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Estrogen Enhances Activity of Wnt Signaling During Osteogenesis by Inducing Fhl1 Expression

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ABSTRACT

Estrogen is a crucial hormone for osteoclast inhibition and for preventing osteoporosis. However, the hormone's role in osteoblast growth and differentiation remains unclear. The complexity of estrogen's role in guiding osteoblast behavior arises partly from crosstalk with other signaling pathways, including Wnt signaling. In this study, we show that the Wnt agonist, LiCl, induced *Fhl1* gene expression, which substantially enhanced osteoblast differentiation. Staining with alizarin red revealed that MC3T3-E1 mineralization was enhanced by overexpression of *Fhl1*. In addition, *Fhl1* promoted the expression of the osteogenic markers, *Runt-related transcription factor 2* (Runx2), osteocalcin (*OCN*), and *osteopontin* (*OPN*), whereas MC3T3-E1 cells with gene knockdown of *Fhl1* exhibited limited mineralization and expression of Runx2, *OCN*, and *OPN*. We further demonstrate evidences from quantitative reverse transcription real-time polymerase chain reaction and reporter assay that *Fhl1* expression was synergistically stimulated by estrogen (E2) and LiCl, but reduced by the estrogen-receptor inhibitor fulvestrant (ICI 182,780). However, estrogen could not enhance osteogenesis while *Fhl1* expression was knocked down. Because estrogen and Wnt signaling frequently interact in developmental processes, we propose that Fhl1 can be an acting molecule mediating both signaling pathways during osteogenesis. J. Cell. Biochem. 116: 1419–1430, 2015.

KEY WORDS: ESTROGEN; WNT SIGNALING; FHL1; OSTEOBLAST; DIFFERENTIATION; OSTEOCALCIN; OSTEOPONTIN; RUNT-RELATED TRANSCRIPTION FACTOR 2

Estrogen and Wnt signaling pathways are crucial for regulating developmental processes and maintaining tissue homeostasis in animal species. Moreover, these pathways often interact in response to physiological stress and disease, resulting in various cellular behaviors. In the brain, estrogen signaling activates Wnt by downregulating dickkopf-1 (Dkk1), a Wnt antagonist, to prevent neurodegeneration [Zhang et al., 2008]. In the uterus, estrogen prompts beta-catenin activation in the uterine epithelium to induce uterine epithelial cell growth [Galea et al., 2013]. In breast cancer, estrogen receptor α (ERα) enhances cell growth via Wnt signaling [Lim et al., 2011]. Crosstalk between these pathways is also observed during osteoblast activity regulation.

Estrogen signaling plays critical roles in skeletal homeostasis, during which estrogen maintains a dynamic equilibrium between osteoclasts and osteoblasts through a process known as bone remodeling. Although the major physiological effect of estrogen's action on bone remodeling is to inhibit osteoclast formation, such effect is mediated partly through regulating osteoblast activity. For example, estrogen enhances expression of TGF-β, Fas ligand, and osteoprotegerin, but suppresses the expression of cytokines such as interleukin-6 during osteoblastic remodeling, thereby reducing osteoclast formation and increasing osteoclast apoptosis [Bord et al., 2003; Hughes et al., 1996; Koka et al., 1998; Krum et al., 2008]. However, studies on the effects of estrogen signaling on the growth and differentiation of osteoblasts have occasionally produced inconsistent results. For example, as observed in postmenopausal woman and ovariectomized animals, the numbers of osteoclasts and osteoblasts, as well as the rate of bone resorption and formation, increase because of estrogen deficiency, even though the rate of bone resorption exceeds formation [Jochems et al., 2005; Raisz, 2005; Rodan and Martin, 2000]; nevertheless, female mice with osteoblast-specific ERα gene ablation exhibited remarkably reduced osteoblast activity and bone volume [Maatta et al., 2013; Melville et al., 2014]. Similarly, a human patient who exhibited resistance to estrogen signaling caused by a mutated estrogen receptor had delayed bone maturation [Smith et al., 1994]. Conflicting results have also been

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reported by several in vitro studies [Almeida et al., 2013; Galea et al., 2013; Matsumoto et al., 2013; Nasu et al., 2000; Okazaki et al., 2002], that estrogen promotes osteoblast differentiation. Therefore, the influence of estrogen on osteoblast activity is complex, possibly because of interactions between estrogen and other signaling pathways, such as Wnt.

Functional crosstalk between estrogen signaling and the Wnt pathway has recently been determined to be a crucial aspect of osteoblast differentiation [Armstrong et al., 2007; Galea et al., 2013; Liedert et al., 2010; Sunters et al., 2010]. We previously reported that Fhl1, a molecule downstream of Wnt signaling, can execute Wnt activity to promote myogenesis [Lee et al., 2012] and inhibit chondrogenesis [Lee et al., 2013]. FHL1, a member of the four-and-a-half LIM protein (Fhl) family [Greene et al., 1999; Morgan and Madgwick, 1996], is predominantly expressed in skeletal muscle and also expressed at lower levels in the heart and other tissues. Additionally, *FHl1* is expressed at high levels in embryonic lungs, neural tubes, and limb buds during early development [Chu et al., 2000]. *Fhl1* responds to Wnt/β-catenin signaling through its 2 TCF/LEF elements in the upstream region, and subsequently promotes myogenesis. Because Wnt signaling is highly associated with estrogen signaling in osteogenesis, Fhl1 may interact with estrogen signaling in the differentiation of osteoblasts as well. Conversely, previous study has reported that estrogen represses *Fhl1* expression in rat muscle cells [Wang et al., 2010]; by contrast, Fhl1 negatively regulates estrogen signaling in some cancer cell lines [Ding et al., 2011; Zhang et al., 2012]. This relationship was inconsistent with our expectations, prompting us to investigate how estrogen signaling influences Fhl1 activity in differentiating osteoblasts. To more comprehensively understand the relationship between estrogen signaling and Fhl1, a lentiviral system was adopted to overexpress or downregulate the *Fhl1* gene in MC3T3-E1, an osteoblastic precursor cell, in the presence or absence of estrogen. In addition, we explored their relationship using a luciferase reporter system, verifying whether the estrogen receptor affected *Fhl1* transcription by using fulvestrant (ICI 182,780) to block estrogen receptor activity. Finally, the differentiations of MC3T3-E1 under different circumstances were investigated through alizarin red staining (ARS) and marker gene analysis.

