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EGFR Signaling Suppresses Osteoblast Differentiation and Inhibits Expression of Master Osteoblastic Transcription factors Runx2 and Osterix

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ABSTRACT

The epidermal growth factor receptor (EGFR) and its ligands regulate key processes of cell biology, such as proliferation, survival, differentiation, migration, and tumorigenesis. We previously showed that, EGFR signaling pathway is an important bone regulator and it primarily plays an anabolic role in bone metabolism. In this study, we demonstrated that EGF-like ligands strongly inhibited osteoblast differentiation and mineralization in several lines of osteoblastic cells. Real-time RT-PCR and promoter reporter assays revealed that EGF-like ligands suppressed the expression of both early and late bone marker genes at the transcriptional level in the differentiating osteoblasts via an EGFR-dependent manner. This inhibitory effect of EGFR signaling was not dependent on its mitogenic activity. Furthermore, we demonstrated that EGFR signaling reduced the expression of two major osteoblastic transcription factors Runx2 (type II) and Osterix in osteoblast differentiating cells. EGFR-induced decrease in Runx2 transcriptional activity was confirmed by Runx2 reporter and chromatin immunoprecipitation assays. EGFR signaling increased the protein amounts of transcription co-repressors HDAC4 and 6 and over-expression of HDAC4 decreased Runx2 amount in differentiating osteoblasts, implying that HDACs contribute to the down-regulation of Runx2 by EGFR. Moreover, activation of EGFR in undifferentiated osteoprogenitors attenuated the expression of early bone markers and Osterix and decreased Runx2 protein amounts. Together with our previous data, that EGFR stimulates osteoprogenitor proliferation and that blocking EGFR activity in osteoblast lineage cells results in fewer osteoprogenitors and an osteopenic phenotype, we conclude that EGFR signaling is important for maintaining osteoprogenitor population at an undifferentiated stage. J. Cell. Biochem. 112: 1749–1760, 2011.

KEY WORDS: EGFR; OSTEOBLAST DIFFERENTIATION; Runx2; OSTERIX; HDAC

The signaling network of epidermal growth factor (EGF)-like ligands and their receptors is one of the best-studied signaling systems. It modulates cell functions in a variety of ways, including proliferation, survival, adhesion, migration, and differentiation. The EGF-like ligands include EGF, amphiregulin, and transforming growth factor alpha (TGFα), which only binds to the EGF receptor (EGFR), and heparin binding EGF (HB-EGF), betacellulin, and epiregulin, which bind to both EGFR and ErbB4. All of the ligands are synthesized as transmembrane proteins that are inserted into the plasma membrane and are then cleaved by cell surface proteases to release the mature growth factors characterized by a consensus EGF motif that binds to EGFR. EGFR is a 170 kD plasma membrane glycoprotein with both extracellular ligand-binding and intracellular protein tyrosine kinase domains. EGFR is just one in a subfamily of four closely related cell membrane proteins known as class I/EGFR receptors: EGFR (also referred to as ErbB1), ErbB2, ErbB3, and ErbB4. A distinct group of growth factors called neuregulins binds with ErbB3 and ErbB4. ErbB2 has no known ligand and ErbB3 has a defective kinase domain due to substitutions of essential residues. Therefore, both receptors act primarily as subunits for other ErbBs [reviewed in Citri and Yarden, 2006].

Osteoblastic cells, such as UMR 106-01, primary osteoblastic cell cultures [Ng et al., 1983], and MC3T3 [Zhu et al., 2007], possess EGFR. In vivo, strong immunostaining for EGFR was observed in cells of osteoblastic lineage, including osteoprogenitors, osteoblasts, and osteocytes in growing red deer antler [Barling et al., 2005] and

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mandibles of human fetuses [Davideau et al., 1995]. In situ hybridization with ^{35}S-labeled EGFR antisense riboprobe in rat mandibles also showed hybridization signals with osteoprogenitors, osteoblasts and some osteocytes [Davideau et al., 1995]. However, in vivo radioautography using ^{125}I-EGF in rat femoral and alveolar bones indicated that EGF-binding cells were very similar in structure and morphology to osteoprogenitors but mature osteoblasts, osteocytes, and osteoclasts were poorly labeled [Cho et al., 1988; Martineau-Doize et al., 1988]. In contrast, our previous report demonstrated that osteoclasts do not express functional EGFR and do not respond to EGF treatment [Zhu et al., 2007]. However, EGF-like ligands do have the ability to stimulate bone resorption and osteoclast formation indirectly through decreasing the expression of osteoprotegrin (OPG) and increasing the expression of monocyte chemoattractant protein 1 (MCP1) in osteoblasts [Zhu et al., 2007]. OPG is a soluble decoy receptor blocking the interaction of RANKL with its receptor (RANK) on osteoclasts and inhibiting osteoclastogenesis [Simonet et al., 1997; Yasuda et al., 1998]. MCP1, a CC chemokine, induces multinucleated osteoclast formation, stimulates osteoclast fusion and activity, and has chemoattractant activity towards osteoclasts [Kim et al., 2005, 2006; Li et al., 2007].

The function of EGFR signaling in bone homeostasis has not been well characterized. We have found that parathyroid hormone, an anabolic hormone for bone metabolism, rapidly and dramatically stimulates amphiregulin expression in osteoblastic cells and amphiregulin has the ability to stimulate proliferation of osteoprogenitors derived from rat calvarie but, meantime strongly inhibit their osteoblast differentiation [Qin et al., 2005]. The similar effects were also observed with another EGF-ligand, betacellulin [Genetos et al., 2009]. Moreover, animal studies showed that blocking EGFR activity in osteoblast lineage cells results in fewer osteoprogenitors and osteoblasts and leads to defective bone formation and an osteopenic phenotype [Zhang et al., 2011]. However, the detailed molecular mechanism of how EGF-like ligands regulate osteogenesis has not been studied. Here we show that, all EGF-like ligands are able to suppress osteoblast differentiation in osteoblastic cell lines in an EGFR-dependent manner and we explore mechanisms that mediate this inhibitory effect.

MATERIALS AND METHODS

CHEMICALS

Recombinant human EGF, TGFα, HB-EGF, amphiregulin, and heregulin were purchased from R&D Systems. PD153035 was obtained from Calbiochem.

