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Amphiregulin Is a Novel Growth Factor Involved in Normal Bone Development and in the Cellular Response to Parathyroid Hormone Stimulation*[S]

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Parathyroid hormone (PTH) is the major mediator of calcium homeostasis and bone remodeling and is now known to be an effective drug for osteoporosis treatment. Yet the mechanisms responsible for its functions in bone are largely unknown. Here we report that the expression of amphiregulin (AR), a member of the epidermal growth factor (EGF) family, is rapidly and highly up-regulated by PTH in several osteoblastic cell lines and bone tissues. Other osteotropic hormones (1α,25-dihydroxyvitamin D_3 and prostaglandin E_2) also strongly stimulate AR expression. We found all EGF-like ligands and their receptors are expressed in osteoblasts, but AR is the only member that is highly regulated by PTH. Functional studies demonstrated that although AR is a potent growth factor for preosteoblasts, it completely inhibits further differentiation. AR also strongly and quickly stimulated Akt and ERK phosphorylation and c-*fos* and c-*jun* expression in an EGF receptor-dependent manner. Moreover, AR null mice displayed significantly less tibial trabecular bone than wild-type mice. Taken together, we have identified a novel growth factor that is PTH-regulated and appears to have an important role in bone metabolism.

Parathyroid hormone (PTH),[1] an 84-amino acid polypeptide hormone secreted by the parathyroid glands, plays an essential role in calcium and phosphate homeostasis and bone remodeling. Paradoxically, PTH has two opposite effects on bone if administered in different ways. Although continuous administration causes bone loss, intermittent injection increases bone volume and bone density (1–3). To date, PTH is the only osteoporosis treatment that promotes bone formation. The primary target of PTH in bone is the osteoblast, which expresses a single PTH receptor (PTH1R), a G protein-coupled seven-transmembrane domain receptor (4). Upon PTH binding, both protein kinase A and protein kinase C signaling pathways are activated, and consequently the expression of many genes are altered (5). Recently, we performed microarray experiments to study the gene expression profile changes in PTH-treated UMR 106-01 cells, a rat osteoblastic osteosarcoma cell line (6). A total of 125 known genes were identified as PTH-regulated. Amphiregulin (AR), a member of the epidermal growth factor (EGF) family, was one of those genes.

The EGF family members are divided into two classes (7). The first class, also known as EGF-like ligands, all bind to the EGF receptor (EGFR/ErbB1), which includes EGF, AR, and transforming growth factor alpha (TGF-α), which bind to EGFR exclusively, and heparin-binding EGF (HB-EGF), betacellulin, and epiregulin, which bind both EGFR and ErbB4. The second class, collectively termed neuregulins, bind directly to the receptors ErbB3 and/or ErbB4. The EGFR (ErbB1) is a receptor tyrosine kinase and lies at the beginning of a complex signal transduction cascade that modulates cell proliferation, survival, adhesion, migration, and differentiation (8). Upon ligand binding, the EGFR undergoes dimerization and phosphorylation at tyrosine residues in its intracellular domain, thus activating several important cellular signal transduction pathways. The major signaling routes are the Ras-Raf-mitogen-activated protein kinase (9) and phosphatidylinositol 3-kinase-Akt pathways (10). It is now known that ErbB2 is the preferred coreceptor for the EGFR, and the heterodimeric receptor complex signals more potently than a homodimer of the EGFR (8).

AR was first isolated from conditioned medium of MCF-7 human breast carcinoma cells exposed to phorbol 12-myristate 13-acetate (11, 12). AR is bifunctional because it inhibits the growth of many human tumor cells but stimulates the proliferation of other cells such as normal fibroblasts and keratinocytes (13, 14). Similar to EGF, AR is produced as a precursor transmembrane protein that undergoes proteolytic cleavage to yield the mature protein. So far, there have been no reports of AR production or function in bone. However, EGF has been shown to have several effects on bone cells or on bone: it stimulates osteoblast proliferation (15), decreases alkaline phosphatase (16) and collagen production (17), changes bone nodule formation (18), and yet, has catabolic effects on bone

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[S] The on-line version of this article (available at http://www.jbc.org) contains a supplemental table and a figure.

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[1] The abbreviations used are: PTH, parathyroid hormone; AR, amphiregulin; BMP, bone morphogenetic protein; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; HB-EGF, heparin-binding epidermal growth factor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; MEM, Eagle's minimal essential medium; microCT, micro-computed tomography; 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; PGE_2, prostaglandin E_2; RANKL, receptor activator of nuclear factor-κB ligand; RT, reverse transcription; TGF-α, transforming growth factor-α.

(19), *i.e.* similar to AR, bifunctional effects. Nevertheless, the production, detailed mechanism, and the significance of the EGF signaling pathway in bone are not well understood.

In this paper, we demonstrate that AR is a general immediate response gene for PTH action in bone. It has profound effects on osteoblasts because it strongly stimulates the growth of preosteoblasts while it inhibits the differentiation and mineralization of mature osteoblasts. Its role in normal bone development and in PTH function will be discussed.

EXPERIMENTAL PROCEDURES

Chemicals—Synthetic human PTH(1–38) was purchased from Bachem (Torrance, CA). Compound 32, compound 56, wortmannin, and 1α,25-dihydroxyvitamin D_3 (1α,25-$(OH)_2D_3$) were purchased from Calbiochem. Rat PTH(1–34), recombinant human AR, human EGF, prostaglandin E_2 (PGE_2), and cycloheximide were obtained from Sigma. Antibodies for ERK1/2 and phospho-ERK1/2 were obtained from Santa Cruz (Santa Cruz, CA). Antibodies for Akt and phospho-Akt were obtained from Cell Signaling (Beverly, MA).

Cell Culture—UMR 106-01 cells were maintained in Eagle's minimal essential medium (MEM) supplemented with 5% (v/v) fetal bovine serum. For experiments, the cells were seeded in 100-mm dishes at 1.2×10^4 cells/cm^2 in the same medium overnight and then switched to serum-free MEM for 2 days before the addition of appropriate agents. Rat primary calvarial osteoblastic cells were obtained from neonatal rat calvariae by sequential digestions with collagenase and trypsin as described previously (20). Cells were cultured in MEM containing 10% fetal bovine serum until confluence at day 7. Then the medium was switched to differentiation medium (BGJb medium containing 10% fetal bovine serum, 10 mM β-glycerophosphate, and 50 μg/ml ascorbic acid). MC3T3 cells were maintained in MEM plus 10% (v/v) fetal bovine serum. After confluence, cells were cultured in the same differentiation medium. Before PTH treatment, both types of cells were serum starved for 1 day.

