

Effects of prolactin on osteoblast alkaline phosphatase and bone formation in the developing rat

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Coss, Djurdjica, Lili Yang, Chiaoyun Benson Kuo, Xiaolei Xu, Richard A. Luben, and Ameae M. Walker. Effects of prolactin on osteoblast alkaline phosphatase and bone formation in the developing rat. *Am J Physiol Endocrinol Metab* 279: E1216–E1225, 2000.—The purpose of the current study was to determine whether maternal prolactin (PRL) had any effects on bone formation in the developing rat pup. Because the most prevalent forms of PRL in rats are unmodified and phosphorylated PRL, both recombinant PRL and a molecular mimic of phosphorylated PRL (PP-PRL) were administered to pregnant animals. Blood samples from the dams showed normal estrogen and progesterone and no effect of extra PRL on parathyroid hormone (PTH), calcium, or alkaline phosphatase (AP). In newborn pups, however, there was a 30% decrease in blood AP in both PRL-treated groups, whereas PTH and calcium levels were not different from controls. When primary rat osteoblasts were exposed to both PRLs, AP activity was reduced, with PP-PRL being the more potent form of the hormone. Histological examination of pup bone formation showed reduced calvarial bone and reduced endochondral ossification in pups exposed to PP-PRL. These results are the first to show a direct inhibitory effect of PRL on osteoblast function.

phosphorylated prolactin; intramembranous bone formation; endochondral bone formation

IT HAS LONG BEEN APPRECIATED that both physiological and pathological hyperprolactinemia is associated with alterations in bone physiology. Thus 1) tumor-induced hyperprolactinemia in people (16, 21, 30, 31) and rats (2) is associated with osteopenia; 2) elevation of prolactin (PRL) by the administration of neuroleptic drugs (13) also results in osteopenia (3); 3) prolonged lactation results in significant bone loss (9, 23, 26, 35); and 4) aging is correlated with increased PRL (36) and bone loss (28) and, in postmenopausal women, the degree of osteopenia can be predicted by PRL levels (22).

However, because hyperprolactinemia in the adult frequently results in hypogonadism (29), most authors have concluded that prolactin's effects on bone are likely to be indirect (1, 17). More recently, however, PCR analyses of human osteosarcoma cell lines (4) and primary cultures of mouse osteoblasts (7) have rekindled interest in the idea that PRL may also have direct

effects on bone metabolism by demonstrating the presence of PRL receptors in these cells. Although most studies have focused on the potential role of PRL in the remodeling of the adult skeleton, our aim was to analyze potential effects of PRL on initial bone development. The perinatal period is a time of substantial bone formation, as evidenced by the threefold higher levels of alkaline phosphatase (AP) in newborn vs. adult animals (6) and is therefore an unusually sensitive period in which to study potential influences on bone metabolism.

In most studies, PRL is treated as if it were a single hormone. With increasing evidence of different biological activities of different forms of PRL (10, 14, 25, 32, 38), however, it is important to begin to consider the potentially different activities of each form in each tissue. In the rat, the two PRL forms, which together account for 90–99% of the material released from the pituitary, are unmodified and phosphorylated PRL (18, 19). The relative proportion of unmodified and phosphorylated PRL released from the pituitary varies with physiological state (19), including the duration of gestation (18).

In this study, we have analyzed the effects of elevations in unmodified and phosphorylated PRL by use of recombinant versions of both. In the case of unmodified PRL, the recombinant version was human methionyl wild-type PRL (5). In the case of phosphorylated PRL, a molecular mimic was used. In this mimic, the serine, phosphorylated inside intact pituitary secretory granules (37), was replaced by an aspartate residue. Because this molecular mimic of phosphorylated PRL contains no phosphate, it cannot be dephosphorylated during *in vitro* or *in vivo* experiments. The molecular mimic is human methionyl S179D PRL and has been shown to accurately mimic naturally phosphorylated PRL *in vitro* (5, 38). Also, as shown by analysis of signaling molecules, the human methionyl S179D PRL competes with human methionyl PRL for signaling through the rat PRL receptor (8). We have dubbed this S179D molecular mimic “pseudophosphorylated” PRL (PP-PRL).

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Administration of PP-PRL during pregnancy resulted in decreased calvarial bone and decreased endochondral ossification in the pups at the time of birth, without effect on maternal gonadal steroids or measures of maternal bone formation or turnover rate. Administration of the wild-type PRL had no histologically obvious effect on the amount of bone. When direct effects of each PRL were tested on osteoblasts in primary culture, both PRLs reduced AP activity, but PP-PRL was more potent. Together, these results support a direct role for PRL in the regulation of bone formation.

MATERIALS AND METHODS

Immunocytochemistry. Neonatal rats were killed, and calvariae were removed and fixed for 3 h in cold fixative [2% paraformaldehyde, 0.01 M periodate, 0.75 M lysine, and 6% sucrose in phosphate buffer-PLP (27)]. Tissues were then cryoprotected by overnight incubation in 15% sucrose. The tissues were mounted with tissue-freezing medium, and 6- μ m frozen sections were cut on a Zeiss cryotome (Heidelberg, Germany). Sections were washed with wash buffer (20% goat serum, 0.2% BSA in 0.01 M PBS), and nonspecific binding was blocked by incubation in goat serum. Sections were incubated with U6 anti-prolactin receptor monoclonal antibody (kindly provided by Paul Kelly, Institut National de la Santé et de la Recherche Médicale, Faculté de Médecine Necker, Paris, France), diluted in wash buffer to 5 μ g/ml, for 2 h. Separate sections were incubated with nonimmune mouse IgG-1 κ diluted to the same concentration in wash buffer, as a control. A secondary antibody-only control was also performed. After three washes, sections were incubated with fluorescein goat anti-mouse IgG (Molecular Probes, Eugene, OR) diluted to 20 μ g/ml in wash buffer for 1 h. Sections were washed three times in wash buffer and twice in PBS before they were mounted with Prolong antifade (Molecular Probes) and examined on the fluorescence microscope. Both traditional microscopy and scanning confocal fluorescence microscopy were used, and illustrations were chosen from the most informative images. For osteoblasts, this was mainly from the traditional microscope, because phase contrast could confirm the identity of the cells. For chondrocytes, identity is clear from their position in the cartilagenous matrix, and the confocal image better illustrates cell membrane fluorescence. All immunocytochemical localizations were performed a minimum of 3 times.

Protein expression. Wild-type recombinant PRL and S179D PP-PRL were produced and characterized as previously described (5). Both proteins were expressed and purified in parallel and were expressed at similar levels. Each preparation was tested for its biological activity by Nb2 bioassay (5, 34). Nb2 cells are T lymphoma cells isolated from the lymph node of an estrogen-treated rat that proliferate in response to PRL (34). Proteins were concentrated to 1 mg/ml of saline using Amicon Centripreps (Amicon, Danvers, MA).

