Potential Involvement of Twist2 and Erk in the Regulation of Osteoblastogenesis by HB-EGF-EGFR Signaling

Takashi Nakamura, Hidetoshi Toita, Akimasa Yoshimoto, Daigo Nishimura, Tomoyo Takagi, Takuya Ogawa, Tatsuo Takeya, and Norihiro Ishida-Kitagawa

Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara 630-0192, Japan

ABSTRACT. Epidermal growth factor (EGF) family members play important roles in the skeletal system. In this study, we examined the role of EGF receptor (EGFR) signaling in osteoblastogenesis in vitro. The expression of HB-EGF and epiregulin (EPR) was transiently induced within 24 h after osteogenic stimulation, but when preosteoblastic MC3T3-E1 cells were incubated with HB-EGF or EPR, osteoblast differentiation was inhibited. These effects were Ras-dependent, and ERK modulated Runx2 activity through the localization of Smad1 and the induction of Twist2. PI3-kinase was also required for the induction of Twist2. However, the inhibition of individual signaling pathways was not sufficient to overcome HB-EGF-mediated inhibition of osteoblast differentiation. Additionally, HB-EGF treatment promoted the proliferation of preosteoblasts, and this was associated with the downregulation of p27 at the protein level. These results suggest that HB-EGF-EGFR signaling inhibits the differentiation of osteoblasts by suppression of Runx2 transcriptional activity and enhances proliferation of preosteoblasts by downregulation of expression of p27.

Key words: EGFR/osteoblasts/proliferation/differentiation/cell culture

Introduction

Bone homeostasis is tightly regulated by both systemic and local mechanisms (Harada and Rodan, 2003; Karsenty, 2006; Tettelbaum and Ross, 2003), and these signaling networks modulate the activity of two cell types within the bone parenchyma: osteoclasts and osteoblasts. Osteoclasts are hematopoietic in origin and mediate bone resorption, but mesenchymally-derived osteoblasts play an essential role in skeletal development and bone formation (Karsenty et al., 2009). Many signaling pathways involved in osteoblast development have been identified. Bone morphogenic protein (BMP) and several transcription factors including Smad, Runx2, and Osterix, are essential for osteoblast formation (Bialek et al., 2004). Runx2 functions upstream of Osterix and induces the expression of osteoblastic marker genes, including major bone matrix protein genes, such as Collagen type I and Osteocalcin. The activity of Runx2 is both positively and negatively regulated. In particular, C/EBPβ, Smad1, and Smad5 bind and activate Runx2, while other factors, e.g. Twist, inhibit the transcriptional activity of Runx2 (Bialek et al., 2004; Franceschi et al., 2007).

Epidermal growth factor receptor (EGFR or ErbB1) signaling modulates both the proliferation and differentiation of osteoblasts (Canalis and Raisz, 1979; Hata et al., 1984; Kumegawa et al., 1983). EGFR is a transmembrane glycoprotein with intrinsic tyrosine kinase activity that becomes activated after binding EGF family members, including EGF, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (AR), betacellulin (BTC), transforming growth factor-α (TGF-α), epiregulin (EPR) and epigen (Iwamoto and Mekada, 2006; Kochupurakkal et al., 2005). Osteoblast proliferation and differentiation are abnormal in mice lacking EGFR, and this leads to impaired bone formation and skeletal structure (Sibilia et al., 2003; Wang et al., 2004). Primary osteoblasts isolated from calvaria of mice lacking EGFR proliferate less and undergo maturation more readily in vitro than those isolated from wild-type mice. Furthermore, transgenic mice overexpressing EGFR ligands demonstrate variable, ligand-specific effects on bone formation. Overexpression of EGF led to osteoblast hyperproliferation and hyperplasia and a reduction in cortical bone thickness.
(Chan and Wong, 2000), whereas BTC overexpression resulted in increased cortical bone mass in transgenic mice (Schneider et al., 2009a). Thus, EGFR ligands may promote osteoblast proliferation and/or differentiation, leading to proper bone formation. Determining the mechanism by which EGFR family members differentially regulate intracellular signaling networks downstream of EGFR in osteoblasts is essential to gain a better understanding of osteoblastogenesis. Toward this end, in this study we examined the role of EGFR signaling in osteoblast homeostasis.