In Vivo Injection of PTH—Four-week-old male Sprague-Dawley rats, about 75 g, were purchased from Hilltop (Scottdale, PA). Rats were injected subcutaneously with vehicle (0.9% saline solution) or hPTH(1–38) (8 μg/100 g) and euthanized using CO_2 at 0.5, 1, 4, or 8 h after injection. The primary spongiosa samples from distal femur were harvested as described previously (21). The animal protocols were approved by Robert Wood Johnson Medical School Institutional Animal Care and Use Committee.

Analysis of mRNA Abundance by Real Time RT-PCR—Cells or tissues were harvested at the indicated time points after hormone treatments. Total RNA was isolated using Tri Reagent (Sigma) followed with an RNeasy kit (Qiagen). A TaqMan Reverse Transcription kit (Applied Biosystems) was used to reverse transcribe mRNA into cDNA. Following this, PCR was performed on Opticon (MJ Research) using a SYBR Green PCR Core kit (Applied Biosystems). Each analysis was performed two or three times with independent sets of cells or tissues from hormone treatment to RT-PCR to obtain the mean value ± S.E. shown in the figures. The primers used for the RT-PCR are summarized in the supplemental table. For UMR 106-01 and MC3T3 cells, rat and mouse β-actin was used as an internal control. For femoral samples and primary osteoblastic cells, rat glyceraldehyde-3-phosphate dehydrogenase was used as an internal control.

DNA Synthesis Measurement—Incorporation of [^{3}H]thymidine into macromolecules was used to assess DNA synthesis. Rat primary calvarial osteoblastic cells were seeded at 4×10^4 cells/well in 24-well plates. Cells were then serum starved for 1 day before being treated with appropriate agents overnight. Cells were incubated with [^{3}H]thymidine (1 μCi/ml) for the final 3 h of incubation. DNA containing incorporated radiolabel was collected onto glass fiber filters using a semiautomatic cell harvester (Skatron), whereas unincorporated [^{3}H]thymidine was removed by exhaustive water elution. The incorporation was assayed by liquid scintillation counting. Experiments were performed with three samples/group and repeated twice. Statistical analysis was performed by Student's *t* test.

Cell Counting—Rat primary calvarial osteoblastic cells were seeded into 6-well plates at 6.2×10^4 cells/well. On the next day (day 1), the medium was switched to serum-free MEM. On days 2 and day 4, fresh MEM containing different amounts of AR was added. The cells were detached from the plates with trypsin/EDTA, and the cell numbers were counted using a hemacytometer on day 6. Experiments were performed with three samples/group and repeated twice. Statistical analysis was performed by Student's *t* test.

Cell Cycle Analysis—Rat primary calvarial osteoblastic cells were seeded into 100-mm dishes at 5×10^5 cells/dish. Cells were serum starved the next day for 1 day before treatment with AR or EGFR inhibitors overnight. One million cells were trypsinized, washed once with phosphate-buffered saline, and fixed in 70% ethanol for at least 1 h on ice. Fixed cells were washed with phosphate-buffered saline and incubated with propidium iodine solution containing ribonuclease A. The cell cycle of stained cells was analyzed by Beckman Coulter XL.

Alizarin Red Staining and Alkaline Phosphatase Activity Staining—For alizarin red staining, cells were fixed in cold 70% ethanol for 1 h and stained with 40 mM alizarin red for 10 min. Then cells were washed five times for 20 min each with water and then photographed. For alkaline phosphatase activity staining, cells were stained with the 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium liquid substrate system (Sigma) for 10 min, and the reaction was stopped by washing with water.

Immunoblotting—Preparation of cell lysates and Western blot analyses were performed as described previously (22).

Microcomputed Tomography (microCT) Measurement of AR Null and Wild-type Mice—AR null mice (B6/129) were described previously (23). A breeding colony was established using heterozygous mice to provide littermate controls. The femora and tibiae were harvested from 4-week-old AR null mice and their wild-type littermates and were subjected to microCT analyses. The animal protocol was approved by Robert Wood Johnson Medical School Institutional Animal Care and Use Committee. The trabecular architecture of the proximal tibial metaphysis was measured using a microCT 20 (Scanco Medical AG, Bassersdorf, Switzerland). After an initial scout scan, a total of 100 slices with an increment of 9 μm was obtained on each bone sample starting 0.3 mm below the growth plate. The area for analysis was outlined within the trabecular compartment, excluding the cortical and subcortical bone. A three-dimensional analysis was done to determine bone volume, trabecular number, trabecular thickness, trabecular separation, and connectivity density. Cortical bone parameters were measured on the mid shaft of the femur. Ten slices of the diaphysis were made, and the same segmentation parameters were used for analysis. The periosteal surface was outlined, and a two-dimensional analysis was done to determine bone volume and periosteal perimeter. The endocortical surface was outlined and the analysis repeated to determine endocortical perimeter. The mean cortical thickness was determined by distance measurements at eight different points on the cortical slice. Data were analyzed by Dunnett's one-way analysis of variance using SigmaStat software (SPSS Sciences, Chicago).

RESULTS

PTH Stimulates AR Expression Both in Vitro and in Vivo—Recently we identified 125 PTH-regulated genes in UMR 106-01 cells using microarray technology (6). AR (also known as schwannoma-derived growth factor) mRNA was shown to be stimulated more than 2-fold after 4 h and 12 h with 10^{-8} M rat PTH(1–34) treatment. Real time RT-PCR quantitatively proved that AR is indeed an early rPTH-responsive gene in this osteoblastic cell line (Fig. 1*A*). The mRNA expression of AR dramatically increased about 23-fold after 1 h of 10^{-8} M rPTH(1–34) treatment. This stimulation then decreased, as the fold change was about 11-fold at 4 h of PTH treatment, but was maintained at a significant level even at 12 h (8-fold). As shown in Fig. 1*B*, the stimulation of AR expression is PTH dose-dependent. Significant stimulation was observed at 10^{-10} M rPTH(1–34) (4-fold) and higher concentrations (15-fold at 10^{-9} M and 23-fold at 10^{-8} M). No obvious PTH effect was detected at 10^{-11} and 10^{-12} M. The real time RT-PCR product was sequenced, and the result completely matches the rat AR cDNA sequence (data not shown).

