

## Prostaglandin G/H synthase-2 is required for maximal formation of osteoclast-like cells in culture

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### Article

We examined the effect on osteoclast formation of disrupting the prostaglandin G/H synthase genes *PGHS-1* and *-2*. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production was significantly reduced in marrow cultures from mice lacking PGHS-2 (*PGHS-2*<sup>-/-</sup>) compared with wild-type (*PGHS-2*<sup>+/+</sup>) cultures. Osteoclast formation, whether stimulated by 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-D) or by parathyroid hormone (PTH), was reduced by 60–70% in *PGHS-2*<sup>-/-</sup> cultures relative to wild-type cultures, an effect that could be reversed by providing exogenous PGE<sub>2</sub>. Cultures from heterozygous mice showed an intermediate response. PGHS inhibitors caused a similar drop in osteoclast formation in wild-type cultures. Co-culture experiments showed that supporting osteoblasts, rather than osteoclast precursors, accounted for the blunted response to 1,25-D and PTH. This lack of response appeared to result from reduced expression of RANK ligand (RANKL) in osteoblasts. We cultured spleen cells with exogenous RANKL and found that osteoclast formation was 50% lower in *PGHS-2*<sup>-/-</sup> than in wild-type cultures, apparently because the former cells expressed high levels of GM-CSF. Injection of PTH above the calvaria caused hypercalcemia in wild-type but not *PGHS-2*<sup>-/-</sup> mice. Histological examination of bone from 5-week-old *PGHS-2*<sup>-/-</sup> mice revealed no abnormalities. Mice lacking PGHS-1 were similar to wild-type mice in all of these parameters. These data suggest that PGHS-2 is not necessary for wild-type bone development but plays a critical role in bone resorption stimulated by 1,25-D and PTH.

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# Prostaglandin G/H synthase-2 is required for maximal formation of osteoclast-like cells in culture

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We examined the effect on osteoclast formation of disrupting the prostaglandin G/H synthase genes *PGHS-1* and *2*. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production was significantly reduced in marrow cultures from mice lacking *PGHS-2* (*PGHS-2*<sup>-/-</sup>) compared with wild-type (*PGHS-2*<sup>+/+</sup>) cultures. Osteoclast formation, whether stimulated by 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-D) or by parathyroid hormone (PTH), was reduced by 60–70% in *PGHS-2*<sup>-/-</sup> cultures relative to wild-type cultures, an effect that could be reversed by providing exogenous PGE<sub>2</sub>. Cultures from heterozygous mice showed an intermediate response. *PGHS* inhibitors caused a similar drop in osteoclast formation in wild-type cultures. Co-culture experiments showed that supporting osteoblasts, rather than osteoclast precursors, accounted for the blunted response to 1,25-D and PTH. This lack of response appeared to result from reduced expression of RANK ligand (RANKL) in osteoblasts. We cultured spleen cells with exogenous RANKL and found that osteoclast formation was 50% lower in *PGHS-2*<sup>-/-</sup> than in wild-type cultures, apparently because the former cells expressed high levels of GM-CSF. Injection of PTH above the calvaria caused hypercalcemia in wild-type but not *PGHS-2*<sup>-/-</sup> mice. Histological examination of bone from 5-week-old *PGHS-2*<sup>-/-</sup> mice revealed no abnormalities. Mice lacking *PGHS-1* were similar to wild-type mice in all of these parameters. These data suggest that *PGHS-2* is not necessary for wild-type bone development but plays a critical role in bone resorption stimulated by 1,25-D and PTH.

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## Introduction

Prostaglandin G/H synthase (PGHS), also called cyclooxygenase (COX), is the rate-limiting enzyme in the conversion of arachidonic acid released from membranes to prostanoids (1). The 2 enzymes for PGHS are encoded by separate genes and are differentially expressed. *PGHS-1* (*COX-1*) is constitutively expressed; *PGHS-2* (*COX-2*) is an inducible primary-response or immediate early gene (2). *PGHS-1* and *PGHS-2* may also differentially regulate prostaglandin production by using different substrates (3–5). Under conditions of limiting substrate, *PGHS-2* appears to be the primary source of prostaglandin production (6). *PGHS-2* is the enzyme that is largely responsible for prostaglandin responses in osteoblasts stimulated by multiple agonists (7).

Mice in which either the *PGHS-1* or *PGHS-2* gene is disrupted have been engineered (8–10). *PGHS-1*-deficient (*PGHS-1*<sup>-/-</sup>) mice survive normally. *PGHS-2*-deficient (*PGHS-2*<sup>-/-</sup>) mice develop nephropathy that may limit life span. *PGHS-2*<sup>-/-</sup> female mice have multiple defects in reproductive processes, including ovulation (absence of corpora lutea), fertilization, implantation, and decidualization, that are not caused by a deficiency

of gonadotropins or ovarian hormones (11). The effect of a deficiency in *PGHS-1* or *PGHS-2* on bone has not yet been examined in these mice.

Bone resorption is a highly regulated process involving interactions of osteoclastic precursors with osteoblasts or stromal cells (12–14). Prostaglandins are potent stimulators of resorption in organ culture (15). Many factors that stimulate prostaglandin production also stimulate resorption in organ culture (16–20). Stimulated osteoclast formation in marrow cultures is frequently found to be prostaglandin dependent (18, 21–31). We have examined the effects on osteoclast formation of disruption of 1 (*PGHS-2*<sup>+/+</sup>) or both (*PGHS-2*<sup>-/-</sup>) *PGHS-2* alleles using different in vitro systems to separate effects on osteoclast support cells and hematopoietic osteoclast precursors. We have also examined effects of disruption of *PGHS-1* alleles (*PGHS-1*<sup>-/-</sup>).

## Methods

**Materials.** PGE<sub>2</sub> and indomethacin were purchased from Sigma (St. Louis, Missouri, USA). Parathyroid hormone (PTH; bovine 1-34) came from Bachem California (Torrance, California, USA) or Sigma. 1,25-dihydroxyvitamin D<sub>3</sub> (1,25-D) was purchased from

Biomol Research Laboratories (Plymouth Meeting, Pennsylvania, USA). NS-398 came from Cayman Chemical Co. (Ann Arbor, Michigan, USA). FCS and  $\alpha$ -MEM were obtained from GIBCO BRL (McLean, Virginia, USA). Recombinant murine macrophage-CSF (M-CSF), murine granulocyte-macrophage CSF (GM-CSF), and polyclonal GM-CSF antibody were obtained from R&D Systems Inc. (Minneapolis, Minnesota, USA). PGE<sub>2</sub> antibody for radioimmunoassay was purchased from Lawrence Levine (Brandeis University, Waltham, Massachusetts, USA). All other chemicals were obtained from Sigma. RANK ligand (RANKL) was kindly provided by Dirk Anderson (Immunex Corp., Seattle, Washington, USA).

**Animals.** *PGHS-1* and *PGHS-2* knockout mice were developed by homologous recombination in embryonic stem cells (8, 10). Because female *PGHS-1*<sup>-/-</sup> and *PGHS-2*<sup>-/-</sup> females have fertility and parturition problems, *PGHS-2*<sup>-/-</sup> mice are produced by crossing heterozygous (+/-) mice. Because *PGHS-2*<sup>-/-</sup> mice in a pure C57BL/6 background produce very few *PGHS-2*<sup>-/-</sup> offspring, we mated +/- offspring of crosses between C57BL/6<sup>+/-</sup> and C57BL/6 × 129/sv<sup>+/+</sup> to produce -/- mice for *PGHS-1* and *PGHS-2* studies. Because of genetic variability, we analyzed individual littermates from multiple litters and performed multiple experiments to confirm reproducibility. Mice were genotyped and ears were notched for identification after weaning. Mice were sacrificed at 5–8 weeks of age. All animal protocols were approved by the Animal Care and Use Committees of the University of Connecticut Health Center.

