Epidermal Growth Factor Receptor (EGFR) Signaling Promotes Proliferation and Survival in Osteoprogenitors by Increasing Early Growth Response 2 (EGR2) Expression

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Background: Maintaining bone architecture requires continuous generation of osteoblasts from osteoprogenitors. Our previous study of mice with epidermal growth factor receptor (EGFR) specifically inactivated in osteoblast lineage cells revealed that EGFR stimulates bone formation by expanding the population of mesenchymal progenitors. EGFR ligands are potent regulators for the osteoprogenitor pool, but the underlying mechanisms are largely unknown. Here we demonstrate that activation of EGFR increases the number of osteoprogenitors by promoting cell proliferation and suppressing either serum depletion-induced or TNFα-induced apoptosis mainly through the MAPK/ERK pathway. Mouse calvarial organ culture revealed that EGF elevated the number of proliferative cells and decreased the number of apoptotic cells, which led to increased osteoblasts. Microarray analysis of MC3T3 cells, an osteoprogenitor cell line, revealed that EGFR signaling stimulates the expression of MCL1, an antiapoptotic protein, and a family of EGR transcription factors (EGR1, -2, and -3). The up-regulation of MCL1 and EGR2 by EGF was further confirmed in osteoprogenitors close to the calvarial bone surface. Overexpression of NAB2, a co-repressor for EGRs, attenuated the EGFR-induced increase in osteoprogenitor number. Interestingly, knocking down the expression of EGR2, but not EGR1 or -3, resulted in a similar effect. Using inhibitor, adenovirus overexpression, and siRNA approaches, we demonstrate that EGFR signaling activates the MAPK/ERK pathway to stimulate the expression of EGR2, which in turn leads to cell growth and MCL1-mediated cell survival. Taken together, our data clearly demonstrate that EGFR-induced EGR2 expression is critical for osteoprogenitor maintenance and new bone formation.

The integrity of bone architecture is maintained by constant bone remodeling, which requires an exquisite balance between bone resorption by osteoclasts and bone formation by osteoblasts. Osteoblasts, the cells synthesizing the bone matrix and initializing mineralization in the bone, arise from a postulated osteoprogenitor cell line and then differentiate into osteoblasts. Osteoprogenitors lead to low bone formation activity, which is directly correlated with age-related osteoporosis and other disease-related bone loss. Many cytokines, growth factors, and hormones are required for regulating the pool of osteoprogenitors by influencing their proliferation, differentiation, and survival. Among them, epidermal growth factor (EGF)-like ligands have been known for a long time to be potent mitogens for MSCs and osteoprogenitors. EGFR signaling stimulates the expression of MCL1, an antiapoptotic protein, and a family of EGR transcription factors (EGR1, -2, and -3). The up-regulation of MCL1 and EGR2 by EGF was further confirmed in osteoprogenitors close to the calvarial bone surface. Overexpression of NAB2, a co-repressor for EGRs, attenuated the EGFR-induced increase in osteoprogenitor number. Interestingly, knocking down the expression of EGR2, but not EGR1 or -3, resulted in a similar effect. Using inhibitor, adenovirus overexpression, and siRNA approaches, we demonstrate that EGFR signaling activates the MAPK/ERK pathway to stimulate the expression of EGR2, which in turn leads to cell growth and MCL1-mediated cell survival. Taken together, our data clearly demonstrate that EGFR-induced EGR2 expression is critical for osteoprogenitor maintenance and new bone formation.

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EGFR Stimulates Osteoprogenitor Growth and Survival via EGR2

EGFR has been shown to stimulate osteoblasts and osteoclasts, which are involved in bone formation and resorption. The role of EGR2 in this process has been investigated, and it was found that EGR2 expression is essential for maintaining the osteoprogenitor pool.

**Experimental Procedures**

Chemicals—Recombinant human EGF and TNFα were purchased from PeproTech. TGFβ, HB-EGF, and amphiregulin were obtained from R&D Systems. U0126, wortmannin, PD98059, and SB202190 were purchased from Calbiochem. Gefitinib was a product of LC Laboratories. Ethidium bromide (EB) and acridine orange (AO) were purchased from Sigma-Aldrich.