To study whether AR is a ubiquitous PTH target gene, we tested two other PTH-responsive osteoblastic cell preparations: rat calvarial primary osteoblastic cells (Fig. 1*C*) and mouse MC3T3 cells (Fig. 1*D*). The primary osteoblastic cells undergo proliferation, differentiation, and mineralization phases *in vitro*. Rat PTH(1–34) strongly induced AR expression in those cells, with the highest induction (23-fold) in the mineralization phase and the lowest induction (5-fold) in the proliferation phase. In all phases the strongest induction of AR occurred at

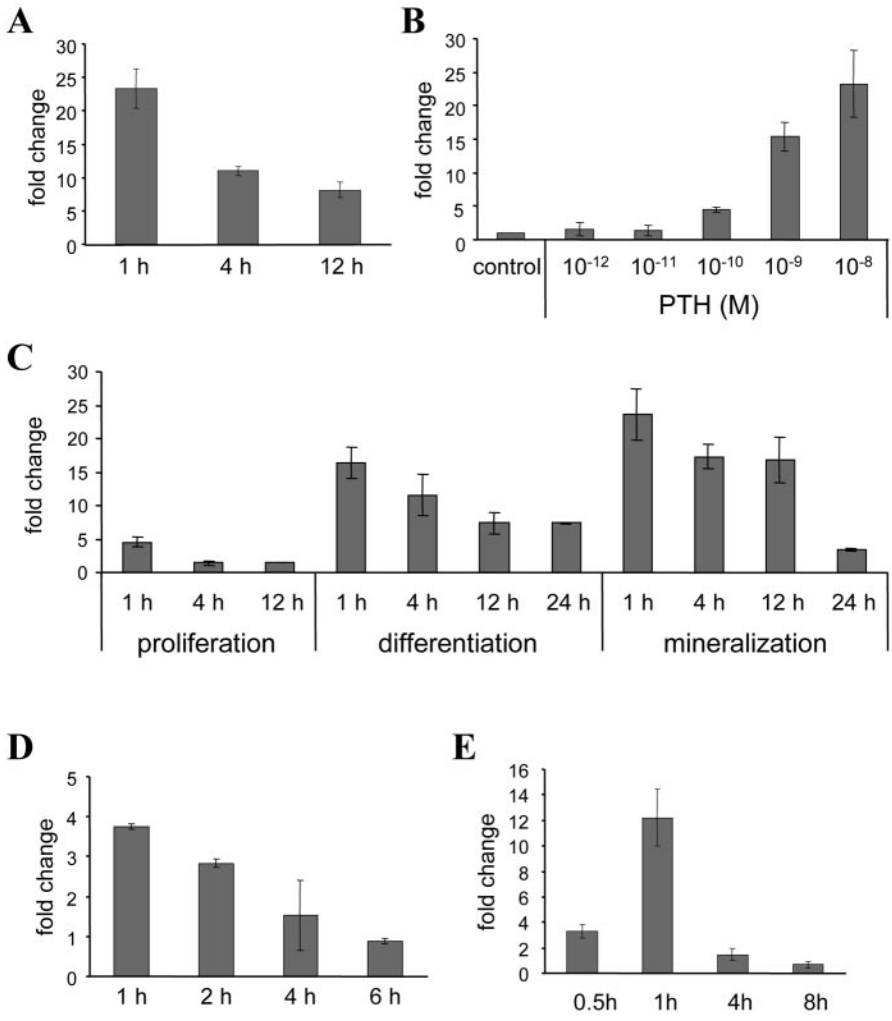

FIG. 1. **PTH rapidly stimulates AR mRNA expression in bone and osteoblastic cells.** Real time RT-PCR analysis was used to assess the fold change in AR mRNA after PTH treatment in UMR 106-01 cells (*A* and *B*); primary calvarial osteoblastic cells at day 6 (proliferation phase), day 14 (differentiation phase), and day 21 (mineralization phase) in culture (*C*); MC3T3 cells at day 8 after switching to differentiation medium (*D*); and rat distal femur metaphyses (*E*). In *A*, *C*, and *D*, the concentration of PTH used was 10^{-8} M. *B*, dose response of AR expression after 1 h of PTH treatment in UMR 106-01 cells. Except for the *in vivo* injection experiment (*E*, human PTH(1–38), 8 μg/100 g)), the PTH used was rat PTH(1–34).

1 h. It is worth noting that the basal expression of AR did not change among three phases (data not shown). In MC3T3 cells, a significant increase in AR expression was also observed at early time points (4-fold at 1 h and 3-fold at 2 h) of PTH treatment.

It is known that intermittent injection of PTH into young rats increases bone and mineral density (24). Expression of several genes, such as c-*fos, IL-6*, and *myc*, which are important for PTH actions in bone, have been found to be regulated by acute PTH injection in rat osteoblast-enriched femoral metaphyseal primary spongiosa (21). To investigate whether PTH regulates AR *in vivo*, we injected human PTH(1–38) (8 μg/100 g) into young male rats and harvested the femoral metaphyses at various time points. As shown in Fig. 1*E*, the level of AR mRNA was elevated dramatically to about 12-fold after 1 h of PTH injection and decreased to about 2-fold after 4 h, demonstrating that amphiregulin is actually regulated by PTH *in vivo* in a very rapid fashion similar to that seen in culture.

PTH Induction of AR Is a Primary Response—To determine whether PTH induction of AR requires new protein synthesis, UMR 106-01 cells were treated with 10^{-8} M rPTH(1–34) in the presence or the absence of cycloheximide, a reagent that inhibits its protein synthesis. Fig. 2 shows that cycloheximide had no effect on the PTH induction of AR because in cycloheximide-pretreated UMR cells PTH increased AR mRNA expression 16-fold, similar to the 18-fold increase in the ethanol-pretreated cells. This result suggests that one or more existing transcription factors that are post-transcriptionally regulated by PTH are likely responsible for the AR induction. The fact

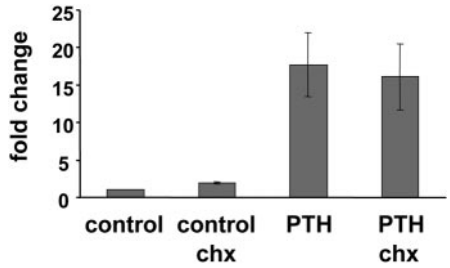

FIG. 2. **The stimulation of AR expression by PTH is a primary response.** UMR 106-01 cells were treated with ethanol (0.1% v/v) or cycloheximide (*chx*, 30 μg/ml) for 1 h. Cells were then treated with 10^{-8} M rat PTH(1–34) for another 1 h before harvesting RNA for real time RT-PCR analyses. The AR mRNA level in the cells treated only with ethanol was set as 1.

that there was no superinduction of AR in the cycloheximide-pretreated cells also tends to exclude the possibility that PTH regulates AR by stabilizing its mRNA. In addition, in a separate study, we have shown that PTH stimulates activity of the AR promoter in a cAMP-response element-binding protein-dependent manner.[2]

AR Is a Target Gene for Other Osteotropic Hormones—In addition to PTH, 1α,25-$(OH)_2D_3$ and PGE_2 are other agents that play important roles in bone remodeling via osteoblasts. Next, we investigated whether these hormones could also regulate AR expression. Rat primary osteoblastic cells were serum

[2] L. Qin and N. C. Partridge, manuscript submitted.