Animals. Sprague-Dawley female rats were maintained in constant light-dark conditions (12:12-h light-dark cycle) and were fed ad libitum. After mating and observation of a vaginal plug, rats were randomly assigned, 10 to each of 3 different groups: a control group, a group treated with wild-type recombinant PRL, and a group treated with PP-PRL. Alzet minipumps (Alza, Palo Alto, CA, model 2004), containing the appropriate protein, were implanted at *day 0.5* on the morning after observation of the plug. The pumps delivered 6 μ g/24 h throughout pregnancy. One-half of the dams were killed on *day 19.5* of gestation and one-half after delivery. On

days 6.5 and *11.5* of gestation, blood was obtained from the tail and, on *day 19.5*, from the trunk. To reduce the potential influence of stress, no individual animal was bled more than once from the tail. After delivery, pups were weighed and killed by metaflane overdose, and representative pups from each litter were fixed by immersion in PLP fixative. Others were decapitated after being killed for the collection of trunk blood. Trunk blood from 5–6 pups was pooled for each litter. All procedures were approved by the University of California Riverside Committee on Laboratory Animal Care and were in accordance with National Institutes of Health guidelines.

Determination of circulating levels of recombinant PRLs. To eliminate any possible distortion of RIA values caused by cross-reactivity of antisera with rat lactogens, circulating levels of administered recombinant proteins were determined by immunoprecipitation of biosynthetically 35 S-labeled recombinant proteins (15). Use of excess antibody ensured maximal precipitation from serum samples. Greater than 85% of counts present in samples taken on *days 1–16* remained in immunoprecipitable PRL. Knowing the specific activity of the radiolabeled PRL allowed us to determine that administration of 6 μ g/24 h resulted in circulating levels of 50 ng/ml by *day 4* of pump implantation; levels remained constant thereafter. Steady-state levels after pump implantation were the same for each form of PRL. Clearance for the 1st h after a bolus injection was determined in anesthetized animals (45 mg/kg pentobarbital sodium) treated with bromocryptine (500 μ g/kg to reduce endogenous PRL release) before cannulation. Radiolabeled PRLs were injected as 750 ng in 300 μ l of saline containing 0.05% bovine serum albumin.

AP, calcium, parathyroid hormone, and progesterone in blood. Sigma kit 104-LL was used for measurements of blood AP. This assay uses *p*-nitrophenyl phosphate as substrate and measures all types of AP activity. Parathyroid hormone (PTH) levels were measured using a PTH RIA/IRMA kit from Nichols Diagnostic Institute (San Juan Capistrano, CA). Progesterone and estrogen levels were measured by RIA with kits from Diagnostic Products (Los Angeles, CA). Calcium levels were measured by atomic absorption spectrophotometry on a Perkin-Elmer 400.

All samples presented in a given figure were measured in the same assay, thereby limiting errors to intra-assay variation. Measurements for dams' parameters were performed on plasma or serum, whereas for pups' parameters, measurements were performed on plasma. Heparin was used as the anticoagulant. All assays were validated for use on heparinized plasma samples.

Histological staining. Fixed tissue from neonatal rats from the three treatment groups was dissected, dehydrated in an ethanol series, and embedded with the same orientation in Paraplast. Serial 6- μ m sections were cut until at least one-third of the way across the calvaria or halfway across the digit. Equivalent sections were stained with hematoxylin and eosin or Von Kossa's stain or Alizarin red. Multiple sections per pup, two pups per dam, and four dams per group were examined. For measurements, calvarial width at three places per section was ascertained.

Primary bone cell culture. Calvariae were removed from nontreated neonatal rats and digested with collagenase [2.4 mg/ml, (200 units/mg) and 10 μ g/ml *N* α -*p*-tosyl-L-lysine chloromethylketone trypsin inhibitor in HEPES buffer containing 0.1% BSA and 0.5% glucose] for 2 h at 37°C. Supernatant from the first 15 min of digestion was discarded to enrich the eventual preparation with osteoblasts. The subsequent supernatant, containing released osteoblasts, was then transferred to a new tube. Cells were washed and plated in DMEM with 10% fetal bovine serum. Cells were maintained in log

phase and were grown at 37°C in a humidified atmosphere of 5% CO₂. Cells were used for experiments at passages 2 or 3.

RT-PCR. Total cellular RNA was extracted with TRI-reagent (Molecular Research Center, Cincinnati, OH). First strand complementary DNA was synthesized from 5 µg total RNA from osteoblasts and Nb2 cells and 1 µg RNA from rat pituitary by use of reverse transcriptase (Promega, Madison, WI) for 1 h at 37°C after random priming. Twenty microliters of the product were diluted to 50 µl, and 2 µl were used in the PCR reaction. Five micromoles final concentration of each primer (5'-CCCACCCACCATAACTGATG and 3'-TCCAG-CAGATGGGTATCAAATC for PRL receptor and, in a separate reaction for rat PRL, 5'-CCTCCTGCTGATGATGTCAAACC and 3'-GGGAGTTTCTGGGCTTGTTCCT) were added to the 50-µl final volume PCR buffer with 2 U Ampli-taq Gold (Perkin-Elmer, Branchburg, NJ) per reaction and 1.5 and 3 mM MgCl₂ for PRL and PRL receptor, respectively. Thirty-six cycles of amplification were performed with the following profile: 1 min at 95°C, 1 min at 63°C, and 2 min at 72°C, followed by extension for 10 min at 72°C. Amplification products were resolved on a 1.5% agarose gel.

Cell proliferation assay. Osteoblasts were plated in 96-well plates at 4,000 cells/well (such that they would reach confluence at or near the beginning of treatment) or 1,000 cells/well (so as to be proliferating during treatment) in 100 µl of DMEM with 10% horse serum. Horse serum was used because horse PRL cannot activate rat receptors (34). Cells were allowed to attach overnight, and on the next day, 100 µl of twofold-concentrated PRL in the same medium were added to the wells. After 72 h, 20 µl/well 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium (MTS) solution (Promega, Madison, WI) were added, and the color was allowed to develop for 2 h. Absorption was read on an ELISA reader using a 492-nm filter. MTS color development was shown to be linear for primary osteoblasts between 1,000 and 14,000 cells/well.

Cell AP assay. For AP activity, cells were treated as described for the proliferation assay and were then washed and lysed after 72 h of treatment. Lysis was in 200 µl 0.1% Triton X-100 in 25 mM carbonate buffer, pH 10.3. One hundred microliters of cell extract were then added to 200 µl of 250 mM carbonate buffer, pH 10.3, containing 1.5 mM MgCl₂ and 15 mM *p*-nitrophenyl phosphate. Color development was quantified by absorption at 405 nm.

Statistical analyses. Statistical significance was assessed by using Duncan's modified *t*-test for multiple comparisons against a single control group or by one-way analysis of variance.

RESULTS

PRL receptors on cells of bone and cartilage. Although previous investigators have demonstrated the presence of PRL receptor mRNA and protein in primary osteoblasts (7), potential contamination of such cultures by other cell types made it important that PRL receptors be localized to morphologically recognizable osteoblasts in intact bone. Figure 1 shows immunocytochemical evidence of the presence of PRL receptors on osteoblasts of normal rat skull bones. No osteoclast staining was observed. A comparison of the specific antibody- and nonspecific antibody-treated sections clearly shows specific osteoblastic staining in the former. Osteoblasts adhere to the surface of the developing bone and have a substantial cytoplasm. Bone matrix reflects fluorescent emission, and hence, bone to which the positively stained osteoblasts adhere has a

higher background fluorescence than that in the two controls. This is eliminated by the use of confocal fluorescence microscopy, as is illustrated in Fig. 1B. Figure 1B also illustrates a more punctate staining of the receptors.

