The epidermal growth factor receptor (EGFR) and its ligands function in diverse cellular functions including cell proliferation, differentiation, motility, and survival. EGFR signaling is important for the development of many tissues, including skin, lungs, intestines, and the craniofacial skeleton. We have now determined the role of EGFR signaling in endochondral ossification. We analyzed long bone development in EGFR-deficient mice. EGFR deficiency caused delayed primary ossification of the cartilage anlage and delayed osteoclast and osteoblast recruitment. Ossification of the growth plates was also abnormal resulting in an expanded area of growth plate hypertrophic cartilage and few bony trabeculae. The delayed osteoclast recruitment was not because of inadequate expression of matrix metalloproteinases, including matrix metalloproteinase-9, which have previously been shown to be important for osteoclast recruitment. EGFR was expressed by osteoclasts, suggesting that EGFR ligands may act directly to affect the formation and/or function of these cells. EGFR signaling regulated osteoclast formation. Inhibition of EGFR tyrosine kinase activity decreased the generation of osteoclasts from cultured bone marrow cells.

Skeletal elements develop by two distinct mechanisms: intramembranous and endochondral ossification (1). Endochondral ossification is a process by which a cartilaginous template is first formed and then replaced by bone. During embryogenesis, condensations of mesenchymal cells form, within which chondrocytes develop, proliferate, and differentiate to form a cartilage template that contains distinct zones of resting, proliferative, and hypertrophic chondrocytes. The proliferation and differentiation of chondrocytes within the cartilage template are spatially ordered, with proliferating cells at the two ends of the template and progressively more mature cells forming hypertrophic cartilage in the middle. Hypertrophic chondrocytes secrete a specialized extracellular matrix (ECM) containing collagen X, which becomes calcified. Endochondral ossification begins with the invasion of the calcified hypertrophic cartilage by blood vessels, accompanied by osteoclasts and osteoblasts (primary ossification center). The function of osteoclasts is to remove the hypertrophic cartilage ECM and that of osteoblasts is to replace it with bone ECM. Longitudinal bone growth is accomplished by the continuing proliferation and maturation of chondrocytes at the ends of the cartilage template (the growth plates) to form more hypertrophic cartilage and its continual removal and replacement by bone (growth plate ossification or formation of primary spongiosa). Normal endochondral bone development requires the exquisite coordination of hypertrophic cartilage formation, vascular invasion, and the development and function of osteoclasts and osteoblasts (2, 3).

The epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases includes EGFR/ErbB1, HER2/ErbB2, HER3/ErbB3, and HER4/ErbB4 (4, 5). EGFR binds several ligands including epidermal growth factor (EGF), transforming growth factor-α, betacellulin, epiregulin, and amphiregulin. During mouse development, EGFR and ligands are expressed in many tissues, including skeletal tissues such as embryonic mandible, Meckel’s cartilage, and limbs (6–10). EGFR-deficient mice have abnormal craniofacial cartilage and intramembranous bone formation resulting in abnormal development with narrow, elongated snouts, underdeveloped jaw, and a high incidence of cleft palate, as well as abnormal development in many epithelial organs including skin, intestines, and lungs (11–16).

EGFR expression has been detected in the axial and appendicular skeleton at the bone-cartilage junction (17), suggesting a role for this signaling pathway in endochondral bone formation. Overexpression of EGF in transgenic mice using the β-actin promoter results in growth retardation and overproliferation of osteoblasts, consistent with a role for EGFR in osteoblastic cell growth (18). A recent study showed that EGFR-deficient mice have impaired endochondral ossification, probably secondary to a defect in hypertrophic chondrocyte maturation and osteoblastic cell proliferation (19). However, in cultured fetal rat long bones, EGF stimulates bone resorption, suggesting that EGFR signaling also plays a role in osteoclast function (20). In this study, we showed that impaired recruitment of osteoclasts contributed to the impaired endochondral bone formation in EGFR-deficient mice and that EGFR signaling is necessary for osteoclast formation from bone marrow progenitors.
EXPERIMENTAL PROCEDURES

