Transgenic mice over-expressing the human PTN gene under the control of human osteocalcin promoter had a higher mineral content in contrast to the wild type mice, which could compensate for the ovariectomy-induced bone loss [9]. Mice with non-restricted PTN over-expression (under β-actin promoter) had a higher calcium content in the bones and advanced intramembraneous ossification [10]. When the PTN was over-expressed under a human β-actin promoter, the transgenic mice displayed an ivory-like solid bone surfaces with increased thickness of the cortical bone as well as the bone volumes of cortical and cancellous bones [7]. However, the long-term effects of PTN over-expression on long bone development, particularly its effects on bone fracture healing, is not known. In this study we have comparatively studied the long bone development for 1-24 months in the restricted PTN over-expressing transgenic mice (under the control of human osteocalcin promoter) and the wild-type (BDF1) mice. The fracture healing and bone defect repair was also studied in skeletonly matured (6-12 months old) PTN and BDF1 mice.

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Materials and Methods

Animals

The PTN mice are transgenic mice over-expressing the human PTN gene, which was generated by inserting six extra copies of the human PTN gene under the control of the human osteocalcin promoter in the BDF-1 strain of mice [7]. BDF-1 mice served as the wild-type control group. The BDF1 mice are the F1 generation of hybrid progeny of C57 B1/6/Ola/Hsd (black) and DBA/2/Ola/Hsd (grey) strains of mice. Both the PTN and BDF1 mice were produced and characterized in Japan as described previously [7] and were gifts from Prof. T. Hashimoto-Gotoh, Kyoto Prefectural University of Medicine, Japan. The homozygous pairs of PTN mice and the BDF-1 mice were bred and maintained under licence from the British Home Office in accordance with the Animals (Scientific Procedures) Act (1986) at the Queen's University Belfast biomedical research facility and housed in rooms maintained at 22°C with a 12h/12 h light/dark cycle. The mice had free access to water and autoclaved foods.

Bone Sample Preparation

Male PTN and BDF1 mice were sacrificed at ages 1, 2, 4, 6, 12 and 24 months, with an average of 8 mice in each group (range 8–12). Both femurs and tibiae were collected and muscle attachments were carefully removed. Eight femurs and tibiae per group were used for mechanical testing. The remaining bones (except the 24 month group) were subject to radiography, peripheral quantitative computed tomography (pQCT) and histology examinations.

Radiography Analysis

AP (anterior-lateral) radiographic view of each bone sample was obtained using a high-resolution digital radiography system (Faxitron MX-20 with DC-2 option, Faxitron X-ray Corporation, Illinois, USA). The exposure condition was 26 KV, 10 ms at 2 × magnifications. The digital radiographies were saved in TIFF format and analysed using NIH Scion image software (http://rsb.info.nih.gov/nih-image/index.html). The length of the tibia (from midpoint of tibial plateau to the midpoint of distal tibia) and femur (from the midpoint of the femoral condyles to the tip of the greater trochanter) diameters of both femoral and tibial metaphysis (the widest part at the proximal tibia and distal femur), diaphysis (midpoint of the tibia or femur) and cortical thickness (mean of 4 measurements of randomly selected cortex in the diaphyseal regions) were measured and compared.

Peripheral Quantitative Computed Tomography (pQCT)

After radiographic examination, the bone samples were preserved in 95% ethanol and scanned using pQCT (Stratec XCT 960M, Norland Medical Systems, Fort Atkinson, WI, USA). In brief, two femurs or tibiae were placed in the holder at a time and the measurements were taken from a slice (1 mm thick) from the diaphyseal midpoint (approx. 3–4 mm from the knee surfaces) identified at the scout view window. Scans were analysed using the manufacturers supplied software program "XMICE vl.3" for total bone mineral density (BMD) and cortical and trabecular BMD. A threshold of 1.3 attenuation units differentiated mouse bone from soft tissues; a threshold of 2.0 differentiated high-density cortical bone from low-density trabecular bone. Calibration of the pQCT was routinely performed with a set of hydroxyapatite standards.

Mechanical Testing

Mechanical testing was performed on fresh samples from 1–24-month-old PTN and BDF1 mice, 3-point bending tests were performed on a Minimat testing machine with a 9 mm support span (Minimat, 20 N load beam, Rheometric Scientific, NJ, USA) at room temperature (22°C). The load was applied at a rate of 3 mm/min and the load and displacement responses were measured by the in-built devices and recorded. The biomechanical properties, ultimate load and stiffness were determined from the load-displacement curves.