In sections of digits formed by endochondral ossification, specific labeling of chondrocytes was also found (Fig. 2). Chondrocytes, in lacunae separated by cartilagenous matrix, are present throughout the cartilage matrix. Chondrocytes in both reserve and proliferating regions of the developing long bone stained positively for PRL receptors (Fig. 2).

Effects of PRL administration on pup bone development. Administration of PP-PRL to dams throughout pregnancy resulted in reduced pup calvarial bone. Calvariae of the control group were 52.12 ± 1.72 µm thick, those in the wild-type PRL-exposed group were 53.87 ± 1.72 µm thick, and those from the PP-PRL group were 24.88 ± 1.28 µm thick. (PP-PRL different from the other two groups with *P* value < 0.0001.) Also evident in the PP-PRL-exposed group was reduced endochondral ossification (Fig. 3, C and F). For the latter, the cartilagenous precursor was formed essentially normally, and hypertrophy of the diaphyseal chondrocytes was evident. There was, however, no evidence of the formation of a bony collar or diaphyseal endochondral ossification, both of which were evident in the controls. By contrast, administration of wild-type PRL had no obvious effect (Fig. 3, B and E). Sections stained with Von Kossa's silver stain or Alizarin red to demonstrate calcified matrix showed no differences in the degree of calcification of the matrix present (data not shown).

Clearance of wild-type PRL and PP-PRL. As illustrated in Fig. 4, both PRLs were cleared from the circulation at very similar rates. Their differential efficacies are therefore not due to different half-times in the circulation.

Maternal parameters during pregnancy. Administration of either wild-type PRL or PP-PRL had no effect on progesterone levels on any of the 3 days measured during treatment (Table 1). Also unaltered were PTH levels, serum calcium, AP, or estrogen at day 19.5 of pregnancy (Fig. 5), i.e., after 19 days of treatment with this level of additional PRL.

Pup parameters. Pups in all three groups were the same weight at birth (6.6 ± 0.1 g control; 6.5 ± 0.1 g wild-type PRL; 6.7 ± 0.1 g PP-PRL), consistent with the formation of equivalent cartilagenous skeletons.

Plasma concentrations of PTH were very variable in the newborn pups, and there was no significant difference among groups (Fig. 6A). Plasma calcium levels, which were about one-third those seen in the dams, also showed no statistically significant difference from controls (Fig. 6B).

AP levels, however, were significantly decreased in both PRL-treated groups, by ~30% (Fig. 6C). PRL treatment also resulted in decreased plasma osteocalcin (Fig. 6D). The trend suggested an increased efficacy of PP-PRL, but high variability among groups of pups rendered this statistically not significantly different.

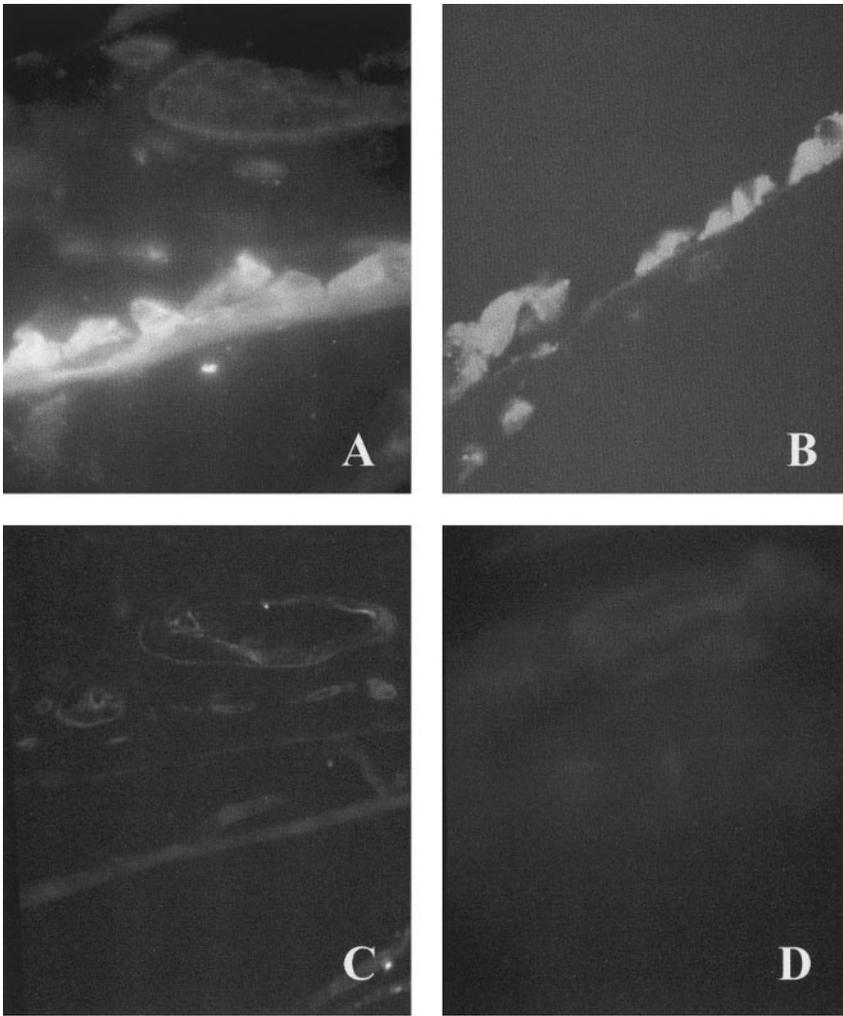


Fig. 1. Immunocytochemical localization of prolactin (PRL) receptors on calvarial osteoblasts. *A* and *B*: specific staining with anti-PRL receptor; *C*: nonspecific staining with nonimmune isotype-matched control IgG; *D*: staining with the secondary fluorescent antibody alone. *C* and *D* were adjacent sections to section shown in *A*, and *B* is a confocal image of a similar section. Magnification factor is 2,500.