Reagents—AG1478 was purchased from Calbiochem (San Diego, CA). Mouse macrophage colony stimulating factor (mouse M-CSF) was purchased from R&D (Minneapolis, MN). Recombinant murine RANK ligand (rm-RANKL) was purchased from PeproTech, Inc. (Rocky Hill, NJ). Methylmercuric (50 μM) or mercuric ions (0.1 mM) were added to the medium. MG132 was purchased fromCalbiochem (La Jolla, CA) and hygromycin B was purchased from Calbiochem (La Jolla, CA). All the other reagents were purchased from the suppliers.

Histological Analyses—The generation of the EGFR-null allele by homologous recombination in ES cells was previously described (11). EGFR+/+ and EGFR+/− mice were genotyped by PCR for the targeted allele. Mice heterozygous for the null allele (EGFR+/−) were mated and the embryos from the vaginal plug were collected. EGFR-/+ Embryos or newborn pups were recognized by their obvious phenotype of open-eyed (15). Bones were fixed in 4% paraformaldehyde in phosphate-buffered saline overnight and decalcified in 0.5M EDTA (pH 7.4) for 1–3 days at 4°C prior to processing for paraffin embedding. E16.5 bones were decalcified. For general morphology, sections were stained with Masson Trichrome stains using a kit from Sigma according to the instructions provided by the manufacturer.

In Situ Hybridization—Complementary DNAs corresponding to Cbf-1 (Runx2), osteocalcin, collagen-type I, matrix metalloproteinase-9 (MMP-9), MMP-13 (collagenase-3), and MMP-14 (MT-MMP) were used to generate [35S]UTP-labeled antisense riboprobes using a transcription kit (Stratagene, La Jolla, CA). In situ hybridization was performed as described previously (21). Briefly, slides were deparaffinized, treated with proteinase K (20 μg/ml) for 5 min at ambient temperature, and hybridized with 35S-labeled antisense riboprobes in hybridization buffer (50% deionized formamide, 300 μM NaCl, 20 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.5 mg/ml yeast tRNA, 10% dextran sulfate, and 1×Denhardt’s) in a humidified chamber at 55°C over-night. After hybridization, the slides were treated with RNase A, washed to a final stringency of 50% formamide, 2×SSC at 60°C, dipped in emulsion, exposed for 1–4 weeks, developed, and counterstained with hematoxylin and eosin.

Gelatin Substrate Gel Analyses—The long bones were dissected from E16.5 and E18.5 EGFR+/− and EGFR+/− embryos and homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml each aprotinin, leupeptin, and pepstatin). Insoluble aggregates and nuclei were removed by centrifugation and protein concentration of the supernatant was quantified using the BCA protein assay reagent kit (Pierce). Samples (10 μg of protein) were added to non-denaturing loading buffer and separated on 10% SDS-polyacrylamide gels containing 1 mg/ml gelatin (Sigma). Gels were washed two times in 2.5% Triton X-100 at ambient temperature for 30 min each, incubated in substrate buffer (50 mM Tris-HCl, pH 8.0, 5 mM CaCl2, 0.02% sodium azide) at 37°C overnight. Gels were then fixed and stained in 30% isopropl alcohol, 10% acetic acid, and 0.1% Coomassie Blue.