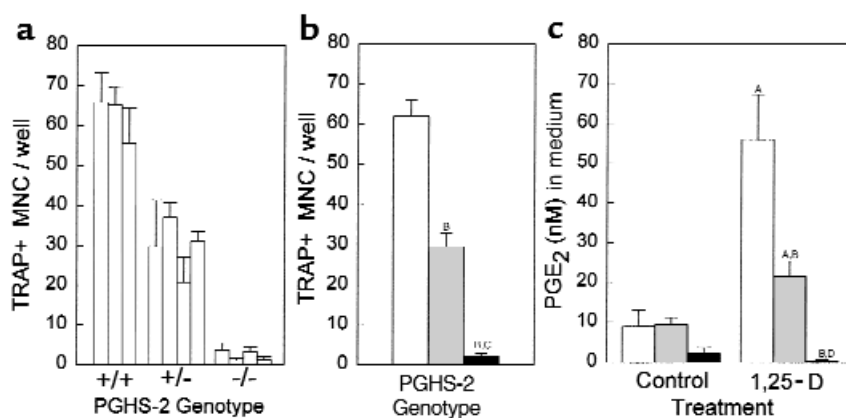
For the PTH injections, 6-week-old C57BL/6 × 129

mice were injected subcutaneously above the right hemicalvaria every 6 hours for 3 days with 0.025 mL of 10  $\mu$ g of PTH (Bachem California) or vehicle (1 mM HCl with 1 mg/mL BSA). Venous blood was obtained by cavernous sinus puncture. Serum creatinine concentrations were measured by spectrophotometer using a kit from Sigma. Total serum calcium and phosphate were measured in calorimetric assays using kits from Sigma.

**Genotyping of mice.** Tail DNA was extracted following a standard protocol, and was analyzed by PCR using primer sequences as described previously (8, 10). The conditions for PCR for *PGHS-2* were 1 cycle of 2 minutes at 92°C; and 30 cycles of 30 seconds at 94°C, 30 seconds at 65°C, and 5 minutes at 70°C. For *PGHS-1*, the PCR conditions were 1 cycle of 2 minutes at 92°C; 3 cycles of 1 minute at 94°C, 1 minute at 58°C, and 10 minutes at 65°C; and 30 cycles of 30 seconds at 94°C, 30 seconds at 60°C, and 5 minutes at 65°C. Products were electrophoresed on a 1% agarose gel in 1× trisborate/EDTA buffer at 100 V.

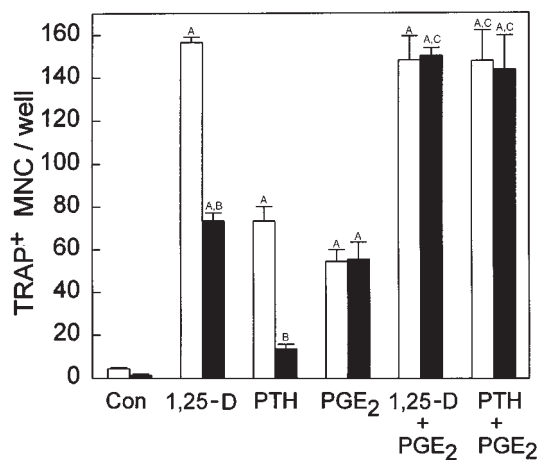
**Primary osteoblastic cells.** Calvariae were excised from 1–4 mice (5–7 weeks of age), dissected free of loose connective tissue, and washed with PBS at pH 7.4. Calvariae were digested with 0.5 mg/mL of crude collagenase P (Roche Molecular Biomedicals Inc., Indianapolis, Indiana, USA) in a solution of 1 mL trypsin/EDTA and 4 mL PBS for 10 minutes at 37°C with gentle rocking. The digestion procedure was repeated to provide 5 populations of cells (fraction 5 was digested for 20 minutes). After each digestion, released cells were removed, and the reaction was stopped with 10% FCS. Cells from populations 2–5 were pooled and then cultured to confluence in 100-mm dishes at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>, in phenol red-free DMEM with 10% heat-inactivated FCS, 100 U/mL penicillin, and 50  $\mu$ g/mL streptomycin. Cells were then resuspended and used in coculture experiments or replated in 6-well dishes at 5,000 cells/cm<sup>2</sup>.

**Bone marrow cell cultures.** Tibiae and femurs were aseptically dissected, the bone ends were cut off with scissors, and the marrow was flushed with  $\alpha$ -MEM. Collected marrow cells were washed with  $\alpha$ -MEM and then plated in  $\alpha$ -MEM containing 10% FCS at 10<sup>6</sup> cells/well in 24-well plates (Corning-Costar Corp., Cambridge, Massachusetts, USA). Cells were cultured for 7 days at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. On days 3 and 6, 0.4 mL of the 0.5 mL of medium in each well was replaced with fresh medium.



**Figure 1**

TRAP<sup>+</sup> MNC formation and PGE<sub>2</sub> production in 1,25-D-stimulated bone marrow cultures from *PGHS-2*<sup>+/+</sup>, *PGHS-2*<sup>+/-</sup>, and *PGHS-2*<sup>-/-</sup> mice. Cultures were treated for 8 days with vehicle (Control) or 1,25-D (10 nM). (a) Each bar represents the mean  $\pm$  SE of 3 replicate wells from 1 mouse cultured from 1 mouse and treated with 1,25-D. All mice were from 2 litters born within a day of each other. No TRAP<sup>+</sup> MNC were seen in control cultures. (b) The mean ( $\pm$  SE) number of TRAP<sup>+</sup> MNC for each genotype was calculated from the mean for individual mice: *PGHS-2*<sup>+/+</sup> (white bar), *PGHS-2*<sup>+/-</sup> (gray bar), and *PGHS-2*<sup>-/-</sup> (black bar). (c) Medium from 1 well per mouse was taken at the end of the culture period and assayed in duplicate for PGE<sub>2</sub>. Genotypes are as in b. <sup>A</sup>Significant difference from control group;  $P < 0.01$ . <sup>B</sup>Significant difference from *PGHS-2*<sup>+/+</sup> genotype;  $P < 0.01$ . <sup>C</sup>Significant difference from *PGHS-2*<sup>+/-</sup> phenotype;  $P < 0.01$ . <sup>D</sup>Significant difference from +/- phenotype;  $P < 0.05$ .



**Figure 2**

Effects of exogenous PGE<sub>2</sub> on TRAP<sup>+</sup> MNC formation in bone marrow cultures treated with 1,25-D and PTH. Marrow was pooled from several *PGHS-2*<sup>+/+</sup> mice (white bars) or *PGHS-2*<sup>-/-</sup> mice (black bars) and was cultured for 7 days with vehicle (Con), 1,25-D (10 nM), or PTH (10 nM), with and without PGE<sub>2</sub> (1 μM). Bars represent mean ± SE for TRAP<sup>+</sup> MNC formation in 6 wells. <sup>A</sup>Significant difference from control group; *P* < 0.01. <sup>B</sup>Significant difference from comparably treated +/+ genotype; *P* < 0.01. <sup>C</sup>Significant effect of addition of PGE<sub>2</sub>; *P* < 0.01.

