Overexpression of the Transcriptional Factor Runx2 in Osteoblasts Abolishes the Anabolic Effect of Parathyroid Hormone *in Vivo*

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There is convincing evidence that Runx2 could be a regulator of the anabolic action of parathyroid hormone (PTH) in bone. We therefore decided to determine how Runx2 overexpression in osteoblasts affects the anabolic response to PTH. Transgenic osteoporotic female mice overexpressing Runx2 (TG) and their wild-type littermates (WT) were treated with PTH (100 µg/kg/day, 7 days a week) or with the vehicle for 6 weeks. Unexpectedly, Runx2 overexpression blunted the increase in the mineral density and volume of bone induced by intermittent PTH in WT mice. Our findings also indicate that PTH failed to increase bone formation in TG mice overexpressing Runx2. This abolition of the effect of PTH by Runx2 overexpression was attributable to a decrease in the differentiation of osteoblastic cells both *in vivo* and *in vitro*. Finally, we showed that less cAMP was induced by PTH and that there were fewer PTH binding sites in TG than WT osteoblasts. In conclusion, our findings demonstrate that *in vivo* a high level of Runx2 abolishes the anabolic effect of PTH, probably via a decrease in the sensitivity of TG osteoblasts to PTH, and that the level of expression of Runx2 is critical if PTH is to produce its anabolic effect on bone *in vivo*. (Am J Pathol 2007; 170:1676–1685; DOI: 10.2353/ajpath.2007.061069)

Parathyroid hormone (PTH) is the first anabolic agent to demonstrate effectiveness in the treatment of osteoporosis. A number of studies in animals and humans have demonstrated that short-term, intermittent administration of low doses of PTH has a significant anabolic effect on bone.¹ At a cellular level, the increase in bone mass is

associated with increased activity of existing osteoblasts and increased differentiation of osteoblast precursors or recruitment of lining cells.² When PTH binds to its receptor, PTH-R1, this triggers a wide range of molecular events by activating the adenylate cyclase and phospholipase C pathways. PTH mediates the *trans*-activation of cAMP response element-binding protein,³ which is required for the PTH-induced stimulation of the activator protein-1 family of transcription factors.⁴ However, the molecular mechanisms that mediate the osteoblastic effects of PTH remain to be clearly elucidated, in particular its possible interaction with *Runx2*, a master gene for osteoblast functions. *Runx2* is a member of the runt family of transcription factors the expression of which is absolutely necessary for osteoblastic precursors to differentiate and mature osteoblasts to be activated.⁵ *Runx2*-null mice failed to develop a skeleton and had no functional osteoblasts because of arrested maturation.^{6,7} In transgenic (TG) mice overexpressing *Runx2* specifically in cells of the osteoblastic lineage, the maturation of osteoblasts was affected, and their osteoclastogenic properties increased, suggesting that elevated *Runx2* expression in osteoblastic cells can enhance bone resorption *in vivo*.^{8,9} There are limited data available about the regulation and expression of *Runx2* by PTH. There is evidence that low concentrations of PTH increase the level of expression of *Runx2 in vitro* and *ex vivo*, whereas high PTH concentrations decrease the *Runx2* level *in vitro*.¹⁰ It has been also demonstrated that PTH, acting via the cAMP and PKA pathways, prevents osteoblast apoptosis by inactivating the proapoptotic protein Bad and stimulating the transcription of the anti-apoptotic gene *Bcl-2*.¹¹ It has been suggested that *Runx2* and cAMP response element-binding protein may be key mediators of the PTH-dependent inhibition of osteoblasts apoptosis. Bellido and colleagues¹¹ suggested that the intermittent PTH treatment that is a prerequisite for anabolism is achieved as a result of transient falls in the intracellular level of

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Runx2. This hypothesis was supported by the demonstration that intermittent PTH treatment of osteoblastic cells *in vitro* is followed by the ubiquitinylation and degradation of Runx2 via the proteasome. The fall in Runx2 may be a critical event in shortening the anabolic effect of PTH.^{11,12} The level of expression of Runx2 in osteoblasts might therefore be critical for the anabolic response to intermittent PTH treatment. We report here our investigation of the skeletal response to PTH in a mouse model of osteoporosis in which Runx2 was overexpressed in cells of the osteoblastic lineage.⁹ Our findings indicate that Runx2 plays a key role in the skeletal response to PTH.

Materials and Methods

Animal and Experimental Protocol

Mice overexpressing Runx2 were produced as previously described.⁹ The mice were housed under controlled conditions at 24°C on a 12-hour light/12-hour dark cycle. Four-week-old TG females and their wild-type (WT) female littermates from the ninth generation (CB6F9) were used for the experiment. The mice were subjected to subcutaneous injections with human recombinant PTH fragment 1-34 (Lilly, Indianapolis, IN) at 100 µg/kg body weight/day or with the vehicle (acidified saline), 7 days/week for 6 weeks ($n = 22$ to 9 per group). Body weight was measured weekly, and dose was adjusted accordingly. To make it possible to evaluate the dynamic bone formation parameters by histomorphometry, the mice were given two fluorochrome markers by intraperitoneal injection [3 days before necropsy, 20 mg/kg oxytetracycline (Pfizer, Amboise, France), and 1 day before necropsy, 20 mg/kg calcein (Sigma-Aldrich, St. Louis, MO)]. Twenty-four hours after the last PTH injection, the animals were anesthetized by intraperitoneal administration of 6% sodium pentobarbital. The animals were sacrificed by exsanguination under anesthesia. The animals were allowed free access to food and distilled water in full compliance with the French government animal welfare policy.