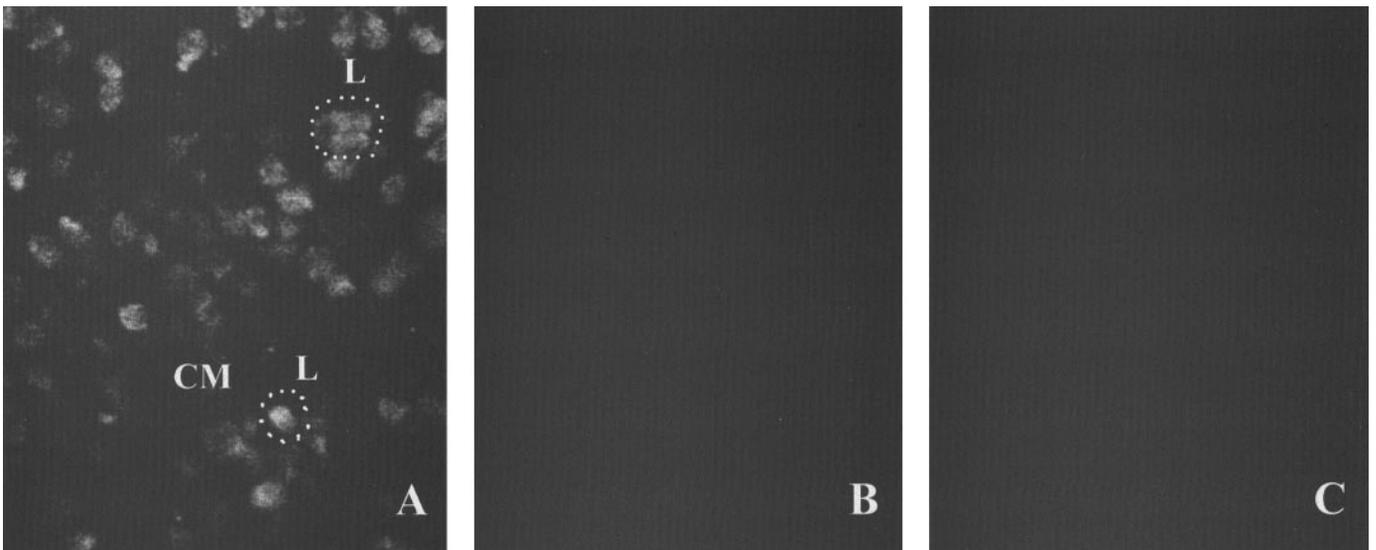


Fig. 2. Immunocytochemical localization of PRL receptors to chondrocytes. *A*: confocal image of cartilage stained with anti-PRL receptor. *B* and *C*: equivalent confocal images obtained on adjacent sections from tissues incubated in nonimmune isotype-matched control IgG (*B*) and secondary fluorescent antibody alone (*C*). Circle of dots outlines a lacuna (L). The lower one is in a region of resting cartilage; the upper one is in a region of proliferating cartilage and shows two chondrocytes within a single lacuna; CM, cartilagenous matrix. Magnification factor is 2,500.

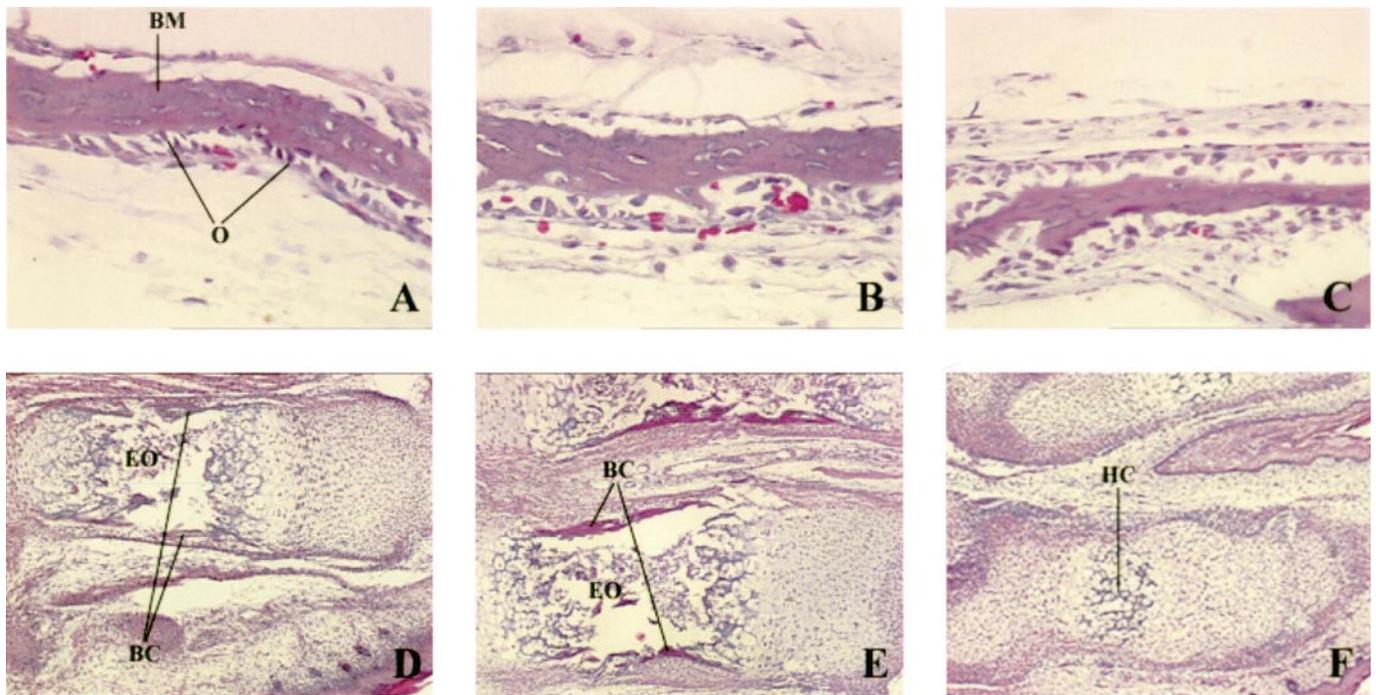


Fig. 3. Calvarial bone and digit cartilage/endochondral ossification in newborn rat pups in the 3 treatment groups. *A* and *D*: control pups (no exposure to additional PRL); *B* and *E*: tissues from pups exposed to wild-type PRL; and *C* and *F*: pups exposed to pseudophosphorylated (PP)-PRL. BM, bone matrix; O, osteoblast; BC, bony collar; EO, endochondral ossification; HC, hypertrophic cartilage. Magnification factor is 300 for *A-C* and 60 for *D-F*. The nos. of sections/pup, pups/dam and dams/group analyzed are given in MATERIALS AND METHODS, and the results of quantitation are given in RESULTS. This effect on bone formation has been observed in 3 separate pregnancy experiments.

Primary osteoblast cultures. Because blood levels of calcium and AP can be aggregates of effects on several tissues, we investigated direct effects of the two forms of PRL on osteoblasts in primary culture.

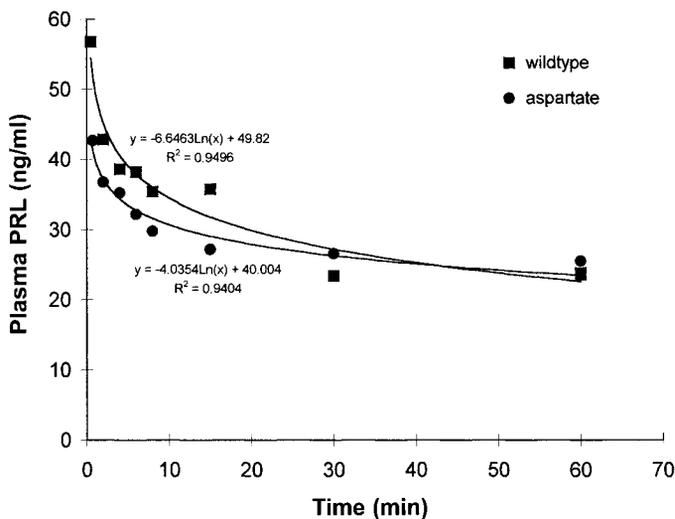


Fig. 4. Short-term clearance of wild-type PRL and PP-PRL from the circulation. A bolus of 750 ng of radiolabeled PRL was infused. Blood samples were obtained at the times indicated, and the concentration of administered PRL was calculated from the specific activity. The graph shows the result from 1 animal for each PRL, but each is representative of 10 replicates. Both animals were in estrus, as determined by vaginal smear analysis.

