Mice expressing a constitutively active PTH/PTHrP receptor in osteoblasts show reduced callus size but normal callus morphology during fracture healing

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Background The parathyroid hormone-/parathyroid hormone-related protein (PTH/PTHrP) receptor plays a crucial role in endochondral bone formation and possibly also in fracture healing. Patients with Jansen’s metaphyseal chondrodysplasia (JMC) have a gain-of-function mutation in the PTH/PTHrP receptor. Transgenic mice expressing JMC PTH/PTHrP receptor mutants in osteoblasts are characterized by increased trabecular bone formation and reduced osteoblastic activity at periosteal sites. We have analyzed the bone phenotype and studied the fracture healing process in this model.

Methods We performed bone density analysis of tibiae from 17-week-old transgenic mice and controls. Also, tibial fractures were produced in 14-week-old mice. Fracture healing was examined by radiographic and histological analysis.

Results Transgenic mice had a lower total bone mineral content (BMC), by a factor of one-third. The changes were bone compartment-specific with an increase in trabecular bone volume and a decrease in cortical thickness. The calluses in the transgenic mice were smaller, with a reduction in BMC and mean cross-sectional area by a factor of one-half. Despite the smaller size, however, the morphology and progression through the healing process were similar in both transgenic and wild-type littermates.

Interpretation We conclude that the constitutively active PTH/PTHrP receptor has compartment-specific effects on bone formation when expressed in osteoblasts. During fracture healing, however, both the periosteal and the endochondral processes are activated, leading to fracture healing that is temporally and morphologically normal, although the callus tissue is less prominent.

Fracture healing is a complex process that involves an inflammatory response followed by production of callus tissue and new bone through both endochondral and intramembranous bone formation processes (Bolander 1992, Buckwalter et al. 1996). The appearance of chondrocytes in the center of the callus, and the subsequent proliferation and hypertrophy of these cells as they lay down a cartilaginous matrix resembles the events that occur in the developing growth plate (Kronenberg 2003).

In the growth plate, the PTH/PTHrP receptor is an important regulator of the progression of chondrocytes through the differentiation pathway by mediating the effects of parathyroid hormone-related protein (PTHrP) (Lanske et al. 1996, Vortkamp et al. 1996). PTHrP is produced by round proliferating chondrocytes adjacent to the perichondrium and binds to the PTH/PTHrP receptor in prehypertrophic chondrocytes. The activation of the PTH/PTHrP receptor delays hypertrophic differentiation to allow elongation of the growth plate (Chung et al. 1998). Patients with Jansen’s metaphyseal...
chondrodysplasia (JMC), which is caused by gain-of-function mutations in the PTH/PTHrP receptor (Schipani et al. 1995), are severely dwarfed and their growth plates show a failure of proliferative chondrocytes to mature in a timely manner (Jansen 1934, Juppner and Schipani 1996).

The PTH/PTHrP receptor also mediates the systemic effects of parathyroid hormone (PTH) (Juppner et al. 1991, Kronenberg et al. 1998). PTH has a crucial role in regulating calcium and phosphate homeostasis and bone turnover. PTH also has important pharmacological effects. When injected subcutaneously once a day in patients with osteoporosis, it stimulates bone formation, increases bone mineral density (Kurland et al. 2000), and reduces the risk of fracture (Neer et al. 2001). In animal models, PTH has been reported to increase both callus size and the mechanic stability of fractures induced in rat femurs (Holzer et al. 1999, Alkhiary et al. 2005). The pharmacological effects of PTH on bone tissue are believed to be mediated by the action of the PTH/PTHrP receptor on osteoblastic precursors.

By taking advantage of the availability of a transgenic animal expressing a constitutively active PTH/PTHrP receptor under the control of the mouse collagen type $\alpha_1$ (I) promoter, we decided to investigate the role of this receptor in fracture healing in greater depth. This mouse model has an interesting bone phenotype, characterized by increased trabecular bone formation and a thinning of the cortical bone (Calvi et al. 2001). In addition, transgenic mice are smaller and have shorter limbs than their wild-type littermates. In this study, we characterized the bone phenotype of these transgenic mice in greater detail and studied the fracture healing process in these animals.