Measurement of Bone Mineral Density (BMD) by Dual-Energy X-Ray Absorptiometry

Dual-energy X-ray absorptiometry analysis of all animals was performed under anesthesia. Total body, whole femur, and whole tibia BMD (mg/cm²) were measured using a PIXImus instrument (software version 1.44; GE Lunar, Madison, WI) and operating in ultrahigh resolution mode (resolution 0.18 × 0.18 mm). The precision and reproducibility of the instruments had previously been scanned. The coefficient of variation was <2% for all of the parameters evaluated. A phantom was scanned daily to normalize and monitor the stability of the measurements.

Histology and Histomorphometry

The left femur from each animal was excised at sacrifice and the surrounding soft tissue stripped off. After storing in 70% ethanol, dehydrating in xylene at 4°C, and then embedding without demineralization in methylmethacrylate, 5-µm-thick coronal sections were cut parallel to the long axis of the femur, using an SM 2500S microtome (Leica, Wetzlar, Germany). Sections were stained with aniline blue to investigate the microarchitectural parameters. Two 10-µm-thick sections were taken for measuring the dynamic parameters. The measurements were performed in an area of ~2 mm² of trabecular bone located in the secondary spongiosa. The histomorphometric parameters were recorded at this standard sampling site in accordance with the Guidelines of the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research.¹³ The trabecular bone volume (BV/TV), the trabecular bone thickness (Tb.Th), and the trabecular separation (Tb.Sp) were measured using the software package developed for bone histomorphometry (Microvision, Evry, France). The dynamic parameters were measured in 10-µm-thick, unstained sections examined under UV light. The mineral apposition rate (mean interlabel thickness divided by the time between the two labeling periods) was measured using the same image analyzer (Microvision) by a semi-automatic method. The mineralizing surfaces (MS/BS) were measured using an objective eyepiece Leitz integrateplatte II (Leica) at ×128 magnification. The bone formation rate (BFR) was calculated according to the American Society for Bone and Mineral Research nomenclature.¹³ For cortical bone, we measured the average bone and medullar diameters at the femoral metaphysis and calculated the cortical thickness (Cort.Th).

Quantitative Reverse Transcriptase-Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNAs from the metaphysis of tibia were isolated using Trizol reagent (Invitrogen, Carlsbad, CA) and cleaned using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was isolated from a primary osteoblastic cell lysate using NucleoSpin (Macherey-Nagel, Easton, PA) according to the manufacturer's instructions.

The RT reaction was performed using the Reverse-ITmax RTase blend (Abgene, Epsom, Surrey, UK). Quantitative real-time PCR expression analysis was performed using a Roche light cycler, Absolute SYBR Green capillary mix (Abgene). Aldolase A and 18S were used for normalization. The sets of primer used for real-time PCR were: aldolase A (forward: 5'-TGAA-GCGCTGCCAGTATGTGA-3'; reverse: 5'-GGTCGCTC-AGAGCCTTGTAGA-3'), 18S (forward: 5'-CGGCTACC-ACATCCAAGGAA-3'; reverse: 5'-GCTGGAATTACCG-CGGCT-3'), collagen type 1 (forward: 5'-CTTGTTGG-TTTTGTATTCGATGAC-3'; reverse: 5'-GCGAAGGCA-ACAGTCGCT-3'), alkaline phosphatase (ALP) (forward: 5'-AAGGCTTCTTCTTGCTGGTG-3'; reverse: 5'-GCCTTA-

CCCTCATGATGTCC-3'), osteocalcin (forward: 5'-CTCAC-AGATGCCAAGCCCA-3'; reverse: 5'-CCAAGGTAGCGC-CGGAGTCT-3'), endogenous Runx2 (forward: 5'-TTGAC-CTTTGTCCCAATGC-3'; reverse: 5'-AGGTTGGAGGCAC-ACATAGG-3'), transgene (forward: 5'-AGAGACCACAAT-AACCAGCACG-3'; reverse: 5'-GGCGGCCATATGACTAC-AAAG-3'), PTH-R1 (forward: 5'-GAAGTCCAATGCCAGTG-TCCA-3'; reverse: 5'-ACTCCTCCAGGGATTTTGT-3'), RANKL (forward: 5'-GGCCACAGCGCTTCTCAG-3'; reverse: 5'-GAGTGACTTTATGGGAACCCGAT-3'), and OPG (forward: 5'-AGTCCGTGAAGCAGGAGTG-3'; reverse: 5'-CCATCTGGACATTTTGTGAAA-3').