CELL CULTURE

MC3T3-E1 subclone 4 cells were maintained in growth medium (αMEM supplemented with 10% fetal bovine serum (FBS) plus 100 IU/ml penicillin and 100 μg/ml streptomycin). To differentiate these cells into mature osteoblastic cells, MC3T3 cells were seeded in differentiation medium (growth medium with 50 μg/ml L-ascorbic acid (AA)) at a density of 50,000 cells/cm^2. Media were changed every 2 days. At day 8, 5 mM NaH_2PO_4 was added into media for bone nodule formation and von Kossa staining. To obtain primary mouse bone marrow osteoblastic cultures, bone marrow cells were flushed from femora and tibiae of 1–2 month old mice and plated at a density of 300,000 cells/cm^2 in growth medium. The medium was changed to differentiation medium on day 5 and every 2–3 days afterwards. Human bone marrow aspirates purchased from Allcells were purified through a Ficoll gradient (GE Healthcare) to obtain mononuclear cells. These cells were seeded in a T175 flask in αMEM medium with 15% FBS plus 1% glutamine, 0.1 mM L-ascorbic acid phosphate in order to obtain human bone marrow stromal stem cells (BMSSCs). For osteoblast differentiation, these cells were grown to confluency and then switched to the same medium containing 10 nM dexamethasone and 1.8 mM KH_2PO_4. Rat primary calvarial osteoblastic cells were obtained from neonatal rat calvariae by sequential digestions with collagenase and trypsin as described previously [Shalhoub et al., 1992]. Cells were cultured in MEM containing 10% FBS until confluence at day 7. Then the medium was switched to differentiation medium (BGJb medium containing 10% FBS, 10 mM β-glycerophosphate, and 50 μg/ml AA). For all treatment, 8 nM EGF-like ligands were added to the media unless otherwise specified. All experiments with animals were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

RNA ISOLATION, cDNA SYNTHESIS AND REAL-TIME RT-PCR (qRT-PCR)

Total RNA was isolated from cells using Tri Reagent (Sigma). TaqMan® Reverse Transcription kit (Applied Biosystems) 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 PCR primers are summarized in the Supplementary Table.

CELL CYCLE ANALYSIS

MC3T3 cells were seeded in differentiation medium at a density of 50,000 cells/cm^2 with or without EGF. Two days later, cells were trypsinized, washed once with PBS, and fixed in 70% ethanol for at least 1 h on ice. Fixed cells were washed with PBS and incubated with propidium iodide solution containing ribonuclease A. The cell cycle of stained cells was analyzed by Beckman Coulter XL.

CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY

ChIP analyses were performed using a modification of the Upstate Biotechnology procedure. Briefly, MC3T3 cells were cultured in growth or differentiation medium with or without EGF treatment for 3 days and harvested in ice-cold PBS containing protease inhibitors after 1% formaldehyde treatment. Cell lysates were sonicated to generate 400–1,000 bp DNA fragments and then diluted 10-fold. Two ml aliquots were precleared with 80 μl salmon sperm DNA/protein A-agarose 50% gel slurry for 1 h and immunoprecipitated by rabbit IgG or anti-Runx2 (Santa Cruz Biotechnology) overnight. Immune complexes were collected, washed sequentially in low salt, high salt, and lithium chloride followed by 2 washes in Tris/EDTA buffers. Protein/DNA complexes were eluted with ChIP elution buffer (1% SDS and 50 mM $NaHCO_3$) and heated at 65°C for 4 h to reverse crosslinking. Supernatant obtained from sonicated chromatin without antibody was used as the input control.

Following treatment with proteinase K for 1 h at 45°C, the DNA was purified, resuspended in 50 μl Tris–EDTA buffer and 2 μl aliquots were used for PCR to quantify Runx2-bound DNA amounts. PCR primers used for analysis of the osteocalcin promoter region are: forward 5′-GCAATCACCAACCACAGCATCCTT-3′ and reverse 5′-ACCCTCCAGCATCCAGTAGCA-3′. PCR products were resolved in 2% agarose gels.

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAY

MC3T3 cells were seeded in growth medium overnight and then co-transfected with indicated firefly and control Renilla luciferase constructs using Genejammer (Stratagene) according to the manufacturer's protocol in either growth or differentiation medium followed by EGF treatment. Cell lysates were harvested at the indicated times and analyzed for luciferase activity using the dual luciferase assay reagent (Promega) and an OptiCompII luminometer (MGM Instruments). Firefly luciferase activity was normalized to Renilla luciferase activity to account for potential differences in transfection and cell lysis efficiency. The promoter constructs of 1.4 kb IBSP [Tu et al., 2004], 657 bp osteocalcin [Ducy and Karsenty, 1995], 600 bp Runx2 P1 [Drissi et al., 2002], and 665 bp Osterix [Lu et al., 2006] were described previously. The expression vector of HDAC4 (pCMV-FLAG HDAC4) was kindly provided by Dr. Xiang-Jiao Yang (McGill University Health Centre, Montreal, Canada).

IMMUNOBLOTTING

Cell lysate was solubilized in RIPA buffer (50 mM Tris, pH 7.4, 100 mM NaCl, 1% sodium deoxycholate, 1% Triton-X 100, and 0.1% SDS) with protease inhibitor. Cell lysate (50 μg) was separated by SDS–PAGE and transferred onto PVDF membrane. Immunoreactive protein bands were visualized using anti-Runx2 (MBL) and anti-HDAC1, 3, 4, 5, and 6 (Cell Signaling Technology) and corresponding secondary antibodies, followed by chemiluminescence (Amersham ECL™ Western Blotting Detection Reagents, GE healthcare).

STATISTICAL ANALYSIS

All results are expressed as means ± SEM of triplicate measurements with all experiments being repeated independently at least three times. Statistical analyses were carried out using Student's *t*-test (Microsoft Excel 2007).

RESULTS

ALL EGF-LIKE LIGANDS STRONGLY SUPPRESS OSTEOBLAST DIFFERENTIATION IN MULTIPLE IN VITRO OSTEOGENESIS SYSTEMS

Our previous studies demonstrated that, amphiregulin stimulates the proliferation of osteoprogenitors derived from rat calvarie but inhibits their osteoblastic differentiation [Qin et al., 2005]. In order to investigate whether this is a ubiquitous phenomenon for all EGF-like ligands and osteoblastic cells, we tested other ligands, such as EGF, HB-EGF, and TGFα, and other in vitro osteogenesis systems, such as MC3T3 cells, mouse bone marrow primary osteoblastic cells, and human BMSSCs. MC3T3 is a preosteoblastic cell line that undergoes osteoblast differentiation in the presence of L-ascorbic acid. After 10 days of culturing in differentiating medium, MC3T3 cultures formed numerous mineralized bone nodules (Fig. 1A,B). Continuous treatment with TGFα, EGF, amphiregulin, or HB-EGF completely eliminated the formation of mineralized bone nodules. qRT-PCR analysis revealed that the mRNA expression of early differentiation marker genes, alkaline phosphatase (AKP) and integrin binding sialoprotein (IBSP), increased rapidly during the initial differentiation stage (days 2 and 4). The AKP expression remained stable after day 4 but IBSP expression further increased in the late differentiation period. EGF treatment strongly decreased the expression of these genes to the levels similar to or lower than that of cells cultured in growth medium throughout the entire culture period (Fig. 1C). The expression of the late differentiation marker gene, osteocalcin, was dramatically increased after 4 days of differentiation and was about 54-fold higher at day 8 compared to the growth medium control at the same day. EGF treatment knocked down this expression to 5-fold higher than control (Fig. 1C). Other EGF-like ligands, TGFα, amphiregulin, and HB-EGF showed similar effects (data not shown).

We also tested the effect of EGF-like ligands on the osteogenesis of mouse bone marrow primary osteoblastic cells. After 20 days in differentiation medium, these cells became mature osteoblasts with mineralized bone nodules. EGF-like ligands were able to strongly suppress this differentiation process and very few nodules were observed at day 20 (Fig. 1D). The mRNA levels of differentiation markers, AKP, IBSP, and osteocalcin, were dramatically decreased to about 3–20% by various EGF-like ligands, compared with untreated cells (Fig. 1E). Interestingly, TGFα seems to have the strongest effect in suppressing the expression of these genes.