Materials and Methods

Cells and reagents

MC3T3-E1 cells were maintained in minimum essential medium alpha (α-MEM; Wako) supplemented with 10% FBS, penicillin, and streptomycin. The following antibodies were used: anti-β-actin monoclonal antibody (AC-74; Sigma Aldrich), rabbit anti-ERK, anti-pERK (Promega), anti-cyclin D1 (DCS-6; MBL), anti-Rb (BD Biosciences), anti-p21 (Santa Cruz), anti-p27 (MBL), anti-anti-pERK (Promega), recombinant human epiregulin protein (rEpr) and anti-human IgG, protein-A (GE Healthcare UK Ltd.), and anti-goat IgG (Chemicon). Ascorbic acid and glycerol 2-phosphate were obtained from Sigma. Recombinant human HB-EGF protein (rHB-EGF), α2 anti-smooth muscle (Promega), rabbit anti-ERK, mouse anti-β-actin (AC-74; Sigma Aldrich), rabbit anti-ERK1, ERK2, MEK1, MEK2, MEK1 S218E, MEK CA), Twist2 was obtained by RT-PCR and cloned into the retroviral expression vector pCX4-puro (Dr. T. Akagi; KAN Research Institute, Kyoto, Japan). Constitutively active MEK1 (MEK1 S218E: MEK CA) was generated by site-directed mutagenesis with Glu substitutions at Ser218 and Ser222. The dominant-negative form of Ha-Ras (Ras S17N, DN-Ras) was kindly provided by Dr. K. Kaibuchi (Nagoya University, Japan) and was subcloned into pCX4-puro. For the production of retroviruses, Plat-E cells were co-transfected with pGL, pE-Eco (Takara), and retroviral vectors provided by Dr. K. Kaibuchi (Nagoya University, Japan) and was subcloned into pCX4-puro. For the production of retroviruses, Plat-E cells were co-transfected with pGL, pE-Eco (Takara), and retroviral vectors. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl and 50 mM MgCl2) and staining with a NBT/BCIP solution. For Alizarin red staining, cells were fixed with cold 70% ethanol and stained with a 40 mM Alizarin red solution.

RNA isolation and RT-PCR

Total RNA was extracted from cells using ISOGEN (Nippon Gene), according to the manufacturer’s instructions. cDNA was made by reverse transcription using SuperScript™II (Invitrogen) after DNase treatment of the RNA samples. PCR was performed using rTaq DNA Polymerase (Takara) with the gene-specific primer pairs as follows: Hb-egf, 5’-AAATACCGCAGCTCAGGA-3’ (forward) and 5’-GGGATAGGAACGAGAA-3’ (reverse); Egf, 5’-CCAGGGCAACGTACAA-3’ (forward) and 5’-GGTCATAACCAGGAA-3’ (reverse); Tgfα, 5’-CAGGATTGGTCGCACCCT-3’ (forward) and 5’-CAACGATTGCACCTTAGA-3’ (reverse); Tgfβ1, 5’-AACCCAGACACAGCATC-3’ (forward) and 5’-GGCATTTGAGTTTGTTGTA-3’ (reverse); Opn, 5’-AGGACTGACACTG-3’ (forward) and 5’-CTCCCTGGCTCTTCTTGGA-3’ (reverse); Twist2 was obtained by RT-PCR and cloned into the retroviral expression vector pCX4-puro (Dr. T. Akagi; KAN Research Institute, Kyoto, Japan). Constitutively active MEK1 (MEK1 S218E: MEK CA) was generated by site-directed mutagenesis with Glu substitutions at Ser218 and Ser222. The dominant-negative form of Ha-Ras (Ras S17N, DN-Ras) was kindly provided by Dr. K. Kaibuchi (Nagoya University, Japan) and was subcloned into pCX4-puro. For the production of retroviruses, Plat-E cells were co-transfected with pGL, pE-Eco (Takara), and retroviral vectors provided by Dr. K. Kaibuchi (Nagoya University, Japan) and was subcloned into pCX4-puro. For the production of retroviruses, Plat-E cells were co-transfected with pGL, pE-Eco (Takara), and retroviral vectors. Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl and 50 mM MgCl2) and staining with a NBT/BCIP solution. For Alizarin red staining, cells were fixed with cold 70% ethanol and stained with a 40 mM Alizarin red solution.