Isolation of Bone Marrow Mononuclear Cells and Osteoclast Formation Assay—Bone marrow mononuclear cells were isolated from 9- to 10-week-old C57BL/6 mice by a modified procedure from a previously described method (22). Briefly, mice were killed by cervical dislocation. Femora were cut longitudinally, and bone marrow cells were flushed out into a cell culture dish by slowly injecting MEM at one end of the bone using a 27-gauge needle. The cell suspension was filtered through a cell strainer (Falcon, 70 μm nylon) and pelleted by centrifugation at 1000 rpm at 4°C. Cells were resuspended in MEM containing 10% fetal calf serum, plated in a 100-mm cell culture dish at a density of 1 × 107 cells/ml, and incubated at 37°C in 5% CO2 overnight. The next morning the non-adherent cells were collected, centrifuged, and purified on a Ficoll-Hypaque gradient. The monocyte cell layer was aspirated carefully from the medium and washed with phosphate-buffered saline. The cells were counted, resuspended in MEM containing 2.5% fetal bovine serum, and plated in a 24-well plate at 4 × 104 cells/well. To each of these wells, a final concentration of the compounds was added. The cultures were maintained under 3% CO2 for 6 days.

Bone Marrow Cell Culture Proliferation Assay—Bone marrow mononuclear cells were isolated as described above and seeded in 96-well culture plates at a density of 20,000 cells/well with MEM supplemented with 2.5% fetal bovine serum, 25 ng/ml M-CSF, 25 ng/ml RANKL. Either vehicle (MeSO4) or different concentrations of AG1478 (1.25, 2.5, and 5 μM) were added to the wells. Cells were then incubated at 37°C in 5% CO2. After 48 h, the number of viable cells was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide uptake method. Briefly, 10 μl of a 5-mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution was added to each well and the cultures were incubated in 5% CO2 for 4 h. 100 μl of 10% SDS in 0.1 M HCl was then added to each well overnight and the color reaction was determined by absorbance at 570 nm. Six wells were used for each treatment, and experiments were repeated 3 times.

Bone Resorption Assay—Osteoclast resorption was performed on calcium phosphate-coated discs (BD Biosciences Biocat OsteoLogic discs). Bone marrow cells were isolated and cultured on osteologic discs in the presence of M-CSF and RANKL in MEM with 10% fetal calf serum. After osteoclasts form, the medium was changed to resorption medium (MEM adjusted to pH 7.0 with HCl with 10% fetal calf serum), and either MeSO4 or AG1478 (5 μM) was added. After 2 days, the discs were bleached to remove cells, washed in water, and air-dried. Resorption pits were visualized on a light microscope. Images were taken with a digital camera and analyzed using Adobe Photoshop. Images were visualized in gray scale and inverted. Areas of resorption pits were outlined, and under the histogram function, the percentage of pixels in the top 25% of the gray scale range contained in the outlined area was calculated as the percent resorption area. Statistical analysis was done using the Student’s t test.

RESULTS

EGFR−/− Mice Show Delayed Primary Endochondral Ossification and Lengthened Growth Plate Hypertrophic Cartilage Zones—EGFR-null mice generally die within the first postnatal day because of severe respiratory distress (14). Therefore, we studied skeletal development during embryonic development.

Delayed Osteoclast Recruitment into EGFR−/− Cartilage

Mice heterozygous for the null allele (EGFR+/−) were mated and the embryos from the vaginal plug were collected. EGFR-/+ Embryos or newborn pups were recognized by their obvious phenotype of open-eyed (15). Bones were fixed in 4% paraformaldehyde in phosphate-buffered saline overnight and decalcified in 0.5M EDTA (pH 7.4) for 1–3 days at 4°C prior to processing for paraffin embedding. E16.5 bones were decalcified. For general morphology, sections were stained with Masson Trichrome stains using a kit from Sigma according to the instructions provided by the manufacturer.
In the data that follows, we show the humerus; however, similar phenotypes were seen in the radius, ulna, and in the hind limbs. Primary ossification in the wild type and EGFR+/+ mice occurred normally with humeri at E16.5 showing completed invasion of capillaries into the calcified hypertrophic cartilage (HC), with the resultant removal of the middle section of the HC and replacement of this area with vascularized tissues (Fig. 1A). In contrast, in the EGFR−/− humeri, the middle section of the EGFR−/− HC remained intact, indicating delayed primary ossification (Fig. 1B).