Human BMSSCs have the ability to differentiate into mature osteoblastic cells in the presence of L-ascorbic acid phosphate and dexamethasone in vitro. Addition of EGF in the differentiation medium decreased the mineralization process as visualized by alizarin red staining (Fig. 1F). The mRNA levels of AKP and IBSP in these EGF-treated cells were also significantly decreased compared to those in control after 3, 7, and 14 days of differentiation (Fig. 1G).

TIME- AND DOSE-DEPENDENT SUPPRESSION OF BONE MARKER GENES BY EGF

In order to understand the mechanisms by which EGF-like ligands suppress osteoblast differentiation, we examined the time course and dose responsiveness of this inhibition on bone marker genes AKP and IBSP. After 4 days of differentiation, MC3T3 cells were treated with EGF for various times and doses. Since MC3T3 cells normally express abundant bone nodules after 8–10 days in differentiation medium, we consider that at day 4 these cells are in the middle stage of osteoblast differentiation. Figure 1C also indicates that at day 4 the expression of early marker genes is already substantially elevated but the expression of late marker gene just starts to increase. As shown in Figure 2A, the inhibition of IBSP and AKP expression was a late event with significant inhibition not observed until 8 h. The inhibition level continued to increase until 48 h. With this duration, EGF dose-dependently suppressed mRNA expression of both AKP and IBSP with maximal suppression observed above 0.5 nM (Fig. 2B).

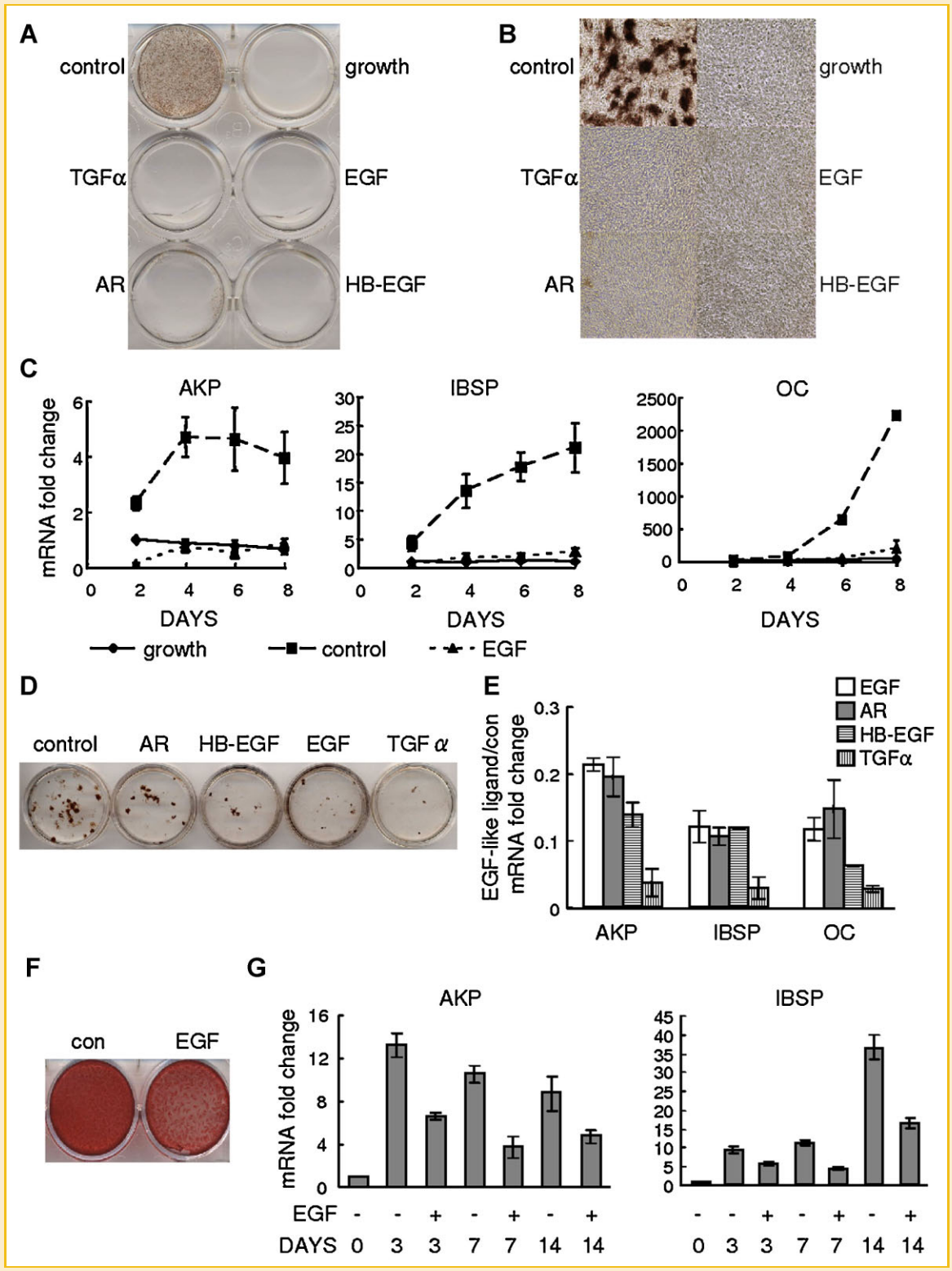

Fig. 1. EGF-like ligands suppress osteoblast differentiation and mineralization. A–C: MC3T3 cells were seeded in six-well plates in osteogenic differentiation medium in the absence (control) or in the presence of EGF-like ligands (AR, amphiregulin). Media were changed every 2 days with addition of fresh EGF-like ligands. After 10 days, von Kossa staining was performed to visualize mineralized bone nodules. The plate was either scanned (A) or observed under a microscope (100×, B). As a negative control, wells containing MC3T3 cells in growth medium are labeled as growth. RNAs were isolated on days 2, 4, 6, and 8 after differentiation and used for qRT-PCR (C) to measure the relative amounts of bone markers (OC, osteocalcin). The expression level of each bone marker in the growth medium at day 2 was set as 1. D,E: Mouse primary bone marrow osteoblastic cells were cultured in differentiation medium with EGF-like ligands starting from day 5. On day 21, von Kossa staining was performed to visualize bone nodules (D) and RNAs were obtained to quantify bone marker gene expression using qRT-PCR (E). The expression level of each bone marker in control was set as 1. F,G: Human BMSSCs were cultured in osteogenic differentiation medium with or without EGF for 14 days. At the end of the culture, alizarin red staining was performed to show mineralized calcium deposits (F). RNAs were harvested on the indicated days to measure the relative amounts of bone markers (G).