Western blotting and subcellular fractionation

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 2 mM PMSF, 2 mM Na3VO4, 20 mM NaF, 100 U/μl of aprotinin, and 1% Triton X-100). Proteins were separated by SDS-PAGE, transferred to a PVDF membrane, and detected by Western blotting using an ECL detection kit (GE Healthcare UK Ltd.). Nuclear and cytoplasmic extracts were prepared using NE-PER™ nuclear and cytoplasmic extraction reagents (PIERCE), according to the manufacturer’s instructions.

Immunofluorescence microscopy

Cells were grown on coverslips and stimulated with BMP2 for 6 h with or without rHB-EGF and PD98059. Cells were then fixed with 3.7% paraformaldehyde for 10 min at room temperature (RT), permeabilized with 0.2% Triton X-100/PBS, and blocked with 5%
skim milk/PBS for 1 h at RT. Cells were then incubated with anti-
phosphoSmad1/5/8 for 1 h and visualized with goat-anti-rabbit Alexa
fluor 546 (Molecular Probes). Nuclei were counterstained with
Hoechst dye. All images were obtained using a LSM510 laser
scanning microscope (Carl Zeiss, Inc.).

**Transient transfection and luciferase assay**

The mouse *ALP* promoter (−1838/+81) in the pGL3 vector
(Promega) was kindly provided by Dr. T. Komori (Nagasaki
University, Japan). MC3T3-E1 cells were transfected with the
plasmids using LipofectAMINE2000 reagent (Invitrogen), accord-
ing to the manufacturer’s instructions. Cells were harvested 48 h
after transfection, and luciferase activity was determined using a
Dual-Luciferase Reporter Assay System (Promega).

**Results**

**EGFR signaling inhibits osteoblastogenesis in a Ras-dependent manner**

To better understand the role of EGFR signaling in osteo-
blast homeostasis, we first examined the expression of EGFR
family members in MC3T3-E1 cells, a well characterized
osteoblast-like cell line. When cultured in media containing
ascorbidic acid and glycerol 2-phosphate that promotes osteo-
blastic differentiation, *Hb-egf* and *Epiregulin (Epr)* mRNA
expression were transiently increased within 24 h (Fig. 1A).
*EGFR* mRNA was constitutively expressed under the same
conditions (data not shown). ALP activity is a marker of
osteoblast differentiation, and, under the culture conditions
used, it became detectable after 3–5 days and continued to
increase until 10 days after the addition of differentiation
stimuli. Additionally, mineralizing activity, a marker of
mature osteoblasts, became apparent after 20 days (Fig. 1B).

We next wished to examine whether the observed upregu-
lation of HB-EGF and Epr affected osteoblast maturation
and/or differentiation using recombinant human HB-EGF
(rHB-EGF) and Epr (rEpr). When MC3T3-E1 cells were
grown in osteogenic media supplemented with rHB-EGF,
both ALP and mineralizing activities were substantially
decreased, and there was an associated downregulation of
*Alp* and *Ocn* mRNA expression (Fig. 1B and 1C). ALP
activity was also decreased when MC3T3-E1 cells were
differentiated in the presence of rEpr (data not shown), indi-
cating that EGFR signaling downregulates differentiation
signals in MC3T3-E1 cells. All ErbB-family receptors can
activate Ras and the downstream MAPK signaling cascade
consisting of Raf, MEK, and ERK (Montagut and Settleman,
2009). Accordingly, rHB-EGF stimulation induced ERK
phosphorylation in MC3T3 cells (Fig. 1D). The ability of
HB-EGF to inhibit osteoblast differentiation was abrogated
when either a neutralizing antibody against human HB-EGF
(αHB-EGF) or an EGFR inhibitor (AG1478) were included
in the culture medium (Fig. 1E). Thus, rHB-EGF stimulation
downregulates *Alp* and *Ocn* expression downstream of
EGFR-initiated signaling.