Cell Culture—MC3T3-E1 subclone 4 cells were maintained in the growth medium (αMEM supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml streptomycin). Rat primary calvarial osteoprogenitors were obtained from neonatal calvaria by sequential collagenase and trypsin digestion as described previously (22) and cultured in the growth medium. Generation of 3.6kb collagen1α-Cre Egr2flox/flox mice and their wild-type siblings was described previously (14). To obtain primary mouse bone marrow mesenchymal progenitor cultures from these mice, bone marrow cells were flushed from femora and tibiae of 1–2-month-old mice and plated in the growth medium containing 2 mM glutamine. Flow cytometric characterization and experiments were performed at passage 5.

All animal work performed in this study was approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

5-Bromo-2′-deoxyuridine (BrdU) Assay—MC3T3 cells were seeded in 96-well plates at 15,800 cells/cm² and serum-starved for 1 day. Cells were then treated with inhibitors for 30 min before the addition of 50 ng/ml EGF. On the next day, cells were incubated with BrdU labeling solution for 4 h and assayed with cell proliferation BrdU-colorimetric ELISA (Roche Applied Science).

EB/AO Staining for Apoptotic Cells—MC3T3 cells were seeded in 6-well plates at 15,800 cells/cm² and serum-starved overnight before the addition of 50 ng/ml EGF-like ligands. One day later, cells were washed with PBS and stained with 5 μg/ml EB and 5 μg/ml AO in 1× PBS. Apoptotic cells were identified as cells having condensed chromatin (green at early apoptotic stage and red at late apoptotic stage). Living cells had a characteristic green chromatin staining with a normal morphology of the nucleus (23). The number of apoptotic and total cells was counted in three fields per well under a fluorescence microscope. For TNFα-induced apoptosis, cells were cultured in the growth medium and switched to αMEM with 2% FBS followed by pretreatment of EGF-like ligands 30 min before the addition of 10 ng/ml TNFα.

Culture and Histology of Calvariae—Calvariae were cultured as relatively intact tissue as described previously (24). Briefly, calvariae harvested from neonatal mouse pups and cleaned free of surrounding soft tissue were cut into two equal halves at the sagittal suture. Each hemi-calvariae was placed in DMEM supplemented with 1% fetal bovine serum and 1% antibiotics. The calvariae were mounted with a change of medium every 2 days. Calvariae were then analyzed for expression of osteocalcin and osteopontin using immunohistochemistry.
fixed in 10% neutralized buffered formalin overnight, decalcified in 10% EDTA for 2 days, and processed and embedded in paraffin. Five-μm-thick sections were cut parallel to the plane of the sagittal suture, and comparisons were made at equivalent positions in each sample. Sections were stained with hematoxylin and eosin (H&E) for general histology. Immunohistochemistry of Ki67, MCL1, and EGR2 (Abcam) was performed according to the manufacturer’s instructions. For TUNEL and tartrate-resistant acid phosphatase staining to detect apoptotic cells and osteoclasts, sections were stained with an Apoptag in situ peroxidase kit (Millipore) and a leukocyte acid phosphatase kit (Sigma-Aldrich), respectively. All sections were photographed under the microscope at 200× magnification. Images were quantified for bone surface measurement and counted for the numbers of osteoblasts and osteoclasts, Ki67, and apoptotic osteoprogenitors using Bioquant Osteo software.

**Immunoblotting**—Cells were lysed in a modified radioimmuno precipitation buffer (25 mM Tris-HCl, pH 7.4, 75 mM NaCl, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 0.05% SDS, 2 mM EDTA, 50 mM β-glycerophosphate, 1 mM Na3VO4, 1 mM PMSF, and protease inhibitor mixture). Fifteen μg of protein from each sample were separated on the 10% SDS-PAGE gels. The protein samples were then transferred to polyvinylidene difluoride membranes (Bio-Rad). Protein amounts were measured by immunoblotting with the following antibodies: rabbit anti-MCL1, mouse anti-phospho-AKT, rabbit anti-AKT, rabbit anti-EGR1 (Cell Signaling), rabbit anti-EGR2 (Abcam), mouse anti-phospho-ERK, rabbit anti-ERK, and mouse anti-β-actin (Santa Cruz Biotechnology) antibodies. The bands were visualized using corresponding secondary antibodies conjugated with horseradish peroxidase followed by chemiluminescence (Amersham Biosciences ECL™, Western blotting detection reagent, GE Healthcare).