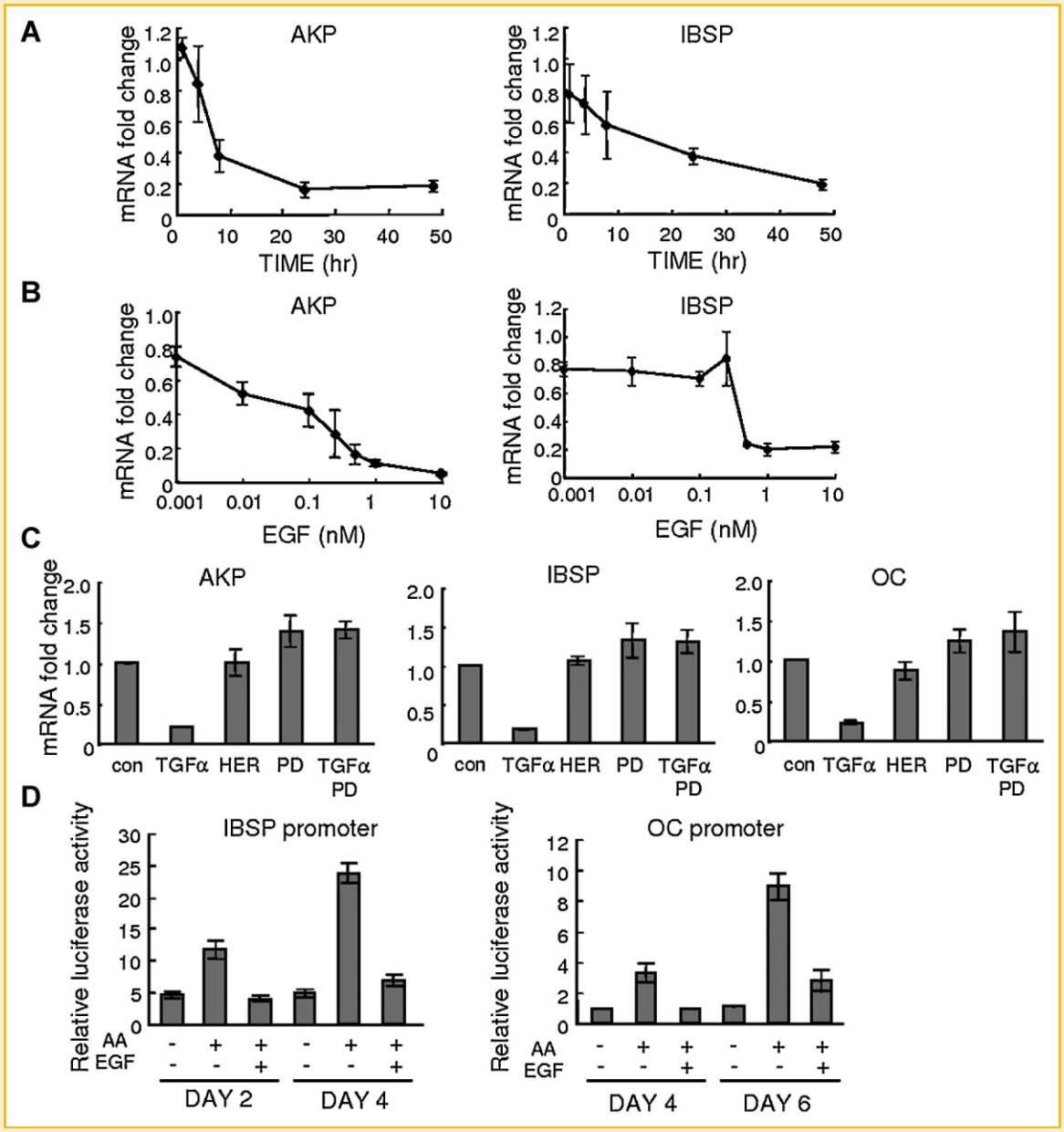

Fig. 2. EGF-like ligands inhibit the expression of bone marker genes in MC3T3 cells. A: Time course of AKP and IBSP expression in response to EGF in osteoblast differentiating MC3T3 cells. Cells were seeded in differentiation medium for 4 days. EGF was then added to the media and RNAs were harvested at the indicated times for qRT-PCR analysis. B: Dose response of AKP and IBSP expression to EGF in differentiating MC3T3 cells. Cells were seeded in differentiation medium for 4 days. Various amounts of EGF were added to the media and RNAs were harvested 48 h later for qRT-PCR. C: EGF-like ligands suppress bone marker gene expression in an EGFR-dependent manner. MC3T3 cells were cultured in differentiation medium for 4 days. Then cells were pretreated with DMSO (0.1% v/v) or PD 153035 (PD, 10 μM) for 1 h followed by TGFα or heregulin (HER) treatment. After 48 h, RNAs were harvested and subjected to qRT-PCR analysis. D: EGFR signaling inhibits promoter activities of bone marker genes in differentiating MC3T3 cells. Cells were transiently transfected with an IBSP promoter- or an osteocalcin promoter-driven luciferase construct and then subjected to osteoblast differentiation in the absence or in the presence of EGF for the indicated times and dual luciferase assays were performed.

EGF-LIKE LIGANDS SIGNAL THROUGH EGFR AND INHIBIT DIFFERENTIATION MARKER EXPRESSION AT THE TRANSCRIPTIONAL LEVEL

Among all EGF-like ligands, EGF, TGFα, and amphiregulin only bind and signal through EGFR, but HB-EGF can bind and signal through both EGFR and ErbB4. Another set of ligands, neuregulins, can activate ErbB3 and ErbB4 but not EGFR [Citri and Yarden, 2006]. To test whether ErbB3 and 4 also have the inhibitory effect on osteogenesis, we treated differentiating MC3T3 cells with heregulin, a representative member of neuregulins, and found it had no effect on the expression of bone marker genes (Fig. 2C). In contrast, similar molar amounts of TGFα dramatically decreased the expression of AKP, IBSP, and osteocalcin to 21%, 18%, and 23%, respectively, of untreated cells. Moreover, this decrease was abolished by addition of

the EGFR-specific inhibitor PD153035. These data clearly suggest that, EGFR is the main receptor mediating the inhibitory action of EGF-like ligands on osteoblast differentiation.

Next, we investigated whether EGFR signaling regulates bone marker gene expression at the transcriptional level using promoter reporter assays. We transfected a full-length 1.4 kb IBSP promoter-driven luciferase construct into MC3T3 cells followed by culturing the cells in differentiation medium. Compared to cells cultured in growth medium, the promoter activity of this construct increased about 2.5-fold at day 2 and 4.8-fold at day 4 but these increases were completely abolished by EGF treatment (Fig. 2D). A similar result was obtained with a 657 bp osteocalcin promoter (Fig. 2D). These data are consistent with the fact that EGF-like ligands suppress the mRNA levels of IBSP and osteocalcin and further demonstrate that EGFR signaling regulates bone marker expression at the transcriptional level.