We next examined the role of Ras signaling on the inhibi-
tion of osteoblast differentiation following EGFR ligation.
We prepared MC3T3-E1 cells expressing a dominant-
negative form of Ras (DN-Ras) and stimulated the cells
with BMP2 (Fig. 1F). At baseline, ALP activity was enhanced
in these cells compared to mock-transfected cells, and the
addition of HB-EGF to the mock-transfected cells led to
decreased ALP activity. In contrast, no change in ALP
activity was seen following HB-EGF treatment of DN-Ras
expressing cells. Thus, Ras inhibits osteoblastogenesis and
is required for the function of EGFR signaling.

**EGFR signaling inhibits Runx2 activity through Twist2 and Smad**

Runx2 is a key regulator of osteoblast differentiation, and
*Alp* and *Ocn* mRNA expression are controlled by Runx2. As
shown in Fig. 1C, treatment of cells with rHB-EGF down-
regulated the expression of *Alp* and *Ocn* mRNA, we exam-
ned the expression profiles of Runx2. However, Runx2
mRNA levels were unchanged (Fig. 2A) even in rHB-EGF-
treated cells, suggesting that HB-EGF stimulation regulates
the activity of Runx2 rather than its expression. Runx2
activity is directly modulated by transcriptional regulators
such as ATF4, SATB2, and Twist 1/2. We therefore exam-
ned the expression of these genes in rHB-EGF-treated
cells. In the presence of rHB-EGF, Twist2 mRNA was tran-
siently induced at day 1, but there was no change in Twist2
expression under control conditions (Fig. 2A). Twist2 is a
negative regulator of Runx2 activity, and the timing of the
expression of Twist2 after rHB-EGF treatment coincides
with the changes in *Alp* mRNA expression in differentiating
MC3T3-E1 cells in the presence or absence of HB-EGF
(Fig. 1C). In contrast, most of the other genes examined
were constitutively expressed in differentiating cells, sug-
gesting that *Twist2* could be involved in the downregula-
tion of *Alp* and *Ocn* downstream of EGFR signaling. Accord-
ingly, *Twist2* mRNA expression was suppressed by the
 treatment of cells with either a MEK (PD98059) or PI3-
kinase inhibitor (LY294002) (Fig. 2B). These data suggest
that the induction of *Twist2* expression is mediated by the
MEK and/or PI3K pathway(s). Further analysis was carried
out to examine the effect of EGFR signaling on Runx2
activity using a reporter plasmid in which luciferase expres-
sion is driven by the *Alp* promoter, which contains a Cbfa1/
Runx2-binding site (pALP(−1838/+81)-luc) (Harada *et al*.,
1999). Treatment of cells with rHB-EGF significantly sup-
pressed luciferase activity (Fig. 2C). When cells were trans-
fected with ERK1/2, Constitutively active MEK1 (MEK
CA) and Twist2, luciferase activity was also suppressed,
suggesting that HB-EGF induced Twist2 and MAPK signal-
ing inhibited osteoblast differentiation by suppressing
Runx2 transcriptional activity. When cells were treated with PD98059, the suppression of luciferase activity by rHB-EGF was not restored (Fig. 2C). LY294002 inhibited Runx2 transcriptional activity even in the absence of rHB-EGF, suggesting that PI3K was essential for the osteoblast differentiation. These data suggested that ERK and Twist2 are sufficient to inhibit Runx2 activity, MAPK signaling is not essential for the inhibition of differentiation by HB-EGF.

Runx2 integrates BMP/Smad1 signaling through the formation of a Runx2-Smad1 complex in a specific region of the nucleus (Lian et al., 2006). Additionally, in a previous study EGFR signaling induced Smad1 phosphorylation at its linker region through the ERK/MAPK phosphorylation, and BMP signaling inhibited the nuclear translocation of Smad1 and BMP receptors (Kretzschmar et al., 1997). Consistent with these results, BMP2 stimulation of MC3T3-E1 cells enhanced the nuclear localization of Smad1, and this was suppressed by rHB-EGF treatment (Fig. 3A). Inhibition of MEK signaling with PD98059 restored Smad1 nuclear localization to a level comparable to that observed in differentiating cells (Fig. 3A), and this was confirmed by sub-