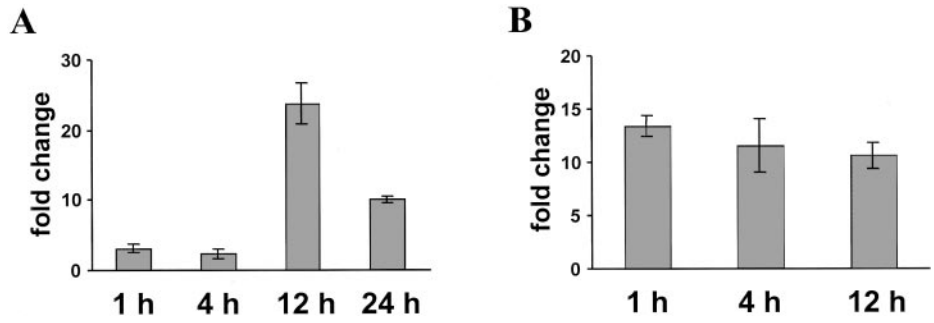

FIG. 3. **1α,25-$(OH)_2D_3$ (*A*) and PGE_2 (*B*) stimulate AR expression in primary osteoblastic cells.** Rat calvarial primary osteoblastic cells at day 13 in culture (differentiation phase) were serum starved for 1 day and then treated with control, 10^{-8} M 1α,25-$(OH)_2D_3$, or 10^{-6} M PGE_2 for the indicated time points before RNA purification for real time RT-PCR analysis to determine the fold changes in AR mRNA levels.

starved for 1 day then treated with either 1α,25-$(OH)_2D_3$ or PGE_2 for various time periods. RNAs were harvested to analyze the AR mRNA level by real time RT-PCR (Fig. 3). Interestingly, both hormones have strong effects on AR induction, albeit the expression patterns are different from those induced by PTH. The AR induction by 1α,25-$(OH)_2D_3$ peaked to 24-fold at 12 h but was low at earlier time points (3-fold at 1 h and 2-fold at 4 h) (Fig. 3*A*). Note that AR has been recently found to be up-regulated by 1α,25-$(OH)_2D_3$ in squamous cell and breast carcinoma cells in a similar time-dependent pattern (25). PGE_2 quickly and continuously elevated the expression of AR, resulting in 13-, 12-, and 11-fold increases at 1, 4, and 12 h, respectively (Fig. 3*B*).

Expression of EGF-like Ligands and Their Receptors in Osteoblastic Cells—To date, there is no report of the expression of EGF family ligands and receptors in osteoblastic cells. Because we have demonstrated that AR is regulated by several osteotropic hormones, it became important to determine the profile of other EGF ligands and their receptors in osteoblastic cells. Using RT-PCR, we were able to detect the expression of EGF, TGF-α, HB-EGF, betacellulin, epiregulin, EGFR, and ErbB2 in both UMR 106-01 and rat calvarial primary osteoblastic cells (data not shown). Furthermore, we investigated whether expression of those ligands and receptors is regulated by PTH. Real time RT-PCR revealed that although the expression of EGFR, ErbB2, EGF, epiregulin, and betacellulin was unaffected by PTH treatment in UMR 106-01 cells, TGF-α and HB-EGF expression showed more than 2-fold increases at 12 h and 1 h of 10^{-8} M rPTH(1–34) treatment, respectively (supplementary figure). Those results are consistent with our previous microarray analysis results in which the expression of TGF-α showed a 2.5-fold increase at 12 h of PTH treatment, and the expression of HB-EGF was not detected under any conditions. Nevertheless, these fold inductions by PTH are much lower than that of AR as shown above (23-fold at 1 h), suggesting that PTH mainly regulates AR but not other EGF-like ligands and receptors in bone.

AR Has Strong Proliferative Effects on Osteoblastic Cells, and the EGFR Signaling Pathway Is Important for Normal Growth of Osteoblastic Cells—AR is a bifunctional growth factor because it stimulates proliferation in some cells and inhibits its growth in others (13, 14). Next we investigated the effect of AR on the proliferation of osteoblastic cells. Rat calvarial osteoblastic cells in the proliferation phase were used for all of the following experiments. Those cells are considered as committed preosteoblasts because they do not express differentiation-specific markers. These primary cells were obtained from calvariae of neonatal rats and cultured in medium with various amounts of AR for 4 days. Fig. 4A clearly indicates that the cell number/well increased as the medium AR concentration increased. Even in the presence of the lowest AR concentration (5 ng/ml), a significant increase in cell number (35%) was observed ($p < 0.001$). [^{3}H]Thymidine incorporation into DNA showed a similar result (Fig. 4*B*). There was a concentration-dependent increase in DNA synthesis of rat primary osteoblastic cells. Specifically, 50 ng/ml (5 nM) AR produced a 2.7-fold increase in [^{3}H]thymidine incorporation over untreated cells. This effect was slightly higher than 25 ng/ml (4 nM) EGF and comparable with 10% fetal bovine serum.

AR binds to and signals through the EGFR in breast cancer cell lines, fibroblasts, and keratinocytes. The addition of EGFR-specific inhibitors, 1 μM compound 32 (C32) or compound 56 (C56), not only eliminated the proliferative effect of AR on rat primary osteoblastic cells, but decreased incorporation of [^{3}H]thymidine to almost 50% of control cells (Fig. 4*C*, *left part*), suggesting that this effect of AR was through the EGFR. Similar to a previous report that PTH inhibits the proliferation of osteoblastic cells (26, 27), 10^{-8} M rPTH(1–34) inhibited DNA synthesis by 50% in primary osteoblastic cells. The addition of C32 or C56 had an additive effect with PTH, further inhibiting [^{3}H]thymidine incorporation to only 25% of control (Fig. 4*C*, *middle part*). Moreover, 1 μM C32 or C56 on its own strongly inhibited [^{3}H]thymidine incorporation to about 50% of control cells (Fig. 4*C*, *right part*). Note these cells have been serum-depleted for 1 day before addition of inhibitors. This result clearly indicates that EGF-like ligands, including AR, produced by osteoblastic cells could have autocrine or paracrine effects on normal growth of osteoblastic cells through EGFR signaling pathways. Similar results were also observed with UMR 106-01 cells (data not shown).