Cell Cultures

A primary culture of mouse osteoblastic cells was obtained by sequential collagenase IV (Sigma-Aldrich) digestion of calvaria from 2- to 5-day-old mice. Cells were expanded in T75 flasks (Falcon; BD Biosciences Discovery Labware, Bedford, MA) until confluence in α -modified essential medium (α -MEM) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells have been harvested with trypsin/ethylenediaminetetraacetic acid (Invitrogen) treatment and replated in differentiation medium (growth medium supplemented with 50 μ mol/L ascorbic acid and 10 mmol/L β -glycerophosphate; Invitrogen). For molecular analysis and determination of the alkaline phosphatase activity, cells were seeded at 1×10^6 cells/plate and at 9×10^4 cells/well in 10-cm plates and 24-well plates, respectively, and grown for the indicated times. For mineralizing experiments, cells were plated in 10-cm plates at 1×10^6 cells/plate and grown for 14 days. The medium was changed every 3 to 4 days. Nodules of mineralized extracellular matrix were identified by alizarin red staining (Sigma-Aldrich) after fixing the cells in 70% ethanol. To assess the proliferation of the osteoblastic cells, cells were cultured in 96-well plates (4×10^3 cells/well) for 2 days in the presence of 50 μ mol/L ascorbic acid. Proliferation was determined during the treatment period using a 5-bromo-2'-deoxyuridine enzyme-linked immunosorbent assay to measure the incorporation of 5-bromo-2'-deoxyuridine (GE Healthcare, Buckinghamshire, UK). Appropriate negative controls were included.

Binding Assays

Radioligand binding experiments were performed as previously described.¹⁴ Cells were washed with phosphate-buffered saline and harvested by scraping with a rubber policeman and quickly frozen at -20°C . Each cell pellet was resuspended in 5 ml of buffer A (10 mmol/L Tris-HCl and 4 mmol/L ethylenediaminetetraacetic acid, pH 7.40) containing protease inhibitors (10 μ g/ml pepstatin, 2 μ g/ml aprotinin, 10 μ g/ml chymostatin, and 10 μ g/ml leupeptin) (Sigma-Aldrich). Then, cell lysates were homogenized by 30 strokes with a Dual tissue grinder (Kontes Glass, Vineland, NJ). The homogenates were centrifuged at $700 \times g$ (Jouan CR 412 centrifuge; Jouan, Winchester, VA) for 10 minutes at 4°C to remove nuclei

and debris. Supernatants were centrifuged at $40,000 \times g$ (L7-55 ultracentrifuge, 70.1 Ti rotor; Beckman Coulter, Inc., Fullerton, CA) for 30 minutes at 4°C , and the supernatants were aspirated, and the membrane-enriched pellets were suspended in buffer B (50 mmol/L Tris-HCl, 0.15 mol/L NaCl, 2 mmol/L CaCl_2 , 5 mmol/L KCl, 5 mmol/L MgCl_2 , 4 mmol/L ethylenediaminetetraacetic acid, and 20% glycerol, pH 7.50) with the same protease inhibitors at a protein concentration of 2 mg/ml and stored at -80°C . The radioreceptor assays were performed within 1 month.

To assess binding, various concentrations of [¹²⁵I]-[Nle,⁸,¹⁸Tyr³⁴]PTH-(1-34) amide (Amersham Biosciences) and unlabeled [Nle]PTH-(1-34) (Bachem, Bubendorf, Switzerland) were added to 0.1 ml of buffer B with 1 mmol/L phenylmethyl sulfonyl fluoride and 0.3% bovine serum albumin. The reaction was initiated by addition of 2 μ g of membrane protein. After a 1-hour incubation at 21°C , separation of bound from free radioligand was achieved by applying samples to 25-mm polyvinylidene difluoride filters (0.45 μm ; Millipore, Billerica, MA), which had been presoaked with buffer B containing 10% fetal calf serum before mounting them on a 12-port vacuum manifold (Millipore). Filters were washed with 3 ml of chilled buffer B (two times) and transferred to tubes, and radioactivity was counted with a γ -counter (Perkin-Elmer, Selton, CT).

Determination of Alkaline Phosphatase Activity

Primary osteoblasts were seeded at 9×10^4 cells/24-well plates for various times. Cells were washed twice and scraped into deionized water. ALP activity and protein concentration were determined using ADVIA1650 (Bayer Corp., Diagnostics Div., Tarrytown, NY). Protein concentrations were determined by the bicinchoninic acid method (Pierce, Chichester, UK). Results were expressed in IUs and normalized for protein content (IU/mg protein).

cAMP Measurements

Cells were cultured in α -MEM supplemented with 10% fetal bovine serum and ascorbic acid in 24-wells plates for 4 days at a density of 9×10^4 cells/well. Cells were washed twice with α -MEM, starved overnight in 1% fetal bovine serum, and then incubated with 1 mmol/L 3-isobutyl-1-methylxanthine for 30 minutes in serum-free culture medium. The effect of PTH was evaluated in the presence of 1 mmol/L 3-isobutyl-1-methylxanthine for 15 minutes. Cells were scraped off and pelleted by centrifuging. Cellular cAMP content was measured by radioimmunoassay and normalized for protein content, which was determined by a bicinchoninic acid assay (Pierce).