MATERIALS AND METHODS

CELL CULTURE

MC3T3-E1 cells were grown at 37°C in α-MEM, containing 10% fetal bovine serum bovine serum albumin (BSA), penicillin G (100 U/mL), and streptomycin (100 μg/mL) in a 5% CO_2 atmosphere. For osteoblast differentiation, MC3T3-E1 cells were induced with osteogenic media, comprising the above growth medium supplemented with ascorbic acid-2-phosphate (vitamin C-P 0.2 mM) (Sigma–Aldrich, St. Louis, MO) and β-glycerol phosphate (10 mM) (Sigma–Aldrich). The osteogenic media were replaced every 2–3 days. The 17β-estradiol (E2) (Sigma–Aldrich) and estrogen antagonist fulvestrant (ICI 182,780, Sigma–Aldrich) hormones were dissolved in ethanol and subsequently diluted 1000-fold with a medium for cell culture. The final working concentration was 10 nM for 17β-estradiol (E2) and 100 μM for fulvestrant.

ALIZARIN RED STAINING AND CERYLPYRIDINIUM CHLORIDE ASSAY

Mineralization in osteogenic cultures was determined through alizarin red (Sigma–Aldrich) staining. Alizarin red selectively binds to calcium, staining it red. MC3T3-E1 cells were seeded in 6-well plates, and grown in osteogenic media for 14–28 days. Cells were then washed with phosphate buffered saline (PBS), fixed with 70% ethanol for 1 h, washed 3 times with distilled water, and then stained with 40 nM alizarin red (pH 4.2) for 10 min. Subsequently, the cultures were washed 3 times with deionized water. To quantify matrix mineralization, sample-bound alizarin red stain was solubilized with 10% cetylpyridinium chloride (CPC) buffered to pH 7.0 with 10 mM sodium phosphate, and quantified using a spectrophotometer to measure the absorbance at 562 nm.

RNA EXTRACTION AND RNA QUANTIFICATION

Total RNA from cultured MC3T3-E1 cells was isolated using an acid guanidinium thiocyanate–phenol–chloroform extraction method. Samples of cDNA were synthesized from 1 μg of total RNA through reverse transcription (RT) by using random hexamer primers. The cDNA was used as the template for semiquantitative polymerase chain reaction (PCR) and real-time PCR analysis; PerlPrimer software was used to design specific primers. The quantitative RT and real-time PCR (QRT-PCR) procedure was performed using the SYBR green master mix kit (Fermentas, Waltham, MA) in an ABI 7500 instrument (Applied Biosystems, Carlsbad, CA). The β-*actin* gene was used to normalize the amount of template for each sample. Each reaction was performed in triplicate, and the results were averaged. Primer sequences for tested genes were as follows: β-*actin*, 5′-CAGCCTTCCTTCTTGGGTATG-3′, and 5′-AGTACTTGCGCTCAGGAGGAG-3′; *Fhl1*, 5′-CTGCCTGAAGTGCTTTGACA-3′, and 5′-ATCTTGCCATCCTTGGACAC-3′; *Wnt3a*, 5′-CTGGCAGCTGTGAAGTGAAG-3′, and 5′-TACGTGTAACGTGGCCTCAG-3′; *Runx2*, 5′-CCCAGCCACCTTTACCTACA-3′, and 5′-TATGGAGTGCTGCTGGTCTG-3′; *Ocn*, 5′-CTGACCTCACAGATCCCAAGC-3′, and 5′-TGGTCTGATAGCTCGTCACAAG-3′; and *Opn*, 5′-TCTGATGACCGTCACTGC-3′, and 5′-AGGTCCTCATCTGTGGCATC-3′.

LENTIVIRAL VECTOR CONSTRUCTION

To insert the *Fhl1-myc*-tagged cDNA into the pLKO AS3W.GFP lentiviral vector (National RNAi Core Facility, Academia Sinica, Taiwan), we amplified a cDNA fragment according to a previous study [Lee et al., 2012]. We used 5′-CCCAAGCTAGCTAGTTAAGC-3′ and 5′-ACTAGAATTCACAGTCGAGGC-3′ primers (underlined bases were used to generate a cohesive end) to create a NheI site at the 5′ terminus, and an EcoRI site at the 3′ terminus. The resultant cDNA fragment was digested with NheI and EcoRI and ligated into pLKO AS3W.IRES-eGFP, and then the final vector was designated pLKO-Fhl1. The entire coding sequences of *Wnt3a* were isolated by the RT-PCR from mouse tissue and cloned into the EcoRV site of pBluescript by using a gene-specific primer pair (5′-GCTAGCATGGCTCCTCTCGGATAC-3′ and 5′-AGTACTTGCAGGTGTGCACGTC-3′). Subsequently, the *Wnt3a* cDNA in pBluescript was amplified and subcloned into the pcDNA6.0 vector for expression. The *Wnt3a* cDNA was

PCR-amplified using a specific primer pair (5′-GCTAGCATGG-CTCCTCTCGGATAC-3′ and 5′-AGTACTTGCAGGTGTGCACGTC-3′), and subsequently inserted into the EcoRV site of pcDNA6.0 vector linked with a *myc*-epitope at the C-terminus. The *Wnt3a-myc*-tagged cDNA was digested with EcoRI and PmeI and ligated into the same restriction sites of pLKO AS3W.IRES-eGFP. The resultant vector was designated pLKO-Wnt3a. A lentiviral vector, pLKO AS3W.eGFP. IRES-bsd (pLKO-eGFP; National RNAi Core Facility), was employed as a negative control.