**RNA Isolation, cDNA Synthesis, and Real-time RT-PCR**—Total RNA was isolated from cells using Tri Reagent (Sigma). TaqMan® reverse transcription kit (Applied Biosystems, Inc.) was used to reverse-transcribe mRNA into cDNA. Following this, PCR was performed using a Power SYBR® Green PCR master mix kit (Applied Biosystems). The primer sequences for the genes used in this study are: mouse β-actin (forward, 5′-TCTCTCTGAGGCAAGTACTCT-3′; reverse, 5′-CGGAC-TCATCGTACTCTGCTT-3′), Egr1 (forward, 5′-CCTTCC-AGTTGCGAATCTGCA-3′; reverse, 5′-ACAAATGTCAC-GGCAAAAGGC-3′), Egr2 (forward, 5′-TCGGATCTGAT-GCAACT-3′; reverse, 5′-TCGGATCTGATGCAAACT-3′), Egr3 (forward, 5′-CATCGTCTGACCAACGAGAA-3′; reverse, 5′-GGAAGGAGAGTGCAGGAA-3′), and Mcl1 (forward, 5′-GTGGCCACAAAACTTTAAGGAG-3′; reverse, 5′-GGCTTCTTGTGACAGGCAAACT-3′). Relative expression was calculated for each gene by the 2−ΔΔCT method with β-actin for normalization.

**Microarray Analysis**—MC3T3 cells cultured in growth medium were serum-starved overnight and treated with 50 ng/ml EGF for 1, 4, and 24 h. Total RNA was isolated using Tri Reagent (Sigma) followed by purification using an RNeasy kit (Qiagen). Double-stranded cDNA was synthesized and used for linear amplification by in vitro transcription with an Ambion WT expression kit (Invitrogen), and expression profiles were created using the Mouse Gene 1.0ST array (Affymetrix) containing probes for 28,853 mouse genes in the Penn Molecular Profiling Facility. The Partek Genomics Suite software was used to screen for differentially expressed genes. To qualify as an EGF-regulated gene at a certain time point, the average expression level of a candidate gene was required to be 1.45-fold higher or lower in the EGF-treated samples than in the control, and the multiple testing-corrected p value threshold for likelihood of difference of EGF-treated and control samples was less than 0.05.

**Adenovirus Infection**—MC3T3 cells were seeded in 6-well plates at 8,400 cells/cm² in growth medium overnight and infected with adenoviruses containing control (Ad-DNR, Vector Biolabs), NAB2 (Ad-NAB2, a gift from Dr. Ehrengruber and Dr. Oursler (25)), or EGR2 (Ad-EGR2, Vector Biolabs) at 50–100 multiplicity of infection in αMEM supplemented with 2% FBS. One day later, cells were serum-starved overnight followed by EGF treatment. Cells were harvested 1 h later for Western blot, 24 h later for BrdU assays, and 5 days later for cell number counting.

**siRNA Knockdown of Egr Genes**—MC3T3 cells at 50–60% confluency in 6-well plates were transfected with two transfections of 20 nM siRNAs for Egr1 (MMC.RNAI.N007913.12.2 and MMC.RNAI.N007913.12.4), Egr2 (MMC.RNAI.N010118.12.1 and MMC.RNAI.N010118.12.4), or Egr3 (MMC.RNAI. N018781.12.1 and MMC.RNAI.N018781.12.2) or mock siRNA (TriFecta siRNA kit, Integrated DNA Technologies) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. One day later, cells were serum-starved overnight and then treated with 50 ng/ml EGF.

**Statistical Analysis**—All results are expressed as means ± S.E. of triplicate measurements with all experiments being repeated independently at least three times. Unpaired Student’s t test was used to evaluate the statistical difference between control and treated groups. In cases of multiple groups, differences were first analyzed with one-way analysis of variance followed by Bonferroni’s post test. Values of p < 0.05 were considered significant.