Statistical Analysis

The results are expressed as SEM. For BMD measurements on the whole body and tibia, results were reported as the percentage change from baseline. Statistical anal-

ysis was performed using two-way analysis of variance. Where significant overall differences were detected by analysis of variance, Fisher's two-tailed unpaired *t*-test was used to compare differences between the treatments. *P* values of less than 0.05 were considered to be significant. The statistical analysis program used was Statview (SAS, Cary, NC).

Results

In Vivo Runx2 Overexpression in Osteoblasts Induces Bone Loss in Mice

In our previous publication describing adult Runx2-overexpressing mice, we reported significant changes not only in BMD and bone volume but also in the level of expression of various genes of which the expression is related to osteoblastic differentiation or activity and to osteoclastogenesis. This histomorphometric and molecular analysis reported previously⁹ has been performed on long bones from 4-month-old adults of the first generation (F1).

In the present study, we used 1-month-old TG female mice from the ninth generation. At the beginning of the experiment, TG mice displayed significantly lower BMD than WT mice for the whole body ($35.0 \pm 0.1 \mu\text{g}/\text{mm}^2$ for WT mice and $32.5 \pm 0.1 \mu\text{g}/\text{mm}^2$ for TG mice, $P = 0.014$) and for the tibia ($39.5 \pm 0.1 \mu\text{g}/\text{mm}^2$ for WT mice and $33.0 \pm 0.1 \mu\text{g}/\text{mm}^2$ for TG mice, $P = 0.0003$). During the 6-week treatment period, the gain in bone mass observed for the whole body in TG mice treated with the vehicle was significantly lower than that found in WT mice (Figure 1A) ($44.9 \pm 0.6 \mu\text{g}/\text{mm}^2$ for WT mice and $37.6 \pm 0.9 \mu\text{g}/\text{mm}^2$ for TG mice, $P < 0.0001$). At the tibia, the gain in bone mass was the same in WT and TG mice, whereas the BMD was significantly lower in TG than in WT mice ($50.7 \pm 0.9 \mu\text{g}/\text{mm}^2$ for WT mice and $40.5 \pm 1.5 \mu\text{g}/\text{mm}^2$ for TG mice, $P < 0.0001$).

Histomorphometric analysis of femur showed that in the vehicle-treated mice, BV/TV (Figure 2A) was significantly lower in the TG mice than in the WT mice ($P < 0.0001$), attributable to a significant increase in trabecular separation (Figure 2B). Cortical bone (Figure 2D) was also affected by the transgene. Indeed, the cortex was significantly thinner and the cancellous diameter greater in TG than in WT animals (-20% , $P < 0.0001$ for the cortical thickness and -12.6% , $P < 0.0008$ for the cancellous diameter). Furthermore, osteoclast number (OcN/BV, Figure 2D) and BFR (Figure 3C) were unaffected by the transgene expression. These data indicated that trabecular bone remodeling was the same in both genotypes.

Daily Administration of hPTH(1-34) Increased Bone Density and Bone Mass in WT Mice but Not in Mice Overexpressing Runx2

To assess the involvement of Runx2 overexpression in the anabolic response to PTH, we treated 1-month-old

Figure 1. Overexpression of Runx2 in osteoblasts abolished the increase in BMD induced by PTH at the whole body level (A) and in the tibia (B). BMD was measured by dual-energy X-ray absorptiometry before and at the end of the 6-week treatment. WT and TG mice were intermittently treated for 6 weeks with vehicle (VEH, black bars) or 100 $\mu\text{g}/\text{kg}$ body weight/day PTH (PTH, white bars). Data are presented as the percentage of change from baseline, and values are means $\pm$ SEM ($n = 9$ to 22 per group). * $P < 0.01$ versus vehicle, § $P < 0.01$ versus WT.

female mice for 6 weeks with daily injections of rhPTH1-34 (100 $\mu\text{g}/\text{kg}$) or of the vehicle. At this dose, the serum calcium was not modified in WT and in TG mice (data not shown). As expected, daily intermittent treatment with PTH caused a significantly greater increase in BMD in WT mice than in the vehicle-treated mice at all of the skeletal sites measured [whole body, $P < 0.01$; femur, $P < 0.01$ (data not shown); tibia, $P < 0.01$] (Figure 1). Interestingly, PTH failed to induce any greater increase in BMD in TG mice than the vehicle.