At E18.5, ossification in the wild type/heterozygous humeri continued in the longitudinal direction resulting in the formation of an area of trabecular bone (primary spongiosa) and a growth plate that contained an area of hypertrophic cartilage of relatively small size (Fig. 1C). However, at E18.5, the EGFR−/− humeri showed a lengthened HC zone at the growth plate and ossification that had not proceeded very far longitudinally (Fig. 1D). This delay in ossification continued until birth, with continuing accumulation of HC at the growth plates in newborn EGFR−/− mice compared with their wild type or heterozygous littermates (Fig. 1E and F). Trabecular bone formation was also impaired in EGFR−/− mice. In E18.5 wild type or heterozygous humeri, the primary spongiosa area was large and contained many long trabeculae (Fig. 1G), but in the EGFR−/− humeri, there were only a few short trabeculae (Fig. 1H). This impairment in bone formation persisted until birth, and the differences in trabecular bone were also seen in newborn mice (Fig. 1, I and J). We concluded that primary ossification of the cartilage templates and the subsequent ossification of the growth plates of the long bones are impaired in the EGFR−/− mice. This occurs in the absence of overall growth retardation in utero. Because the EGFR−/− mice die soon after birth, and those that survive for a few days to weeks are severely growth retarded, we did not analyze postnatal development.

Because heterogeneous mice did not show a bone phenotype, the EGFR wild type and heterozygote embryos were used interchangeably in the results that follow. For simplicity they are referred to as wild type.

**EGFR−/− Mice Have Delayed Osteoclast Recruitment into Hypertrophic Cartilage**—We next determined the EGFR-dependent mechanisms in endochondral ossification. A key event in primary ossification of the hypertrophic cartilage anlage is the recruitment of osteoclasts. Mononuclear hematopoietic precursors are disseminated via the bloodstream and deposited in the mesenchyme surrounding the bone rudiments. There, they proliferate and differentiate into TRAP+ cells that are the precursors of multinucleated osteoclasts (3). These (pre)osteoclasts invade the HC together with blood vessels and initiate the resorption of HC (25). During this migration into HC the mononucleated (pre)osteoclasts fuse together to form the multinucleated mature osteoclasts. We saw many TRAP+ cells inside versus outside the calcified HC between wild type and EGFR−/− bones (Fig. 2G). There were no apparent differences in the size of the TRAP+ cells. There were also no differences in the number of nuclei per TRAP+ cell between wild type and EGFR−/− mice (data not shown). The difference in the number of TRAP+ cells inside the calcified HC between EGFR−/− and wild type bones diminished by E18.5 (Fig. 2, C–G). These results indicate that the delayed primary ossification of EGFR-deficient HC is coupled with a delay in osteoclast recruitment.

**Impaired Bone Formation in EGFR−/− Mice Is Due in Part to Delayed Osteoblast Recruitment**—During primary ossification, concurrent with vascular invasion and osteoclast recruitment, osteoblasts also migrate from the bony collar into the hypertrophic cartilage. Because bone formation was also impaired in the EGFR−/− humeri, we asked whether the recruitment of osteoblasts into the EGFR−/− HC was also delayed. We assayed for the expression of the transcription factor Cbfa-1, a marker of osteoblast differentiation (26–28) and for the expression of osteocalcin, a marker of mature osteoblasts (29). In E16.5 wild type and heterozygous humeri, many Cbfa-1-positive cells were found in the middle of the HC zone, whereas in the EGFR−/− bones, these cells were found more at the periphery (Fig. 3, A and B). Similarly, osteocalcin-positive...
cells were found in the middle of wild type hypertrophic cartilage (Fig. 3, C–F). Similarly, osteocalcin (oc) expressing cells are found at the periphery of hypertrophic cartilage in E16.5 EGFR+/− (D, arrows) and in the middle of wild type hypertrophic cartilage (G, arrows). By E18.5, there were abundant osteocalcin-positive cells in the metaphysis of EGFR+/− humerus. However, these cells are found mainly at the cartilage-bone junction (F, arrows), and very few are found in the primary spongiosa, which also contain very few trabecular spicules (P, arrowheads). In contrast, in the E18.5 wild type humerus there are abundant osteocalcin-positive cells both at the cartilage-bone junction (E, arrows) and in the primary spongiosa on trabecular spicules (E, arrowheads). Collagen type I expression was found in both wild type and EGFR+/− E18.5 humerus (G and H, arrows). Bar, 200 μm.