Flow cytometry experiments were performed to study the effect of AR on the regulation of the cell cycle of primary osteoblastic cells. As shown in Table I, after serum starvation, 80.6% of cells were in G_1 phase of the cell cycle. AR treatment decreased cells in G_1 to about 68.5% and increased cells in both S phase and G_2+M phase. Simultaneous addition of C32 completely abolished the effect of AR on cell cycle regulation. However, C32 itself showed no effect on cell cycle regulation under these conditions.

In summary, the above experiments demonstrate that AR has potent stimulatory effects on the proliferation of preosteoblastic cells. This is consistent with previous findings that EGF stimulates the growth of osteoblastic cell lines *in vitro* (15).

AR Prevents Osteoblastic Cells from Differentiation and Mineralization—Next we studied the effect of AR on osteoblast differentiation. *In vitro*, rat calvarial primary osteoblastic cells proliferate and usually reach confluence at day 7 after seeding. Then, changing the medium to BGJb containing ascorbic acid and β-glycerophosphate induces these cells to differentiate and mineralize. Around day 20, mineralized bone nodules prevail in the culture and are easily observed under the microscope or by alizarin red staining (Fig. 5*B*, *control panel*). However, addition of AR in the medium from either day 1 (AR_d1) or day 7 (AR_d7) to day 20 completely inhibited differentiation, as no bone nodules could be seen in the culture (Fig. 5*B*, *right two panels*). Staining the cultures to detect alkaline phosphatase activity, a differentiation marker, indicates that AR-treated cells expressed very little alkaline phosphatase (Fig. 5*B*). RNAs were harvested from

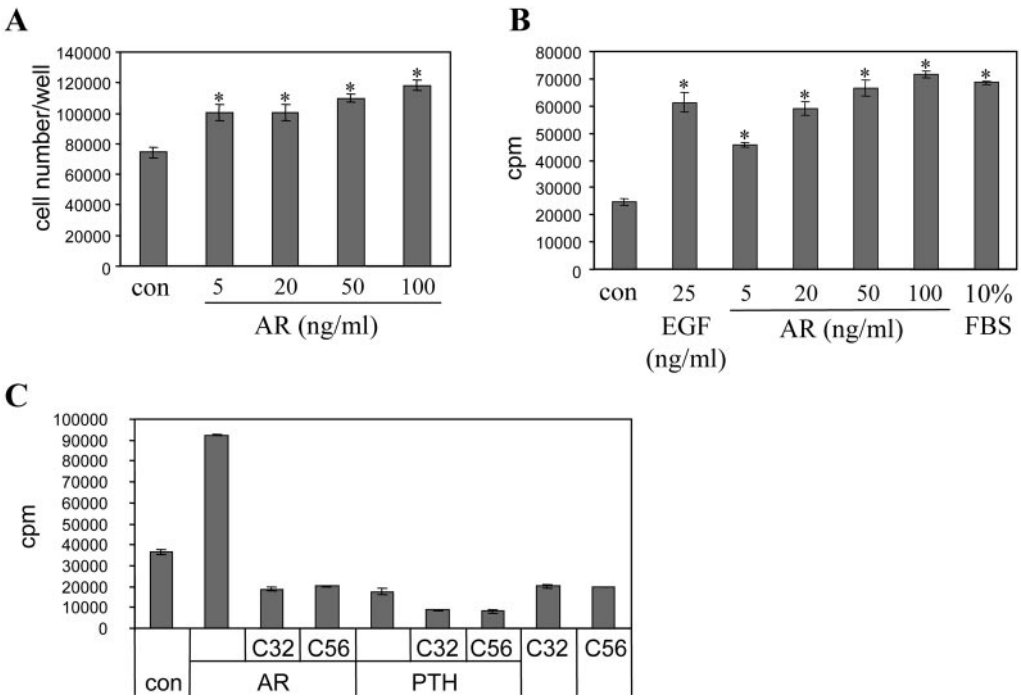

FIG. 4. **AR is a potent growth factor for osteoblasts.** Cell counts (*A*) and [^{3}H]thymidine incorporation (*B*) were performed with rat primary osteoblastic cells in proliferation stage to demonstrate that AR stimulates proliferation (for details, see "Experimental Procedures"). In *A* and *B*, * represents $p < 0.001$. *C*, the proliferative effect of AR requires EGFR. After 1 day of serum starvation, primary osteoblastic cells were pretreated with the following for 1 h: dimethyl sulfoxide (0.1% v/v), 1 μM C32, or 1 μM C56. Cells were then treated with the following overnight: 50 ng/ml AR, 10^{-8} M PTH(1–34), or control medium. [^{3}H]Thymidine was added for the last 3 h of the assay. The incorporated [^{3}H]thymidine amounts in each well were then measured.

TABLE I
Effect of AR on the cell cycle progression of rat primary osteoblastic cells

Treatment group	Cell percentage		
	G_1	S	G_2 + M
Control	80.6	11.9	7.1
50 ng/ml AR	68.5	16.3	16.3
50 ng/ml AR + 1 μM C32	79.0	10.5	11.3
1 μM C32	78.4	11.4	9.2

day 20 cultures to analyze the expression of several bone markers. As shown in Fig. 5*C*, the mRNA levels of MMP-13, alkaline phosphatase, osteocalcin and osteonectin were decreased about 3-, 7-, 50-, and 2-fold, respectively, in AR-treated osteoblastic cells compared with untreated cells. Note that the difference in the starting time of AR treatment (AR_d1 *versus* AR_d7) had no effect on preventing differentiation.

AR Stimulates Akt Phosphorylation, ERK Phosphorylation, and c-fos and c-jun Expression in Osteoblastic Cells—The phosphorylated EGFR transmits signals through a variety of intracellular substrates, depending partly on the cell type and the bound ligand. To study the intracellular events subsequent to AR-binding osteoblastic cells, we analyzed Akt and ERK phosphorylation, two major signal pathways activated by the EGFR.