**RESULTS**

**EGFR Signaling Stimulates the Proliferation and Survival of Osteoprogenitors**—We previously demonstrated that EGF and amphiregulin are strong mitogens for rat primary calvarial osteoprogenitors (7). However, the mechanisms underlying this phenomenon have not been investigated. In this study, we first confirmed the mitogenic effect of EGFR signaling in an osteoprogenic cell line, MC3T3. Continuous EGF treatment significantly increased the number of MC3T3 cells 1.52- and 1.60-fold at days 3 and 4, respectively (Fig. 1A). This increase is partially due to the enhanced proliferation because the amount of BrdU incorporated into the EGF-treated cells was 29% more than that incorporated into the vehicle-treated cells (Fig. 1B). This increase was completely abolished by pretreatment of cells with gefitinib, an EGFR inhibitor, U0126, a MEK1/2 inhibitor, or wortmannin, suggesting that both MAPK/ERK and PI3K/AKT pathways are essential for mediating the stimulatory effects of EGFR on osteoprogenitor proliferation. Moreover, blocking these pathways, especially the ERK path-
FIGURE 1. EGFR signaling promotes proliferation and survival in osteoprogenitor cells. 

**A**, cell counts were performed at the indicated days with MC3T3 cells cultured in αMEM with 2% FBS in the presence or absence of 50 ng/ml EGF. *p < 0.01; **p < 0.001 versus control (con).**

**B**, BrdU incorporation assay indicates that EGF stimulates osteoprogenitor proliferation through MAPK/ERK and PI3K pathways. MC3T3 cells were serum-starved for 1 day and pretreated with the following for 1 h: DMSO (0.1% v/v), 5 μM gefitinib (GEF), 20 μM U0126, 1.5 μM wortmannin (WM), and 10 μM SB202190 (SB). Cells were then treated with either vehicle or EGF overnight before the addition of BrdU for the last 4 h. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus DMSO without EGF; #, p < 0.01 versus SB202190 without EGF.

**C**, activation of EGFR signaling stimulates the phosphorylation of ERK and AKT in osteoprogenitors. MC3T3 cells were pretreated with either DMSO or inhibitors followed by 1 h of EGF treatment. Cells were then harvested and subjected to Western blot analyses of phospho-ERK (p-Erk) and phospho-AKT (p-Akt).

**D**, representative images of EB/AO staining. MC3T3 cells were serum-starved overnight followed by the addition of control (SS) or EGF for 1 day. An arrow and an arrowhead point to an early (green) apoptotic cell and late (red) apoptotic cells with condensed chromatin, respectively, whereas live cells exhibit normal nuclear green fluorescence.

**E**, representative images of EB/AO staining. MC3T3 cells were serum-starved overnight followed by the addition of control (SS) or EGF for 1 day. An arrow and an arrowhead point to an early (green) apoptotic cell and late (red) apoptotic cells with condensed chromatin, respectively, whereas live cells exhibit normal nuclear green fluorescence.

**F**, EGFR signaling suppresses osteoprogenitor apoptosis induced by serum depletion mainly through the MAPK/ERK pathway. MC3T3 cells were serum-starved overnight followed by the addition of control (SS) or EGF for 1 day. An arrow and an arrowhead point to an early (green) apoptotic cell and late (red) apoptotic cells with condensed chromatin, respectively, whereas live cells exhibit normal nuclear green fluorescence.

**G**, EGFR signaling attenuates the TNFα-induced apoptosis in osteoprogenitors. MC3T3 cells were serum-starved overnight followed by the addition of control (SS) or EGF for 1 day. An arrow and an arrowhead point to an early (green) apoptotic cell and late (red) apoptotic cells with condensed chromatin, respectively, whereas live cells exhibit normal nuclear green fluorescence.

**H**, EGFR signaling attenuates the TNFα-induced apoptosis mainly through the MAPK/ERK pathway. *p < 0.05; **, p < 0.01 versus their respective control.

**I** and **J**, mesenchymal progenitors from Col-Cre EgfrWa5/flox mice did not respond to the EGF survival signal under either serum depletion (**I**) or TNFα-induced (**J**) conditions. WT, wild type. *, p < 0.05; **, p < 0.01 versus FBS or no treatment control; $, p < 0.05; #, p < 0.01 versus SS or TNFα control.
way, significantly decreased the basal level of cell proliferation (Fig. 1B). However, inhibiting p38 kinase by SB202190 did not affect either the basal or the EGF-induced proliferation. As shown in Fig. 1C, EGF acted through EGFR in osteoprogenitors to strongly and rapidly activate ERK and AKT phosphorylation, which can be abolished only by their pathway-specific inhibitors but not by other inhibitors.