**Osteoclasts Express EGFR**—The delay in osteoclast recruitment into EGFR+/− HC may be because of either a direct effect of EGFR signaling in osteoclasts and/or osteoclast precursors or an indirect effect because of EGFR function on other cells that in turn regulate osteoclasts. To determine whether EGFR ligands can act directly on osteoclasts, we determined expression of EGFR by osteoclasts. We isolated wild type bone marrow cells from CD1 mice and cultured them in vitro with M-CSF and RANK ligand to induce the formation of osteoclasts. We then determined the expression of EGFR in cultured osteoclasts by RT-PCR and Northern blotting. Total RNAs were isolated from primary cultures of osteoclasts and from the heads of wild type and EGFR+/− E18.5 embryos for positive and negative controls, respectively. RT-PCR was performed using a forward primer in exon 1 and a reverse primer in exon 4 to distinguish wild type EGFR mRNA and any residual mRNA from the targeted allele, which has a disrupted exon 2. We observed the expression of EGFR mRNA in osteoclasts.
and wild type embryonic heads (Fig. 4A). As expected, no PCR product corresponding to EGFR was seen in the EGFR−/− embryonic heads. On Northern blot, two transcripts of 9.6 and 5.0 kb in size, were detected in RNA from osteoclasts and wild type embryonic head, but not from EGFR−/− head.

Delayed Primary Osteoclast Recruitment into EGFR-null Mice Is Not Because of Deficiency in MMPs—Previous studies showed that MMPs were necessary for the migration of (pre)osteoclasts into calcified hypertrophic cartilage during primary endochondral ossification (23). In particular, MMP-9 (gelatinase B) is required for the timely recruitment of these cells to the periphery of the HC, consistent with the location of MMP-9 expressing cells at the cartilage-bone junction (Fig. 5, A and C). MMP-9 expression was found in cells at the calcified hypertrophic cartilage, including at the cartilage-bone junction in wild type (A and C, arrows), and in cells at the outer edge of the calcified hypertrophic cartilage in EGFR−/− (A and D, arrows). E–H, bright field (E and F) and dark field (G and H) images of tissue sections of E18.5 humerus from wild type (E and G) or EGFR−/− (F and H) mice hybridized with 35S-labeled MMP-9 antisense probe. Similar number and distribution of MMP-9 expressing cells were found in both wild type and EGFR−/− humerus. I, gelatin zymogram of tissue lysates from the long bones of wild type and EGFR−/− mice showing a slightly decreased level of both latent and activated gelatinase B (Gel Ba) in EGFR−/− bones and a normal level of latent and activated gelatinase A (Gel Aa). Bar (A–H), 200 μm.

**FIG. 4.** RT-PCR and Northern blot analysis of EGFR mRNA in cultured osteoclasts. A, RT-PCR with EGFR-specific primers of total RNA isolated from cultured osteoclasts, wild type embryonic heads, and EGFR−/− heads. The expected 499-bp PCR product was seen in RNA from osteoclast and wild type embryonic head, but not in EGFR−/− head. B, Northern blot of total RNA isolated from cultured osteoclasts, wild type embryonic heads, and EGFR−/− heads hybridized with a 32P-labeled EGFR probe. Two transcripts, 9.6 and 5.0 kb in size, were detected in RNA from osteoclasts and wild type embryonic head, but not from EGFR−/− head.