Cultures were treated with 1,25-D (10 nM), PTH (10 nM), or PGE<sub>2</sub> (1 μM), added at the beginning of the culture and at each medium change. Osteoclast-like cell (OCL) formation was measured by TRAP<sup>+</sup> multinucleated cell formation. At the end of the culture period, cells were washed with PBS and fixed with 2.5% glutaraldehyde for 30 minutes. Cells were stained for TRAP using a leukocyte acid phosphatase A kit (Sigma). The number of TRAP<sup>+</sup> MNC per well was counted under a microscope.

**Cocultures.** Spleen cells were prepared by macerating spleen tissues with a needle. The spleen cells (10<sup>6</sup> cells/well) were cocultured with primary calvarial osteoblasts (10<sup>4</sup> cells/well) in 0.5 mL α-MEM supplemented with 10% FCS in 24-well plates. Cultures were treated with 1,25-D, PTH, or PGE<sub>2</sub>, and were maintained for 7 days following the same protocol described for bone marrow cultures, except that the media was completely changed at day 3. At the end of the culture period, the cells were stained for TRAP, and TRAP<sup>+</sup> MNC per well were counted.

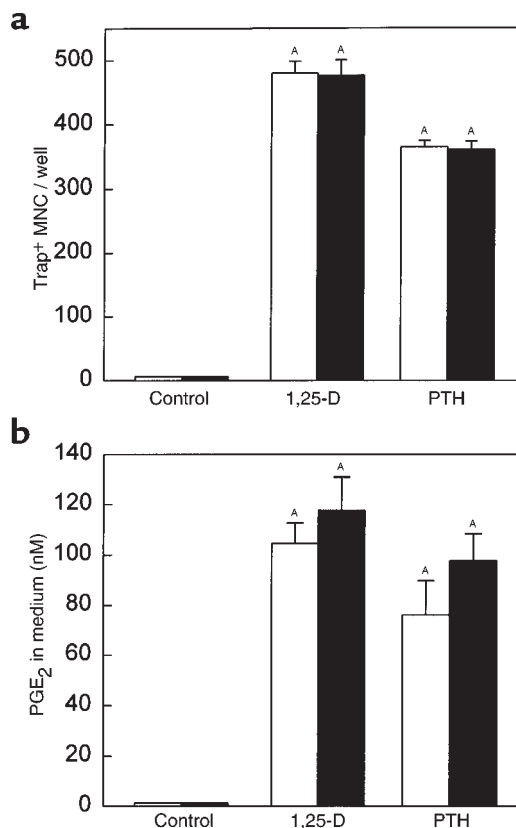
**Spleen cell cultures.** Spleen cells were prepared and cultured as described above for 6 days in the presence of RANKL (10 ng/mL) and M-CSF (10 ng/mL).

**Pit formation assay.** Marrow cells, cultured as described above for 7 days with 1,25-D or PGE<sub>2</sub>, were resuspended and allowed to settle onto the surface of devitalized bovine cortical bone slices (4.4 × 4.4 × 0.2 mm) for 90 minutes in PBS. Bone slices were rinsed vigorously and incubated for 24 hours at 37°C in α-MEM (with 0.7 g/L of sodium bicarbonate) and 10% FCS. After incubation, samples were fixed with 2.5% glutaraldehyde in PBS for 30 minutes, cells were stained for TRAP, and

bone slices were stained with 1% toluidine blue in 1% borax. The number of resorption pits per bone slice was counted using reflective light microscopy.

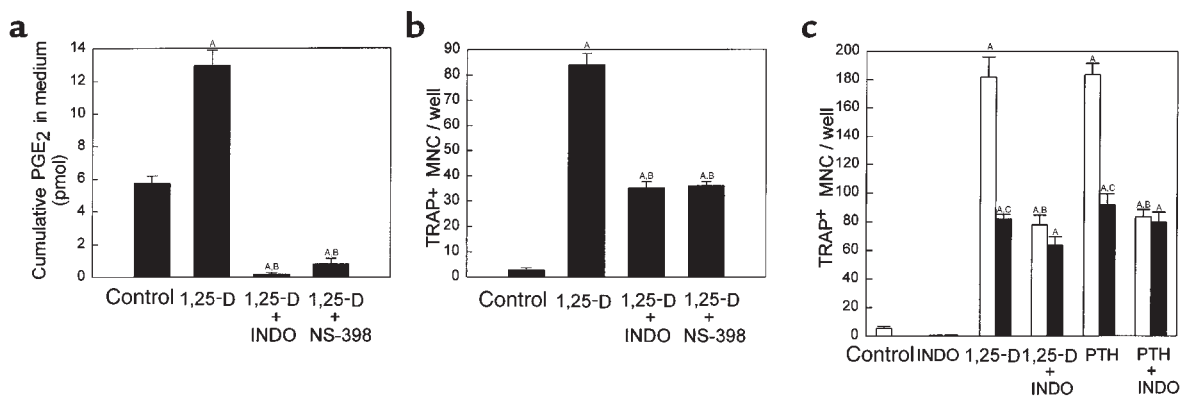
**RT-PCR.** Total RNA was extracted according to the method of Chomczynski and Sacchi as described previously (32). Total RNA was converted to cDNA by reverse transcriptase (Superscript II; GIBCO BRL) and random hexamer. PCR amplification was done using *Taq* polymerase (AmpliTaq; Perkin-Elmer Corp., Norwalk, Connecticut, USA) in a thermal cycler (Perkin-Elmer Corp.). After a hot start, temperature cycling was as follows: denaturation at 94°C for 1 minute, primer annealing at 65°C for 1 minute, and extension at 72°C for 2 minutes for 10 cycles. In subsequent cycles, the primer annealing temperature was decreased stepwise by 5°C every 5 cycles. After the last cycle, the mixture was incubated at 72°C for 7 minutes. To verify that amplification was in the linear range, we performed PCR amplification for 27–35 cycles.

PCR primers for murine GAPDH and GM-CSF were purchased from CLONTECH Laboratories Inc. (Palo Alto, California, USA). Primers for murine



**Figure 3**

Effect of *PGHS-1* gene disruption on TRAP<sup>+</sup> MNC formation and PGE<sub>2</sub> production in marrow culture. Marrow from *PGHS-1* knockout mice (black bars) or from wild-type littermates (white bars) was cultured with vehicle (Control) or 1,25-D (10 nM) for 7 days. (a) Bars represent mean ± SE for TRAP<sup>+</sup> MNC in 4 wells. (b) Bars represent mean ± SE for medium PGE<sub>2</sub> produced during the last 2 days of culture in 4 wells. <sup>A</sup>Significant difference from control group; *P* < 0.01.