Another factor possibly contributing to the increase of cell numbers is EGFR-induced cell survival. To explore this possibility, the numbers of apoptotic cells under serum-depleted condition were measured in the absence or presence of EGF. EB/AV staining revealed that EGF, amphiregulin, TGFβ, and HB-EGF suppressed the apoptotic cell number by about 60–70% (Fig. 1, D and E). The same survival effect of EGFR signaling was also observed with primary calvarial cells (Fig. 1E). In contrast with the proliferation mechanism described above, an inhibitor assay indicated that MAPK/ERK is the most likely pathway that mediates the survival effect of EGFR signaling (Fig. 1F). It is worth noting from this experiment that PI3K/AKT contributes significantly to the basal survival.

TNFα is a proinflammatory cytokine secreted by a variety of cell types in bone, and it functions as an inducer of apoptosis for osteoblasts (26). In line with previous findings, we found that it strongly increased apoptosis in MC3T3 cells at concentrations of 2.5–10 ng/ml (Fig. 1G). Interestingly, both EGF and TGFβ were able to suppress this increase and reduced the percentage of apoptotic cells. Inhibitor assay revealed a similar mechanism as serum depletion-induced apoptosis in which only the MAPK/ERK pathway mediates the survival effect of EGFR signaling (Fig. 1H).

We have previously constructed osteoprogenitor/osteoblast-specific EGFR knock-out mice (Col-Cre EgfrWt/lox mice (14)) and demonstrated that EGFR stimulates bone formation by maintaining the population of mesenchymal progenitors, including bone marrow MSCs and committed osteoprogenitors. To explore whether cell survival could be a possible factor accounting for the low mesenchymal progenitor number observed in these mice, we isolated the bone marrow cells and cultured them for several passages. Flow cytometry analysis indicated that these cultured cells are indeed mainly mesenchymal progenitors because of the surface antigen profile (Sca-1+CD29−CD45−, data not shown). Interestingly, although EGF treatment greatly rescued cell death in wild-type cells induced by either serum depletion or TNFα, it had no effects on cells derived from EGFR knockout mice (Fig. 1, I and J), suggesting that an innate inhibition in cell survival could be one reason for the decreased number of osteoprogenitors in these mice.

**EGFR Signaling Stimulates the Proliferation and Survival of Osteoprogenitors in Calvariae**—All experiments described above were performed with cell culture. To study the physiological role of EGFR signaling, we harvested calvariae from newborn mouse pups and cultured them in either growth medium or serum-depleted growth medium in the presence or absence of EGF. After 6 days, we found that serum starvation significantly decreased the total osteoblast number in the calvariae (Fig. 2A), which was mainly due to the decreased number of proliferative osteoprogenitors (Fig. 2B) and increased number of apoptotic osteoprogenitors (Fig. 2C). Culturing the calvariae with EGF elevated the number of osteoblasts about 4.9-fold with a 3.9-fold increase in the Ki67+ proliferative cells and a 67% decrease in TUNEL+ apoptotic cells. These results further demonstrate that EGFR signaling plays a critical role in maintaining the population of osteoblast lineage cells in calvariae.
ial organ. Interestingly, we notice that serum depletion also decreased the number of calvarial osteoclasts but that EGF elevated this tartrate-resistant acid phosphatase-positive population (Fig. 2D). The effect of EGF on osteoclast is non-cell autonomous because it was diminished in calvariae from CollabCre EgfrWa5/flox mice (Fig. 2E). These results are consistent with our previous study (27) demonstrating that EGFR signaling indirectly enhances osteoclastogenesis by acting on osteoblastic cells to decrease the expression of osteoprotegerin (OPG), a decoy receptor of receptor activator of NF-κB ligand (RANKL), and increase the expression of monocyte chemoattractant protein 1 (MCP1), a chemokine stimulating osteoclast fusion and activity.

EGFR Signaling Stimulates MCL1 Expression in Osteoprogenitors—To investigate the underlying mechanisms of EGFR activation on osteoprogenitors, we treated MC3T3 cells with EGF and harvested RNA at 1, 4, and 24 h for microarray analysis. Among all the genes regulated by EGF, we identified Mcl1, coding for a pro-survival protein, as the only apoptosis-related gene whose expression was significantly regulated by EGF (supplemental Table 1). qRT-PCR indicated that Mcl1 mRNA increased rapidly after 30 min of EGF treatment and peaked around 1 h (Fig. 3A), and Western blot confirmed this at the protein level (Fig. 3B). Although serum depletion strongly decreased the amount of MCL1, EGF-like ligands were able to enhance the MCL1 expression to a level similar to that in 10% FBS-containing medium (Fig. 3C). A similar increase of MCL1 was also observed in EGF-treated primary calvarial cells (Fig. 3E). Interestingly, EGF-induced MCL1 was only diminished by EGFR and MEK inhibitors but not by PI3K inhibitor (Fig. 3, D and E). These results are consistent with our aforementioned data that EGFR signaling regulates apoptosis mainly through the MAPK/ERK pathway. We further confirmed our in vitro findings by immunohistochemistry of mouse ex vivo calvarial culture, where enhanced MCL1 expression was observed in osteoprogenitors after EGF treatment from WT mice but not from Col-Cre EgfrWa5/flox mice after being cultured in the presence or absence of EGF.