PREPARATION OF LENTIVIRUS CONCENTRATES AND CELL INFECTION

HEK293T cells were cultured in 60-mm plates for transfection of the lentiviral vectors. The pCMV-deltaR8.91 packing plasmid (National RNAi Core Facility), the pMD.G envelope plasmid (National RNAi Core Facility), and the lentiviral vector plasmid containing pLKO AS3W.Fhl1. eGFP were cotransfected into HEK293T cells by using Turbofect reagent (Fermentas). The transfection medium was replaced with a fresh medium containing 1% BSA at 24 h post-transfection. The viral supernatant was harvested at 24, 48, and 72 h after the high-BSA medium exchange, and then filtered through a 0.45-μm filter (Merck Millipore, Darmstadt, Germany). Virus particles were collected through ultracentrifugation at 50,000*g* at 4°C for 2 h, and then resuspended in Dulbecco′s Modified Eagle Medium (DMEM). The virus suspension was stored at −70°C until use. Polybrene (8 μg/mL, Sigma–Aldrich) was added to the viral suspension to increase transduction efficiency. After a 2-day incubation with viral supernatant plus polybrene, lentiviral particle medium was replaced with α-MEM containing 10% FBS and cells were maintained in this medium for 5 more days. Virus-transduced cells containing eGFP cDNA enabled quantifying transduction efficiency according to the intensity of green fluorescence of cells.

RNA INTERFERENCE

The pLKO.1 plasmids, expressing *Fhl1a-shRNA* (TRCN0000113520, target sequence 5′-CATGAGACCAAGTTCGCCAAA-3′) and *Fhl1b-shRNA* (TRCN0000113521, target sequence 5′-GCAGTTAAGAAC-TTGCCAGAA-3′) to knockdown *Fhl1* expression, were purchased from the National RNAi Core Facility. Plasmid expressing *eGFP-shRNA* (TRCN0000072178, target sequence: 5′-CAACAGCCA-CAACGTCTATAT-3′) was used as the negative control. The supernatants of *shRNA*-containing lentivirus were generated through cotransfection with the pLKO.1 plasmid containing the *shRNA* sequences, the pCMV-deltaR8.91 packing plasmid, and the pMD.G envelope plasmid, as mentioned previously. Stable transfectants were selected and grown in a medium containing 2 μg/mL of puromycin (Sigma–Aldrich).

IMMUNOBLOTTING

MC3T3-E1 cells were seeded on 35-mm plates and infected with lentivirus containing *Fhl1*. Cells expressing GFP were selected and cultured for 21 days in α-MEM medium, and harvested in Laemmli sample buffer. Samples were subjected to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a PVDF membrane (Millipore). After blocking with PBS containing 5% nonfat milk and 0.1% Tween 20, membranes were probed with and anti-myc antibody (GeneMark, Taiwan) followed by goat anti-mouse IgG-alkaline phosphatase (AnaSpec, Fremont, CA). The protein band was stained with BCIP/NBT substrate.

CELL VIABILITY ASSAY

The effects of E2, fulvestrant, lentivirus-transduced Fhl1, and shFhl1 on the cell viability of MC3T3-E1 were evaluated using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. To measure the effects of hormones, MC3T3-E1 cells were seeded on 96-well plates with 3,000 cells per well and cultured for 24 h. After rinsing with PBS, cells were cultured in a fresh medium supplemented with 10 nM E2 or 100 μM fulvestrant for 24, 48, 72, and 96 h. An MTT solution (5 mg/mL of MTT in medium) was then added to the cell cultures. To measure the cytotoxic effects of lentivirus, 1 week after lentiviral transduction, 3,000 cells per well were seeded onto 96-well plates and cultured in growth medium for 24 h. Cells viability was then analyzed through MTT assay at 24, 48, 72, and 96 h. The MTT solution was added to the culture solution to a volume equal to 10%. Further incubation at 37°C for 4 h yielded purple MTT formazan crystals. Once solubilized with DMSO, the absorbance in each culture was measured at 570 nm with the background (650 nm) subtracted.

LUCIFERASE REPORTER GENE ASSAY

After treatment with 20 mM NaCl, 20 mM LiCl, 10 nM E2, or 20 mM LiCl plus 10 nM E2, MC3T3-E1 cells were transfected with the TOPFlash luciferase-reporter plasmids or with the 2.5 kb Fhl1 promoter luciferase-reporter plasmids [Lee et al., 2012] by using X-tremeGENE 9 reagent (Roche, Nutley, NJ) in 12-well plates. After 2 days of transfection, cells were harvested and lysed with cell lysis buffer (Biovision, Milpitas, CA). Cell lysates were analyzed using the luciferase reporter assay kit (Biovision) according to the manufacturer′s instruction. Luciferase activities were measured in a microplate reader (Infinite 200, Tecan) and normalized to the protein content.

ASSAY OF ALKALINE PHOSPHATASE ACTIVITY

One week following lentiviral transduction, 10,000 cells per well were seeded onto 96-well plates and cultured in growth medium for 24 h. Cells were replaced with osteogenic medium the next day and incubated for 3 days. After the medium was removed, the cells were rinsed with PBS and fixed with 4% formaldehyde for 3 min. After washing, the cells in each well were reacted with BCIP/NBT substrate to determine alkaline phosphatase (ALP) activity.

STATISTICAL ANALYSIS

All experiments were performed as duplicates, and repeated 3 times. Results were expressed as means ± standard deviation. Data from the various treatments were compared using the *t*-test, and differences less than $P < 0.05$ were considered statistically significant.

RESULTS

ENDOGENOUS FHL1 EXPRESSION IN MC3T3-E1 IS INCREASED FOLLOWING DIFFERENTIATION

We investigated endogenous *Fhl1* expression in MC3T3-E1 during differentiation to determine whether *Fhl1* expression was changed during osteogenesis. As shown in Figure 1A, culturing cells