To determine that expression of the PRL receptor was maintained when rat osteoblasts were placed in primary culture, we performed RT-PCR for the message. The primers chosen did not distinguish between the long and short forms of the receptor. As can be seen in Fig. 7, cells at *passage 3* express message for the PRL receptor, but not for PRL. RNA from Nb2 cells and pituitary was used as the positive control for receptor and RNA from pituitary as the positive control for PRL. Lack of osteoblast expression of PRL makes it more likely that osteoblast PRL receptors respond to circulating rather than locally produced PRL. Expression of the PRL receptor protein was confirmed by immunocytochemistry (data not shown).

Figure 8 shows the effect of the two forms of PRL on AP activity in primary osteoblasts. Essential absence of PRL for the 0 ng/ml concentration was ensured by culturing the cells in 10% horse serum; horse PRL does not interact with rat PRL receptors (34). At low osteo-

Table 1. Progesterone levels during gestation

| Day | Control | WT-PRL | PP-PRL |
|------|--------------|--------------|--------------|
| 6.5 | 374.3 ± 37.5 | 350.7 ± 19.8 | 322.6 ± 27.8 |
| 11.5 | 369.3 ± 20 | 379.9 ± 19.9 | 389.0 ± 42.6 |
| 19.5 | 377.3 ± 23.3 | 283.3 ± 27.8 | 327.8 ± 17.8 |

Values are means ± SE; n = 5 animals per group per time point. Day, day of gestation; WT-PRL, wild-type prolactin (PRL)-treated animals; PP-PRL, pseudophosphorylated PRL-treated animals.

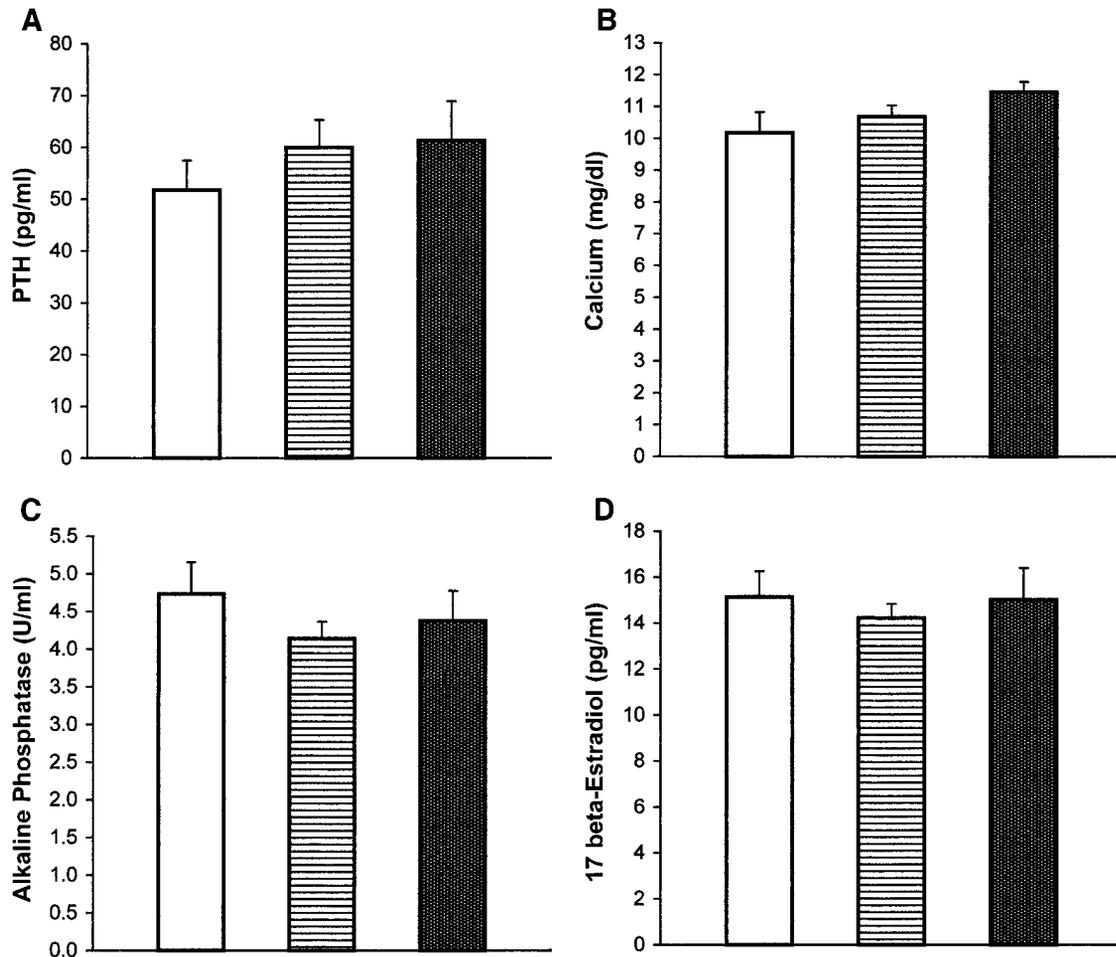


Fig. 5. Maternal levels of parathyroid hormone (PTH), serum alkaline phosphatase (AP), serum calcium, and serum estrogen on *day 19.5* of gestation. A: PTH; B: serum calcium; C: serum AP; D: serum estrogen. Values are means \pm SE; $n = 5$ dams/group. Open bars, control; hatched bars, wild-type PRL treated; solid bars, PP-PRL treated.

blast density, when cells were treated during proliferation, added wild-type PRL had no effect on AP activity, whereas the PP-PRL markedly reduced AP activity in a dose-dependent manner >10 ng/ml. At higher cell density, when cells were treated at confluence, both PRLs inhibited AP activity >10 ng/ml, with the PP-PRL being more potent at supraphysiological levels. In the low density experiments, 100% AP activity was 20 mU/mg of protein and in the high density experiments was 127 mU/mg of protein. Neither preparation of recombinant PRL had any effect on osteoblast proliferation at either cell density as measured in the MTS assay (data not shown). Because cell number increased during the 72-h incubation and cell number was the same in the 0 ng/ml PRL and 1,000 ng/ml PRL samples, there was no evidence of any toxicity in the preparations. In other experiments, standard PRL, provided by the National Institute of Diabetes and Digestive and Kidney Diseases (human PRL-B3, AFP 3855A), was also shown to decrease AP activity at high cell density (data not shown).