In UMR 106-01 and two different phases (proliferation and differentiation phases) of primary osteoblastic cells, addition of AR in the medium quickly induced the phosphorylation of Akt, the substrate for phosphatidylinositol 3-kinase (Fig. 6*A*, *lanes 2*, *7*, and *11*). Note that there were very low basal levels of phosphorylated Akt in both cell preparations (*lanes 1*, *6*, and *10*). The addition of the phosphatidylinositol 3-kinase inhibitor wortmannin prior to AR treatment absolutely eliminated the Akt phosphorylation even at very low concentrations (30 nM; *lanes 3*, *4*, and *5*). The ability of AR to induce Akt phosphorylation is comparable with that of EGF because 50 ng/ml (5 nM) AR and 40 ng/ml (7 nM) EGF phosphorylated Akt similarly (compare *lanes 7* and *8*, *lanes 11* and *12*). The EGFR was required for Akt phosphorylation by AR because its inhibitor C32 abolished the phosphorylation (*lanes 9* and *13*).

The same study was performed to detect ERK phosphorylation by AR, and similar results were observed (Fig. 6*B*). AR quickly induced ERK phosphorylation (*lanes 2*, *6*, and *10*) similarly to EGF (*lanes 7* and *11*) in osteoblastic cells. This phosphorylation was mediated by both MEK and EGFR because the MEK inhibitor PD98059 (*lanes 3* and *4*) and the EGFR inhibitor C32 (*lanes 8* and *12*) eliminated ERK phosphorylation by AR.

Because phosphorylated ERK is known to translocate into the nucleus and activate the transcription of c-*fos* and c-*jun* (28), we next investigated whether AR could stimulate c-*fos* and c-*jun* expression. As shown in Fig. 6*C*, AR quickly and transiently increased c-*fos* (30-fold) and c-*jun* (about 12-fold) mRNAs with a peak between 15 and 30 min in UMR 106-01 cells. This stimulation required a functional EGFR because C32 or C56 completely eliminated this effect (Fig. 6*D*). Similar results were obtained with primary osteoblastic cells (data not shown).

AR Null Mice Have Less Trabecular Bone Than Wild-type Mice—Mice lacking functional AR reveal a critical role for AR in ductal morphogenesis in the developing mammary gland, but initial experiments showed no growth abnormalities in these mice (23). We harvested tibiae and femurs from 4-week old AR null mice and their wild-type siblings to study their skeletal parameters. MicroCT measurement revealed that AR null mice have significantly less bone in the trabecular bone compartment in the proximal tibia because parameters such as percent bone volume, trabecular number and thickness, and connectivity density are decreased significantly by 26, 17, 8, and 36%, respectively, in the null mice, whereas trabecular separation is increased significantly by 21% ($p < 0.05$) (Table II). These results strongly suggest that AR plays a critical role

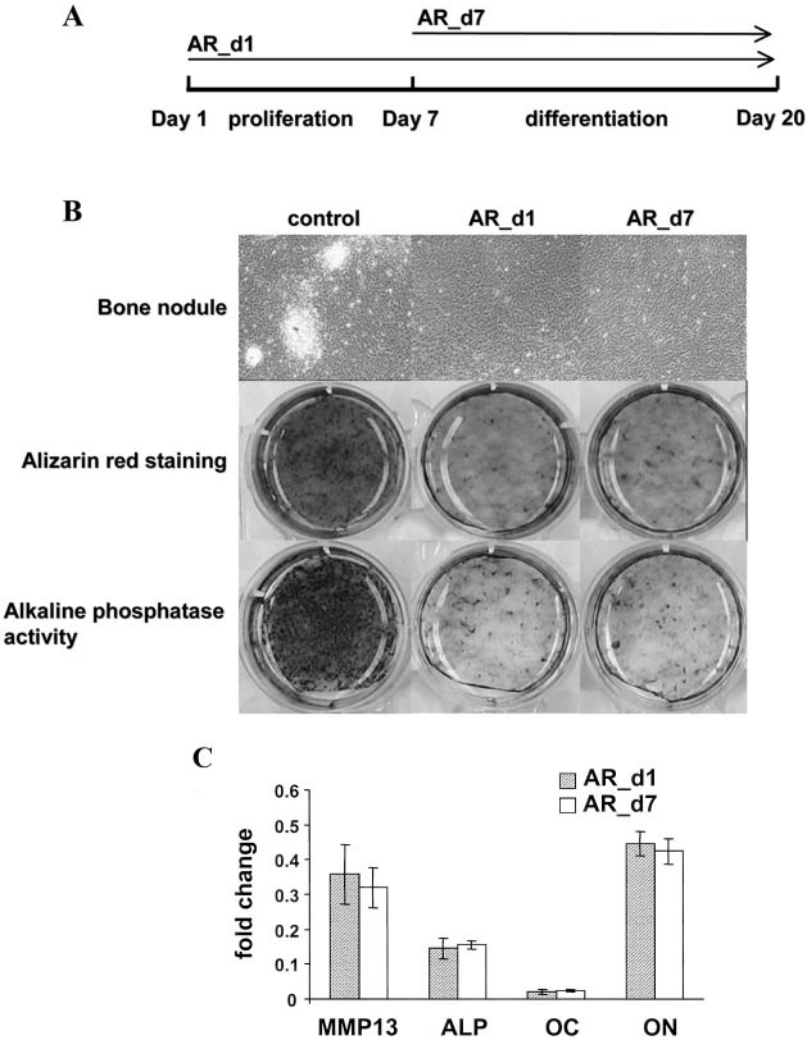

FIG. 5. **AR inhibits osteoblast differentiation and mineralization.** *A*, a schematic diagram showing how primary calvarial osteoblastic cells were treated with AR. The cells were seeded on day 0 and usually confluent on day 7, when the media were changed from 10% MEM to 10% BGJb containing ascorbic acid and β-glycerophosphate. For AR_d1, 50 ng/ml AR was added to the medium from day 1 to day 20. For AR_d7, 50 ng/ml AR was added to the medium from day 7 to day 20. The media were changed every 2 days, and AR was added with the new medium. *B*, on day 20, the cells of each treatment were observed under microscope ($\times$100) (*top panels*) or photographed to show alizarin red staining (*middle panels*) or alkaline phosphatase activity (*bottom panels*). *C*, on day 20, RNA were extracted from cells and subjected to real time RT-PCR to analyze the fold change in several bone markers in the AR-treated samples (*MMP-13*, matrix metalloproteinase-13; *ALP*, alkaline phosphatase; *OC*, osteocalcin; *ON*, osteonectin). The expression level of each bone marker in the untreated sample was set as 1.

in bone metabolism. Meanwhile, microCT studies with cortical bone in the mid shaft femur revealed that there are no differences in skeletal parameters of this area of bone between AR null and wild-type mice (Table III). There was no significant difference in the body weight between these two groups (data not shown).