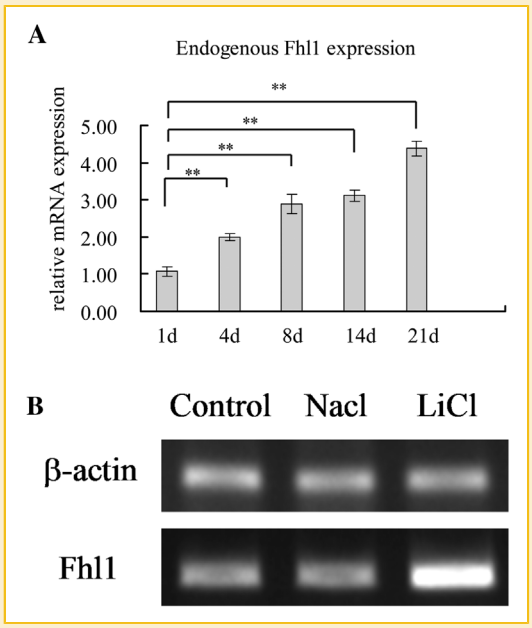

Fig. 1. Endogenous Fhl1 expression was elevated after osteoblast differentiation and treatment with LiCl. (A) MC3T3-E1 cells were cultured in α-MEM, supplemented with vitamin C plus β-glycerol phosphate for osteogenic differentiation from Day 1 to 21. Elevated *Fhl1* expression was apparent from Day 4. (B) MC3T3-E1 cells were cultured in α-MEM. After treatment with the Wnt agonist, LiCl, endogenous *Fhl1* expression was evaluated by semi-quantitative RT-PCR and compared with the NaCl and nontreated controls. *β-actin* expression was used as an internal control.

in a differentiation medium for 1, 4, 8, 14, and 21 days produced gradual increases in *Fhl1* RNA levels, with maximal expression on Day 21; expression was approximately 4- to 5-fold greater than that observed in the control cells without vitamin C stimulation. Moreover, semi-quantitative RT-PCR confirmed that the Wnt agonist, LiCl, enhanced the expression of *Fhl1* when supplemented with vitamin C (Fig. 1B) compared with cell differentiation in vitamin C alone.

CANONICAL WNT3A SIGNALING STIMULATES FHL1 AND OSTEOBLASTIC GENE EXPRESSION

Wnt3a is considered as a canonical Wnt ligand that activates effector cells in a paracrine manner. Because the secreted Wnt3a protein from the supernatant of *Wnt3a*-transfected 293T cells was insufficiently collected, we transduced the *Wnt3a* gene directly into MC3T3-E1 cells. Cells were activated by Wnt3a, which was verified by using the TOPFlash reporter assay (data not shown). Cells were infected with lentivirus containing either the *eGFP* or the *Wnt3a* gene. Day 5 post-transduction, cells were cultured in an osteogenic medium to induce differentiation. In addition to the endogenous elevation following the progression of differentiation, *Fhl1* expressions were significantly enhanced by 45–60% upon *Wnt3a* transduction (Fig. 2A). Different stages of osteogenic gene expression, including *RunX2* (Fig. 2B), *OCN* (Fig. 2C), and *OPN* (Fig. 2D), were analyzed through QRT-PCR. *RunX2* as an early differentiation gene was significantly increased by *Wnt3a* treatment on Days 0 and 3. By contrast, although *OCN* expressions were enhanced approximately 2-fold on Days 0 and 3, *OCN* and *OPN* were dramatically induced by *Wnt3a* on Day 6.

OVEREXPRESSION OF FHL1 INCREASES OSTEOGENESIS OF MC3T3-E1

Osteogenesis can be verified by marker gene expression and calcium mineralization. For mineralization assay, cells must be cultured for periods of 14–28 days. DNA transfection is typically used to test gene functioning in cells. However, for our differentiation study, the plasmid DNA transfection without antibiotic selection did not guarantee that the chromosomal integration of a transgene will remain stable for the experiment duration. Stable DNA transfection using antibiotic-based method facilitates transgenic expression for much longer periods. However, selecting stable clones and expanding surviving clones through many passages requires time. This selecting approach is not suitable for studying MC3T3-E1 differentiation because MC3T3-E1 loses its osteogenic characteristics when subcultured for many passages. Therefore, we adopted a lentiviral-based system to deliver transgenes, because such system can readily and efficiently transfer stable gene expression. We established a lentiviral-Fhl1 (pLKO-Fhl1) expression vector under the control of an ubiquitous CAGGS promoter. The IRES-eGFP cassette was connected with *Fhl1* cDNA to enable the expression of an enhanced green fluorescence protein (eGFP) to be driven by the same CAGGS promoter (Fig. 3A). We used pLKO-eGFP as the control vector for CMV-eGFP delivery by lentivirus. After lentivirus transduction for 7 days, we pooled MC3T3-E1 cells and isolated proteins for Western blotting assay; the exogenous Fhl1 was detected by a myc-tag antibody (Supplementary Fig. S1). Furthermore, as shown in Figure 3B, both pLKO-eGFP and pLKO-Fhl1 exhibited strong green fluorescence in most cells. Virus-infected cells were then stimulated for differentiation. *Fhl1* overexpressed cells (Fig. 3C) substantially enhanced osteoblast differentiation; increases in *RunX2* (Fig. 3D) and *OCN* (Fig. 3E) expression after 0, 3, and 6 days of differentiation were apparent, but an increase in *OPN* expression (Fig. 3F) was observed only on Day 6 compared with the pLKO-eGFP controls. Moreover, ARS showed an enhanced mineralization of *Fhl1* overexpressed cells that were CPC quantitatively assayed approximately 2.5-fold greater than were the controls (Fig. 3G and H). On Day 3 post-induction of osteogenesis, cells were harvested to determine ALP activity, an early differentiation marker of osteogenesis. MC3T3-E1 expressed a basal level of ALP activity on Day 3 of differentiation. However, cells with overexpression of *Fhl1* exhibited a slight blue staining (Fig. 3I).

GENE KNOCK DOWN OF FHL1 DECREASES MC3T3-E1 OSTEOGENESIS

We investigated whether the presence of *Fhl1* expression is necessary for osteogenesis and explored the performance of osteoblasts in the absence of *Fhl1*. Thus, we knocked down endogenous *Fhl1* expression by using lentivirus to deliver *shRNA*. Two *shRNAs* (*shFhl1a* and *shFhl1b*) were used separately to specifically block *Fhl1* expression, and one *shRNA* for *eGFP* (*sheGFP*) was used as a control (see Materials and Methods). The two *shRNAs* of *Fhl1* performed differently, and the repressive activity of *shFhl1a* was apparently superior to that of *shFhl1b*. Compared with the in the *sheGFP* control, treatment with *shFhl1a* and *shFhl1b* reduced endogenous *Fhl1* expression of MC3T3-E1 by 80% (Day 0) to 95% (Day 10) and by 50% (Day 0) to 85% (Day 10), respectively, during the period after differentiation (Fig. 4A). Simultaneously, expressions of the