Histological analysis of femur also revealed marked differences between WT and TG PTH-treated mice. Although PTH induced a significant increase in BV/TV in both WT mice ($P < 0.0001$) and TG mice ($P = 0.0025$)

Figure 2. Intermittent administration of PTH affected the trabecular and cortical compartments of femora differently in WT and in TG mice. WT and TG mice were intermittently treated for 6 weeks with the vehicle (VEH, black bars) or with 100 μg/kg body weight/day PTH (PTH, white bars). Histomorphometric analysis was performed at the end of the 6-week treatment. We determined the trabecular bone volume (BV/TV) (A), trabecular separation (Tb.Sp) (B), trabecular thickness (Tb.Th) (C), osteoclast number per bone volume (OcN/BV) (D), and cortical thickness (Cort.Th) (E). Values are expressed as means ± SEM. * $P < 0.01$ versus vehicle, ** $P < 0.001$ versus vehicle, *** $P < 0.0001$ versus vehicle, § $P < 0.05$ versus WT VEH, §§ $P < 0.02$ versus WT VEH, §§§ $P < 0.0001$ versus WT VEH, and * $P < 0.0001$ versus WT PTH-treated mice.

(Figure 2A), its effect was stronger in WT than in TG mice ($P < 0.0001$). A genotype-related difference in the response to PTH was also observed at the trabecular thickness that was significantly increased in WT mice treated with PTH (+75%, $P < 0.0001$) but not in TG mice (Figure 2C). In contrast, a significant decline in trabecular separation was observed in both WT and TG mice treated with PTH ($P = 0.0005$ and $P = 0.03$, respectively) (Figure 2B). Osteoclast surfaces were also decreased by PTH ($P < 0.0001$ for both group), suggesting that bone resorption was affected equally by PTH in mice of both genotypes (Figure 2D). The response of cortical bone to PTH was different in WT and TG mice. PTH increased cortical thickness in WT mice by more than 50% ($P < 0.0001$), but had no effect on cortical bone thickness in PTH-treated, TG mice (Figure 2E).

The microarchitectural parameters suggest that only bone formation was affected differently by PTH in WT and TG mice. Dynamic formation analysis confirmed this observation and also revealed that the matrix apposition rate, mineralizing surfaces (MS/BS), and consequently BFR were significantly increased ($P < 0.0001$) in WT mice receiving PTH compared with those receiving

Figure 3. PTH stimulates dynamic parameters of bone formation measured at the trabecular bone of femur from WT but not TG mice. PTH (white bars) or vehicle (VEH, black bars) was administered for 6 weeks. Bones were labeled with tetracycline and calcein 3 days and 1 day, respectively, before sacrifice. Matrix apposition rate (MAR) (A), mineralizing surfaces (MS/BS) (B), and BFR (C) are presented as means ± SEM. *** $P < 0.0001$ versus vehicle, and § $P < 0.05$ versus WT VEH.

the vehicle (Figure 3). These dynamic formation parameters remained unchanged in TG mice after PTH administration.

Runx2 Overexpression Affected the Expression of Osteoblastic Genes and Their Response to Anabolic Treatment with hPTH(1-34)

We investigated the effect of PTH treatment on gene expression using total RNA extracted from the total metaphysis of tibia and 24 hours after the last PTH or vehicle injection. Endogenous Runx2 and type-1 collagen genes were expressed at the same level in WT and TG mice treated with vehicle (Figure 4, B–D). However, the osteocalcin mRNA level was significantly lower (–65%) (Figure 4C) in TG than WT mice, as previously reported in adult mice.⁹ The OPG mRNA level was significantly lower in TG than in WT mice treated with the vehicle, and RANKL expression was not significantly affected by genotype (data not shown). In consequence, the OPG/RANKL ratio was significantly lower (–50%) in TG than in WT mice (Figure 4E). We also examined the level of expression of the PTH receptor 1 (PTH-R1) that mediates the anabolic action of PTH in osteoblasts. PTH-R1 expression was lower in TG bone than in WT bone (–42.7%) (Figure 4F).

PTH administration significantly increased transgene expression by 85% ($P < 0.01$, Figure 4A). We also showed that the expression of a number of genes was increased by PTH treatment, although not significantly, in both WT and TG mice. The mRNA level of endogenous Runx2 was increased by PTH equally in WT and TG mice (Figure 4B), but the increase in the mRNA level of type-I collagen tended to be greater in WT mice (+141%) than in TG mice (+80%) (Figure 4D), and the mRNA level of

Figure 4. PTH modified the expression of osteoblastic differentiation marker genes at the mRNA level differently in WT mice and in mice overexpressing Runx2. The mRNA levels of transgene (A), endogenous Runx2 (B), osteocalcin (C), type-1 collagen (COLLA1) (D), OPG/RANKL ratio (E), and PTH-R1 (F) were evaluated by quantitative, real-time PCR. Total RNA was isolated from metaphysis of the tibia from WT ($n = 4$) and Runx2-overexpressing mice (TG, $n = 4$) given either vehicle (VEH, black bars) or PTH (white bars) for 6 weeks. The mRNA levels are expressed as a percentage of the WT control levels. mRNA levels are normalized to aldolase A expression in each sample. Values are presented as means $\pm$ SEM. * $P < 0.05$ versus vehicle, ** $P < 0.01$ versus vehicle, \$ $P < 0.05$ versus WT, and \$\$\$ $P < 0.0001$ versus WT.

PTH-R1 tended to be higher in TG (+150%) than in WT mice (+40%) (Figure 4F).