**Figure 4**

Effect of inhibitors of PGHS-1 and PGHS-2 activity on TRAP<sup>+</sup> MNC formation in bone marrow cultures. Marrow was cultured for 7 days with vehicle (Con), 1,25-D (10 nM), or PTH (10 nM) in the presence or absence of either 0.1  $\mu$ M indomethacin (INDO; an inhibitor of both PGHS-1 and PGHS-2 activity) or 0.1  $\mu$ M NS-398, a selective inhibitor of PGHS-2 activity. Bars represent mean  $\pm$  SE of 4 wells. Comparison of 1,25-D-stimulated cumulative PGE<sub>2</sub> (a) and TRAP<sup>+</sup> MNC formation (b) in *PGHS-2<sup>+/+</sup>* cultures. (c) Comparison of 1,25-D- and PTH-stimulated TRAP<sup>+</sup> MNC formation, with and without indomethacin, in *PGHS-2<sup>+/+</sup>* cultures (white bars) and *PGHS-2<sup>-/-</sup>* cultures (black bars). <sup>A</sup>Significant difference from control group;  $P < 0.01$ . <sup>B</sup>Significant effect of inhibitor;  $P < 0.01$ . <sup>C</sup>Significant difference from  $+/+$  genotype;  $P < 0.01$ .

RANKL (5'-GGGAATTACAAAGTGCACCAG-3' and 5'-GGTCGGGCAATTCTGAATT-3) were designed from published DNA sequences (33). Amplified products were run on a 1% agarose gel, stained with ethidium bromide, and photographed under UV illumination. Images were analyzed with either ScanAnalysis 2.56 (Biosoft, Cambridge, United Kingdom) or NIH Image 1.61.

**PGE<sub>2</sub> assay.** Medium was removed from cultured cells and PGE<sub>2</sub> levels were measured by radioimmunoassay as described previously (34).

**Statistical analysis.** Significant differences among groups within each experiment were determined by ANOVA, followed by post-hoc testing using Bonferroni's method. When data from multiple experiments were pooled, statistical differences between wild-type and knockout genotypes were determined by Student's *t* test.

## Results

**Phenotype of *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* mice.** Mice used for in vitro assays were 5–7 weeks of age and appeared healthy and active. There were no significant differences in body weight or serum creatinine between groups (Table 1). Preliminary data on the histology and histomorphometry of 3 *PGHS-2<sup>+/+</sup>* and 3 *PGHS-2<sup>-/-</sup>* mice at 5

weeks of age showed no differences in the proximal tibia (data not shown).

**Stimulated OCL formation and PGE<sub>2</sub> production in marrow cultures.** In our initial experiments, we cultured marrow individually from all mice in 2 litters born within a day of each other (Figure 1a). Marrow for each mouse was cultured in 3 replicate wells, and the average number of TRAP<sup>+</sup> MNC per well for each mouse was then used to calculate the mean for each treatment group (Figure 1b). There was a 50% reduction in 1,25-D-stimulated (10 nM) TRAP<sup>+</sup> MNC formation in *PGHS-2<sup>-/-</sup>* cultures, and a 96% reduction in *PGHS-2<sup>-/-</sup>* cultures compared with *PGHS-2<sup>+/+</sup>* cultures. Treatment with 1,25-D increased PGE<sub>2</sub> in the medium 6-fold in *PGHS-2<sup>+/+</sup>* cultures (Figure 1c). The 1,25-D-stimulated increase in PGE<sub>2</sub> was 62% lower in *PGHS-2<sup>+/+</sup>* cultures and 99% lower in *PGHS-2<sup>-/-</sup>* cultures than in the *PGHS-2<sup>+/+</sup>* cultures. In subsequent experiments, marrow cultures pooled from several mice of the same genotype were compared.

Induction of TRAP<sup>+</sup> MNC formation by PTH (10 nM) was similarly reduced in *PGHS-2<sup>-/-</sup>* marrow cultures compared with *PGHS-2<sup>+/+</sup>* cultures (Figure 2). In this same experiment, 1,25-D and PTH stimulated 3-fold and 2.5-fold increases, respectively, in PGE<sub>2</sub> levels in the medium of *PGHS-2<sup>+/+</sup>* cultures ( $P < 0.01$ ), but did not increase levels in *PGHS-2<sup>-/-</sup>* cultures (data not shown).

In 6 separate experiments, the mean reduction ( $\pm$  SE) in 1,25-D-stimulated TRAP<sup>+</sup> MNC formation per well in *PGHS-2<sup>-/-</sup>* cultures relative to *PGHS-2<sup>+/+</sup>* cultures was  $72 \pm 9\%$  ( $P < 0.01$ ). In 3 separate experiments, the mean reduction in 1,25-D-stimulated TRAP<sup>+</sup> MNC in *PGHS-2<sup>-/-</sup>* cultures relative to *PGHS-2<sup>+/+</sup>* cultures was  $51 \pm 1\%$  ( $P < 0.01$ ). In 5 separate experiments, the mean reduction in PTH-stimulated TRAP<sup>+</sup> MNC formation in *PGHS-2<sup>-/-</sup>* cultures relative to *PGHS-2<sup>+/+</sup>* cultures was  $64 \pm 5\%$  ( $P < 0.01$ ).

Treatment with PGE<sub>2</sub> (1  $\mu$ M) stimulated TRAP<sup>+</sup> MNC formation in *PGHS-2<sup>+/+</sup>* marrow cultures; this stimulation was not reduced in *PGHS-2<sup>-/-</sup>* cultures (Fig-

**Table 1**

Comparison of body weights and serum creatinine levels in *PGHS-2<sup>+/+</sup>*, *PGHS-2<sup>-/-</sup>*, and *PGHS-2<sup>-/-</sup>* mice

| Genotype                    | Body weight    | Serum creatinine |
|-----------------------------|----------------|------------------|
|                             | (g)            | (mg/dL)          |
| <i>PGHS-2<sup>+/+</sup></i> | 16.7 $\pm$ 1.7 | 0.93 $\pm$ 0.02  |
| <i>PGHS-2<sup>-/-</sup></i> | 16.6 $\pm$ 0.9 | 0.93 $\pm$ 0.01  |
| <i>PGHS-2<sup>-/-</sup></i> | 15.1 $\pm$ 1.9 | 0.96 $\pm$ 0.04  |

Data are mean  $\pm$  SE for 5-week-old C57BL/6  $\times$  129 mice. Each group includes 2 males and 2 females from 2 litters.

**Table 2**Effects of local PTH injection above the calvaria of *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* mice

| Measurement              | Vehicle injected            |                             | PTH injected                |                             |
|--------------------------|-----------------------------|-----------------------------|-----------------------------|-----------------------------|
|                          | <i>PGHS-2<sup>+/+</sup></i> | <i>PGHS-2<sup>-/-</sup></i> | <i>PGHS-2<sup>+/+</sup></i> | <i>PGHS-2<sup>-/-</sup></i> |
| Body weight (g)          | 16.9 ± 0.1                  | 18.2 ± 1.8                  | 15.8 ± 0.5                  | 15.3 ± 0.9                  |
| Serum creatinine (mg/dL) | 0.98 ± 0.09                 | 0.96 ± 0.16                 | 0.88 ± 0.09                 | 1.05 ± 0.13                 |
| Serum calcium (mg/dL)    | 8.7 ± 0.1                   | 9.1 ± 0.2                   | 12.0 ± 0.3 <sup>A</sup>     | 9.0 ± 0.5                   |
| Serum phosphate (mg/dL)  | 7.2 ± 0.5                   | 7.4 ± 0.5                   | 6.6 ± 0.6                   | 8.3 ± 0.5                   |

Data are mean ± SE for 6-week-old mice ( $n = 3$ ). There were 2 males and 1 female in the vehicle-treated groups, and 1 male and 2 females in the PTH-treated groups. <sup>A</sup>Significantly different from vehicle-treated groups and from PTH-treated *PGHS-2<sup>-/-</sup>* mice;  $P < 0.01$ .

ure 2). There was no reduction in PGE<sub>2</sub>-stimulated TRAP<sup>+</sup> MNC formation in *PGHS-2<sup>+/+</sup>* or *PGHS-2<sup>-/-</sup>* cultures in 4 additional experiments. PGE<sub>2</sub> (1 μM) added to 1,25-D-stimulated cultures reversed the reduction in TRAP<sup>+</sup> MNC formation in *PGHS-2<sup>-/-</sup>* cultures (Figure 2). Addition of PGE<sub>2</sub> to PTH-stimulated *PGHS-2<sup>-/-</sup>* cultures enhanced TRAP<sup>+</sup> MNC formation in *PGHS-2<sup>+/+</sup>* cultures and eliminated differences between the wild-type and *PGH-2*-deficient cultures.