DISCUSSION

Preliminary experiments had established that administration at a rate of 6 μ g/24 h of either PRL form to pregnant animals would have no effect on progesterone or estrogen output by the maternal ovary, and this was confirmed in the present study. During the current experiments, therefore, we could be certain that pup bone development would not be influenced by altered levels of maternal steroid hormones. Also unaltered by this protocol were maternal levels of PTH, elevations of which would increase bone turnover rate (11); calcium, an aggregate marker of intestinal uptake; kidney reabsorption and deposition/resorption in bone, the first two of which are reportedly influenced by PRL (2, 24); and AP, a marker of bone formation (33). Thus we had good evidence that changes in fetal bone development were not secondary to changes in maternal bone metabolism or the availability of calcium. The duration of rodent pregnancy is really too short a period in which to study effects on maternal bone, so our results do not rule out the possibility that

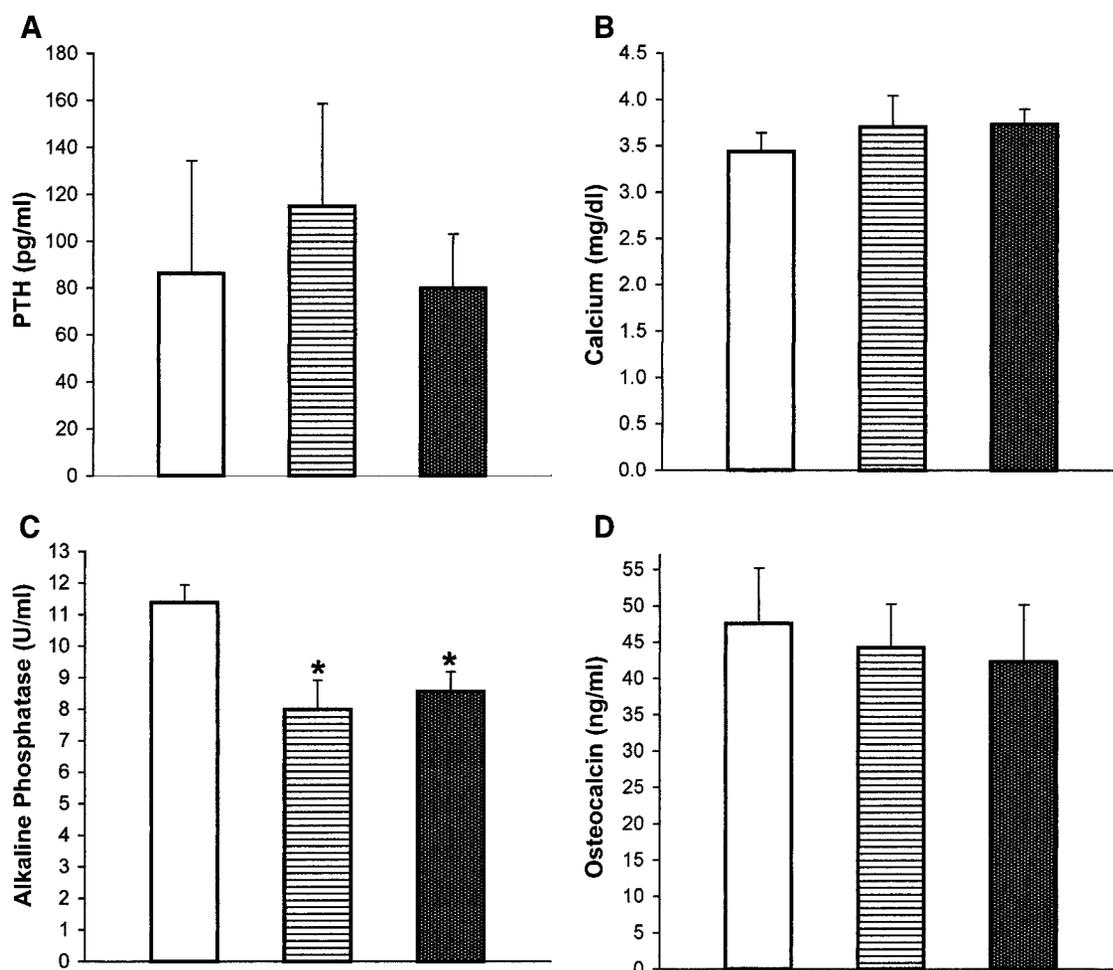


Fig. 6. Levels of PTH, calcium, and blood AP in the newborn pups. A: PTH; B: plasma calcium; C: plasma AP; D: osteocalcin. Values are means \pm SE and are from 5 sets of pooled pup plasma per group. *Significantly different from control, $P < 0.05$. Open bars, control; hatched bars, wild-type PRL treated; solid bars, PP-PRL treated.

longer exposure to the administered PRLs at this level would have had an effect on the maternal skeleton.

Pup blood AP levels are about threefold those in adult animals, indicative of the high rate of bone formation ongoing in the perinatal period (Ref. 6 and results herein). A 30% decrease in pup AP levels after in utero exposure to administered PRL therefore represents a major and important change, suggesting reduced bone formation. Histological analysis at the time of birth, however, showed obvious decreased bone formation only in the pups exposed to PP-PRL. To ensure that the decrease in AP was in fact an effect on osteoblast AP, a series of studies using osteoblasts in primary culture was undertaken. These studies showed both forms of PRL to decrease AP activity in cultures with a high cell density. This effect occurred in the dose range used in the intact animals and is consistent with the lower blood AP observed in both sets of pups at the time of birth. In cultures with a lower cell density more closely approximating the beginnings of bone formation, only the PP-PRL inhibited AP activity. Thus it seems reasonable to suggest that the lowered amount of bone observed in the animals exposed to PP-PRL

was a consequence of an early effect on bone formation, in other words, an effect during initial osteoblast differentiation in calvarial islands or the formation of the long bone bony collar. At the time of birth, both PRLs were likely inhibiting bone formation, but for the wild-type hormone, this had not been going on long enough to see any clear histological evidence of same.

Contrary to our expectations, this direct effect of PRL on osteoblasts was on cell-associated AP activity and not on osteoblast proliferation. Elevated PRL, therefore, seems to influence bone cell differentiation, because osteoblasts express high levels of AP during osteoid formation (33).

Despite the presence of numerous PRL receptors on chondrocytes, elevation of PRL by administration of either form had no very distinct effect on the growth of cartilage. The pups in each group weighed the same, and all stages of interstitial growth and so-called chondrocyte hypertrophy occurred normally in the developing long bones, although there was some evidence of hyperplasia. The defect in endochondral bone formation was observed only when osteoblasts would normally be functional. The common theme in both types

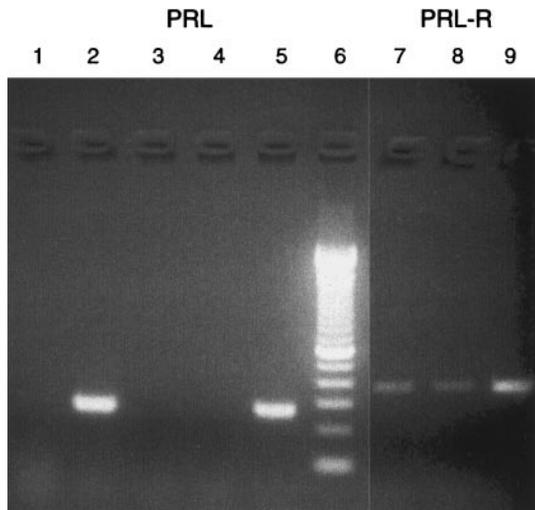


Fig. 7. Maintained expression of the PRL receptor (PRL-R) in primary rat osteoblast cultures. RT-PCR for PRL mRNA (lanes 1–5) or PRL receptor mRNA (lanes 7–9) showing positive amplification products of 270 and 399 bp, respectively. Lane 1, no RT product control; lanes 2, 5 and 9, pituitary; lanes 3 and 7, Nb2 cells; lanes 4 and 8, primary osteoblasts at passage 3; lane 6, 100-bp ladder. This figure is representative of 3 separate RNA isolations from primary osteoblasts.

of bone formation, therefore, seems to be an inhibition of osteoblast function by elevated PRL. The role of the PRL receptor in chondrocytes remains to be established, but cartilage formation and growth do not seem to be adversely affected by elevated PRL.