**Methods**

**Mice**

Transgenic mice (FVB/N background), expressing a constitutively active PTH/PTHrP receptor (HKrk-H223R) under the control of the mouse collagen type $\alpha_1$ (I) promoter (Coll1-Jansen mice), were generated and bred as previously described (Calvi et al. 2001). For each experimental procedure, wild-type littermates were used as controls. The mice were maintained in a virus- and parasite free barrier facility under a 12-h light/12-h dark illumination cycle at Massachusetts General Hospital, and weaned onto autoclaved rodent chow at 18 days of age. The study was approved by the institutional animal care committee (approval number 2002N000352).

**DXA and pQCT analysis of unfractured tibiae**

The right tibiae from 17-week-old transgenic (n 5) and wild-type (n 6) mice were dissected free of soft tissue and subsequently fixed in 4% paraformaldehyde for 24 h before structural analysis. Total length of tibiae, areal bone mineral density (BMD), and bone mineral content (BMC) were measured with Sabre and Sabre Research software for peripheral DXA (pDXA) (Norland Medical Systems, Inc., Fort Atkinson, WI). Computerized tomography was performed with Stratec XCT Research M for peripheral quantitative computerized tomography (pQCT) (software version 5.4B; Norland Medical Systems). Scans were performed at mid-diaphyseal sites of the tibiae to obtain cortical parameters, and at metaphyseal sites to measure trabecular volumetric BMD. Mid-diaphyseal scans were performed at a distance from the proximal growth plate corresponding to 30% of the total length of the tibia. Metaphyseal scans were performed in a standardized manner at a distance from the proximal growth plate corresponding to 2.6% of the total length of the tibia. The trabecular bone region was defined by setting an inner threshold of 400 mg/mm$^3$. It should be emphasized that the DXA technique gives the areal BMD whereas the pQCT gives the real/volumetric BMD. Thus, a factor regulating the outer dimensions of a bone will affect the areal BMD (DXA) but not the volumetric BMD (pQCT).

**Fracture model**

14-week-old mice were anesthetized with isoflurane, and an intramedullary nail (stylus of a 25-gauge intracath) was inserted into the left tibia through a small surgical insertion at the knee joint. The tibiae were then fractured to produce closed, transverse, mid-diaphyseal fractures by controlled blunt trauma using a modification of the technique...
developed for rats (Bonnarens and Einhorn 1984). Surgery was performed on 24 mice, of which 12 were transgenic animals and 12 were wild-type littermates. The mice were allowed to bear weight on the fractured limb and divided randomly into two groups. The first group, consisting of 6 transgenic and 6 wild-type mice, was killed by cervical dislocation at 10 days after surgery for analysis of callus formation. The other group, consisting of 5 transgenic mice (one was found dead in its cage before the time of analysis) and 6 wild-type mice, was killed by cervical dislocation at 21 days after surgery. These time points were chosen because in this rodent model it is known that the soft callus is well developed by 10 days after surgery and usually replaced by 21 days, thus providing a good window to detect disturbances of callus differentiation (Bourque et al. 1992).

**pQCT analysis of callus**

Animals were killed by cervical dislocation either 10 or 21 days after surgery. The fractured tibiae from the transgenic mice and wild-type controls were dissected free of soft tissue, the intramedullary nail was removed, and the tibiae were subsequently fixed in 4% paraformaldehyde for 24 h before structural analysis. The whole callus was scanned to obtain the center, defined as the section with the largest area. Starting from the center of the callus, 5 consecutive pQCT sections were performed, each with a thickness of 5 µm. Analysis of these consecutive sections was used to determine the average cross-sectional area, BMD, and BMC in each fracture.