DISCUSSION

In the present study, we have provided both *in vitro* and *in vivo* evidence demonstrating that AR, an EGF-like ligand, is expressed differentially in osteoblasts after PTH treatment. PTH rapidly stimulated the expression of AR mRNA, reminiscent of a classical immediate early response. In addition, AR expression was induced by other osteotropic agents, such as 1α,25-$(OH)_2D_3$ and PGE_2. This is the first report suggesting an important role for AR in bone development and metabolism and implicates it as the major EGF-like ligand regulated by osteotropic hormones in bone.

Our investigations with primary osteoblastic cell cultures indicate that AR stimulates preosteoblast proliferation but inhibits its further differentiation. The overall effect could be to expand the pool of preosteoblasts and limit the number of mature osteoblasts. According to our current data, a model is proposed to depict the role of AR in PTH action in bone (Fig. 7). The pluripotent mesenchymal stem cells residing in the bone marrow give rise to osteoblastic precursors in addition to differentiation into chondrocytes, adipocytes, myocytes, and endothelial cells. Within an appropriate environment, the committed preosteoblast further differentiates into the mature osteoblast, a cell that deposits bone matrix proteins and finally becomes the osteocyte embedded in mineralized bone. PTH functions mainly on the mature osteoblast and osteocyte because the preosteoblast has a low response to PTH (Fig. 1C). PTH treatment appears to facilitate the final differentiation of the osteoblast and inhibit its apoptosis, thus increasing its bone formation activities. Meanwhile, PTH-treated osteoblasts produce various cytokines and growth factors influencing its surrounding environment. Because the bone marrow is a very heterogeneous and hence complicated system, the targeted cells could include mesenchymal stem cells, hematopoietic stem cells, various stages of precursors for osteoclasts, osteoblasts, chondrocytes, adipocytes, and myocytes and mature osteoclasts and osteoblasts. A classic example is that PTH-treated osteoblasts increase RANKL expression to trigger osteoclastogenesis, thus stimulating bone resorption (29). A recent interesting finding demonstrates that PTH injection into mice expands the hematopoietic stem cell pool through increasing expression of the Notch ligand Jagged 1 on the osteoblast membrane (30). Our microarray studies reveal that more than 10 paracrine or autocrine factors are regulated in osteoblastic cells by PTH treatment (6). Therefore, it seems that PTH has great power to manipulate the microenvironment in bone through its actions on the osteoblast. Some of the manipulations, such as increasing expression of RANKL and Jagged1, may not be directly involved in or may even be contradictory to

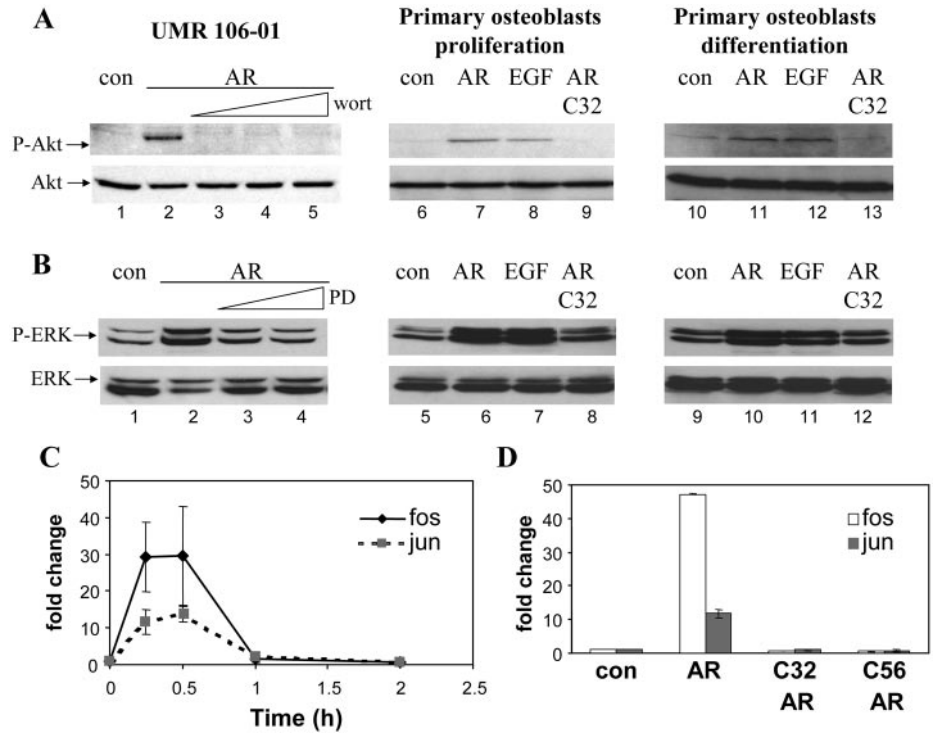

FIG. 6. **AR activates Akt phosphorylation (*A*), ERK phosphorylation (*B*), and c-*fos* and c-*jun* expression (*C* and *D*) in osteoblastic cells.** UMR 106-01 cells (*lanes 1–5* in *A* and *lanes 1–4* in *B*), primary osteoblastic cells at day 6 (*lanes 6–9* in *A* and *lanes 5–8* in *B*) and at day 14 (*lanes 10–13* in *A* and *lanes 9–12* in *B*) were serum starved for 1 day and pretreated with the following for 1 h: dimethyl sulfoxide (0.1% v/v) (*lanes 1*, *2*, *6*, *7*, *8*, *10*, *11*, and *12* in *A* and *lanes 1*, *2*, *5*, *6*, *7*, *9*, *10*, and *11* in *B*), wortmannin (*wort*; *A*, *lane 3*: 0.03 μM; *lane 4*: 0.3 μM; *lane 5*: 3 μM), 1 μM C32 (*lanes 9* and *13* in *A*; *lanes 8* and *12* in *B*), PD98059 (*PD*; *B*, *lane 3*: 50 μM; *lane 4*: 100 μM). Cells were then treated with the following for 10 min before harvesting cell lysates for Western blot analysis: 50 ng/ml AR (*lanes 2*, *3*, *4*, *5*, *7*, *9*, *11*, and *13* in *A*; *lanes 2*, *3*, *4*, *6*, *8*, *10*, and *12* in *B*), 40 ng/ml EGF (*lanes 8* and *12* in *A*; *lanes 7* and *11* in *B*). *A*, immunoblot analysis using antiphosphorylated Akt antibody (*top panel*) and anti-Akt antibody (*bottom panel*). *B*, immunoblot analysis using antiphosphorylated ERK antibody (*top panel*) and anti-ERK antibody (*bottom panel*). *C*, UMR 106-01 cells were serum starved for 2 days and then treated with 50 ng/ml AR for the indicated time points. The levels of c-*fos* and c-*jun* expression were assessed by real time RT-PCR. *D*, UMR 106-01 cells were pretreated with the following for 1 h: dimethyl sulfoxide (0.1% v/v), 1 μM C32, or 1 μM C56. Cells were then treated with control or 50 ng/ml AR for 30 min. The levels of c-*fos* and c-*jun* expression were assessed by real time RT-PCR.