Egr1, -2, and -3 Are Early Immediate Genes of EGFR Signaling in Osteoprogenitors—The microarray data also revealed that Egr1, -2, and -3 genes ranked fifth, third, and first among the most up-regulated genes by EGF at 1 h (supplemental Table 1). qRT-PCR confirmed that the mRNA levels of Egr1, -2, and -3 peaked at 30 min in MC3T3 cells (Fig. 4A) and 1 h in rat calvarial osteoprogenitors (Fig. 4B) after EGF treatment and returned to basal level around 4 h. Western blot further proved that EGR1 and -2 protein amounts significantly increased starting at 30 min after EGF treatment (Fig. 4C). We could not identify any good EGR3 antibody for immunoblot, so we were unable to
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**FIGURE 4.** EGFR signaling promotes the expression of EGR1, -2, and -3 in osteoprogenitors. A and B, qRT-PCR shows the time courses of Egr1, -2, and -3 mRNAs in MC3T3 cells (A) and rat primary calvarial osteoprogenitors (B) after EGF treatment. *, *p < 0.05; **, *p < 0.01; ***, *p < 0.001 versus control (con). C, Western blot assays reveal the increases in EGR1 and -2 protein levels in MC3T3 cells after EGF treatment. arrows indicate EGR1, -2, and -3 block the EGF-induced mRNA expression of their corresponding EGRs. MC3T3 cells were transfected with either DMSO or inhibitors followed by 1 h of EGF treatment. GEF, gefitinib; WM, wortmannin. E, immunohistochemistry of EGR2 (arrows) in mouse calvariae after being cultured in the presence or absence of EGF. SS, serum starvation control.

confirm the EGF regulation of EGR3 at the protein level. Further, inhibitor analysis demonstrated that EGFR signaling regulates EGR1 and -2 through MAPK/ERK but not PI3K/AKT pathway (Fig. 4D). This result was corroborated in the calvariae, where EGF induced EGR2 expression in osteoprogenitors close to bone surface (Fig. 4E).

**EGR2 Mediates EGFR-induced Proliferation**—To investigate whether the actions of EGFR signaling on osteoprogenitors require Egrs, we infected cells with an adenovirus overexpressing NAB2, a transcriptional co-repressor for EGR1, -2, and -3, to block the transcriptional activities of EGRs (28, 29).

Although EGF caused a 1.9-fold increase in the number of cells infected with control virus, a level comparable with uninfected cells, it had no effect on the number of cells infected with adeno-NAB2 (Fig. 5A), indicating that NAB2 abolished the regulatory effect of EGF on osteoprogenitors. To determine whether EGF overexpression was confirmed at the protein level and that EGF did not regulate endogenous or exogenous expression of NAB2 (see Fig. 7B, middle panel). To assign specificity, we next transfected cells with siRNAs to knock down the expression of individual EGRs. qRT-PCR revealed that the mRNA levels of Egr1, -2, and -3 were decreased by their corresponding siRNAs to 42, 59, and 53%, respectively, as compared with those expressed in cells transfected with mock siRNA after EGF treatment (Fig. 5B). Interestingly, although Egr1 and -3 siRNAs had no effects on the increase of cell number induced by EGF, Egr2 siRNA completely abolished this increase (Fig. 5C), suggesting that EGR2, but not EGR1 and -3, mediates the effect of EGFR signaling on expanding the osteoprogenitor population. Note that EGR2 siRNA had no effect on EGR1 and -3 expression (data not shown).