We also provide evidence that Runx2 overexpression affected several of the known genomic effects of PTH. As expected, the administration of PTH induced a significant increase in osteocalcin mRNA in WT mice (+87%) but not in TG mice (+27%) (Figure 4C). OPG and RANKL expression were both affected by the administration of PTH in TG mice (+90 and +60%, respectively; data not shown) but remained unchanged in WT mice. However, the OPG/RANKL ratio was higher in WT mice and was not affected by the treatment in either genotype (Figure 4E).

Runx2 Overexpression Arrests Osteoblast Differentiation

To evaluate the effect of Runx2 overexpression on the time course of osteoblastic differentiation, primary osteoblasts were isolated from neonatal calvaria and induced to differentiate *in vitro* for 14 days. The mRNA levels of several osteoblastic genes were evaluated after 4, 9, and 14 days in culture. The results are shown in Figure 5. We showed that on day 4 in culture, when the cells were still proliferating, only endogenous Runx2 and collagen

Figure 5. Runx2 overexpression affects osteoblast differentiation in primary osteoblastic culture. Primary osteoblastic cells were isolated from calvaria of WT and TG newborn mice and cultured in α -MEM containing 10% fetal calf serum in the presence of 50 μ M/L ascorbic acid and 10 mmol/L β -glycerolphosphate. Total RNA was extracted at day 4 (d4), day 9 (d9), and day 14 (d14) from WT (●) and TG mice osteoblastic cells (○), and gene expression was evaluated by quantitative PCR analysis. The expression of endogenous Runx2 (A), type-1 collagen (COLLA1) (B), osteocalcin (C), ALP (D), PTH-R1 (E), and the transgene (F) was determined. Expression levels were normalized versus 18S. The data are reported as mean $\pm$ SEM of triplicates from one of two independent determinations. * $P < 0.05$ versus d4, ^b $P < 0.01$ versus d4, ^c $P < 0.0001$ versus d4, ^d $P < 0.05$ versus WT, ^e $P < 0.01$ versus WT, and ^f $P < 0.0001$ versus WT.

type-1 mRNA levels were higher in TG osteoblasts than in WT osteoblasts. After 9 days in culture, when the cells were differentiating into osteoblasts, endogenous Runx2, ALP, and PTH-R1 mRNA levels were higher than on day 4 in WT cells but remained unchanged in the TG cells. As expected, after 14 days in culture WT osteoblasts were fully differentiated, and consequently their expression of endogenous Runx2 had decreased, and their expression of the other osteoblastic genes (type-1 collagen, osteocalcin, ALP, and PTH-R1) had significantly increased since day 4 and day 9. In contrast, the mRNA level of these genes was not affected in TG primary cells during this period. The expression of transgene rose from day 4 and continued to increase in culture until day 14. These *in vitro* data confirmed the findings of the *in vivo* molecular analysis and indicated that an arrest had occurred during the maturation process in the TG cells but had not occurred in WT cells, thus leading to less mature osteoblasts.

We confirmed the potential defect in the osteoblastic differentiation process in TG primary cells by determining the alkaline phosphatase activity in the cell lysate (Figure 6A) and the formation of mineralizing nodules (Figure 6B). Alkaline phosphatase activity increased more in WT osteoblasts than in TG osteoblasts at day 14. These data were consistent with the absence of mineralizing nodule

Figure 6. Runx2 overexpression affects osteoblast differentiation and proliferation *in vitro*. Primary osteoblasts isolated from WT (black bars) and TG (white bars) newborn mice were cultured in α -MEM containing 10% fetal calf serum in the presence of 50 μ mol/L ascorbic acid and 10 mmol/L β -glycerophosphate. **A:** ALP activity was determined after 4 and 14 days in culture. Data are expressed as mean $\pm$ SEM, and are representative of three independent experiments (six wells per group). *** P < 0.0001 versus day 4, and 5P < 0.01 versus WT at day 14. **B:** Representative pictures of the formation of alizarin red-stained matrix nodules at 14 days of culture. These pictures are representative of three independent experiments. **C:** Proliferation was evaluated by 5-bromo-2'-deoxyuridine incorporation during a 12-hour period. Data are presented as mean $\pm$ SEM of six replicates of one representative experiment. ** P < 0.001 versus WT.

formation (alizarin red staining) in TG primary cell cultures, whereas it did occur in WT cultures. In addition, we showed that at day 4 proliferation was significantly lower in TG osteoblasts than in WT osteoblasts (Figure 6C).