**FIG. 5.** Expression of MMP-9 in the humeri of wild type/homozygous and EGFR−/− mice. A–D, bright field (A and B) and dark field (C and D) images of tissue sections of E16.5 humerus from wild type (A and C) or EGFR−/− (B and D) mice hybridized with 35S-labeled MMP-9 antisense probe. MMP-9 expression was found in cells inside the vascularized hypertrophic cartilage, including at the cartilage-bone junction in wild type (A and C, arrows), and in cells at the outer edge of the calcified hypertrophic cartilage in EGFR−/− (B and D, arrows). E–H, bright field (E and F) and dark field (G and H) images of tissue sections of E18.5 humerus from wild type (E and G) or EGFR−/− (F and H) mice hybridized with 35S-labeled MMP-9 antisense probe. Similar number and distribution of MMP-9 expressing cells were found in both wild type and EGFR−/− humerus. I, gelatin zymogram of tissue lysates from the long bones of wild type and EGFR−/− mice showing a slightly decreased level of both latent and activated gelatinase B (Gel Ba) in EGFR−/− bones and a normal level of latent and activated gelatinase A (Gel Aa). Bar (A–H), 200 μm.

(MT1-MMP), which are highly expressed in osteoclasts (30–32), was consistent with the TRAP staining results. In E16.5 wild type humeri, MMP-9 expression was found in cells corresponding to osteoclasts in the vascularized bone marrow cavity and at the cartilage-bone junction (Fig. 5, A and C). In the E18.5 EGFR−/− humeri, MMP-9 expression was found in cells at the periphery of the HC, consistent with the location of osteoclasts in these skeletal elements at this time (Fig. 5, B and D). There were no significant differences in the amount of MMP-9 mRNA per cell, judged by the intensity of the signal. By E18.5, there were just as many MMP-9 expressing cells at the cartilage-bone junction of the growth plate and in the bone marrow cavity in the EGFR−/− bones compared with wild type bones. This is consistent with the observation that the differences in the number of osteoclasts between wild type and EGFR−/− bones have diminished by this time. Substrate gel
analyses showed that there was slightly less MMP-9 protein in extracts of EGFR-null bones compared with wild type (Fig. 5f). There was no apparent change in the ratio of active versus latent forms of MMP-9 proteins. These results indicated that MMP-9 expression and activity in osteoclasts were not significantly altered by EGFR deficiency, even though there might be an overall decrease in the level of MMP-9 because of decreased osteoclast number. Thus deficiency in MMP-9 activity was unlikely to be the primary cause for the delayed recruitment of osteoclasts into EGFR-null iliac. Similar results were observed for the expression of MMP-14 (MT1-MMP). In E16.5 wild type humeri, MMP-14 was expressed by cells with similar distribution to osteoclasts, at the cartilage-bone junction and in the bone marrow cavity (Fig. 6, A and C). In contrast, cells expressing MMP-14 were located at the periphery of the unvascularized HC in E16.5 EGFR−/− bones (Fig. 6, B and D). At E18.5 the number and location of MMP-14 expressing cells were comparable in wild type, heterozygous, and EGFR−/− bones (data not shown).

MMP-13 (collagenase-3) may also be important for the vascularization of hypertrophic cartilage during primary endochondral ossification. It can degrade native collagen, which is a major component of the hypertrophic cartilage ECM (33, 34). MMP-13 is highly expressed in hypertrophic chondrocytes and osteoblasts (35) and therefore may act to degrade collagen in the hypertrophic cartilage ECM during endochondral ossification. In E16.5 wild type humeri, MMP-13 was expressed by late hypertrophic chondrocytes and by cells at the vascularization front, some of which may be osteoblasts (Fig. 6, E and G). In the E16.5 EGFR−/− bones, there was high expression of MMP-13 in hypertrophic chondrocytes in the calcified HC (Fig. 6, F and H). These results indicated that deficiency in MMP-13 expression was not the cause for the delayed primary ossification and osteoclast recruitment into EGFR−/− hypertrophic cartilage.