**Fig. 1.** EGFR signaling suppresses osteoblast differentiation through Ras. A) Expression of EGF family members mRNA in osteoblasts. MC3T3-E1 cells were induced to differentiate into osteoblasts, and the mRNA expression of EGF family members was monitored by RT-PCR at the indicated time points. GAPDH was used as an internal control. *Hb-egf*, heparin-binding EGF-like growth factor; *Tgfα*, transforming growth factor-α; *Areg*, amphiregulin; *Epr*, epiregulin; *Btc*, betacellulin. P, positive control. mRNA from mouse heart, liver, and lung was used as a positive control. B) Effect of rHB-EGF on osteoblastogenesis. MC3T3-E1 cells were seeded on 24-well plates, and osteoblast differentiation was induced. Alkaline phosphatase (ALP) activity and mineralizing activity were measured by ALP staining (upper panel) and Alizarin red staining (lower panel), respectively, at the indicated time points. C) Expression of osteoblastic marker genes in MC3T3-E1 cells. Cells were cultured in osteogenic media in the presence of rHB-EGF, and the mRNA expression of the indicated genes was monitored by RT-PCR. GAPDH was used as an internal control. D) Activation of ERK in osteoblast differentiation. The phosphorylated form of ERK was visualized by Western blotting. MC3T3-E1 cells were stimulated with rHB-EGF and indicated inhibitors as shown in D) for 3 days and ALP staining was carried out. E) Involvement of Ras in osteoblast differentiation. A dominant-negative form of Ras was expressed in MC3T3-E1 cells, and ALP staining was performed three days after osteogenic stimulation in the presence or absence of rHB-EGF. Mock, empty vector.
EGFR Signaling in Osteoblasts

57

These findings suggest that the normal induction of twist2 expression and Smad translocation that occurs during osteoblast differentiation is inhibited by ERK signaling downstream of HB-EGF. However, treatment of cells with both PD98059 and rHB-EGF did not lead to greater osteo-

Fig. 2.  EGFR signaling inhibits Runx2 activity through the induction of Twist2.  A) Induction of Runx2 regulatory genes by rHB-EGF. The expression of genes known to be under the control of Runx2 was monitored in rHB-EGF-treated MC3T3-E1 cells by RT-PCR. GAPDH was used as an internal control. B) The effect of inhibitors of Twist2 induction. MC3T3-E1 cells were cultured for one day with the indicated inhibitors, and the expression of Twist2 was measured by RT-PCR. PD, PD98059, MEK inhibitor; LY, LY294002, PI3K inhibitor. C) A reporter assay using a plasmid containing the mouse Alp promoter (−1838/+81). Cells were transfected with the pALP(−1830/+81)-luc plasmid and cultured for 48 h in the absence (AA−) or presence (AA+) of ascorbic acid (AA), and the effect of rHB-EGF (HB+) on luciferase activity was quantified. ERK1 and ERK2 (ERK1/2), constitutively active MEK1 (MEK CA) and Twist2 (Twi2) were co-transfected with pALP-luc plasmid and cells were treated with PD98059 (PD) and LY294002 (LY) when needed. All experiments were performed in triplicate. Bars represent the means±SEM (n=3). *p<0.05 versus AA+. Vec: vector

cellular fractionation experiments (Fig. 3B).
Fig. 3. EGFR signaling inhibits Runx2 activity by inhibiting Smad nuclear localization. A) Intracellular localization of Smad1. Localization of Smad1 in control and rHB-EGF-treated cells was examined by fluorescent microscopy using anti-Phospho-Smad1. Nuclei were counterstained with Hoechst dye. Bar, 100 μm. B) Nuclear localization of Smad1 determined by subcellular fractionation. Nuclear extracts and cytoplasmic fractions were prepared from the cells 6 h after stimulation, and were immunoblotted using anti-Phospho-Smad1 antibody. AA, ascorbic acid; HB, rHB-EGF; PD, PD98059. C) ALP activity in PD98059-treated cells. MC3T3-E1 cells were cultured with the indicated concentrations of PD98059 in the presence of rHB-EGF (HB+). ALP staining was performed after three days of culture.
EGFR Signaling in Osteoblasts