**Effect of *PGHS-1* deficiency on stimulated OCL formation in marrow cultures.** There was no reduction in 1,25-D- or PTH-stimulated TRAP<sup>+</sup> MNC formation in marrow cultures from *PGHS-1<sup>-/-</sup>* mice compared with *PGHS-1<sup>+/+</sup>* cultures (Figure 3a). In addition, there was no reduction in 1,25-D- or PTH-stimulated PGE<sub>2</sub> production in *PGHS-1<sup>-/-</sup>* cultures (Figure 3b).

**Effects of nonsteroidal anti-inflammatory drugs (NSAIDs) on stimulated OCL formation in marrow cultures.** To assess further the relative roles of *PGHS-2* and *PGHS-1* in stimulated OCL formation in marrow cultures, we compared *PGHS-2<sup>+/+</sup>* marrow cultures treated with 0.1 μM

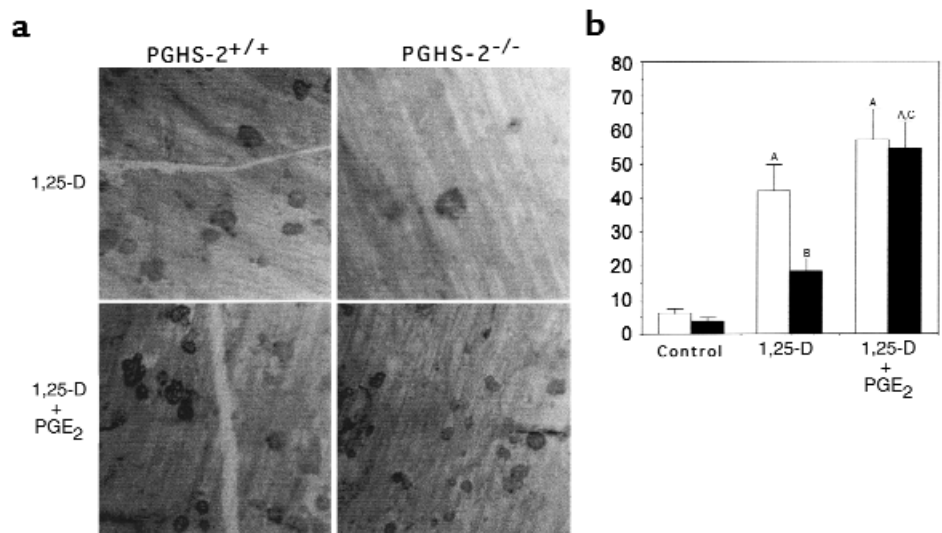
indomethacin, which inhibits both *PGHS-1* and *PGHS-2* activity, and cultures treated with NS-398 (0.1 μM), a selective inhibitor of *PGHS-2* in osteoblastic cells (35). Both NSAIDs inhibited PGE<sub>2</sub> production in 1,25-D-stimulated marrow cultures (Figure 4a) and decreased TRAP<sup>+</sup> MNC formation by 60–65% (Figure 4b). We also examined the effects of indomethacin on 1,25-D- or PTH-stimulated *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* marrow cultures to determine if the residual stimulated OCL formation in *PGHS-2<sup>-/-</sup>* cultures could be reduced by inhibiting *PGHS-1* activity (Figure 4c). Treatment with indomethacin caused no additional reduction in OCL formation in *PGHS-2<sup>-/-</sup>* cultures (Figure 4c).

**Effects of *PGHS-2* deficiency on pit formation.** We assessed the ability of OCL formed in marrow cultures to form resorption pits on cortical bone slices (Figure 5). 1,25-D-stimulated an 8-fold increase in the number of pits formed by marrow cells from *PGHS-2<sup>+/+</sup>* mice. 1,25-D-stimulated pit formation was reduced by 60% in marrow cultured from *PGHS-2<sup>-/-</sup>* mice; this reduction was reversed by treatment with PGE<sub>2</sub>. There was no difference in pit area among groups (data not shown).

**Stimulated OCL formation in cocultures of spleen and osteoblastic cells.** We cocultured *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* spleen cells (as a source of osteoclastic precursors) with primary osteoblasts derived from calvariae of *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* mice. 1,25-D- and PTH-stimulated (Figures 6a and 6b, respectively) TRAP<sup>+</sup> MNC formation was reduced 65% and 80%, respectively, in cocultures of *PGHS-2<sup>-/-</sup>* osteoblasts with either *PGHS-2<sup>+/+</sup>* or *PGHS-2<sup>-/-</sup>* spleen cells. Stimulated OCL formation was not inhibited when *PGHS-2<sup>-/-</sup>* spleen cells were cultured with *PGHS-2<sup>+/+</sup>* osteoblasts. Addition of PGE<sub>2</sub> reversed the inhibition of TRAP<sup>+</sup> MNC formation in cocultures with *PGHS-2<sup>-/-</sup>* osteoblasts. The effect of PGE<sub>2</sub> on TRAP<sup>+</sup> MNC formation was additive to the effect of PTH in cocultures with *PGHS-2<sup>+/+</sup>* osteoblasts. Similar results were seen in 2 more experiments with PTH, and in a second experiment with 1,25-D.

**Figure 5**

Formation of resorption pits on cortical bone slices by cultured marrow cells from *PGHS-2<sup>+/+</sup>* mice (white bars) and *PGHS-2<sup>-/-</sup>* mice (black bars). Cultures were treated with either vehicle (Control) or 1,25-D (10 nM) with and without PGE<sub>2</sub> (1 μM). An osteoclast resorption pit was defined as having multiple overlapping resorption lacunae. (a) Photomicrograph of resorption pits on cortical bone. (b) Number of resorption pits counted on 6 bone slices (mean ± SE). <sup>A</sup>Significant difference from control group;  $P < 0.01$ . <sup>B</sup>Significant difference from 1,25-D-treated *PGHS-2<sup>+/+</sup>* cells;  $P < 0.05$ . <sup>C</sup>Significant effect of addition of PGE<sub>2</sub>;  $P < 0.01$ .



**Injection of PTH over the calvaria.** To determine if there were in vivo consequences of the differences in OCL formation seen in vitro, we examined the ability of PTH injected subcutaneously above the calvaria to raise serum calcium levels in *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* mice. Following an established model (36, 37), 6-week-old mice (3 in each group) were injected 4 times a day for 3 days above the right hemicalvaria with 10  $\mu$ g of PTH or vehicle. Serum was obtained 2 hours after the last injection. There was no significant difference in body weight, serum creatinine, or serum phosphate among the groups (Table 2). There was no difference between serum calcium levels in *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* mice injected with vehicle. PTH injection produced marked hypercalcemia in the *PGHS-2<sup>+/+</sup>* mice but did not elevate

serum calcium in the *PGHS-2<sup>-/-</sup>* mice (Table 2).