Runx2 Overexpression Blunts the Osteoblastic Response to hPTH(1-34)

The molecular analysis of primary osteoblastic cells, and on *in vivo* treated bone described above (Figures 4F and 5E), provided evidence of reduced PTH-R1 expression in TG osteoblasts but not in WT osteoblasts. To verify the possible correlation between the reduced expression of PTH-R1 in osteoblasts and the lack of response of the TG animals to PTH, we analyzed the ability of the PTH receptors present at the surface of the osteoblastic WT and TG cells to interact with its ligand. Interestingly, 125 I-PTH binding was significantly lower in TG cells than in WT cells (P < 0.0001) (Figure 7A). However, the affinity of the receptor for PTH was similar in both genotypes (k_d = 9.6 ± 0.7 nmol/L for WT and k_d = 10.8 ± 0.9 nmol/L for TG). In primary cultures of osteoblasts, rhPTH(1-34) treatment at low doses (5 and 10 nmol/L) induced marked stimulation of cAMP production in WT control cells (Figure 7B), whereas the production of cAMP was signifi-

Figure 7. Primary osteoblasts (POBs) overexpressing Runx2 are less sensitive to PTH than WT osteoblastic cells. **A:** Radioligand-binding experiments for PTH receptor were realized on WT (black bar) and TG (white bar) osteoblast cell membranes. Results are mean $\pm$ SEM of five replicates from one experiment. *** P < 0.0001 versus WT. **B:** cAMP production was stimulated by PTH in POBs. Confluent POB cells from WT (●) and TG (○) mice were exposed to hPTH-(1-34) at the indicated concentrations for 15 minutes after 20 minutes of pretreatment with 1 mmol/L 3-isobutyl-1-methylxanthine. Dots and bars represent mean $\pm$ SEM of triplicates from one of two independent experiments. * P < 0.01 versus vehicle, *** P < 0.0001 versus control, and 5P < 0.05 versus WT.

cantly lower in TG cells at both doses. These data confirmed that TG cells are much less sensitive to PTH than WT cells.

Discussion

The increase in bone formation after PTH administration *in vivo* has been attributed to increased production of osteoprogenitors and differentiation of osteoblasts and decreased apoptosis of existing osteoblasts. Several reports have clearly shown that Runx2 activity is required for the anabolic action of PTH *in vivo*. However, when we assessed BMD in TG mice that overexpress Runx2, PTH failed to induce any significant increase of BMD at any skeletal site. In our study we used a dose of 100 μ g/kg PTH, which is higher than the doses often used (40 to 80 μ g/kg) in *in vivo* studies on bone.^{15,16} On the other hand, other investigators¹¹ have shown that doses between 30 and 300 μ g/kg have the same positive effect on bone mass after 28 days of treatment. Thus, as expected, trabecular and cortical bone were affected by intermittent PTH treatment in WT mice as already described.^{15,16} However, in our model of *in vivo* Runx2 overexpression, both sites were either unaffected or less affected by the treatment. TG mice made no response at all to PTH at cortical sites, and PTH had milder effects at the trabecular compartment in TG mice than in WT mice. These site-specific differences might be explained by the fact that trabecular bone is more sensitive to anabolic PTH treatment than cortical bone. The structural changes that are usually observed in WT mice under PTH treatment are mainly the result of an increase in bone formation.^{15,16} Matrix apposition rate, MS/BS, and BFR were not increased by PTH in treated TG mice, whereas they were increased in their WT controls. These observations support the idea that PTH increased neither osteoblast differentiation nor matrix synthesis in TG osteoblasts.

The major question raised by these findings is how to explain the absence of response to PTH in TG mice.

Krishnan and colleagues¹⁰ have presented evidence that exposure to PTH for 6 or 24 hours causes a transient but significant increase in Runx2 expression both *in vitro* and *ex vivo*. These data do not corroborate our quantitative PCR findings. Under our experimental conditions, endogenous Runx2 expression was unaffected by the treatment. This apparent discrepancy can easily be explained by the fact that *in vivo* investigation does not make it possible to assess the early events induced by PTH. Another possible explanation is simply that *in vivo*, Runx2 is not strongly regulated by PTH at the transcriptional level¹⁷ and that Runx2 activity is mainly regulated by PTH at the posttranscriptional level.^{18,19}

In our study, we observed a trend toward an increase in PTH-R1 expression in response to PTH treatment in both genotypes. To date, contradictory data have been published and have indicated both *in vivo* and *in vitro* that the regulation of PTH-R1 expression by PTH itself is highly dependent on the context.^{20–24} Beside this, the most interesting thing to note is that our data support the idea that the smaller response of TG mice to PTH might be related to the low level of PTH-R1 expression in TG bone and to the decrease in the number of cells expressing PTH-R1. These results obtained *in vivo* have been reproduced *in vitro*, and they indicate that the expression of PTH-R1 is lower in TG primary osteoblasts than in WT cells at both the RNA and the protein levels. We concluded from all these findings that the lower PTH-R1 expression in TG osteoblasts and their lower sensitivity to PTH both arise from a block in osteoblast differentiation. This conclusion was supported by the decreased expression of genes generally used as early and late markers of osteoblast differentiation. We showed that the transgene was already being expressed as early as day 4 and that its expression continued to rise dramatically until day 14. The level of expression of the transgene was high enough before day 9 to induce arrest in osteoblast differentiation. Indeed, we observed that the expression and activity of alkaline phosphatase, an early marker of osteoblast differentiation, and that of osteocalcin and PTH-R1, two markers of mature osteoblasts, were all lower in TG than in WT osteoblastic cells from day 9. Moreover, mineralized nodule formation *in vitro* indicated that the protein matrix synthesized by TG osteoblasts was unable to mineralize, whereas that synthesized by WT cells did. The apparent discrepancy between the time course of the expression of the transgene and of the type-I collagen gene can be explained by the fact that regulatory *cis*-elements are missing in the shortened 2.3-kb collagen promoter used to drive the expression of the transgene.