THE EFFECT OF EGFR SIGNALING ON BONE MARKER EXPRESSION IS NOT DEPENDENT ON ITS MITOGENIC ACTIVITY

EGFR signaling is a strong mitogen for osteoprogenitors [Ng et al., 1983; Qin et al., 2005]. To investigate whether the effect of EGFR signaling on osteoblast differentiation is dependent on cell proliferation, we analyzed the cell cycle distribution of differentiating MC3T3 cells after EGF treatment. Flow cytometry data revealed that the percentages of cells at each phase after 2 days of EGF treatment were 72.5% (G1), 20.6% (S), and 6.9% (G2 + M), similar to that of untreated cells, 72.6% (G1), 21.8% (S), and 5.6% (G2 + M), implying that EGFR signaling does not stimulate cell proliferation in differentiating cells, and therefore its effect on bone marker expression is not associated with cell cycle progression.

EGFR SIGNALING INHIBITS THE EXPRESSION OF OSTEOBLAST-SPECIFIC TRANSCRIPTION FACTORS Runx2 AND OSTERIX

To delineate the mechanisms by which EGFR signaling suppresses osteoblast differentiation, we analyzed the expression of several osteoblast-specific transcription factors in rat primary calvarial osteoblasts after amphiregulin treatment. We found that while EGFR signaling had no effect on ATF4, Msx2, and Twist1 expression (data not shown), significant decreases in the mRNA levels of both Runx2 and Osterix genes were observed after 4 h (Fig. 3A). After 24 h of treatment, Runx2 and Osterix mRNAs were down-regulated to about 60% and 30%, respectively, of control levels. We observed a similar inhibitory effect on Runx2 and Osterix mRNA levels in differentiating MC3T3 cells, but significant decreases were not detected until 8 h for Osterix and 24 h for Runx2 (Fig. 3B). The dose response experiment indicated that EGF had significant effects on the expression of both genes at 1 nM or above (Fig. 3C). Western blot analysis further revealed that the Runx2 protein amounts increased during MC3T3 cell differentiation but this increase was diminished by EGF treatment (Fig. 3D). Since there is no reliable antibody against Osterix, we could not perform Western blot to measure the Osterix protein level.

The expression of Runx2 is initiated from two promoters, the distal P1 and the proximal P2, which drive expression of the major Runx2 isoforms, type II and type I, respectively [reviewed in Schroeder et al., 2005]. Both Runx2-I and II are expressed in osteoblasts and terminal hypertrophic chondrocytes. While Runx2-I is also existed in non-osseous cells and its expression during osteoblast differentiation stays at a consistent level, Runx2-II expression is restricted to osseous cells and is increased during osteoblast differentiation. In the above experiments (Fig. 3A–C), we used primers detecting both types of Runx2. To determine which Runx2 is regulated by EGFR, we designed primers specific for each type. Interestingly, we did not detect any expression of Runx2-I mRNA in MC3T3 cells using RT-PCR (data not shown). By contrast, the Runx2-II mRNA amount was high in differentiating MC3T3 cells and was decreased to 28% of control after EGF treatment, indicating that type II is the major form expressed in MC3T3 cells and regulated by EGFR signaling (Fig. 3E).

To investigate whether EGFR signaling regulates the transcription of Runx2 and Osterix genes, we transfected MC3T3 cells with a 600 bp Runx2 P1 promoter- or a 655 bp Osterix promoter-driven luciferase construct and then cultured cells in differentiation medium with or without EGF treatment. Both promoters exhibited high relative luciferase activities at 2–4 days after differentiation and these activities were suppressed 4–7-fold after EGF treatment, implying that EGFR signaling regulates Runx2 and Osterix expression at the transcriptional level. These data further demonstrate that EGFR signaling mainly targets Runx2-II that is driven by the P1 promoter.

Runx2 TRANSCRIPTIONAL ACTIVITY IS REDUCED BY EGFR

Runx2 binds to a specific DNA sequence, OSE2, to activate the transcription of osteocalcin [Ducy et al., 1996]. 6xOSE2 driven luciferase construct (6xOSE2-luc) is a Runx2 reporter plasmid containing six repetitive OSE2 sites. We found that, the luciferase activity of this construct is strongly inhibited by EGF in differentiating MC3T3 cells (Fig. 4A). After 3 or 4 days of differentiation, EGF treatment decreased the reporter activity to about 50% of control, the same level as that in cells cultured in growth medium. At day 5, EGF treatment also significantly decreased reporter activity, albeit not to the level of that in cells cultured in growth medium. In vivo ChIP analysis revealed that the binding of Runx2 to this OSE2 site in the osteocalcin promoter in differentiating MC3T3 cells was inhibited by EGF to a level similar to that in proliferating osteoblasts (Fig. 4B), indicating Runx2 transcriptional activity is decreased by EGFR signaling.

EGFR SIGNALING STIMULATES THE EXPRESSION OF TRANSCRIPTION CO-REPRESSORS HDAC4 AND 6

HDACs remove acetyl groups from lysine side chains on histone and non-histone proteins. Through their actions on histones, HDACs promote chromatin condensation and transcriptional repression [Ng and Bird, 2000]. HDACs can also repress Runx2 activity by either protein–protein interaction [Westendorf et al., 2002; Schroeder et al., 2004] or by promoting Runx2 degradation through deacetylation [Jeon et al., 2006]. Since EGFR signaling strongly suppresses bone marker gene expression and Runx2 activity, we next studied whether it has an effect on the expression of the HDACs. Using Western blot analyses, we examined the expression of class I HDACs (1 and 3) and class II HDACs (4, 5, and 6) in differentiating MC3T3 cells. Those HDACs are known to be expressed in osteoblastic