Mouse Femoral Fracture Model

Fourteen PTN transgenic mice (male; mean age 13 months; mean body weight 41.5 g) and 12 BDF1 mice (male; mean age 12.6 months; mean body weight 42.20 g) were used. A standardized femoral fracture was made on the left femur and fixed with four pins and a cross-bar external fixator as previously described [11, 12]. In brief, under general anesthesia, the femoral shaft was exposed and 5 holes were drilled using a drilling jig. Four 0.5 mm steel pins were inserted loosely to the predrilled holes through the fixator bar. A low energy transverse osteotomy at the site of the central perpendicular drill hole was then performed. The fixator bar was then slid down the pins producing compression of the fracture fragments. Animals were x-rayed at days 1, 8 and 16 post-fracture using a standard x-ray jig, killed at day 16 post-fracture and samples were collected for histology examination.

Mouse Tibia Cortical Hole Repair Model

Six-months-old PTN ($N = 7$) and BDF1 ($N = 7$) male mice were used. Under general anesthesia, a 5-mm lateral incision was made on the medial aspect of the tibial diaphysis, 5 mm below the knee joint on both legs. One 1.5 mm (diameter) hole was drilled through one cortex and a collagen sponge (3 × 3 × 3 mm) was implanted with the following solutions: 30 μl of saline, or 30 μl of saline containing 100 ng of rhBMP2, or 100 ng of rhPTN (PeproTech EC Ltd, London, UK). Animals were randomly assigned to a treatment group with at least 4 tibiae receiving the same treatment. The wound was closed and animals were kept for 10 days and then sacrificed. Both tibiae were removed and fixed in 95% ethanol. The specimens were then scanned by a pQCT. A transverse slice at the pinhole level was identified at the scout view, and the total, trabecular and cortical BMD of the chosen slice (1 mm thick) was measured and compared statistically. The specimens were then decalcified and prepared for histology examination.

Histology

The specimens were decalcified at 4°C over a period of 2–3 weeks in 14% EDTA (pH 7.2–7.4). For the fracture specimens, the external fixators were removed on completion of the decalcification. For the tibial cortical hole repair specimens, under dissecting microscopy, a small segment (3–4 mm), including the cortical hole, was cut off from the decalcified tibia. All samples were then processed through graded alcohols, xylene and embedded longitudinally (on their coronal plane for normal and fracture specimens) and transversely (for the hole specimens) in paraffin wax. Sections 7 μm were cut and stained for hematoxylin and eosin (HE) or Sirius Red / Alcian Blue and examined under light microscopy.

Statistical Analysis

All quantitative data were transferred to statistical spreadsheets and analysed using a commercially available statistical program SPSS (Version 11, Chicago, Illinois, USA). Data

Table 1. Length and total bone mineral density of the femurs and tibiae of the PTN and BDF1 mice

Age	PTN	BDF1	*P* value	PTN	BDF1	*P* value
	Length (mm) ± SD	Length (mm) ± SD		Total BMD (mg/cm^3) ± SD	Total BMD (mg/cm^3) ± SD	
Femur						
1 m	11.08 ± 0.18	9.96 ± 0.53	0.009**	439.83 ± 90.48	315.60 ± 86.2	0.006**
2 m	13.47 ± 1.53	13.24 ± 0.77	0.720	453.68 ± 36.62	462.58 ± 51.62	0.66
4 m	13.98 ± 0.98	12.86 ± 0.88	0.140	466.08 ± 24.11	436.7 ± 37.76	0.049*
6 m	14.11 ± 0.71	13.48 ± 0.87	0.070	472.25 ± 82.51	505.9 ± 69.62	0.67
12 m	16.32 ± 0.42	15.97 ± 0.35	0.046*	N/A	N/A	N/A
Tibia						
1 m	13.34 ± 0.58	12.44 ± 0.68	0.009**	324.22 ± 20.78	362.13 ± 50.65	0.099
2 m	15.23 ± 1.70	13.61 ± 0.60	0.020*	447.05 ± 58.2	404.15 ± 35.78	0.042*
4 m	16.25 ± 1.27	14.48 ± 1.50	0.012*	412.28 ± 13.31	391.25 ± 37.64	0.45
6 m	15.65 ± 0.77	15.30 ± 0.72	0.330	514.65 ± 68.54	476.25 ± 44.26	0.083
12 m	18.92 ± 0.44	18.53 ± 0.32	0.014*	591.40 ± 84.90	487.90 ± 43.70	0.0047**

*$P < 0.05$; **$P < 0.01$

from each age group were first tested by two-way ANOVA to identify significant differences between the animal types and the treatment types. If a significant difference ($P < 0.05$) was found, multiple comparisons were then performed using the LSD (least significant difference) method. Difference was considered significant at $P < 0.05$ in all tests.