**Osteoclast Recruitment into EGFR−/− Cartilage**

Delayed Osteoclast Recruitment into EGFR−/− Cartilage

In this study we have investigated the function of EGFR signaling in endochondral bone development by analyzing long bone development in EGFR-null mice. Our data showed that in EGFR-null mice primary ossification of the calcified hypertrophic cartilage anlage was delayed and was associated with a delay in the recruitment of osteoclasts and osteoblasts. Forma-
tion of the primary spongiosa was also abnormal leading to accumulation of growth plate HC and decreased trabecular bone mass. The delay in osteoclast recruitment might be be-

cause of inadequate formation of osteoclasts resulting from EGFR deficiency.

EGFR Signaling Is Necessary for Normal Osteoclast Recruitment and Function during Endochondral Ossification—A critical step in endochondral ossification is the invasion of capillaries into the calcified hypertrophic cartilage zone in the diaphysis of the cartilage anlage. Vascularization of calcified HC is accompanied by the recruitment of osteoclasts and osteoblasts. We found that EGFR deficiency resulted in delayed recruitment of osteoclasts into calcified HC during primary ossification. However, this delay was only temporary, consistent with the model that the lack of EGFR signaling causes defective formation of osteoclasts from precursor cells surrounding the bone rudiments, thus requiring a longer time for sufficient osteoclasts to accumulate. Alternatively, the migration of osteoclasts into calcified HC may also be defective.

Even though osteoclast recruitment into EGFR−/− HC reached the same level as wild type mice at E18.5, ossification of growth plate HC was still not complete, as evidenced by the lengthened HC zones of the EGFR−/− growth plates up until birth. This may be because of the accumulation of HC caused by the delay in primary ossification, and the inability of the subsequently normal number of osteoclasts to overcome the initial difference and therefore ossification in the longitudinal direction would always be behind in the EGFR-null bones. Alternatively, growth plate ossification may also be abnormal because of either abnormal formation of growth plate HC or abnormality in its removal. A recent study also reported increased growth plate HC in the EGFR−/− mice (19). The authors found expression of EGFR in chondroblasts but no differences in growth plate chondrocyte proliferation, and suggested that EGFR negatively regulated hypertrophic chondrocyte maturation. On the other hand, the removal and ossification of EGFR-

FIG. 7. Effect of the inhibition of EGFR signaling on osteoclast formation. A–D, TRAP staining of bone marrow cells cultured for 6 days with RANKL (25 ng/ml), M-CSF (25 ng/ml), and either vehicle or increasing concentrations of the EGFR tyrosine kinase inhibitor AG1478 (1.25, 2.5, and 5 μM). In vehicle-treated cultures, there were a large number of multinucleated TRAP+ cells characteristic of osteoclasts (A). Treatment with AG1478 caused a dose-dependent decrease in the number of multinucleated TRAP+ cells (B–D). E, quantification of the number of osteoclasts developed in control cultures and cultures with different concentrations of AG1478. Each histogram represents the mean number of total osteoclasts counted in three wells of a 24-well plate. F, effects of EGFR signaling inhibition on proliferation of bone marrow mononuclear cells. Wild type bone marrow mononuclear cells were isolated and cultured for 2 days in the presence of RANKL (25 ng/ml), M-CSF (25 ng/ml), and either vehicle or increasing concentrations of the EGFR tyrosine kinase inhibitor AG1478 (1.25, 2.5, and 5 μM). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide uptake was assessed by absorbance and used as a measure of cell number. AG1478 caused a dose-dependent decrease in cell number as reflected by the decrease in absorbance. Each histogram represents the mean value of six replicates. The experiments were repeated three times with similar results.