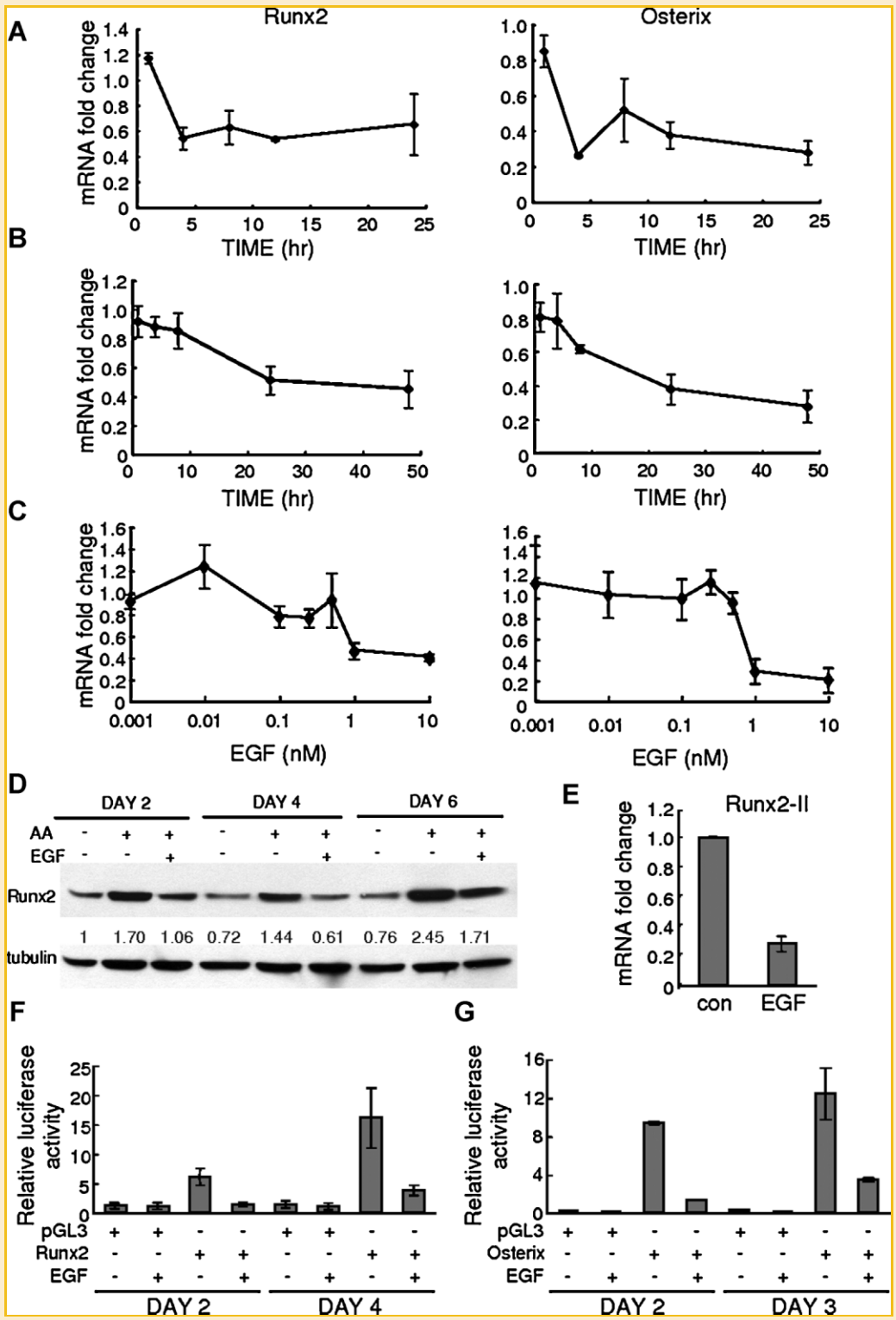

Fig. 3. EGFR signaling inhibits the expression of master osteoblastic transcription factors Runx2 and Osterix in differentiating osteoblastic cells. A: Time course of inhibition of Runx2 and Osterix mRNA levels by amphiregulin in differentiating rat primary calvarial osteoblastic cells at day 14. RNAs were harvested for qRT-PCR analysis at the indicated times. B: Time course of inhibition of Runx2 and Osterix mRNA by EGF in differentiating MC3T3 at day 4. C: Dose response of Runx2 and Osterix mRNA to 48 h of EGF treatment in MC3T3 cells after 4 days of osteoblast differentiation. D: Western blot analysis of Runx2 in MC3T3 cells in growth medium, in differentiation medium with or without EGF at the indicated times. The blot was quantified by densitometric measurement. The data were normalized against tubulin and the corresponding values were shown underneath the blot. E: qRT-PCR analysis of Runx2-II mRNA level after 48 h of EGF treatment in differentiating MC3T3 at day 4. F,G: EGFR signaling inhibits the expression of Runx2 (F) and Osterix (G) at the transcriptional level. MC3T3 cells were transfected with control (pGL3), Runx2, or Osterix promoter constructs and then cultured in differentiation media with or without EGF. Cell lysates were harvested at the indicated times for dual luciferase assays.

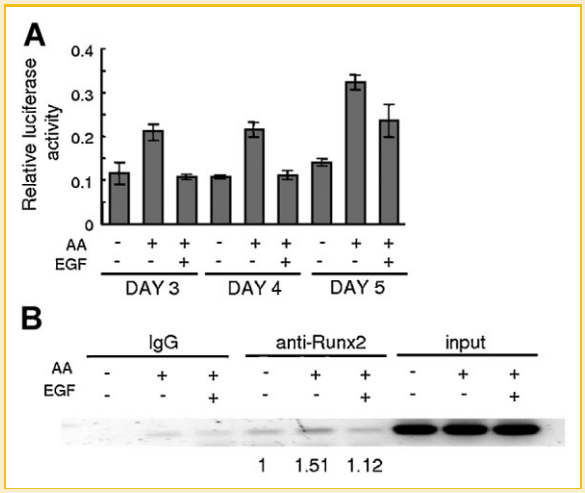

Fig. 4. EGFR signaling blocks Runx2 transcriptional activity. A: EGFR signaling decreases the luciferase activity of Runx2 reporter construct. MC3T3 cells were transfected with a 6xOSE2 driven luciferase construct followed by osteoblast differentiation with or without EGF treatment. Cell lysates harvested at indicated times were subjected to dual luciferase assays. B: ChIP analysis showed that the binding of Runx2 protein to the OSE2 site in the osteocalcin promoter was decreased by EGFR activation. The levels of PCR products derived from Runx2-bound DNA were quantified by densitometry, normalized by input DNA and were shown underneath the gel picture.

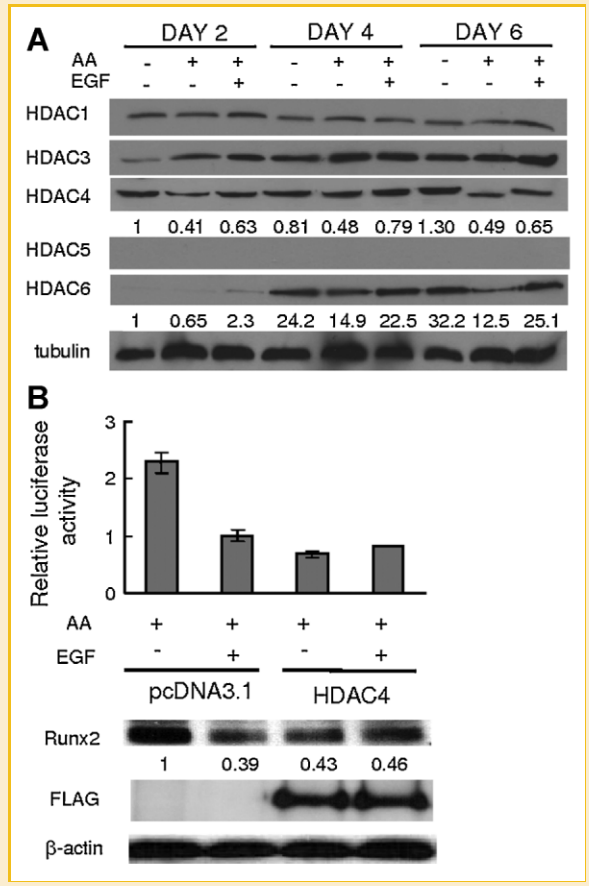

Fig. 5. EGFR signaling stimulates the expression of transcription co-repressors HDAC4 and 6 in differentiating MC3T3 cells. A: Western blots show that protein amounts of HDAC4 and 6, but not HDAC1, 3, and 5, were increased by EGF treatment during MC3T3 cell differentiation. The levels of HDAC 4 and 6 shown at the bottom of blots were obtained by densitometric measurement and normalized by loading control tubulin. B: Overexpression of HDAC4 decreases Runx2 expression. MC3T3 cells were transfected with a Runx2 P1 promoter construct and/or a HDAC4 expression vector and then cultured in differentiation medium with or without EGF. After 48 h, cell lysates were harvested for dual luciferase assay and Western blot analyses. The level of Runx2 shown at the bottom of blot were obtained by densitometric measurement and normalized by loading control β-actin.