Results

Comparative Studies of Bones from the PTN and BDF1 Mice

At 1–12 months, the femurs and tibiae of the PTN mice were longer than those of the aged-matched BDF1 mice (Table 1). The shape of the femur and tibia of the two types was similar. No statistical difference was found in the diameters of metaphysis, diaphysis and mid-shaft cortical thickness of tibiae or femurs at all time points. The PTN mice at 1–4 months appeared to be advanced in bone mineralization, which had well-mineralized cortices compared to the BDF1 mice (Fig. 1). For pQCT examination, the total BMD in the PTN mice was significantly greater than those of the BDF1 mice ($P < 0.05$) at 1 and 4 months in the femur and at 2 and 12 months in the tibia (Table 1). In the tibia, at 4 months the trabeculae at the metaphyseal region was more mature in the PTN mice, showing signs of advanced bone formation and remodelling and cortex maturation, whereas in the BDF1 the trabeculae in the metaphyseal region were still undergoing bone formation and the mineralization of the cortices was not yet completed (Fig. 1). At 1 and 2 months, more trabecular bone was seen at the metaphyseal region in the femur of the PTN mice than those in the age-matched BDF1 mice, but the amount of trabecular bone at the metaphyseal region in both the PTN and BDF1 mice was similar at 4–12 months (Fig. 2). For the mechanical test, in the femur the maximum load of failure in the PTN mice was significantly greater at 4 months ($P < 0.01$) than that of the BDF1 mice, but no statistical difference was seen at other time points (data not shown). In the tibia, the maximum load of failure and stiffness in the PTN mice were significantly greater at 4 months ($P < 0.001$) than those of the BDF1 mice, but

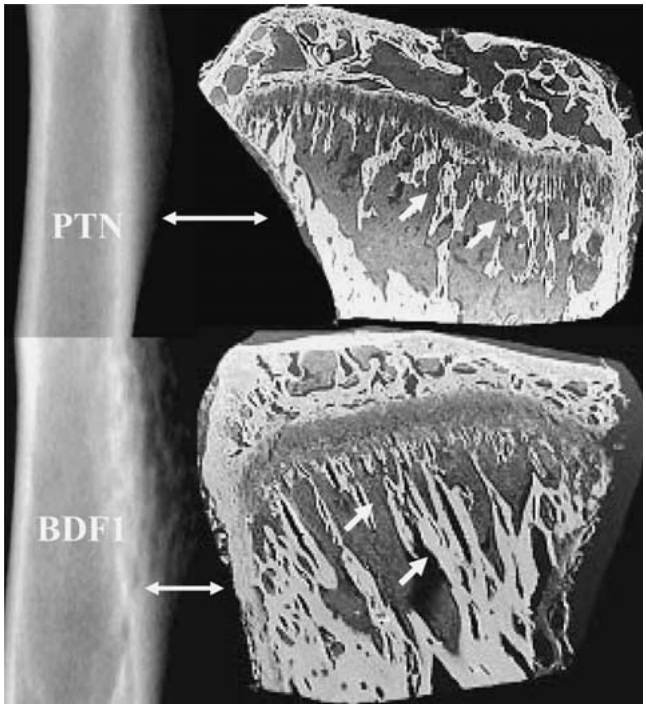

Fig. 1. At 4 months, radiographies from the age-matched PTN and BDF1 mice showed that the tibial cortices were more mature and better mineralized (double-headed arrow) in the PTN mice than those of the BDF1 mice. In the tibiae, the trabecular bones at the metaphysis were more mature at 4 months in the PTN mice than those of the BDF1 mice (arrowheads). Note that the neocortilization was nearly completed at 4 months in the PTN mice whereas in the BDFl mice the cortices were not yet fully mineralized (double-headed arrow). Inverted histological images, original magnification, × 40.