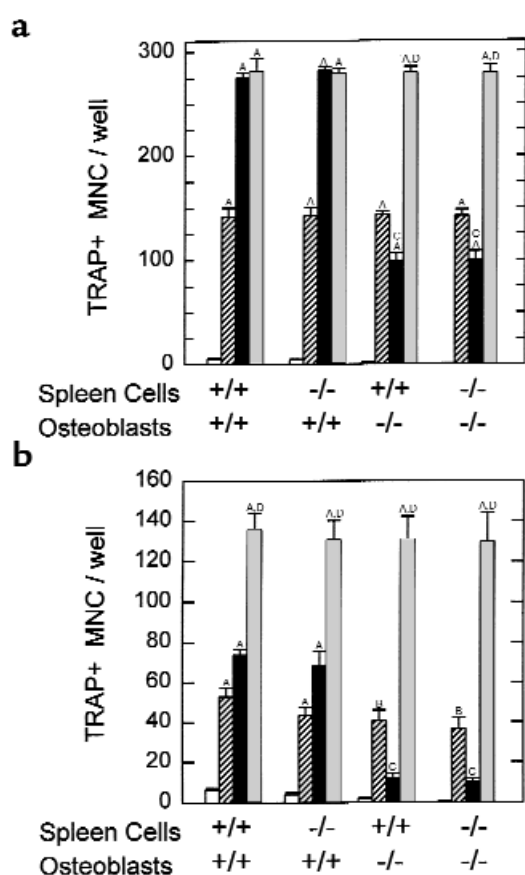
**Regulation of RANKL mRNA expression in osteoblasts.** Because stimulation of RANKL expression in osteoblasts has been shown to be essential for resorption induced by 1,25-D, PTH, and PGE<sub>2</sub> (38–41), we examined the stimulation of RANKL expression in *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* cultures. We measured RANKL mRNA levels in 3 separate experiments by RT-PCR in primary calvarial osteoblast cultures grown to confluence and treated for 24 hours with 1,25-D and PTH. 1,25-D-stimulated RANKL mRNA levels were reduced 47  $\pm$  2% ( $P < 0.01$ ) in *PGHS-2<sup>-/-</sup>* cultures compared with *PGHS-2<sup>+/+</sup>* cultures (data not shown). PTH-stimulated RANKL mRNA levels were reduced 56  $\pm$  8% ( $P < 0.01$ ) in *PGHS-2<sup>-/-</sup>* cultures compared with *PGHS-2<sup>+/+</sup>* cultures (data not shown).

**OCL formation in RANKL and M-CSF-treated spleen cell cultures.** To examine the effects of PGHS-2 deficiency on differentiation of osteoclast precursors without using osteoblastic cells, we used spleen cell cultures treated with RANKL (10 ng/mL) and M-CSF (10 ng/mL) (42, 43). We were unable to detect mRNA for RANKL by RT-PCR in these spleen cell cultures, indicating that few stromal or osteoblastic cells were present (data not shown). There were 2- to 3-fold more TRAP<sup>+</sup> MNCs in *PGHS-2<sup>+/+</sup>* cultures than in *PGHS-2<sup>-/-</sup>* cultures (Figures 7 and 9). In contrast, we found no difference in OCL formation in spleen cell cultures from *PGHS-1<sup>-/-</sup>* mice compared with *PGHS-1<sup>+/+</sup>* cultures (data not shown).

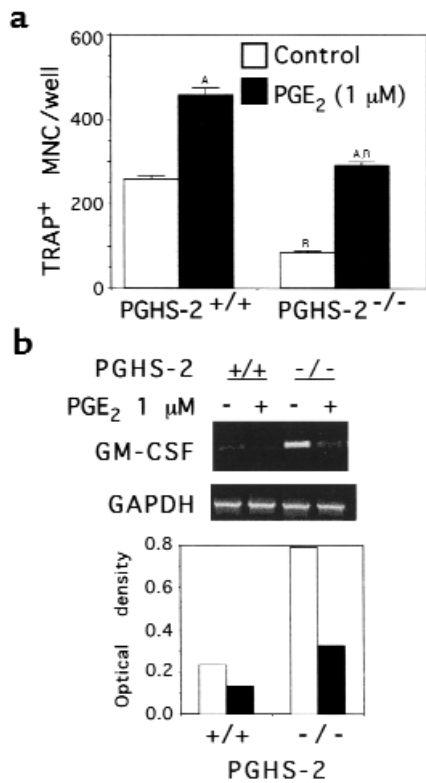
In multiple experiments, we found no TRAP<sup>+</sup> MNC formed in the absence of RANKL and M-CSF. As reported previously (44), addition of PGE<sub>2</sub> (1  $\mu$ M) to cultures treated with RANKL and M-CSF increased TRAP<sup>+</sup> MNC formation (Figures 7 and 8). In the experiment shown in Figure 7, treatment of *PGHS-2<sup>-/-</sup>* spleen cells with PGE<sub>2</sub> did not completely reverse the deficit in TRAP<sup>+</sup> MNC formation that was observed in *PGHS-2<sup>-/-</sup>* cultures compared with PGE<sub>2</sub>-treated *PGHS-2<sup>+/+</sup>* cultures. In 2 similar experiments, however, there was a complete reversal of this deficit (Figure 9). PGE<sub>2</sub> did not stimulate TRAP<sup>+</sup> MNC formation in cultures without RANKL and M-CSF (data not shown). As seen previously (44), addition of PGE<sub>2</sub> to spleen cell cultures decreased the total number of cells present at the end of the culture period (Figure 8).

PGE<sub>2</sub> levels in medium were barely detectable by our radioimmunoassay for PGE<sub>2</sub> (lower limit 0.1 nM) in both *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* spleen cell cultures (data not shown). This observation is consistent with the absence of osteoblasts and other stromal cells in these cultures. Treatment with indomethacin (0.1  $\mu$ M) to block prostaglandin production throughout the culture period did not inhibit OCL formation (data not shown).

**Regulation of GM-CSF in RANKL- and M-CSF-treated spleen cell cultures.** Because GM-CSF has been shown to inhibit murine osteoclastogenesis (45, 46), we measured GM-CSF mRNA levels by RT-PCR in the spleen cell cultures. In the experiment shown in Figure 7, the GM-CSF mRNA level was increased 3.5-fold in *PGHS-*



**Figure 6** TRAP<sup>+</sup> MNC formation in cocultures of spleen cells and primary osteoblasts from *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* mice. Osteoblasts were pooled from 4 populations from sequentially digested calvariae. Cocultures were treated with 1,25-D (10 nM) or PTH (10 nM), with or without PGE<sub>2</sub> (1  $\mu$ M) for 7 days and then stained for TRAP. Bars represent mean  $\pm$  SE of quadruplicate cultures. (a) Cultures were treated with vehicle (open bars), PGE<sub>2</sub> (striped bars), 1,25-D (black bars), or 1,25-D + PGE<sub>2</sub> (gray bars). (b) Cultures were treated with vehicle (open bars), PGE<sub>2</sub> (striped bars), PTH (black bars), or PTH + PGE<sub>2</sub> (gray bars). <sup>A</sup>Significant difference from vehicle treatment;  $P < 0.01$ . <sup>B</sup>Significant difference from vehicle treatment;  $P < 0.05$ . <sup>C</sup>Significant effect of *PGHS-2<sup>-/-</sup>* osteoblasts;  $P < 0.01$ . <sup>D</sup>Significant difference from treatment with either agent alone;  $P < 0.01$ .