lineage cells and/or to have the ability to modify Runx2 activity [Westendorf et al., 2002; Schroeder et al., 2004; Jeon et al., 2006; Lee et al., 2006). As shown in Figure 5A, all HDACs except HDAC5 are expressed in differentiating MC3T3 cells. Note that the protein amounts of HDAC4 and 6 were decreased in differentiating osteoblasts (with AA) comparing to proliferating osteoblasts (without AA) after the same period of culture. However, EGF treatment significantly increased amounts of HDAC4 (1.54-, 1.65-, and 1.33-fold increase after 2, 4, and 6 days of differentiation) and HDAC6 (3.54-, 1.54-, and 2-fold increase after 2, 4, and 6 days of differentiation) but not HDAC1 and 3. Interestingly, over-expression of HDAC4 strongly suppressed the Runx2 P1 promoter activity and the endogenous Runx2 protein amount in the differentiating MC3T3 cells (Fig. 5B). Furthermore, it abolished the inhibitory effect of EGF on the Runx2 promoter activity and protein expression (Fig. 5B), suggesting that one possible mechanism for EGFR's inhibitory effect on Runx2 expression and activity is through up-regulation of transcription co-repressors.

EGFR SIGNALING ATTENUATES THE EXPRESSION OF EARLY BONE MARKERS AND REDUCES Runx2 PROTEIN IN UNDIFFERENTIATED OSTEOPROGENITORS

All experiments described above were performed in osteoblastic differentiating cells. MC3T3 cells cultured in growth medium are osteoprogenitors and express relatively abundant levels of AKP and IBSP. We found that, in the absence of differentiation signal, EGF-like ligands still have the ability to significantly decrease the mRNA expression of AKP and IBSP, indicating that their effects on differentiation markers are not directly correlated with the differentiation process (Fig. 6A). Similar to the differentiating cells, MC3T3 cells grown in proliferating medium expressed no detectable amount of Runx-I mRNA (data not shown). However, the mRNA level of Runx-II was not changed after 48 h of EGF treatment (Fig. 6B). Interestingly, Runx2 protein amount was decreased significantly as shown by Western blot (Fig. 6C), suggesting that EGFR signaling may regulate Runx2 expression at either translational or protein degradation level. In contrast, Osterix mRNA expression was strongly suppressed about 4-fold by EGF (Fig. 6B).

DISCUSSION

EGF-like ligands and their receptor EGFR are one of the best-studied signaling networks and plays vital roles in tissue development and tumorigenesis. However, its role in skeletal development and remodeling has been long neglected. Here, we demonstrated that all

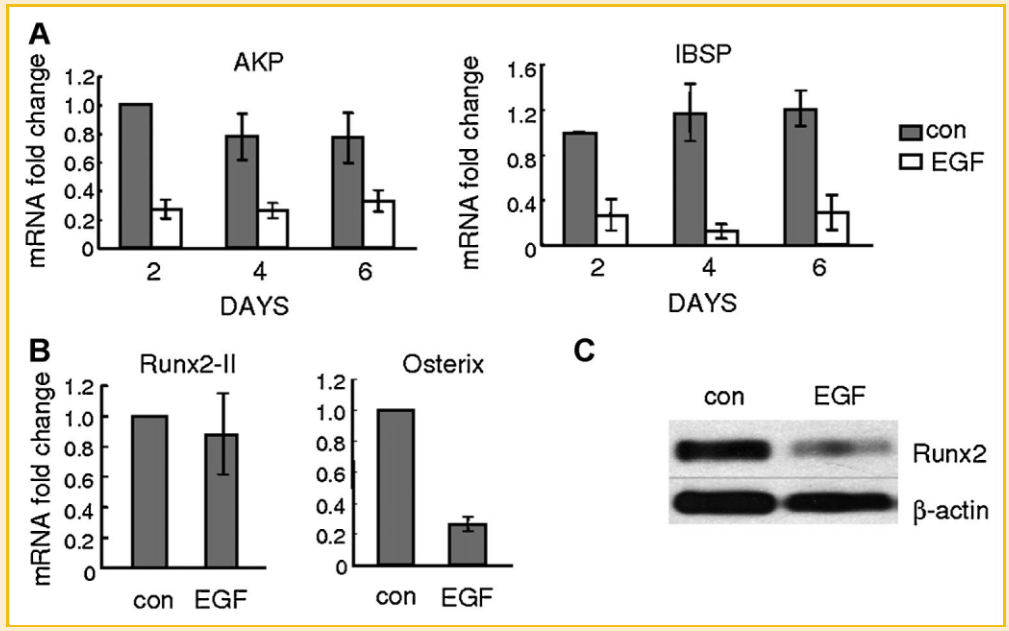

Fig. 6. EGFR signaling prevents proliferating osteoprogenitors from entering osteoblast differentiation. A: EGF suppresses the expression of AKP and IBSP in the proliferating MC3T3 cells. Cells cultured in growth medium were treated with TGFα and media were changed every 2 days with addition of fresh EGF. RNAs were harvested at the indicated times for qRT-PCR. B,C: qRT-PCR analyses of Runx2-II and Osterix mRNA expression (B) and Western blot analysis of Runx2 protein (C) in MC3T3 cells cultured in growth medium in the absence (con) or in the presence of EGF for 48 h.

EGF-like ligands suppress osteoblast differentiation and inhibit the expression of bone markers at the transcriptional level in an EGFR-dependent manner. In this study, we tested several in vitro osteogenesis systems, such as MC3T3 cells, mouse bone marrow osteoblastic cells, and human BMSSCs, and obtained similar results, implying that this inhibitory effect is a ubiquitous phenomenon. Meanwhile, activation of EGFR signaling in osteoprogenitors decreases the amounts of early bone markers and transcriptional factors, and thus prevents these cells entering into differentiation process. Together with previous studies that EGF-like ligands are growth factors for osteoprogenitor cells [Ng et al., 1983; Qin et al., 2005], we conclude that one physiological function of osteoblastic EGFR signaling is maintaining the pool of osteoprogenitors at an undifferentiated stage in bone. This is consistent with our in vivo animal data that blocking EGFR activity in osteoblast lineage cells results in fewer osteoprogenitors and leads to defective bone formation and an osteopenic phenotype [Zhang et al., 2011].

To analyze the molecular mechanisms by which EGFR signaling inhibits osteogenesis, we studied the expression of major osteoblast-specific transcription factors and demonstrated that EGFR signaling mainly inhibits Runx2 and Osterix in a dose- and time-dependent manner. Runx2 is required for mesenchymal condensation, osteoblast differentiation, and chondrocyte hypertrophy. Runx2 knockout mice exhibited complete lack of both intramembranous and endochondral ossification due to the absence of osteoblast differentiation [Komori et al., 1997; Otto et al., 1997]. Here, we found that Runx2-II is the major isoform regulated by EGFR signaling at multiple levels. In differentiating MC3T3 cells, activation of EGFR down-regulates the transcription of Runx2-II as shown by qRT-PCR, promoter reporter assay and Western blot (Figs. 3 and 4). In proliferating MC3T3 cells, EGFR signaling might regulate Runx2-II at translational and/or protein degradation levels, resulting in a decrease in Runx2 protein amount with no change in the mRNA level (Fig. 6).