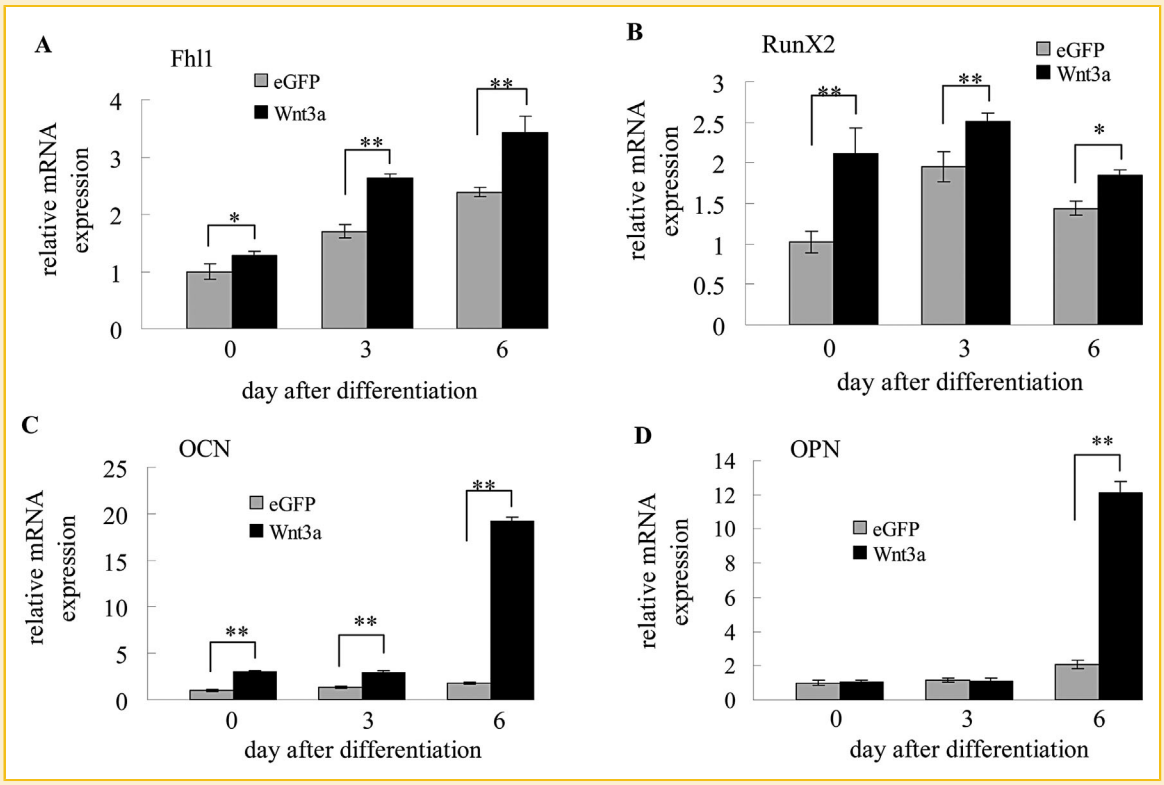

Fig. 2. Canonical Wnt3a signaling stimulated *Fhl1* and osteoblastic gene expression. MC3T3-E1 cells were infected with lentivirus containing either the *eGFP* or *Wnt3a* gene. Day 5 post-transduction, cell differentiation was induced by treatment with vitamin C and β-glycerol phosphate. Cells were harvested on Days 0, 3, and 6 post-induction. Expressions of (A) *Fhl1* and differentiation markers, (B) *RunX2*, (C) *OCN*, and (D) *OPN* expression were then analyzed by QRT-PCR. Student's *t*-test was used; $^{*}P < 0.05$ and $^{**}P < 0.01$.

osteoblastic differentiation markers *RunX2*, *OCN*, and *OPN* were also reduced according to the repression by *shFhl1*. In most assays, *shFhl1a* had a 2- to 3-fold greater activity than did *shFhl1b* in decreasing marker gene expression (Fig. 4B–D). Particularly, expression of *RunX2*, an early stage marker, was inhibited on Days 0, 3, and 6. Expression of *OCN*, a middle stage marker, was inhibited on Days 3, 6, and 10, whereas *OPN* (marker for the late stage) expression only decreased on Day 10.

After 28 days of culturing, we examined the mineralization in osteoblasts by using ARS. The *shFhl1*-expressing cells showed considerable reductions in calcium deposition (Fig. 4E). A CPC assay revealed that mineralization was only 10% in *shFhl1a* cells and 30% in *shFhl1b* cells comparing with that in the *sheGFP* control (Fig. 4F). Alkaline phosphatase activity was assayed on Day 3 post-induction for osteogenesis. Again, *shFhl1a* caused more intense inhibition than did *shFhl1b* to block endogenous ALP activity (Fig. 4G). These results strongly suggested that *Fhl1* expression is necessary for osteoblast differentiation.

ESTROGEN ACTIVATES FHL1 EXPRESSION AND OSTEOBLAST DIFFERENTIATION

Because estrogen activates Wnt signaling in osteoblastic processes, and Fhl1 is a downstream molecule of the Wnt/β-catenin pathway, we investigated whether estrogen signaling affects *Fhl1* expression in MC3T3-E1. We first identified whether the Fhl1 promoter can be affected by estrogen treatment by using a luciferase reporter assay. We linked the 2.5-kb Fhl1 promoter to a luciferase-reporter gene. This reporter plasmid was previously verified to highly respond to β-catenin and LiCl in C2C12, a myogenic cell line [Lee et al., 2012]. TOPFlash plasmid containing 8× Tcf/β-catenin response elements was used as a positive control in response to the canonical Wnt/β-catenin signaling. Similar to previous results we acquired from C2C12, both TOPFlash and Fhl1-luciferase could be activated by the Wnt agonist, LiCl, in MC3T3-E1 cells (Fig. 5A). Moreover, 17β-estradiol could act alone or synergistically with LiCl to activate Fhl1-luciferase but not TOPFlash reporter activity. This result indicated that estrogen and Wnt signaling can function independently or cooperatively to stimulate *Fhl1* gene expression. We further questioned whether estrogen signaling affects endogenous *Fhl1* and osteogenic gene expressions. MC3T3-E1 cells were stimulated for differentiation using vitamin C and β-glycerol phosphate (β-GP), and then cultured in vitro for 14 and 18 days. Cells were cultured using differentiation medium alone, with estrogen, and with the estrogen-receptor inhibitor fulvestrant, respectively. At 14 days, RNAs were extracted to determine the expression of *Fhl1* and the differentiation markers *OCN* and *OPN* by QRT-PCR. As shown in Figure 5B, *Fhl1* expression was substantially induced by estrogen (approximately 2- to 3-fold), and repressed approximately 40% by fulvestrant. Similarly, estrogen upregulated *OCN* expression by approximately 2- to 3-fold, and fulvestrant inhibited *OCN* expression by approximately 40% (Fig. 5C).