Fig. 4. EGFR signaling enhances osteoblast proliferation. A) Cell proliferation assay. The number of viable cells after rHB-EGF treatment in the absence or presence of ascorbic acid (AA- or AA+, respectively) was counted by a WST assay. Bars represent the means±SEM (n=3). *p<0.01. B) Expression profiles of cell cycle regulatory genes in HB-EGF-treated cells. The expression of Cyclin D1, p21, p27, and Alp mRNA was monitored by RT-PCR at the indicated times (left panel). Alp was used as a marker of differentiation. Western blot analysis using antibodies against the indicated gene products (right panel). GAPDH and β-actin were used as internal controls. C) The effect of p27 on cell proliferation. MC3T3-E1 cells were infected with either an empty retroviral vector (mock) or a vector expressing p27, and cell proliferation was examined by a WST assay. Bars represent the means±SEM (n=3). *p<0.01. D) The effect of exogenous p27 expression on differentiation. Cells prepared as in C) were stimulated by BMP2, rHB-EGF, or both as indicated, and ALP staining was performed after three days.

Thus, ERK signaling contributes to the regulation of Twist2 expression and Smad localization, but this is not sufficient to explain the inhibitory effects of EGFR signaling on osteoblast differentiation. Interestingly, cells treated with PD98059 and grown in osteogenic media demonstrate enhanced Runx2 and ALP activity (Fig. 3C), suggesting that ERK signaling downstream of receptors other than EGFR is involved in the inhibition of osteoblast differentiation.

**EGFR signaling modulates cell cycle regulation and enhances cell proliferation**

EGFR signaling exhibits mitogenic activity, and we next
examined the effect of EGFR signaling on MC3T3-E1 cell proliferation. rHB-EGF enhanced MC3T3-E1 cell proliferation (Fig. 4A), and RT-PCR and immunoblotting analyses of cell cycle regulatory genes in rHB-EGF-treated MC3T3 cells showed that CycD1, Rb, and p21 expression were induced, while p27 was suppressed at the protein level (Fig. 4B). While exogenous expression of p27 in MC3T3-E1 cells suppressed proliferation (Fig. 4C), it did not affect the ability of BMP2 or rHB-EGF to promote or inhibit osteoblast differentiation, respectively (Fig. 4D). Thus, our data indicate that EGFR signaling enhances cell proliferation, but inhibition of proliferation per se is not sufficient to induce differentiation.

Discussion

EGFR family signaling networks have been implicated in regulating bone homeostasis, but the detailed mechanism by which they act in this compartment has remained elusive. Contributing to the difficulty is the presence of multiple genes belonging to the EGF and EGFR families, and different receptor-ligand combinations may have different activities. This is more than a theoretical possibility because EGF, TGF-α, HB-EGF, BTC, and EPR expression are induced in the rat osteoblastic cell line UMR106 and primary rat osteoblasts (Qin et al., 2003; Qin et al., 2005). However, when UMR106 cells are stimulated with the bone-forming agent parathormone (PTH), only AR, HB-EGF, and TGF-α were rapidly induced (Qin et al., 2005; Schneider et al., 2009a). In this study, we found that only Hb-egf and Epr were induced in the mouse preosteoblast cell line MC3T3-E1, and we focused our analysis on EGFR signaling in preosteoblasts and analyzed its role in osteoblastogenesis. Using an in vitro differentiation system, we showed that EGFR signaling induces two distinct signals in preosteoblasts: differentiation-suppressing and proliferation-promoting signals.

The transient induction of HB-EGF and Epr suppressed the expression of Alp and Ocn through osteoblast differentiation, suggesting that they may participate in negative feedback during osteoblast differentiation. Although the expression of Alp and Ocn was suppressed by HB-EGF, the expression of Coll and Opn, which are also known as osteoblastic marker genes, was not affected. In this study, we focused on the early stage of differentiation and Opn is induced in the late stage of differentiation. Furthermore, different from ALP and Ocn which are known to be transcriptional target of Runx2, Opn expression was regulated by Ets transcription factors (Vary et al., 2000). Taken together, constitutive expression of Opn would be due to the differentiation stage which we observed, and the difference of transcriptional factor would be a reason that the expression of Opn was not affected by HB-EGF. Coll is also well known for the osteoblastic marker, but the expression in MC3T3-E1 cells was not changed in osteogenic media for 10 days (Varanasi et al., 2009), suggesting that Coll is not suitable as a marker gene in MC3T3-E1 cells at least for 10 days after differentiation stimulation.