The Runx2 binding site, OSE2, has been identified in the promoter regions of several bone marker genes, such as osteocalcin, IBSP, collagen1α1 and osteopontin, and Runx2 induces the expression of these genes or activates their promoters in vitro [Ducy et al., 1997]. In addition, the expression of AKP is also strongly induced by over-expression of Runx2 in vitro although Runx2 does not directly activate a 2 kb AKP promoter construct [Harada et al., 1999]. Therefore, it is reasonable to propose that one mechanism for EGFR to inhibit bone marker gene expression is by suppressing Runx2 activity. However, in differentiating MC3T3 cells, we observed significant decreases in AKP and IBSP mRNA levels at 8 h after EGF treatment (Fig. 2A) but did not observe a decrease in Runx2 mRNA until 24 h (Fig. 3B), suggesting that either EGFR signaling regulates Runx2 activity at post-transcriptional level at early time points or other transcription factor(s), probably Osterix, mediates EGFR's inhibitory effect at this stage.

Osterix is another essential transcription factor for osteoblast development and proper bone formation. Osterix-deficient mice showed an absence of osteoblasts and defective bone formation [Nakashima et al., 2002]. The current view is that Runx2 directs the entire osteoblast differentiation process, including inducing messenchymal condensation, inducing the expression of osteoblastic markers, and allowing osteoblastic progenitors to differentiate into mature osteoblasts, whereas Osterix functions downstream of Runx2 and is more involved in directing pre-osteoblasts to immature osteoblasts. While many signals regulate

Runx2 activity, few signals have been identified to regulate Osterix. Our data revealed that EGFR signaling dramatically decreases Osterix expression in a dose- and time-dependent manner at the transcriptional level. Moreover, EGF-like ligands have similar effects on Osterix expression in osteoprogenitors before entering differentiation, suggesting this inhibitory effect is not differentiation-dependent. It would be interesting to study how EGFR signaling regulates Osterix expression and whether this regulation mediates the inhibitory effect of EGFR on osteogenesis.

EGFR signaling suppresses the expression of both early and late bone marker genes. The time course data show that significant inhibition occurs after 8 h of treatment, implying that this regulation is a secondary response and most likely de novo protein synthesis is required. However, since the protein synthesis inhibitor, cycloheximide, itself has strong inhibitory effects on the expression of bone marker genes after 8 h of treatment (data not shown), we cannot use this inhibitor to test our hypothesis. The EGF-like ligands/EGFR network signals through multiple intracellular pathways [Yarden, 2001]. In osteoblasts, amphiregulin stimulates Erk1/2 and Akt phosphorylation [Qin et al., 2005], therefore activating both Ras-Raf-MAP-kinase and PI-3-kinase-Akt pathways. However, similar to cycloheximide, inhibitors for these pathways also suppress the expression of bone marker genes after 8 h (data not shown). In fact, these data are consistent with previous data demonstrating that activation of Erk1/2 [Lai et al., 2001; Xiao et al., 2002], p38 [Hu et al., 2003], JNK [Matsuguchi et al., 2009], and Akt [Mukherjee and Rotwein, 2009] are required for osteoblast differentiation. Therefore, at this time, it is difficult for us to identify the exact pathway(s) EGFR uses for blocking differentiation. However, these data do indicate that, for osteoblast differentiation, the activities of both MAPK and Akt pathways must be maintained within a narrow range. While decrease in these activities impairs differentiation, activating their activities by EGFR signaling also blocks osteogenesis. Alternatively, another unknown pathway(s) induced by EGFR could overcome the effect of activation of MAPK and Akt pathways and result in osteogenesis blockage.

Our results suggest that one possible mechanism for EGFR signaling to inhibit Runx2 expression is through up-regulation of transcription co-repressors HDAC4 and 6. HDACs deacetylate lysine residues in histones, increase DNA condensation and prevent transcription. Moreover, they can deacetylate other proteins to repress their activity. For example, HDAC4 and 5 can deacetylate Runx2 and lead to Smurf-mediated degradation of Runx2 [Jeon et al., 2006]. They also act as co-repressors for Runx2 in the TGFβ signaling pathway [Kang et al., 2005]. We found that EGFR signaling moderately stimulates HDAC4 and 6 protein amounts in differentiating osteoblastic cells. We also demonstrated that, HDAC4 can directly repress Runx2 transcription and reduce its protein amount. To our knowledge, this is the first report showing the down-regulation of Runx2 expression by HDAC4.

EGF-like ligands are potent mitogens and it is generally accepted that down-regulation of osteoprogenitor proliferation is a critical step for osteoblast differentiation. EGFR activation of the cell cycle machinery is thought to channel through Ras-Raf-MAP-kinase or PI-3-kinase-Akt pathways culminating in the induction of cyclin D1 expression [Harari and Yarden, 2000], which subsequently binds and activates cyclin-dependent kinases (cdk) 4 and 6. Activated cdk4/6 then phosphorylates Rb resulting in cell cycle progression [Guo et al., 2005]. Some of these cell cycle related genes are important for osteoblast differentiation. For example, hypophosphorylated Rb acts as a co-activator for Runx2 to stimulate osteogenesis [Thomas et al., 2001]. In our study, EGF treatment does not induce cell cycle progression in differentiating osteoblastic cells, indicating that its inhibitory effect on differentiation is not cell cycle-dependent. It would be of interest to study whether the activities of cell cycle machinery proteins, such as cyclin D1, cdk inhibitors p21 and p27, and Rb, are affected by EGFR signaling in the differentiating osteoblasts and, if they are, why cell cycle progression is not changed but terminal differentiation is blocked.

In conclusion, our results characterize the inhibitory effect of EGF-like ligands on osteoblast differentiation and explore the possible molecular mechanisms mediating this effect. In bone, EGFR signaling also regulates osteoclastogenesis [Zhu et al., 2007]. Moreover, we used animal models to investigate the physiologic role of EGFR signaling in bone remodeling and found that EGFR activity correlates with bone contents, suggesting an anabolic role of EGFR signaling in bone metabolism [Zhang et al., 2011]. Thus, our data clearly establish that EGFR signaling is one of those signaling networks that have crucial functions in skeletal homeostasis.

In addition, EGF-like ligands are expressed or over-expressed in most human tumors. Our previous data revealed that, bone metastatic breast cancer MDA-231 cells express high levels of TGFα, HB-EGF, and amphiregulin but low levels of EGF [Zhu et al., 2007]. Since these cells suppress osteoblast differentiation and this could be a contributing factor for osteolytic lesion and bone destruction [Mercer et al., 2004], breast cancer cells-derived EGF-like ligands could be a novel mechanism for cancer-induced bone loss. Together with our previous finding that EGF-like ligands stimulate bone resorption [Zhu et al., 2007], our research strongly implies that EGFR should be investigated as a drug target for cancer bone metastasis treatment.

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