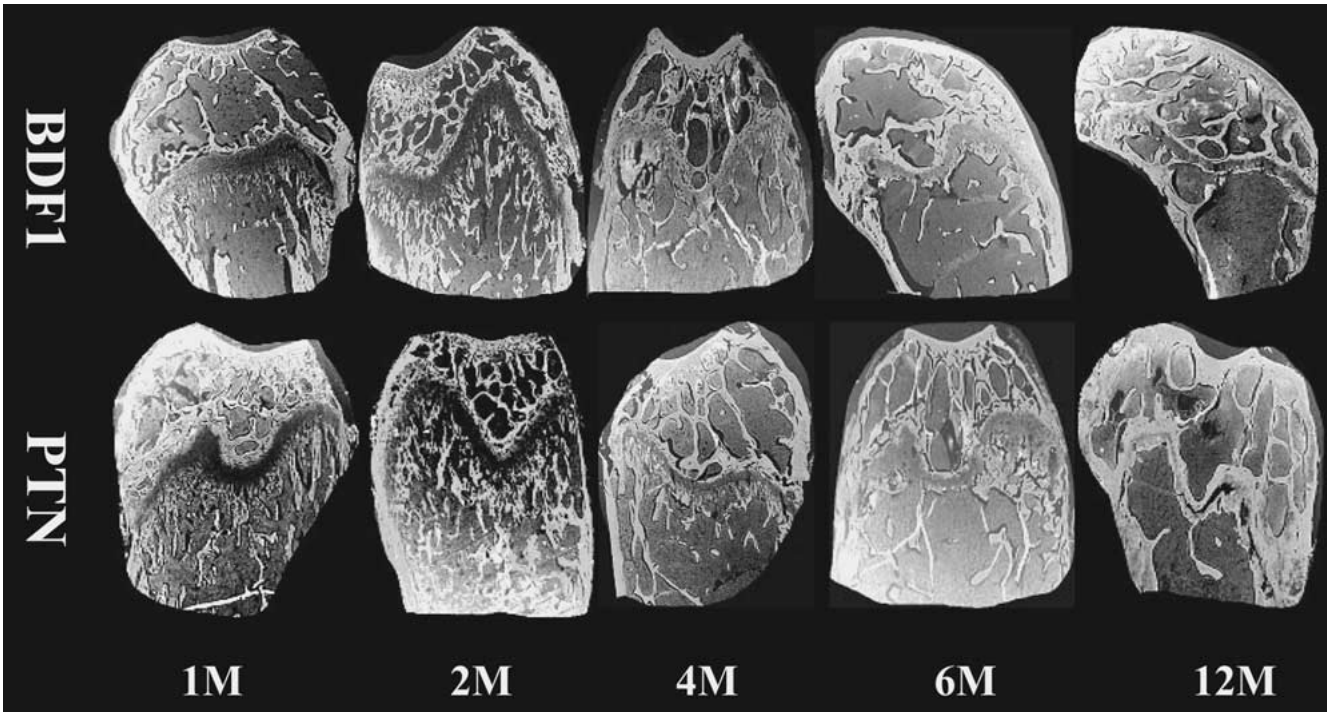

Fig. 2. Representatives of histological appearances of the femurs from the age-matched PTN and BDF1 mice are displayed. At 1 and 2 months, the amount of trabeculae in the metaphyseal regions in the PTN mice was greater than that of the BDF1 mice. At 4–12 months, the morphological appearances were similar in the two animal types. Inverted histological images, original magnification, × 15.

at 12 months the maximum load of failure and stiffness in the BDF1 mice were significantly greater ($P < 0.001$) (Figs. 3 A, B). In both the PTN and BDF1 mice, the stiffness of the tibia and femur reached peak at 12 months and decreased at 24 months (Fig. 3B).

Fracture Healing in the PTN and BDF1 Mice

Eight of the 14 PTN mice (57%) and 4 of the 12 (33 %) BDF1 mice had pinhole fractures during the fracture healing phases of the experiment and were excluded from the study. In the remaining animals, there was no apparent difference in the amount of periosteal callus at days 8 and day 16 post-fracture on radiographs in both types of animals. For histology, all the BDF1 mice that showed radiographic union displayed periosteal callus formation with endochondral ossification at day 16, and signs of callus remodelling (Fig. 4A). In contrast, four out of six PTN mice (67%) with radiographic union had histological appearances of fracture delayed union: the centre of the fracture gap was mainly filled with dense connective tissues, including fibrous and cartilaginous tissues (Figs. 4B, C). In addition, excessive reparative reactions including connective, bony and cartilaginous tissues around the pin inside the bone marrow cavities were noted in 5 out of 6 PTN mice (Data not shown), but only in 1 out of 8 BDF1 mice.