TABLE II
Structural parameters of trabecular bone in the proximal tibia of 4-week-old AR null mice and wild-type littermates measured by microCT

Data were tabulated as the mean ± S.E. The number of animals/group was 8 (female). * $p < 0.05$ *versus* wild type.

Parameter	Wild type	AR null
Percent bone volume (%)	21.44 ± 1.47	15.76 ± 1.19*
Trabecular number (1/mm)	5.75 ± 0.27	4.77 ± 0.23*
Trabecular thickness (μm)	44.49 ± 1.23	40.93 ± 1.08*
Trabecular separation (μm)	178.01 ± 9.82	215.71 ± 10.32*
Connectivity density (1/mm)	281.68 ± 23.34	179.60 ± 14.02*

TABLE III
Structural parameters of cortical bone in the mid shaft femur of 4-week-old AR null mice and wild-type littermates measured by microCT

Data were tabulated as the mean ± S.E. The number of animals/group was 8 (female).

Parameter	Wild type	AR null
Percent bone volume (%)	46.27 ± 0.68	46.01 ± 0.95
Periosteal perimeter (mm)	7.18 ± 0.21	7.16 ± 0.16
Endocortical perimeter (mm)	4.19 ± 0.09	4.08 ± 0.20
Cortical thickness (mm)	0.138 ± 0.005	0.131 ± 0.003

PTH anabolic actions. The latter has come to be defined as an increase in bone formation through the possible mechanisms of a stimulation of osteoblast proliferation, differentiation and inhibition of apoptosis. We think that AR is one of those means for PTH to manipulate the microenvironment in bone. Our data suggest its possible role in osteoblastogenesis, increasing

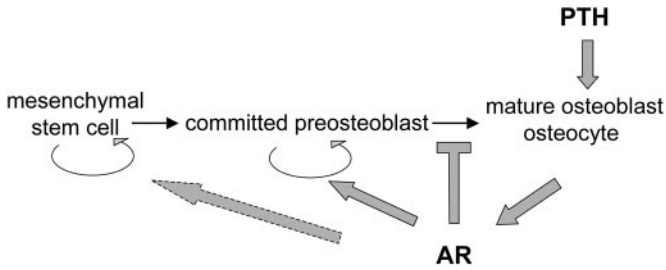

FIG. 7. **A model of the role of AR in PTH action in bone.**

preosteoblast proliferation but inhibiting its maturation (Fig. 7). Because bone marrow mesenchymal stem cells express EGFR (31) and can proliferate and form colonies in a serum-deprived medium as long as EGF is present (32), it is possible that AR has a role in regulating the mesenchymal stem cell pool and therefore influencing several other cell lineages (Fig. 7, *dashed arrow*).

Our studies also suggest detailed mechanisms about how AR stimulates proliferation and inhibits differentiation. AR treatment of preosteoblastic cells rapidly stimulates Akt and ERK phosphorylation and c-*fos* and c-*jun* expression. Because all of those signals lead to cell proliferation, we reason that this is the mechanism for AR to stimulate preosteoblast proliferation and to facilitate the progression of cells from G_1 to S and G_2 phases. Previously EGF was found to oppose the BMP2 induction of osteogenic differentiation markers (33). Later studies demonstrated that BMP stimulates phosphorylation of Smad1, the mediator for BMP signals, and induces its nuclear accumu-

lation. The phosphorylation of Smad1 by ERK in response to EGF inhibits its nuclear accumulation (34). Because AR stimulates phosphorylation of ERKs in both proliferating and differentiating osteoblastic cells, it may use a similar mechanism involving Smad1 to inhibit differentiation.

AR is a member of the EGF family. It is expressed in many human tissues and acts as an autocrine factor for a variety of cancer cell lines and normal cells. Compared with the EGF peptide, the AR mature peptide has an N-terminal extension, but both peptides have amino acid homology and three-dimensional configuration homology and bind to the same receptor (EGFR), indicating that these two peptides or even other EGF family members share redundant functions *in vivo*. There are several lines of evidence to date implying that EGF-like ligands and the EGFR signal cascade play important roles in bone metabolism, especially in bone formation. First, our studies showed that all EGF-like ligands and their receptors are present in osteoblastic cells. Second, although AR is the only one that is highly regulated by PTH, TGF-α and betacellulin were also stimulated by PTH about 2-fold. A previous study observed about a 2-fold increase in EGFR mRNA in UMR 106-01 cells after 48 h of PTH treatment (35). Third, previous (15) and current work in our laboratory has demonstrated that EGF, AR, and TGF-α strongly stimulate osteoblastic cell proliferation (data for TGF-α are not shown). AR also inhibited osteoblast differentiation. Fourth, blocking the EGFR by using inhibitors C32 and C56 significantly inhibits basal DNA synthesis of osteoblastic cells. Fifth, EGF/AR/TGF-α triple knock-out mice are growth-retarded, having a 40% reduction in body weight compared with wild-type mice at weaning (36). EGFR null mice are either embryonic lethal or have severe growth retardation (50–70%) in neonates depending on the genetic background (37–39). Furthermore, mice humanized for EGFR have a low level expression of EGFR in bone and display accelerated osteoblast differentiation and hindered osteoblast proliferation (40). These results suggest an important role of EGF-like ligands and their receptors in bone metabolism.

Because of the multiple members of the EGF family, AR function in bone may be compensated by other members in AR null mice. Consequently, we observed only a mild osteoporosis phenotype in those mice. Our current studies on EGF/AR/TGF-α triple knock-out mice will definitely provide more information about the roles of the EGF family in bone metabolism.

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Mechanisms of Signal Transduction:

Amphiregulin Is a Novel Growth Factor Involved in Normal Bone Development and in the Cellular Response to Parathyroid Hormone Stimulation

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