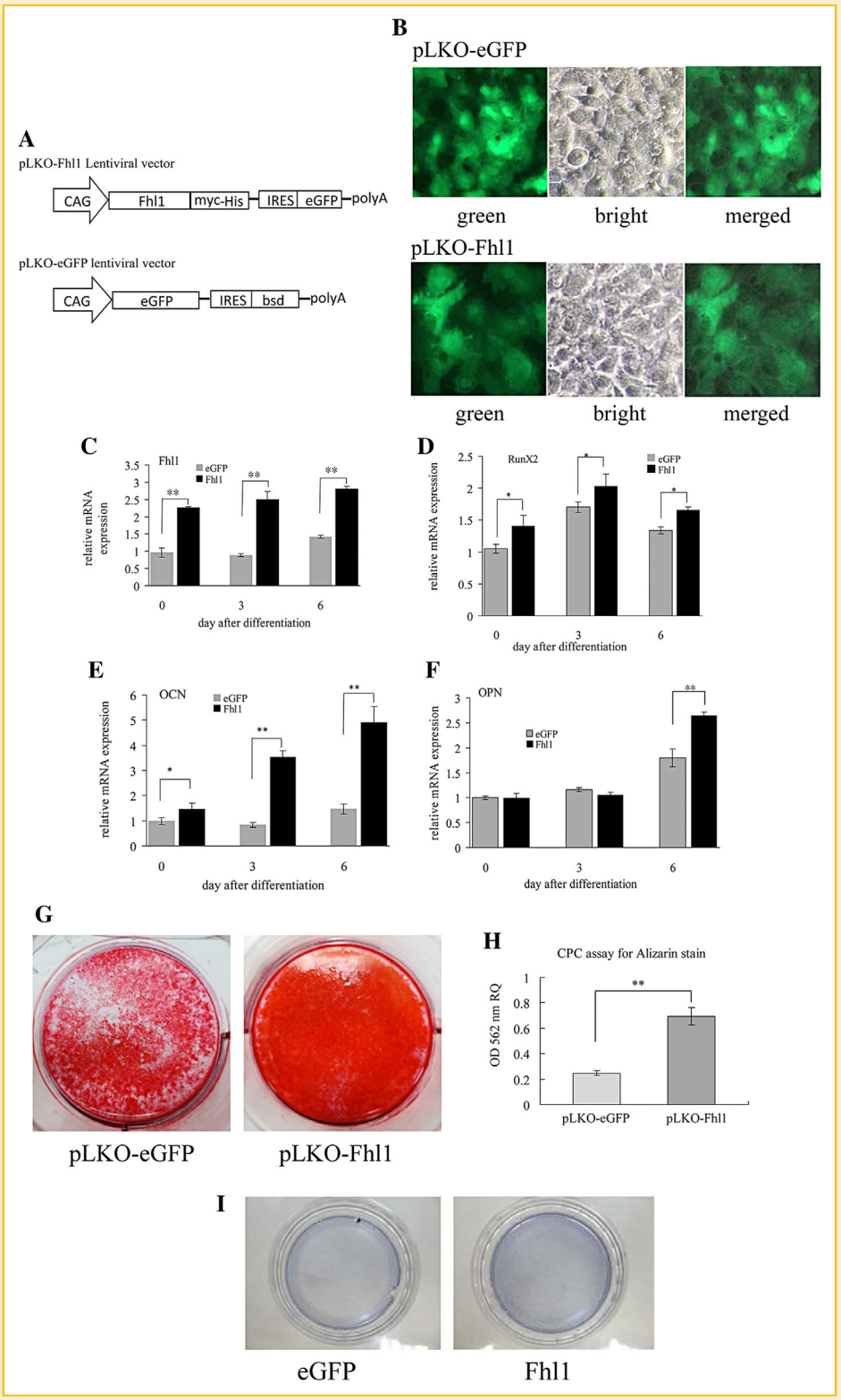

Fig. 3. Overexpression of exogenous *Fhl1* enhanced osteoblast differentiation. (A) MC3T3-E1 cells were infected with lentivirus containing either the *eGFP* or *Fhl1* gene. (B) After a 10-day culture, green fluorescent cells were visualized under a microscope. Cell differentiation was induced by treatment with vitamin C and β-glycerol phosphate. Cells were harvested on Days 0, 3, and 6 post-induction. Expressions of (C) *Fhl1* and differentiation markers, (D) *RunX2*, (E) *OCN*, and (F) *OPN* expression were then analyzed by QRT-PCR. (G) On Day 14 post-induction during osteogenesis, ARS assay was performed. Calcium deposits that were stained by alizarin red were numerous in the *Fhl1* overexpressed (pLKO-Fhl1) cells compared with the control cells (pLKO-eGFP). (H) CPC assay revealed a 2.8-fold more intense red staining of calcium deposits for cells overexpressing *Fhl1* compared to cells expressing exogenous *eGFP* (control). (I) On Day 3 post-induction during osteogenesis, cells were harvested to determine ALP activity. By reacting with the BCIP/NBT substrate, differential blue staining was observed in the eGFP and Fhl1 sample. Student's *t*-test was used; $^{*}P < 0.05$ and $^{**}P < 0.01$.