To distinguish whether EGR2 influences EGFR-regulated proliferation or survival, we first performed a BrdU incorporation experiment. As shown in Fig. 6A, EGF-induced cell proliferation was diminished by NAB2 overexpression. Moreover, either knockdown of EGR2 expression by siRNA or overexpression of EGR2 by an adenovirus abolished the proliferative effect of EGF (Fig. 6B and C). In particular, overexpressing EGR2 increased the basal level of incorporated BrdU amount (Fig. 6C), further confirming that EGR2 is critical for mediating the mitogenic effect of EGFR signaling on osteoprogenitors.
EGFR Mediates EGFR-induced Cell Survival—Next, we investigated whether EGR2 also contributes to the survival effect of EGFR signaling. Our initial adenovirus infection experiment clearly showed that EGF-promoted cell survival was completely blocked by the overexpression of NAB2 (Fig. 7A). In addition, NAB2 overexpression also abolished the EGFr-induced MCL1 expression (Fig. 7B), implying that EGRs might mediate the EGFr regulation of apoptosis-related proteins. Knocking down EGR2 by siRNA in MC3T3 cells further increased the apoptotic cell number in serum-free medium and completely eliminated the decrease of apoptotic cell numbers in the presence of EGF under either serum-depleted condition or TNFα treatment (Fig. 7C). Moreover, decreased EGR2 expression led to a partial inhibition of EGFR-induced increase of MCL1 (Fig. 7D). By contrast, overexpression of EGR2 in MC3T3 cells, which is confirmed by Western blot (Fig. 7F), resulted in a decrease in the number of apoptotic cells in serum-free medium and greatly attenuated the survival effect of EGF on these cells (Fig. 7E), which is likely due to the enhanced amount of MCL1 (Fig. 7F). Taken together, these results strongly suggest that EGFR signaling stimulates EGR2 expression in osteoprogenitors with a subsequent increase in the amount of MCL1, which eventually leads to promoting cell survival.

DISCUSSION

In this study, we show that EGFR signaling is important for sustaining the numbers of osteoprogenitors in both MC3T3 cells and rat primary calvarial osteoprogenitors via promoting their proliferation and inhibiting their apoptosis. These observations were further confirmed in a calvarial organ culture in which EGF treatment greatly increased the number of osteoblasts on the calvarial bone surface by regulating the ratio of proliferative and apoptotic osteoprogenitor cells close to the bone surface. Given the expression of EGF-like ligands in bone marrow cells (data not shown) and osteoblasts (7) and the presence of EGF protein in plasma (30), these results suggest an intriguing mechanism to explain our previous in vivo data that deficiency in EGFR activity in osteoprogenitors and osteoblasts leads to decreased bone marrow mesenchymal progenitor number (14). Indeed, we found in this study that the apoptotic rate of progenitor cells derived from these EGFR knock-out mice cannot be attenuated by EGF treatment and that the amount of MCL1 in those EGFR knock-out progenitors cannot be increased by EGF, suggesting that these progenitor cells are more apoptotic than wild-type cells. In addition, we have demonstrated previously that EGFR signaling suppresses the osteogenic differentiation of mesenchymal progenitors by inhibiting the expression of two major osteoblast transcription factors, Runx2 and Osterix (15). Taken together, we propose that a major role of EGFR signaling in bone is to maintain the size of the mesenchymal progenitor pool and prevent the cells from prematurely differentiating into osteoblasts. The inhibitory effect of EGFR on osteoblast differentiation is reversible (5). Therefore, activation of EGFR signaling does not block the future osteogenesis process once progenitor cells are exposed to the differentiation signals, such as bone morphogenetic proteins (BMPs) and insulin-like growth factors (IGFs) in the bone matrix. Consistent with this view, growth medium containing EGF is considered to be a better medium for ex vivo expansion of human bone marrow MSCs to maintain their stemness and differentiation ability for clinical application than medium with FBS only (5, 8, 31).

Interestingly, we also identified a novel mechanism that a zinc finger protein, EGR2, is a crucial mediator for EGF-induced osteoprogenitor proliferation and survival. EGR2 has been most widely studied in the nervous system, including hindbrain development, peripheral nerve myelination (18), and cognitive processes (32), and in the immune system, such as T-cell activation (33), induction of anergy (34), and development (35). Its role in bone metabolism was first observed by severe bone formation defect in Egr2-null mice around birth (20). A recent study of Egr2 heterozygote mice (21) pointed out that one mechanism contributing to the low bone mass in these mice at postnatal stage is accelerated osteoclast proliferation, which leads to enhanced bone resorption. Bone formation seems to be normal in these mice because osteoblast differentiation and mineralization from bone marrow mesenchymal progenitors and calvarial osteoprogenitors were unchanged. These data could be explained by the remaining one allele of Egr2 gene and insufficient knockdown of EGR2 expression in these mice. An osteoprogenitor- and osteoblast-specific deletion of Egr2 will be more accurate in revealing the role of EGR2 in bone formation.