FIG. 8. Effect of the inhibition of EGFR signaling on osteoclast function. A, images of the resorption pits formed by osteoclasts on calcium phosphate–coated discs. Bone marrow cells were cultured on calcium phosphate–coated discs in the presence of M-CSF and RANKL to form osteoclasts before switching to resorptive media containing vehicle control (Me2SO) or AG1478 (5 μM) for 2 days. B, quantitative analyses of the area of resorption as a percentage of the total area corresponding to an osteoclast. Data are presented as mean ± S.D. (error bars) of 35 osteoclasts analyzed. There was no significant difference between Me2SO (DMSO) and AG1478 treated groups (p = 0.36).
null growth plate HC may also be abnormal, proceeding at a slower rate resulting in accumulation of growth plate HC. We found expression of EGFR on mature osteoclasts, suggesting that these cells can respond directly to EGFR ligands. The function of EGFR in osteoclasts is not known, but it may be to stimulate their proteolytic activity. EGFR ligands have been found to stimulate osteoclastic bone resorption in vivo and in vitro in bone organ cultures (20, 38, 39).

MMPs are necessary for the recruitment of osteoclasts into calcified hypertrophic cartilage during primary endochondral ossification (25). The craniofacial and lung developmental de- calcification hypertrophic cartilage during primary endochondral ossification (20, 38, 39).

Vascular endothelial growth factor (VEGF) is important for vascularization of and osteoclast recruitment into calcified hypertrophic cartilage of the developing bones (25, 40). We found expression of EGFR on mature osteoclasts, suggesting that the delayed recruitment of osteoclasts into EGFR−/− HC. However, this does not exclude EGFR regulation of other ECM degrading proteinases that are important in osteoclast function. In addition, because MMP-13 acts synergistically with MMP-9 (25), the initial delayed recruitment of osteoclasts expressing MMP-9 could functionally blunt HC ECM degradation and exacerbate the delay in HC vascularization.

The delayed recruitment of osteoclasts into EGFR−/− HC, and MMP-14 was unlikely to be the primary mechanism for ossification (25). The craniofacial and lung developmental de- calcification hypertrophic cartilage during primary endochondral ossification.

further delay in vascularization and recruitment of osteoclasts. In addition, because MMP-13 acts synergistically with MMP-9 (25), the initial delayed recruitment of osteoclasts expressing MMP-9 could functionally blunt HC ECM degradation and exacerbating the delay in HC vascularization.

VEGF activity leading to decreased bioavailable VEGF, which leads to further delay in vascularization and recruitment of osteoclasts.

EGFR deficiency leads to impaired trabecular bone formation—Trabecular bone mass is decreased in the EGFR−/− mice. This may partly be because of a delay in the initial recruitment of osteoblasts during primary ossification. However, trabecular spicules continued to be deficient until birth. Fewer osteoblasts were found in the EGFR−/− primary spongia even though they are abundant at the cartilage bone junction. This suggests that formation of osteoblasts in the EGFR−/− bones may be normal, but that their subsequent proliferation/survival and/or function may be impaired. Studies of the LRPs (low density lipoprotein receptor-related protein 5)−/− mice showed that osteoblast differentiation and proliferation were differentially regulated (42). Sibilia et al. (19) reported that primary EGFR−/− valvar osteoblast cultures showed decreased proliferation potential and increased differentiation as measured by their ability to form bone nodules in vitro (19). Thus the decreased trabecular bone mass in the EGFR−/− mice was likely because of decreased osteoblast proliferation. A direct effect of EGFR signaling on osteoblasts is supported by previous studies showing that EGFR was expressed in osteoblasts in vivo (17, 43), and that EGFR stimulated osteoblast proliferation in vitro (44, 45).

EGFR signaling is necessary for the formation of osteoclasts—Previous studies have identified two essential factors for osteoclastogenesis: M-CSF and RANKL (37). M-CSF is necessary for the generation of the monocyte/macrophage cell lineage and RANKL for their differentiation into osteoclasts. We found that induction of osteoclast formation from bone marrow cells in the presence of RANKL and M-CSF was significantly inhibited in the presence of the EGFR tyrrosine kinase inhibitor AG1478. This is consistent with previous studies suggesting a role for EGFR ligands in osteoclast formation.

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