Tibial Cortical Hole Repair in the PTN and BDF1 Mice

For pQCT examination, the total and cortical BMDs at the cortical hole level did not show significant difference in the PTN and BDF1 mice regardless of the treatments. In the BDF1 mice, the trabecular BMD at the cortical hole level appeared to be the greatest in the rhBMP-2-treated group compared to other groups, but it did not reach statistical significance. In contrast, in the PTN mice the trabecular BMD at the cortical hole level was significantly less ($P < 0.05$) in the rhBMP-2-treated group compared to the rhPTN and saline-treated groups (Fig. 5A). The highest trabecular BMD was found in the PTN mice receiving saline or rhPTN treatment among all the groups of both animal types (Fig. 5A). This finding was confirmed by histology: in the BDF1 mice the amount of intramedullary callus at the cortical hole level was similar in all groups (Figs. 5B, D, F); but in the PTN mice the amount of intramedullary callus at the cortical hole level was significantly less in the rhBMP-2 treated group (Fig. 5E) compared to the groups treated with saline (Fig. 5C) or rhPTN (Fig. 5G).

Discussion

This study agreed with previous reports that PTN overexpression in the bone tissues has moderate enhancing effects on mouse long bone development. The PTN mice

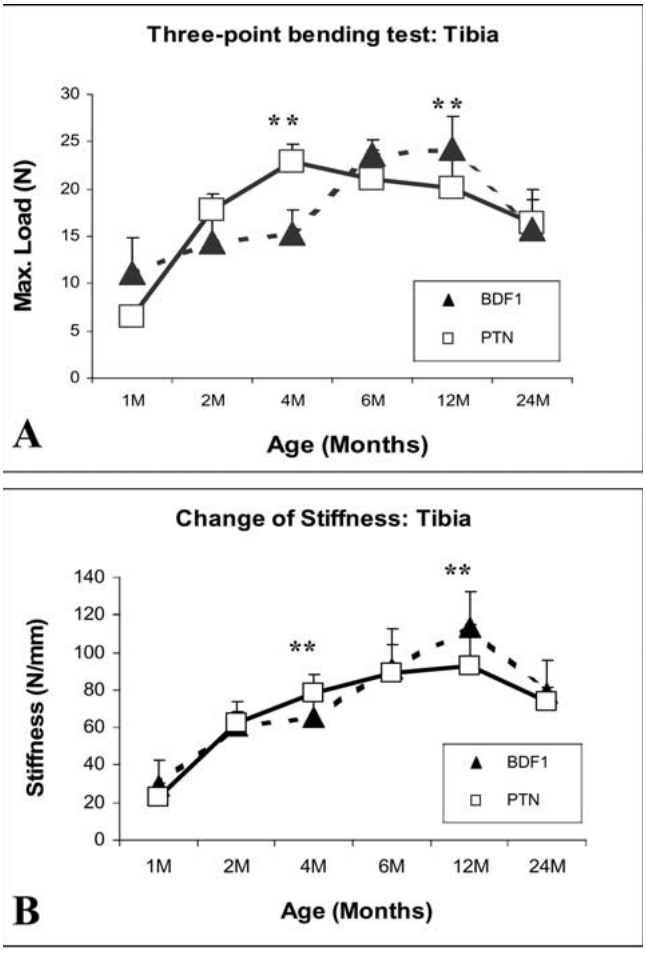

Fig. 3. A. In the tibiae, the maximum load of failure was significantly greater (**$P < 0.001$) in the PTN mice at 4 months but at 12 months it was significantly greater (**$P < 0.001$) in the BDF1 mice. **B**. The tibial stiffness was significantly greater (**$P < 0.001$) in the PTN mice at 4 months, but at 12 months it was significantly greater (**$P < 0.001$) in the BDF1 mice.

had relatively longer tibiae and femurs at 1–12 months of age when compared to the age-matched wild-type BDF1 mice, suggesting that targeted over-expression of PTN in the bone tissues had promoted bone longitudinal growth. The other noticeable findings from the comparative studies were that PTN over-expression appeared to enhance overall bone formation and maturation at early stages (1–4 moths) of long bone development: the PTN mice reached skeletal maturation at around 4–6 months, whereas the wild-type BDF1 mice continued to grow up to 6–12 months, confirmed by mechanical testing results. In the PTN mice, the maximum load of failure of the tibia peaked at 4 months, whereas in the BDF1 mice it peaked at 6 months. However, mechanical testing has also shown that the bone stiffness in the PTN mice was significantly reduced at 12 months of age when compared to the age-matched BDF1 mice, indicating that prolonged PTN overexpression resulted in more brittle bone in later life, when the bone may be more susceptible to fractures.