**Figure 7** Effect of disruption of the *PGHS-2* gene on TRAP<sup>+</sup> MNC formation and GM-CSF mRNA expression in spleen cells cultured without osteoblasts. Cultures were treated with M-CSF (10 ng/mL) and RANKL (10 ng/mL), and then with either vehicle (Control; white bars) or PGE<sub>2</sub> (1 μM; black bars). (a) TRAP<sup>+</sup> MNC formed after 6 days of culture. Data are expressed as mean ± SE for quadruplicate cultures. (No TRAP<sup>+</sup> MNC were formed in cultures without RANKL and M-CSF; data not shown.) <sup>A</sup>Significant difference from vehicle treatment; *P* < 0.01. <sup>B</sup>Significant effect of *PGHS-2*<sup>-/-</sup> genotype; *P* < 0.01. (b) RT-PCR analysis of GM-CSF mRNA levels at the end of the culture period for the experiment shown in a. Ethidium bromide-stained RT-PCR products are shown in the top panel. The optical density ratios of GM-CSF mRNA to GAPDH mRNA are shown in the bottom panel.

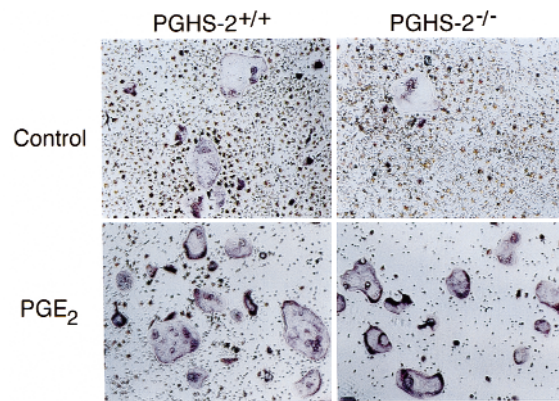
*2*<sup>-/-</sup> spleen cells compared with *PGHS-2*<sup>+/+</sup> cells, and the number of TRAP<sup>+</sup> MNC was 3.2-fold higher in *PGHS-2*<sup>+/+</sup> cultures than in *PGHS-2*<sup>-/-</sup> cultures. In a second and similar experiment, there was a 2-fold increase in GM-CSF mRNA levels in *PGHS-2*<sup>-/-</sup> spleen cell cultures compared with *PGHS-2*<sup>+/+</sup> cultures, and the number of TRAP<sup>+</sup> MNC was 2-fold higher in *PGHS-2*<sup>+/+</sup> cultures than in *PGHS-2*<sup>-/-</sup> cultures (data not shown). Addition of PGE<sub>2</sub> (1 μM) decreased GM-CSF mRNA levels in both *PGHS-2*<sup>+/+</sup> and *PGHS-2*<sup>-/-</sup> cultures (Figure 7).

If the decrease in TRAP<sup>+</sup> MNC formation in *PGHS-2*<sup>-/-</sup> spleen cell cultures relative to *PGHS-2*<sup>+/+</sup> cultures is due to increased GM-CSF production, then a blocking antibody to GM-CSF should increase TRAP<sup>+</sup> MNC formation in *PGHS-2*<sup>-/-</sup> spleen cell cultures and eliminate differences between *PGHS-2*<sup>-/-</sup> and *PGHS-2*<sup>+/+</sup> cultures. Murine GM-CSF (1 ng/mL) inhibited TRAP<sup>+</sup> MNC formation by 90% in RANKL- and M-CSF-stimulated *PGHS-2*<sup>+/+</sup> and *PGHS-2*<sup>-/-</sup> spleen cell cultures (Figure 9).

Polyclonal antibody to murine GM-CSF at a concentration of 0.1 μg/mL blocked the GM-CSF inhibition of TRAP<sup>+</sup> MNC formation in spleen cell culture, and at higher doses (1.0–10 μg/mL) the antibody increased TRAP<sup>+</sup> MNC formation 2-fold, with maximal effects at 1 μg/mL (data not shown). Addition of 1.0 μg/mL anti-GM-CSF antibody to *PGHS-2*<sup>+/+</sup> and *PGHS-2*<sup>-/-</sup> spleen cell cultures reversed the inhibitory effects of GM-CSF and enhanced OCL formation to similar levels in both *PGHS-2*<sup>+/+</sup> and *PGHS-2*<sup>-/-</sup> cultures (Figure 9). PGE<sub>2</sub> alone enhanced OCL formation to a level similar to that induced by the antibody in both *PGHS-2*<sup>+/+</sup> and *PGHS-2*<sup>-/-</sup> cultures. PGE<sub>2</sub> did not add further to the antibody effect, and could not overcome the inhibitory effect of added GM-CSF.

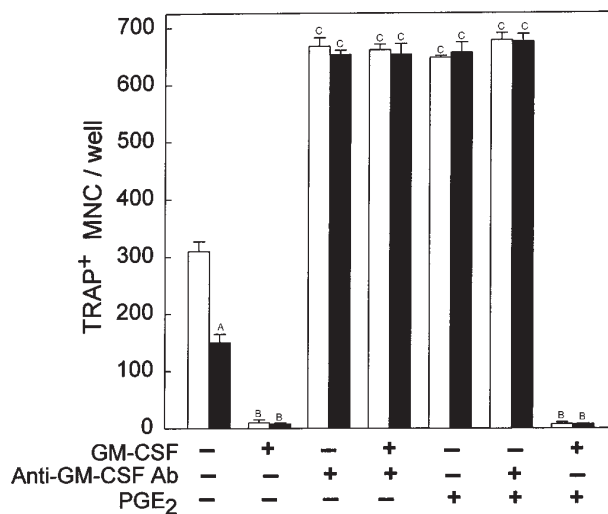
## Discussion

Prostaglandins of the E series are potent stimulators of bone resorption in organ culture, and PGE<sub>1</sub> and PGE<sub>2</sub> (but not PGF<sub>2α</sub>) stimulate osteoclast formation in marrow cultures (47, 48). Agonists reported to stimulate prostaglandin-dependent OCL formation include IL-1 (18, 22, 23), TNF-α (23), PTH (24, 25), 1,25-D (26), IL-11 (27, 28), IL-6 (21), IL-17 (31), phorbol ester (29), and FGF-2 (30). Our results support the conclusion of these earlier studies that prostaglandins play an important role in the response of bone-to-bone resorbing factors, and demonstrate that the critical prostaglandins are produced by PGHS-2. The lack of effect of disrupting *PGHS-1* gene expression is consistent with other studies in which stimulated PGE<sub>2</sub> responses are associated with PGHS-2 induction (7). However, disruption of *PGHS-2* or treatment with NSAIDs only partially blocked PTH- or 1,25-D-stimulated OCL formation in marrow cultures. Therefore, endogenous prostaglandins enhance, but are not required for, PTH- and 1,25-D-stimulated OCL formation. Addition of PGE<sub>2</sub> enhanced the effect of PTH but not 1,25-D in *PGHS-2*<sup>+/+</sup> cultures, suggesting that endogenous prostaglandins are sufficient to maximize



**Figure 8** Effect of PGE<sub>2</sub> on RANKL- and M-CSF-stimulated spleen cell cultures. RANKL-stimulated (10 ng/mL) and M-CSF-stimulated (10 ng/mL) spleen cells from *PGHS-2*<sup>+/+</sup> and *PGHS-2*<sup>-/-</sup> mice were treated for 6 days with vehicle (Control) or PGE<sub>2</sub> (1 μM) and then stained for TRAP.