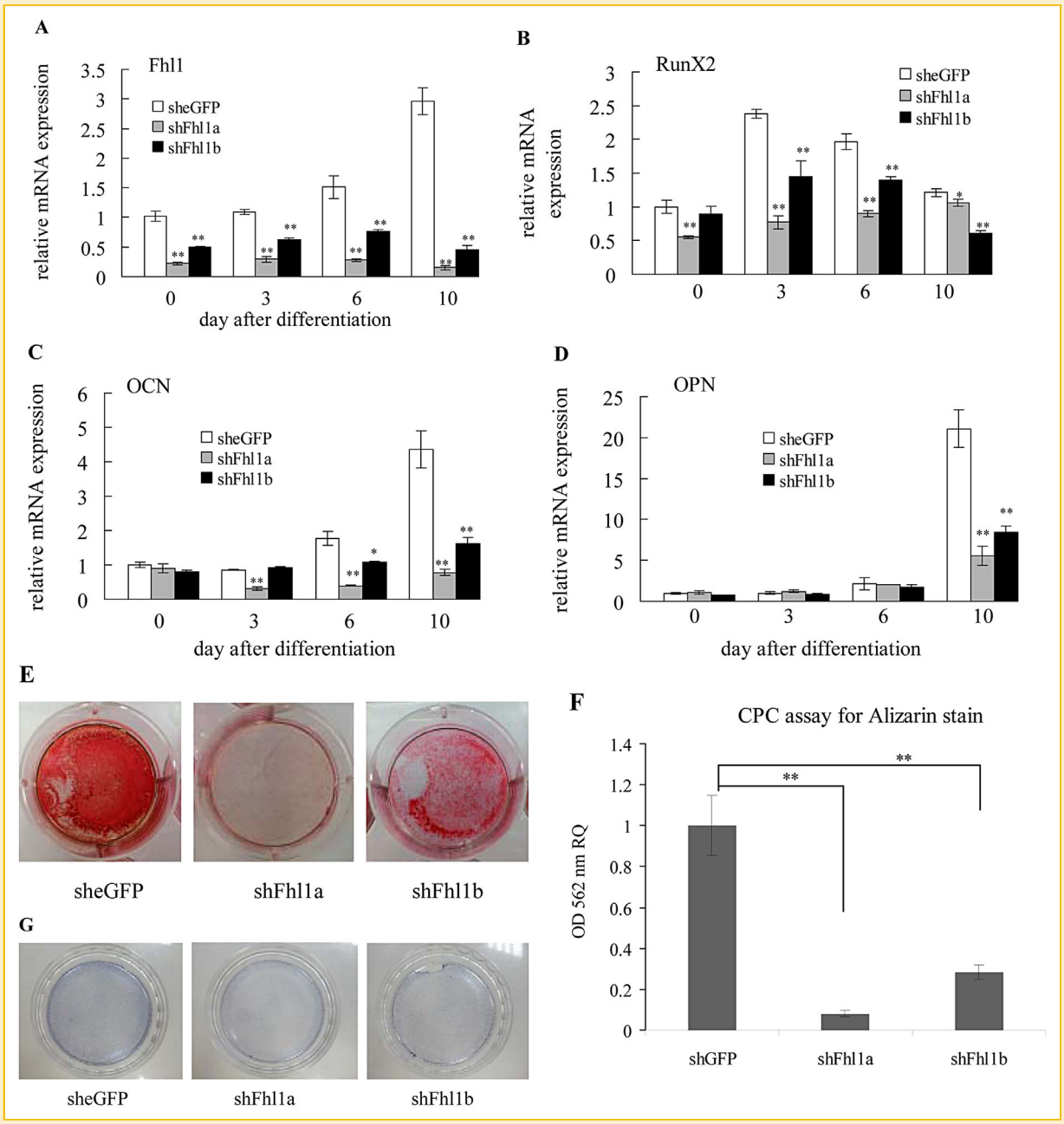

Fig. 4. Gene knockdown of *Fhl1* suppresses osteoblast differentiation. MC3T3-E1 cells were infected with lentivirus expressing shRNA for the *eGFP* gene (*sheGFP*) or *Fhl1* (*shFhl1a* and *shFhl1b*). Differentiation was induced by treatment with vitamin C and β-glycerol phosphate. Cells were harvested on Days 0, 3, 6, and 10 post-induction, and were analyzed by ARS and QRT-PCR. (A) Total *Fhl1* expression was determined by QRT-PCR, which revealed significantly reduced expression of endogenous *Fhl1* in each *shFhl1* cells, compared with the *sheGFP* controls. Differentiation status was determined according to QRT-PCR analysis of the expression of (B) *RunX2*, (C) *OCN*, and (D) *OPN*. (E) Alizarin red stain of calcium deposits was more intense in *sheGFP* cells than it was in *shFhl1* cells. (F) Calcium red stain was quantified by CPC assay. Cells with overexpressed *shFhl1* exhibited approximately 90% (*shFhl1a*) and 70% (*shFhl1b*) less red staining of calcium deposits than did cells expressing exogenous *eGFP*. (G) On Day 3 post-induction during osteogenesis, cells were harvested to determine ALP activity. Less blue staining was observed in *shFhl1a* and *shFhl1b* cells than in *sheGFP* controls. Student's *t*-test was used; $^{*}P < 0.05$ and $^{**}P < 0.01$.

However, *OPN* expression was unaffected by estrogen in 14 day cultures (Fig. 5D), but exhibited an approximately 40% inhibition from fulvestrant. To investigate the mineralization of MC3T3-E1 in the presence of estrogen, we cultured cells for 18 days in differentiation medium and then used ARS to mark calcific deposition. An ARS assay (Fig. 5E) revealed calcific red staining to be more intense in estrogen-treated cells than in control cells (vitamin C + β-GP); moreover, fulvestrant greatly reduced the formation of a calcified matrix. To confirm the ARS assay results, red deposits were dissolved in 10% CPC assay buffer, and the absorbance at 562 nm was measured. The results