PTN is highly expressed during embryonic development and it is normally down-regulated in postnatal growth of bone which ceased expression after skeletal maturity [6]. The mechanism of increased bone formation in early stages of bone development in the PTN mice is likely due to PTN's osteoblast-recruiting action. The additional expression of PTN in the PTN mice has been reported to increase in bone formation [7] and compensating bone mineral loss induced by estrogen deprivation [9]. Increased cell motility (migration) is another possible role of PTN [13, 14]. High levels of PTN expression in the bone tissues under the osteocalcin promoter control could increase the rate of osteoblastic cell migration in the extracellular matrix at which osteoblastic cells can move faster. In several experiments using mouse bone marrow cells, PTN has been shown to have positive effects on osteoblastic cell proliferation [8]. It has also been suggested that PTN overexpression may have caused increased production of type I collagen in the chondrocytes [10]. Taken together, high level PTN in the PTN mice may partly explain the early maturation of growth plate and enhanced endochondral ossification in the PTN mice seen at the early developmental stages (1–4 months).

This study has shown that over-expression of PTN had an adverse effect on fracture healing in the adult mice. We have chosen to use 12-month-old mice to avoid effects of any potential longitudinal growth, as the mice skeleton is fully mature at 12 months of age. As demonstrated by the mechanical testing, the bones of the adult PTN mice were more brittle than those of the BDF1 mice at 12 months, and this may account for the high incidence of pinhole fractures encountered during the process of fracture healing. The 67% PTN mice with fracture had shown a delayed callus formation, implying that the bone formation was impaired or delayed. Recent studies have shown that low concentrations (10 pg/ml) of PTN stimulated osteogenic differentiation of mouse bone marrow cells, and had a modest effect on their proliferation, whereas higher concentrations (ng/ml ranges) had no effects [8]. Furthermore, when PTN was used with BMP-2, it was found that PTN inhibited the BMP-2 mediated osteoinduction at concentrations between 0.05 pg and 100 ng/ml. But when PTN was added after the BMP-2 osteoinduction had been achieved, the same concentrations of PTN enhanced further osteogenic differentiation [8]. In the PTN mice, PTN was present at a relatively high level (due to its over-expression) in the bone tissues before the fracture occurred, which might have inhibited the endogenous BMP-2 osteoinduction at the early stages of fracture healing.

A tibial cortical bone hole repair model was employed to further test the effects of exogenous rhBMP-2