**Figure 9**  
Effects of GM-CSF and a blocking antibody to GM-CSF on TRAP<sup>+</sup> MNC formation in RANKL- and M-CSF-stimulated spleen cell cultures. Spleen cells from *PGHS-2<sup>+/+</sup>* (white bars) and *PGHS-2<sup>-/-</sup>* (black bars) mice were cultured for 6 days with RANKL (10 ng/mL) and M-CSF (10 ng/mL). Cultures were also treated with vehicle (Control), murine GM-CSF (1 ng/mL), murine polyclonal antibody to GM-CSF (1 μg/mL), or PGE<sub>2</sub> (1 μM). Bars represent mean ± SE for quadruplicate cultures. <sup>A</sup>Significant effect of *-/-* genotype; *P* < 0.01. <sup>B</sup>Significant effect of GM-CSF; *P* < 0.01. <sup>C</sup>Significant effect of anti-GM-CSF antibody or PGE<sub>2</sub>; *P* < 0.01.

1,25-D- but not PTH-stimulated OCL formation in these cultures.

Despite the dependence of PTH- or 1,25-D-stimulated OCL formation on prostaglandin production, we have not found PTH-stimulated (49) or 1,25-D-stimulated (50) resorption in organ culture to be inhibited by indomethacin. This lack of dependence on prostaglandins is not due to lack of PGHS-2 induction or endogenous prostaglandin production (51). Perhaps stimulated resorption in organ culture reflects differentiation or activation of a pool of available osteoclastic precursors, and the prostaglandin enhancement of stimulated OCL formation in marrow culture reflects increased formation of new osteoclastic precursors.

Formation of bone-resorbing OCLs requires a contact-dependent interaction between osteoclast precursor cells and stromal or osteoblastic cells (12–14). The molecule mediating this interaction was originally cloned as (RANKL) (33), and was found to be identical to TNF-related activation-induced cytokine (TRANCE) (52). Subsequently, TRANCE/RANKL was found to be identical to osteoclast differentiating factor and to be a ligand for osteoprotegerin (a decoy receptor for RANKL) and is therefore also called ODF or OPGL (38). Induction of RANKL is essential for resorption by 1,25-D, PTH, and PGE<sub>2</sub> (38–41). Our data suggest that PTH- and 1,25-D-stimulated RANKL mRNA expression in osteoclastic support cells is decreased 50–60% in the absence of PGHS-2 expression; this reduction could explain the majority of the decrease in stimulat-

ed OCL formation in *PGHS-2<sup>-/-</sup>* marrow cultures compared with *PGHS-2<sup>+/+</sup>* marrow cultures.

A recent study showed that PGE<sub>2</sub> enhances RANKL-stimulated OCL formation in spleen cell cultures, supporting a role for a direct effect of prostaglandins on hematopoietic precursors of osteoclasts (44). Our data support these observations. In addition, we showed a reduction in RANKL- and M-CSF-stimulated OCL formation in spleen cell cultures from *PGHS-2<sup>-/-</sup>* mice. This reduction was not reversed by indomethacin, and hence cannot be attributed to a difference in PGE<sub>2</sub> production in spleen cell cultures. We suggest that in vivo, prostaglandins modulate the size of the pool of osteoclastic progenitors that are readily available to differentiate in response to stimulators, and that in vitro, in the absence of endogenously produced prostaglandins, these differences are maintained. However, these differences can be overcome by treatment of the spleen cell cultures with exogenous prostaglandins, or the production of prostaglandins by osteoblasts in spleen-osteoblast cocultures.

Our results in spleen cell cultures treated with RANKL and M-CSF suggest that decreased OCL formation in *PGHS-2<sup>-/-</sup>* spleen cell cultures relative to *PGHS-2<sup>+/+</sup>* cultures is due to increased GM-CSF expression. Several previous studies have found that prostaglandins or cAMP analogues downregulate the expression of GM-CSF in marrow stromal cells and lymphocytes (53–55). However, there are conflicting reports on the effects of GM-CSF on osteoclastogenesis in marrow cultures and cocultures. GM-CSF has been found to increase OCL formation in human and primate bone marrow cultures (56, 57) and in some rodent cultures (58–60). On the other hand, there are many studies in murine marrow cultures and coculture systems showing that GM-CSF inhibits OCL formation (45, 46, 61–63), and it has been proposed that GM-CSF inhibits OCL formation by inhibiting expression of integrin αvβ5 (64). Although GM-CSF knockout mice have been found to have abnormalities in granulopoiesis, resistance to infection, and pulmonary physiology (65, 66), we found no reports of abnormal bone histology.

The association of increased GM-CSF expression with decreased OCL formation that was seen in the spleen cell cultures could not be generalized to account for the effect of PGHS-2 deficiency on OCL formation in marrow cultures and cocultures of osteoblasts and spleen cells. Addition of GM-CSF to 1,25-D-stimulated *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* marrow cultures or to PTH-stimulated *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* osteoblast/spleen cocultures completely inhibited OCL formation, but the blocking antibody to GM-CSF had no effect on OCL formation in the absence of exogenous GM-CSF (data not shown). In addition, we were not able to detect GM-CSF mRNA by RT-PCR in marrow cultures from *PGHS-2<sup>+/+</sup>* or *PGHS-2<sup>-/-</sup>* mice (data not shown).

Because prostaglandins produced by PGHS-2 are critical for obtaining maximal OCL responses to 1,25-D and PTH, an in vivo role for prostaglandins in bone resorp-

tion may be most apparent during high bone turnover. Subcutaneous injection of PTH above the calvaria has been shown to increase resorption locally and to cause hypercalcemia in mice (36, 37). *PGHS-2<sup>+/+</sup>* mice subjected to this protocol became hypercalcemic as expected, but *PGHS-2<sup>-/-</sup>* mice did not. These studies suggest that the in vivo bone resorption response to high-dose PTH is blunted in *PGHS-2<sup>-/-</sup>* mice. Although PGHS-2 deficiency did not result in differences in histologic phenotype or differences in serum calcium levels under conditions of unstimulated turnover, the effect of PGHS-2 deficiency on the resorption response to high-dose PTH suggests that a histologic phenotype may become evident when bone resorption is increased, such as occurs after estrogen withdrawal. Although *PGHS-2<sup>-/-</sup>* mice may develop renal nephropathy, *PGHS-2<sup>-/-</sup>* mice remain healthy with age, and have been used to demonstrate effects of reduced PGHS-2 expression in vivo on the incidence of intestinal polyposis (67). In our study, PGE<sub>2</sub> production and OCL formation in *PGHS-2<sup>-/-</sup>* cultures were each intermediate between that found in *PGHS-2<sup>+/+</sup>* and *PGHS-2<sup>-/-</sup>* cultures, suggesting that *PGHS-2<sup>+/+</sup>* mice will be useful for studying the effects of PGHS-2 reduction on bone metabolism in vivo.

Histological examination of bone from 5-week-old *PGHS-2<sup>-/-</sup>* mice revealed no skeletal abnormalities, which suggests that PGHS-2 is not necessary for normal bone development. However, the effects of PGHS-2 expression on bone cells can be replaced by exogenous prostaglandins, and maternally produced prostaglandins could play a role in fetal skeletal development.

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