**Histological analysis of callus**

After pQCT analysis, all 23 of the fractured tibiae were decalcified in 20% buffered EDTA solution, pH 7.2, for 3 weeks. To perform gross histology, all the decalcified tissue was processed and embedded in paraffin blocks. 6-µm histological sections from all the tibiae were then cut and stained with hematoxylin and eosin (H&E) according to standard procedures. In situ hybridization was performed with 35S-labeled cRNA probe for mouse collagen type II mRNA, as previously described (Schipani et al. 1997). Deparaffinized sections were hybridized for 24 h and then rinsed in SSC buffer. The slides were covered with photographic emulsion at 4°C and exposed for 3–14 days depending on signal intensity. Slides were developed and counterstained with H&E, and examined and photographed under bright- and dark-field microscopy.

### Statistics

Statistical analysis was performed using GraphPad Prism software, version 3.03 (GraphPad Software Inc., Australia). Values are expressed as mean (SEM). Differences between wild-type and transgenic groups were calculated using Student’s independent t-test. A probability of p < 0.05 was considered to be statistically significant.

### Results

#### Characterization of Col1-Jansen mice with DXA and pQCT

It has been shown by histological analyses that col1-Jansen transgenic mice have increased trabecular bone, an increased bone marrow stromal cell population, and a thin cortical bone (Calvi et al. 2001). It was also shown by histomorphometry that both bone formation and bone resorption parameters are increased (Calvi et al. 2001). To establish reliable quantitation of callus size and density, it was important to extend these studies by investigating the transgenic bone phenotype using DXA and pQCT. We analyzed hemizygous transgenic (n 5) and wild-type (n 6) animals at 17 weeks.
of age and found that the tibiae of transgenic animals were on average 8% shorter than the tibiae of wild-type littermates. Total bone mineral content (BMC) was reduced by 31% and the areal bone mineral density (BMD) by 20%, respectively, as measured by DXA (Table 1). Interestingly, however—and in agreement with previously published data (Calvi et al. 2001)—pQCT analysis showed that the transgene effect was bone compartment-specific. When measured at metaphyseal sites the trabecular bone content was increased by 35%, whereas measurement of the cortical bone at mid-diaphyseal sites showed that cortical bone content was reduced by 27% (Table 2).

Fracture healing in the Col1-Jansen mouse
We fractured the left tibiae of 14-week-old transgenic (n = 12) and wild-type (n = 12) mice. The animals were allowed full weight bearing. At either day 10 or 21 after surgery, half of the animals were killed by cervical dislocation. At both time points, it was obvious from visual inspection of bone dissected free of soft tissue that the callus was smaller in the transgenic animals than in the wild-type animals (Figure 1).

We then quantified the callus size by analyzing 5 central and consecutive pQCT sections of the callus in all harvested tibiae. This analysis proved impossible at the 10-day time point due to poor demarcation between bone, soft tissue, and the early fracture callus (data not shown). We found that at 21 days after surgery, the bone mineral content (BMC) of the callus was 58% lower in the transgenic ani-

Table 2. Results of pQCT analysis of unfractured tibiae

|               | BMC (mg/mm²) | BMD (mg/mm³) | Area (mm²) | Cortical thickness (mm) | Periosteal circumference (mm) |
|---------------|--------------|--------------|------------|-------------------------|--------------------------------|
|               | WT | TG | WT | TG | WT | TG | WT | TG | WT | TG | WT | TG |
| Cortical bone | 0.91 | 0.66 | 1.17 | 1.08 | 0.77 | 0.61 | 0.22 | 0.20 | 4.18 | 3.71 |
| P-value       | 0.001 | < 0.001 | 0.001 | < 0.001 | 0.007 | 0.0043 | 0.006 | 0.0012 | 0.03 | 0.09 |
| Trabecular bone | 0.33 | 0.45 | (0.012) | (0.003) | P-value | 0.003 |  |  |  |  |

pQCT measurements were performed in wild-type (WT; n = 6) and transgenic (TG; n = 5) mice at 17 weeks of age. The further characterization of the tibiae shows that the phenotypic changes in bone in the transgenic mice were bone compartment-specific. There was a significant reduction in BMC and volumetric BMD, as well as in area and periosteal circumference. Conversely, there was an increase in trabecular volumetric BMD. Results are expressed as mean (SEM).
tissue, suggesting normal progression through the healing process (Table 3).