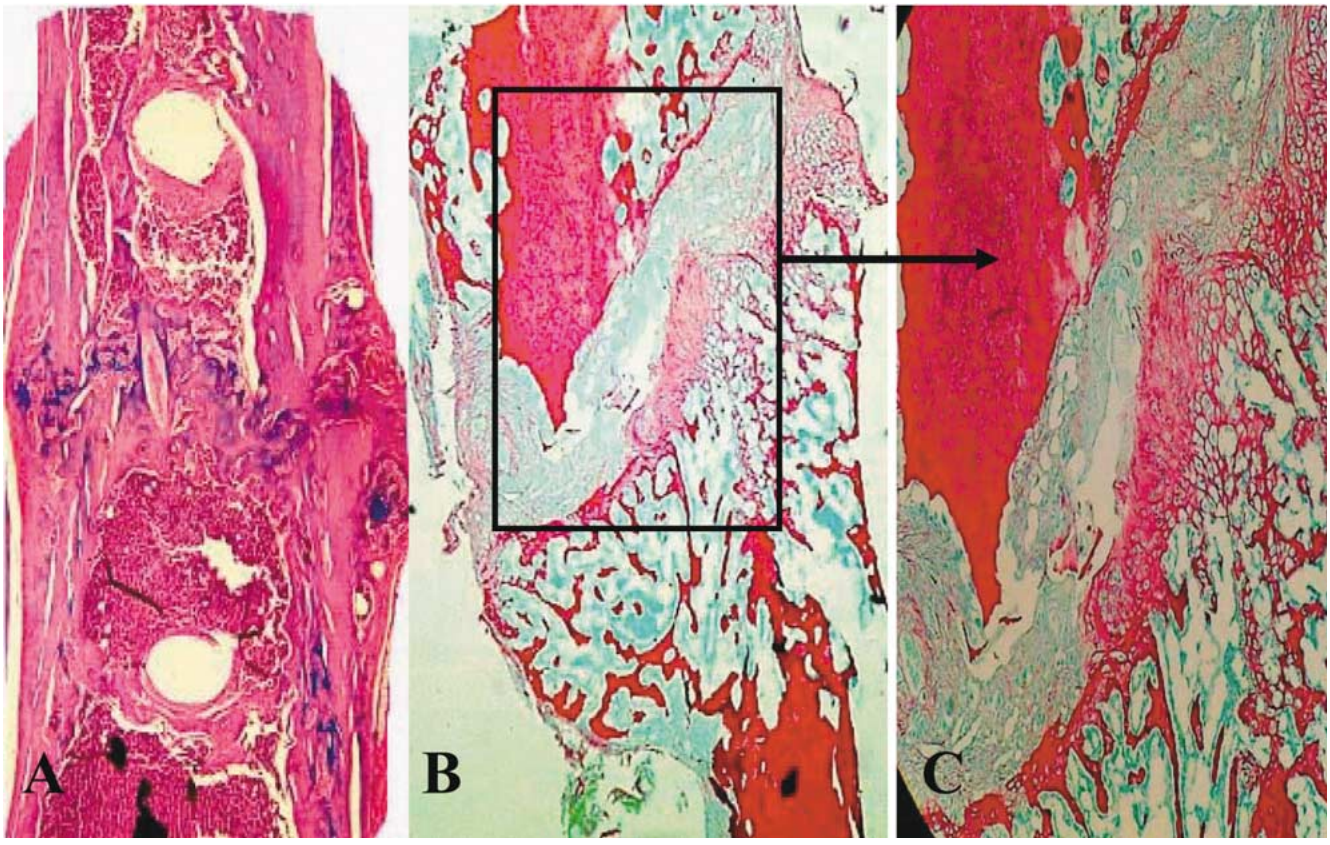

Fig. 4. **A**. A BDF1 mouse with fracture showed radiographic union at day 16 post-fracture. **B**. Histological appearance of a fracture in the PTN mice. **C**. Enlarged boxed area of B showing that the center of the fracture gap was mainly filled with dense connective tissues. **A**: HE staining; **B** and **C** Sirius Red / Alcian Blue staining; original magnification × 20.

and rhPTN on bone healing in the PTN and BDF1 mice. In the BDF1 mice, the trabecular BMD appeared to be the highest in the rhBMP-2-treated group, suggesting that adding exogenous rhBMP-2 (100 ng) may promote bone formation in the BDF1 mice, although the optimal time of sample harvesting and dose of rhBMP-2 treatment remains to be tested. However, in the PTN mice that received the same treatments, a significantly lower trabecular BMD ($P < 0.05$) at the cortical hole level was found in the group with rhBMP-2 treatment compared to the rhPTN and saline groups. This indicates that additional exogenous rhBMP-2 (100 ng) has an inhibitory effect on bone formation when PTN is overexpressed in the bone. Excessive PTN present in the bone tissues may therefore halt bone induction by exogenous or endogenous BMP-2, and lead to an impaired or delayed fracture healing and bone repair. The present finding is in agreement with a previous study that said PTN given in higher concentrations (ng/ml range) had inhibited BMP-2 mediated osteoinduction *in vitro* [8].

In conclusion, it is only recently that the roles of PTN in bone development have begun to come to light. The roles of PTN in bone are speculated to be co-dependent on other factors, and its most likely roles are 1) increasing osteoblastic cell recruitment and attachment; 2) increasing bone cell motility; 3) enhancing osteoblast proliferation and collagen production and 4) inhibiting BMP osteoinductive action [7, 8, 14, 15]. The current study has confirmed that the targeted PTN overexpression in bone tissues has a moderate enhancing effect on mouse long bone development at the early stages (1–4 months) but an adverse effect on bone mechanical proprieties and fracture healing in the adult mice (12 months). The PTN overexpression in the bone tissues has an inhibitory effect on BMP-2-mediated bone induction. Taken together, PTN may play some moderate but important roles in regulating osteogenesis and bone repair.

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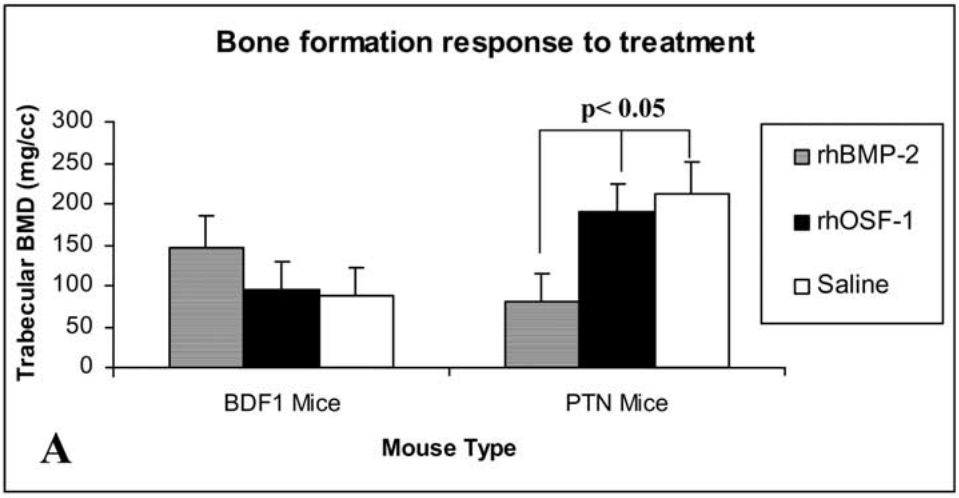