Other investigators have recently suggested that osteoblasts are a target for PRL. This suggestion was based on the presence of PRL receptor mRNA and protein in primary osteoblast cultures and effects on bone metabolism in PRL receptor knockout mice (7). No direct effect of PRL on osteoblast function was demonstrated, however, and interpretation of the results in the knockout animal model is severely complicated by disturbances in the levels of gonadal steroids, calcium, and PTH (7). Thus defects in bone formation may be secondary to other changes. Unexpectedly, with regard to calvariae, the fetal/newborn phenotype is similar in terms of bone mass in the PRL receptor knockout mice and our rat pups exposed to PP-PRL. This could be simply a coincidence related to disturbed bone metabolism resulting from two different mechanisms. Alternatively, it could be a function of a biphasic response to PRL. In other words, some PRL is necessary for osteoblast function, but elevated levels inhibit osteoblast function. If this were the explanation, however, one might have expected to see evidence of a biphasic response in our *in vitro* studies, and none was observed.

The fetal calvariae in the PRL receptor knockout mice were also undercalcified, as demonstrated using Von Kossa's stain (7). We could detect no reduced calcification of the amount of osteoid produced in the PP-PRL-exposed pups by use of the same approach. It is possible, therefore, that alterations in mineralization in this knockout animal are secondary to the documented disturbances in serum calcium and PTH (7).

Analyses of the PRL knockout animal, by contrast, report no similar calvarial phenotype to the PRL receptor knockout (20). Others have suggested that this may be because placental lactogens, which also activate the PRL receptor, can substitute during fetal bone development (7) or are, in fact, the normal ligand for fetal bone PRL receptors. However, given the negative effect of PRL receptor activation demonstrated in our *in vitro* studies, it does not seem likely to us that placental lactogens, which reach microgram-per-milli-

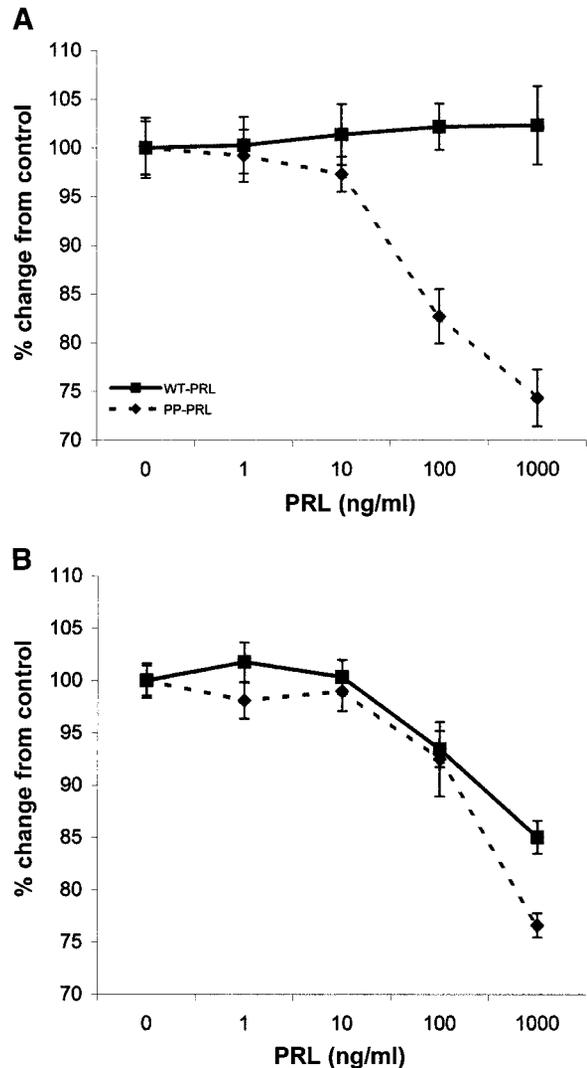


Fig. 8. Effect of the recombinant PRLs on AP activity of primary osteoblast cultures. *A*: effects at low cell density; *B*: effects at high cell density. Data are expressed as a percentage of the value in the non-PRL-treated control cultures (20 mU/mg protein in *A* and 127 mU/mg protein in *B*) to show the percent change. Values are of cell-associated AP after 72 h of PRL treatment \pm SE. WT-PRL, wild-type PRL-treated cells; PP-PRL, PP-PRL-treated cells. This experiment is representative of 8 replicates using either National Institute of Diabetes and Digestive and Kidney Diseases standard PRL or the recombinant proteins in each experiment. In *A*, 100 ng/ml PP-PRL different from 0 ng/ml and all concentrations of WT-PRL, $P < 0.01$. In *B*, 100 ng/ml WT-PRL or PP-PRL different from 0 ng/ml WT-PRL or PP-PRL, $P < 0.016$ and 0.07 , respectively, and 1,000 ng/ml WT-PRL different from 1,000 ng/ml PP-PRL, $P < 0.0007$.

liter concentrations in late pregnancy, would be the normal ligand for fetal bone PRL receptors.

Regardless of what the normal ligand for fetal bone PRL receptors is, our study has demonstrated an adverse effect of elevated maternal PRL on pup bone development, an effect that can be duplicated in the same dose range in vitro. This study therefore reports the first clearly direct effect of PRL on osteoblast function that can be correlated with a reduction in bone formation in vivo.