To investigate whether the size difference was due to disturbances in a specific compartment of the healing fracture, we also performed histological examination of H&E-stained sections from all 23 fractured tibiae. At 10 days after surgery, we found that although the callus appeared smaller in the transgenic mice, the relative size of both the cartilaginous callus and the periosteal bony callus was very similar in transgenic and wild-type specimens (Figure 2). At day 21, we observed a smaller-sized callus in the transgenic animals, but also a full replacement of the soft callus in both transgenic and wild-type mice.

To investigate the distribution of cartilage in the callus, we also performed in situ hybridization using collagen type II as a marker of chondrocytes. We saw no obvious differences in expression of collagen type II mRNA between transgenic and wild-type specimens (Figure 3). We therefore conclude that the amount of callus formed in PTH/PTHrP transgenic animals is less than in wild-type animals, but it is of normal histological appearance and with normal progression through the different stages of fracture healing.

### Discussion

The transgenic model investigated in this paper has been described previously (Calvi et al. 2001). The most striking aspect of this phenotype is the increase in trabecular bone volume and increase in the number of bone marrow stromal cells. On the other hand, the periosteal mineral apposition rate appeared to be impaired in transgenic mice relative to wild-type animals (Calvi et al. 2001). In this study, we have extended these findings by demonstrating the dramatic compartment-specific changes in bone mineral density, such that BMD is increased at trabecular sites but reduced in the cortex.

Since fracture healing is likely to involve recruitment of osteoblast precursors from the periosteum, we hypothesized that fracture healing might be impaired in this mutant animal model. Indeed, we did find that fracture calluses were smaller in transgenic animals than in wild-type littermates. Surprisingly, however, we did not detect any disturbances in the formation of the periosteal bony callus. This finding suggests that although the cellular activity at the periosteum is lower in trans-

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**Table 3. Results of pQCT analysis of callus formation at 21 days after surgery in wild-type (n=6) and transgenic (n=5) mice**

|                | BMD  (mg/mm³) | BMC  (mg/mm) | Area  (mm²) |
|----------------|---------------|--------------|-------------|
| Wild-type      | 0.38 (0.02)   | 0.78 (0.12)  | 2.1 (0.3)   |
| Transgenic     | 0.36 (0.02)   | 0.32 (0.07)  | 0.9 (0.2)   |
| %              | −6.4          | −58          | −56         |
| P-value        | 0.4           | 0.01         | 0.01        |

Data show a normal bone mineral density (BMD) but a significant reduction in both mineral content (BMC) and callus area in the transgenic mice compared to the wild-type mice. Results are expressed as mean (SEM).
studies, it was found that intermittent treatment of animals with PTH during fracture healing caused an increase in callus volume. However, the model used in this paper represents a chronic activation of the PTH/PTHrP receptor in osteoblasts, and this might also explain the discrepancy—at least in part.

We conclude that the constitutively active JMC PTH/PTHrP receptor has dramatic compartment-specific effects on bone formation when expressed in osteoblasts. During fracture healing, however, both the periosteal and the endochondral processes are normally activated, leading to fracture healing that is temporally and morphologically normal, even though the callus tissue is less prominent.

Contributions of authors
RM: primary data analysis, preparation of draft manuscript, manuscript revisions. KBJ: planning of study design, collection of samples, manuscript revisions. T-JC: surgery, manuscript revisions. TE: development of experimental method, planning of study, manuscript revisions. CO: performed QCT and DXA analysis, manuscript revision. ES: planning of study design, providing animal model, financed study, manuscript revision.
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