We also showed *in vitro* that TG primary cells at day 4 in culture exhibited a higher level of endogenous Runx2 and had a lower proliferative capacity than WT cells. The level of Runx2 expression has been shown to be conversely correlated with proliferation and with cyclin expression in mouse osteoblasts.^{25,26} Pratap and colleagues^{25,26} also suggest that Runx2 supports osteoblast maturation by promoting an exit from the cell cycle and activating genes that facilitate bone cell phenotype development. Thus, the repression of proliferation and maturation of osteoblast in TG mice indicates that TG cells

with a high level of Runx2 are somehow blocked at an early stage of differentiation that is not compatible with a normal response to hormonal stimulation.

These *in vitro* molecular data are consistent with our previous findings obtained with bone marrow primary cells isolated from TG mice of the first generation.⁹ Transgene expression induces the same decrease in the level of expression of early and late markers of osteoblastic differentiation, eg, ALP and osteocalcin, indicating that primary osteoblastic cells originating from calvaria are suitable for analyzing the molecular events induced by runx2 overexpression in osteoblastic cells. The measurement of the dynamic formation parameters evaluated at the femur clearly indicated that mice of both genotypes have identical mineralizing surfaces (MS/BS), which reflects the number of active bone-forming cells, and BFR. These data are confirmed by the molecular analysis indicating that type-I collagen expression, the most abundant protein in bone matrix, is not different in WT and TG mice. The decrease in osteocalcin expression in TG bone probably simply reflects the decreased number of fully differentiated osteoblast that are no longer implicated in bone matrix production.

Experiments at the cellular level would be necessary to investigate further the molecular mechanisms underlying the *in vivo* anabolic effects of PTH. However, unlike some other groups, we have chosen not to do this because osteoblasts exposed to PTH *in vitro* obviously do not reproduce the anabolic effects of PTH observed *in vivo*.^{27–29} One mechanism by which PTH has been reported to increase bone formation involves an extension of osteoblast lifespan.^{11,30} We tried to assess osteoblastic apoptosis on demineralized bone slices by terminal dUTP nick-end labeling assay. However, because of the small number of apoptotic cells, we could not detect any effect on osteoblast lifespan in our PTH-treated WT mice (data not shown).

Besides stimulating bone formation, intermittent PTH administration is also known to affect bone resorption. In our study, structural changes such as the reduced trabecular separation and decrease in the number of osteoclasts caused by PTH indicated a decrease in bone resorption in both genotypes. Similar observations have been reported in mice^{31,32} and in rats.^{33,34} The urinary deoxypyridinoline, a biochemical marker of bone resorption, remained unchanged after PTH treatment in both genotypes (data not shown). We also showed that the significantly lower OPG/RANK-L ratio that was observed in TG versus WT mice receiving vehicle persisted after PTH treatment, indicating that the osteoclastogenic properties of WT and TG osteoblasts were equally affected by the treatment. Therefore, the important finding here is that Runx2 overexpression did not affect the action of PTH on bone resorption.

The choice of the promoter to drive the expression of Runx2 is of crucial importance. The key to the genotype differences observed in response to PTH probably lies in the choice of the promoter, which determines the timing and degree of overexpression of the transgene. The osteocalcin promoter and the MMP13 promoter were also used to drive Runx2 in mice.^{35,36} Overexpression of

Runx2 in mature osteoblasts was reported to increase bone mass by increasing osteoblastic activity. We cannot exclude the possibility that in these Runx2-overexpressing mice the response to PTH may have been different and even enhanced. The overexpression of Runx2 driven by the type I collagen promoter interferes with its normal activity affecting BFR and bone resorption in young mice and compromising bone acquisition.⁸ It is therefore possible that the phenotype at baseline might limit the ability of PTH to induce a full anabolic response. We also showed that PTH enhanced the expression of the transgene *in vivo*. This finding was consistent with a previous *in vivo* study reporting that intermittent PTH increases the expression of the luciferase reporter gene driven by the same fragment isolated from the collagen type-1 promoter.³¹ These findings also suggest that PTH treatment reinforces the inhibitory effect of high Runx2 expression on osteoblastic maturation.

We demonstrate in this study that overexpression of Runx2 in differentiating osteoblasts abolishes the anabolic effect of PTH *in vivo*. We also provide evidence that this abolition was attributable to a dramatic reduction in the sensitivity of osteoblastic cells toward PTH, related to decreased osteoblast maturation. These data highlight the importance of the differentiation stage of osteoblasts for the anabolic effect of PTH. Taking together these findings indicate the complexity of the molecular and cellular events that occur in bone cells when PTH increases bone mass.

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