The positive correlation between the expression level of EGR2 and the bone marrow mesenchymal progenitor pool has also been suggested in other studies. Mechanical loading is a great stimulus for bone formation. EGR2 expression in bone is strongly induced by loading, and its expression in cultured osteoblastic cells is also increased by strain (36). Moreover, major signaling pathways associated with loading and bone formation, such as prostaglandins, IGF-I, and Wnt3a, also rapidly increase EGR2 expression in osteoblasts (36). In contrast, glucocorticoid treatment leads to a drastic decrease in bone marrow osteoprogenitor number and an increase in osteoblast and osteocyte apoptosis in mice (2). Interestingly, Egr2 is one of the most strongly inhibited genes in glucocorticoid-treated MC3T3 cells (37). BMP6 is a growth factor stimulating osteoblastogenesis of human mesenchymal progenitors but suppressing their expansion (38). It also strongly decreases EGR2 expression in these cells (39). It is worthwhile to note that although the up-regulation of EGR2 by mitogens is usually rapid and transient, the suppression of EGR2 by the above sig-
nals can be sustained for over 2–4 days, suggesting that EGR2 might be required for maintaining their blocking effect on cell growth.

EGR2 shares a high degree of homology with EGR1 and -3 in the DNA-binding and R1 domains, and they are believed to bind the same consensus promoter sequence. To our surprise, although EGFR signaling dramatically up-regulates all three EGR proteins, only knocking down the EGR2 expression completely abrogated the EGF-induced increase in osteoprogenitor number, indicating a unique function of EGR2 in proliferation and apoptosis that cannot be compensated by other EGRs. The distinct roles of individual EGRs seem to be true in other tissues. For example, thymocytes and splenocytes express EGR1, -2, and -3, but only EGR2, but not EGR1 or -3, is particularly required for the ontogeny of natural killer T (NKT) cells (40). In cognitive processes, although EGR1 and -3 are essential for long term and short term memory, respectively, EGR2 is dispensable for several forms of learning and memory and can act as an inhibitory constraint for certain cognitive functions (32). One possible explanation is that the N-terminal parts of EGRs with less homology might have different interacting proteins or protein complexes, which will determine the unique physiological functions of each EGR protein.

The Egr1 promoter contains five serum-response elements that bind to serum-response factor and ternary complex factor containing ELK1 (41). ELK is phosphorylated by ERK, leading to enhanced DNA binding and serum-response element-mediated transcription. Previous studies have shown that Ras-Raf-MEK-ERK is the major signaling pathway to activate Egr1 expression (42, 43). Similarly, Egr2 promoter contains two serum-response elements (44), and its transcription can also be regulated through MEK/ERK (36). Using an inhibitor approach, we demonstrate that the MAPK/ERK pathway is a major downstream signaling pathway mediating the stimulatory effects of EGF on EGR2 and MCL1 expression and osteoprogenitor survival. We also show that EGR2 regulates the amount of MCL1 because overexpression of EGR2 elevated the MCL1 level and suppressing EGR2 activity through either siRNA or NAB2 overexpression diminished the amount of EGF-induced MCL1. Although most Bcl2 family members are slow turnover proteins, MCL1 has a short half-life at both mRNA and protein levels and thus is highly regulated (45).
EGR2 is likely to regulate MCL protein at multiple levels, such as transcription, phosphorylation, and ubiquitination, and the detailed mechanism needs further investigation. Nevertheless, we delineate the mechanism for EGR2 regulation of apoptosis to be through the MEK/ERK/EGR2/MCL1 pathway. Indeed, the same pathway was recently identified to be essential for mediating macrophage colony-stimulating factor (M-CSF)-induced osteoclast survival (46).

In summary, our studies highlight the importance of EGR2 and its downstream EGR2 signaling in regulating the osteoprogenitor pool. Targeting this growth factor and transcription factor pathway will shed new light on developing novel therapies against bone formation-related diseases, such as osteoporosis.

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