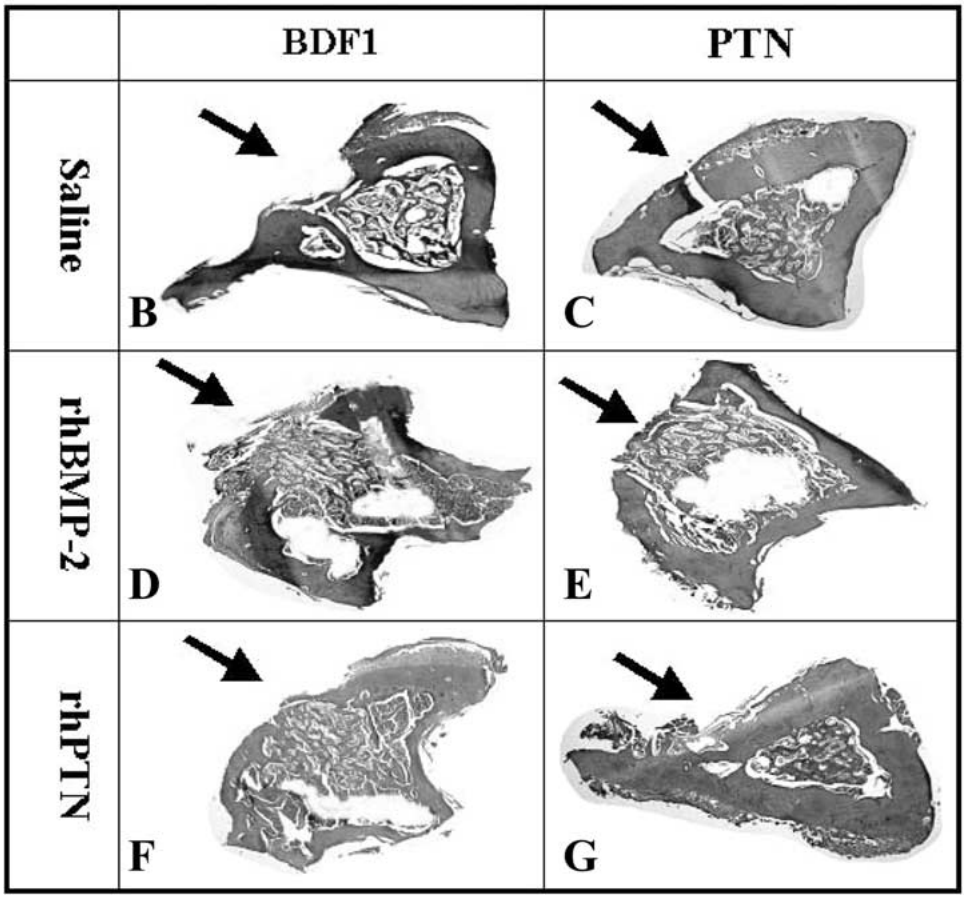

Fig. 5. A. Change of the trabecular BMD at the tibial cortical hole level in response to rhBMP-2, rhPTN or saline treatment in the PTN and BDF1 mice. There was no significant difference in the trabecular BMD among the treatment groups in the BDF1 mice. In the PTN mice, there was a significantly less ($P < 0.05$) trabecular BMD in the group treated with rhBMP-2. **B-G**. Representatives of histological appearances at the tibial cortical hole sites from the transverse sections. **B**. BDF1 mouse treated with saline. **C**. PTN mouse treated with saline. **D**. BDF1 mouse treated with rhBMP-2. **E**. PTN mouse treated with rhBMP-2. **F**. BDF1 mouse treated with rhPTN. **G**. PTN mouse treated with rhPTN. Note that the amount of intramedullary callus was similar in all of the treatment groups in the BDF1 mice. However, in the PTN mice, the group receiving rhBMP-2 had significantly less intramedullary callus formation compared to the other two treatment groups. Arrow indicates the site of cortical hole. B-G, original magnification × 15.

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