HB-EGF induces Ras-mediated intracellular signaling to block osteoblast differentiation and maturation. ERK, which is downstream of Ras, functions in multiple signaling pathways, and Runx2 is a transcription factor with essential roles in osteoblast differentiation during intramembranous and endochondrial bone formation (Karsenty and Wagner, 2002). In this study, we showed that ERK downregulates Runx2 transcriptional activity, and that the inhibition of ERK reduced the induction of Twist2 and restored impaired pSmad nuclear localization by HB-EGF. Twist2 was also shown to suppress Runx2 activity. Expression of DN-Ras completely abrogated the HB-EGF–induced inhibition of differentiation, but the inhibition of ERK signaling with the MEK inhibitor PD98059 did not restore ALP activity, suggesting that ERK and Twist2 are sufficient for the inhibition of osteoblast differentiation; however, ERK is not essential for the inhibition of osteoblast differentiation by HB-EGF. This raises the possibility that an additional signaling molecule/pathway contributes to HB-EGF function. Indeed, treatment of cells with the PI3K/AKT inhibitor LY294002 completely blocked the induction of Twist2 mRNA by rHB-EGF. These data suggested that osteoblast differentiation is controlled by multiple signaling pathways downstream of different stimuli.

EGF family members are prototypical growth factors and induce cell proliferation and migration. In the present study, HB-EGF treatment of MC3T3-E1 preosteoblast cells enhanced cell proliferation, and this was accompanied by modulated expression of the cell cycle regulatory genes p21, cyclin D1, and p27. Expression of p21 and cyclin D1 was upregulated, whereas p27 expression was downregulated. These data are consistent with a previous study showing that p21 is expressed in proliferating cells during osteoblast development in vitro, but p27 is induced during the immediate post-proliferative stage of osteoblast maturation and acts as a key regulator of the transition from osteoblast cell proliferation to differentiation (Drissi et al., 1999). We found that p27 was reduced at the protein level following BMP2 or HB-EGF treatment, but p27 mRNA levels were unchanged, suggesting that p27 is regulated post-translationally in preosteoblasts. However, the exogenous expression of p27 suppressed cell proliferation, and its overexpression was not sufficient to induce differentiation. Thus, the functional relationship between proliferation and differentiation in osteoblastogenesis remains unclear.

In this study, our data show that EGFR signaling negatively regulates osteoblast differentiation via multiple pathways downstream of Ras, and that proliferation is affected by the downregulation of p27. In vivo, EGFR knockout mice and amphiregulin knockout mice showed the phenotype of decreased bone mass. These results suggest three possibili-
ties to the role of EGFR signaling in bone formation:
1) EGFR signaling promotes preosteoblast proliferation.
2) EGFR signaling promotes osteoblast differentiation.
3) EGFR signaling promotes both proliferation and differentiation of osteoblast. In vitro study showed that EGF suppressed osteoblast differentiation and enhanced pre-osteoblast proliferation (Schneider et al., 2009b). It is possible that this discrepancy of the results between in vivo and in vitro study is caused by the stages of osteoblastogenesis in which these experiments were used, namely, presence or absence of proliferating preosteoblasts. Osteoblasts were differentiated from mesenchymal cells, and at first, osteoblasts proliferated (proliferation stage) and produced committed differentiating cells (differentiation stage). The cells used in the in vitro study, including MC3T3-E1 cells, were well committed osteoblasts and the EGFR family members acted as inhibitors in osteoblast differentiation stages. If EGFR signaling predominantly promotes preosteoblast proliferation rather than differentiation in vivo, the phenotypes observed in the transgenic mice may be caused by the lack of proliferation stages following the lack of differentiation, and may result in decreased bone mass.

These are the first data providing a detailed mechanism of the inhibitory activity of EGFR ligands, and they should provide a basis for further study of the involvement of the EGFR signaling network in skeletal cells.

Acknowledgments. We are grateful to Dr. T. Komori (Nagasaki University) for providing the pALP (~183/81)-luc plasmid and Dr. K. Kaibuchi (Nagoya University) for the DN-Ras expression vector. This work was supported in part by Grants-in-aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

Endocrinology, 115: 867–876.

(Rceived for publication, January 6, 2010, accepted, February 1, 2010 and published online, April 24, 2010)