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REFERENCES

- Adler RA, Evani R, Mansouri A, and Krieg RJ Jr. Relative effects of prolactin excess and estrogen deficiency on bone in rats. *Metabolism* 47: 425–428, 1998.
- Adler RA, Farrell ME, Deiss WP, Krieg RJ, and MacLeod RM. Hypercalciuria in a new rat model of hyperprolactinemia. *Metabolism* 40: 292–296, 1991.
- Baastrup PC, Christiansen C, and Transbol I. Calcium metabolism in schizophrenic patients on long-term neuroleptic therapy. *Neuropsychobiology* 6: 56–59, 1980.
- Bataille-Simoneau N, Gerland K, Chappard D, Basle MF, and Mercier L. Expression of prolactin receptors in human osteosarcoma cells. *Biochem Biophys Res Commun* 229: 323–328, 1996.
- Chen TJ, Kuo CB, Tsai KF, Liu JW, Chen DY, and Walker AM. Development of recombinant human prolactin receptor antagonist by molecular mimicry of the phosphorylated hormone. *Endocrinology* 139: 609–616, 1998.
- Christianson G and Johnson B. A study of the enzyme pattern in children and newborn infants. *Acta Paediatr Scand* 49: 626–631, 1960.
- Clement-Lacroix P, Ormandy C, Lepescheux L, Ammann P, Damotte D, Goffin V, Bouchard B, Amling M, Gaillard-Kelly M, Binart N, Baron R, and Kelly PA. Osteoblasts are a new target for prolactin: analysis of bone formation in prolactin receptor knockout mice. *Endocrinology* 140: 96–105, 1999.
- Coss D, Kuo B, Yang L, Ingleton P, Luben R, and Walker AM. Dissociation of Janus kinase 2 and signal transducer and activator of transcription 5 activation after treatment of Nb2 cells with a molecular mimic of phosphorylated prolactin. *Endocrinology* 140: 5087–5094, 1999.
- Cross NA, Hillman LS, Allen SH, Krause GF, and Vieira NE. Calcium homeostasis and bone metabolism during pregnancy, lactation, and postweaning: a longitudinal study. *Am J Clin Nutr* 61: 514–523, 1995.
- D'Angelo G, Martini JF, Iiri T, Fantl WJ, Martial J, and Weiner RI. 16K human prolactin inhibits vascular endothelial growth factor-induced activation of Ras in capillary endothelial cells. *Mol Endocrinol* 13: 692–704, 1999.
- Deftos LJ. Markers of bone turnover in primary hyperparathyroidism. In: *Basic and Clinical Concepts*. New York, NY: Raven, 1994, p. 485–492.
- Deftos LJ. *Clinical Essentials of Calcium and Skeletal Disorders*. Caldo, OK: Professional Communications, 1998.
- Dickson RA and Glazer WM. Neuroleptic-induced hyperprolactinemia. *Schizophr Res* 35: S75–S86, 1999.
- Duenas Z, Torner L, Corbacho AM, Ochoa A, Gutierrez-Ospina G, Lopez-Barrera F, Barrios FA, Berger P, Martinez de la Escalera G, and Clapp C. Inhibition of rat corneal angiogenesis by 16-kDa prolactin and by endogenous prolactin-like molecules. *Invest Ophthalmol Vis Sci* 40: 2498–2505, 1999.
- Giovane C, Schwabach G, and Weiss E. In vivo labeling of overexpressed recombinant proteins in *E. coli*. *Biotechniques* 22: 796–798, 1997.
- Greenspan SL, Neer RM, Ridgway EC, and Klibanski A. Osteoporosis in men with hyperprolactinemic hypogonadism. *Ann Intern Med* 104: 777–782, 1986.
- Greenspan SL, Oppenheim DS, and Klibanski A. Importance of gonadal steroids to bone mass in men with hyperprolactinemic hypogonadism. *Ann Intern Med* 110: 526–531, 1989.
- Ho TW, Kawaminami M, and Walker AM. Secretion of phosphorylated and non-phosphorylated monomer prolactin isoforms during rat pregnancy and pseudopregnancy. *Endocr J* 1: 435–439, 1993.
- Ho TW, Leong FS, Olaso CH, and Walker AM. Secretion of specific nonphosphorylated and phosphorylated rat prolactin isoforms at different stages of the estrous cycle. *Neuroendocrinology* 58: 160–165, 1993.
- Horseman ND, Zhao W, Montecino-Rodriguez E, Tanaka M, Nakashima K, Engle SJ, Smith F, Markoff E, and Dorshkind K. Defective mammapoiesis, but normal hematopoiesis, in mice with a targeted disruption of the prolactin gene. *EMBO J* 16: 6926–6935, 1997.
- Klibanski A and Greenspan SL. Increase in bone mass after treatment of hyperprolactinemic amenorrhea. *N Engl J Med* 315: 542–546, 1986.
- Koloszar S, Gellen J, and Kovacs L. The value of plasma prolactin level determination in the diagnosis of postmenopausal osteoporosis. *Orv Hetil* 138: 71–73, 1997.
- Krebs NF, Reidinger CJ, Robertson AD, and Brenner M. Bone mineral density changes during lactation: maternal, dietary, and biochemical correlates. *Am J Clin Nutr* 65: 1738–1746, 1997.
- Krishnamra N, Wirunratlanakij Y, and Limlomwongse L. Acute effects of prolactin on passive calcium absorption in the small intestine by in vivo perfusion techniques. *Can J Physiol Pharmacol* 76: 161–168, 1998.
- Krown KA, Wang YF, Ho TW, Kelly PA, and Walker AM. Prolactin isoform 2 as an autocrine growth factor for GH₃ cells. *Endocrinology* 131: 595–602, 1992.
- Lopez JM, Gonzalez G, Reyes V, Campino C, and Diaz S. Bone turnover and density in healthy women during breastfeeding and after weaning. *Osteoporos Int* 6: 153–159, 1996.
- McLean IW and Nakane PK. Periodate-lysine-paraformaldehyde fixative. A new fixation for immunoelectron microscopy. *J Histochem Cytochem* 22: 1077–1083, 1974.
- Melton LJ, Chrischilles EA III, Cooper C, Lane AW, and Riggs BL. Perspective. How many women have osteoporosis? *J Bone Miner Res* 7: 1005–1010, 1992.
- Schlechte JA. Clinical impact of hyperprolactinemia. *J Clin Endocrinol Metab* 9: 359–366, 1995.
- Schlechte J, El-Khoury G, Kathol M, and Walkner L. Forearm and vertebral bone mineral in treated and untreated hyperprolactinemic amenorrhea. *J Clin Endocrinol Metab* 64: 1021–1026, 1987.
- Schlechte JA, Sherman B, and Martin R. Bone density in amenorrheic women with and without hyperprolactinemia. *J Clin Endocrinol Metab* 56: 1120–1123, 1983.
- Sinha YN. Structural variants of prolactin: occurrence and physiological significance. *Endocr Rev* 16: 354–369, 1995.
- Stein GS, Lian JB, Stein JL, Van Wijnen AJ, and Montecino M. Transcriptional control of osteoblast growth. *Physiol Rev* 76: 593–629, 1996.
- Tanaka T, Shiu RPC, Gout PW, Beer CT, Noble RL, and Friesen HG. A new sensitive and specific bioassay for lactogenic hormone: measurement of prolactin and growth hormone in human serum. *J Clin Endocrinol Metab* 51: 1058–1063, 1980.
- Tojo Y, Kurabayashi T, Honda A, Yamamoto Y, Yahata T, Takakuwa K, and Tanaka K. Bone structural and metabolic changes at the end of pregnancy and lactation in rats. *Am J Obstet Gynecol* 178: 180–185, 1998.

36. **Vekemans M and Robyn C.** Influence of age on serum prolactin levels in women and men. *Br Med J* 4: 738-739, 1975.
37. **Wang YF, Liu JW, Mamidi M, and Walker AM.** Identification of the major site of rat prolactin phosphorylation as serine 177. *J Biol Chem* 271: 2462-2469, 1996.
38. **Wang YF and Walker AM.** Dephosphorylation of standard prolactin produces a more biologically active molecule: evidence for antagonism between nonphosphorylated and phosphorylated prolactin in the stimulation of Nb2 cell proliferation. *Endocrinology* 133: 2156-2160, 1993.