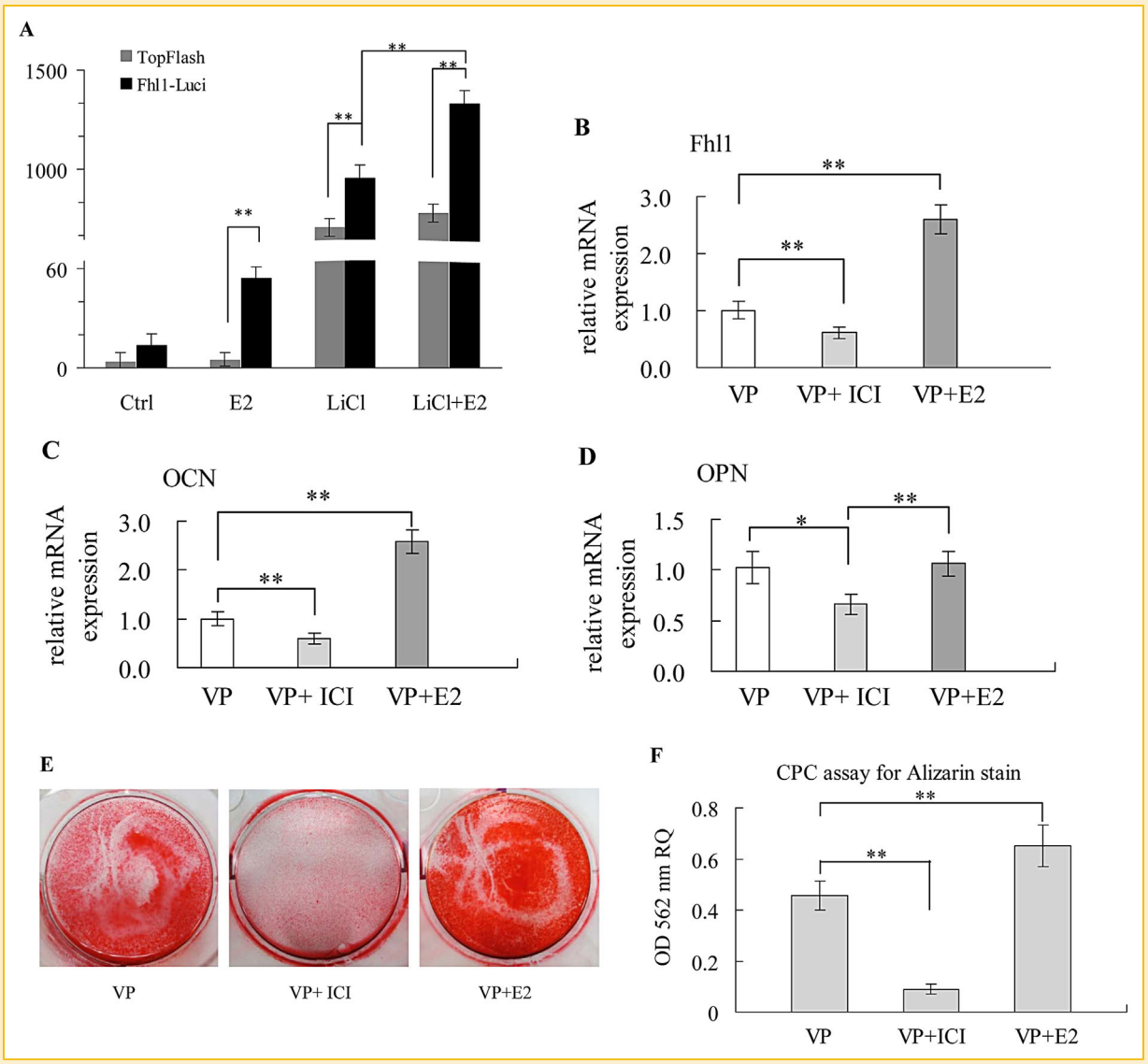

Fig. 5. Estrogen signaling induced *Fhl1* expression and osteoblast differentiation. (A) MC3T3-E1 cells were transfected with the TOPFlash luciferase-reporter or with the 2.5-kb Fhl1 promoter luciferase-reporter plasmid. Cells were then cultured in the growth medium supplemented with estrogen (E2), Wnt agonist (LiCl), or LiCl plus E2. After 2 days of transfection, cells were harvested and the luciferase activities were analyzed. MC3T3-E1 was cultured in an osteogenic-differentiation medium supplemented with vitamin C and β-glycerol phosphate, and either 10 nM 17β-estradiol (E2), or 100 μM fulvestrant (ICI 182,780). The same amount of solvent (ethanol) was supplemented as a negative control. After 14 days, the extent of MC3T3-E1 differentiation was determined by ARS and QRT-PCR. (B) The relative mRNAs of *Fhl1* and the osteogenic markers, (C) *OCN* and (D) *OPN*, were quantified using QRT-PCR. (E) The intensity of ARS indicated the amount of calcium deposition. (F) Mineralization was quantified using a 10% CPC solution (See Materials and Methods). VP: vitamin C plus β-glycerol phosphate. Student's *t*-test was used; $^{*}P < 0.05$ and $^{**}P < 0.01$.

showed that estrogen enhanced mineralization by approximately 40%, and fulvestrant reduced it by approximately 70% (Fig. 5F), results that corresponded with the QRT-PCR results. Thus, we propose that ER-dependent signaling increases osteoblast differentiation, at least partly through Fhl1 activity in MC3T3-E1 cells.

ESTROGEN INCREASES THE OSTEOGENIC GENE EXPRESSION UPON FHL1 OVEREXPRESSION

As mentioned, estrogen can induce endogenous *Fhl1* expression, and therefore assists osteogenic differentiation. We next questioned whether estrogen signaling still enhances osteogenesis when *Fhl1* gene is overexpressed in MC3T3-E1 cells. To address this question, MC3T3-E1 cells were infected by lentivirus carrying either *Fhl1* or *eGFP*, and were cultured in a differentiation medium for 9 days in the presence or absence of 17β-estradiol. QRT-PCR revealed that total *Fh1l* (Fig. 6A) expression was not substantially altered on Day 9 in the presence of E2. However, E2 considerably increased expressions of *OCN* (Fig. 6B) and *OPN* (Fig. 6C) in MC3T3-E1 even though *Fhl1* was overexpressed. These results suggested that estrogen signaling may not only be associated with Wnt signaling, and Fhl1 may be not the only factor estrogen signaling interacts with to promote osteogenic differentiation.

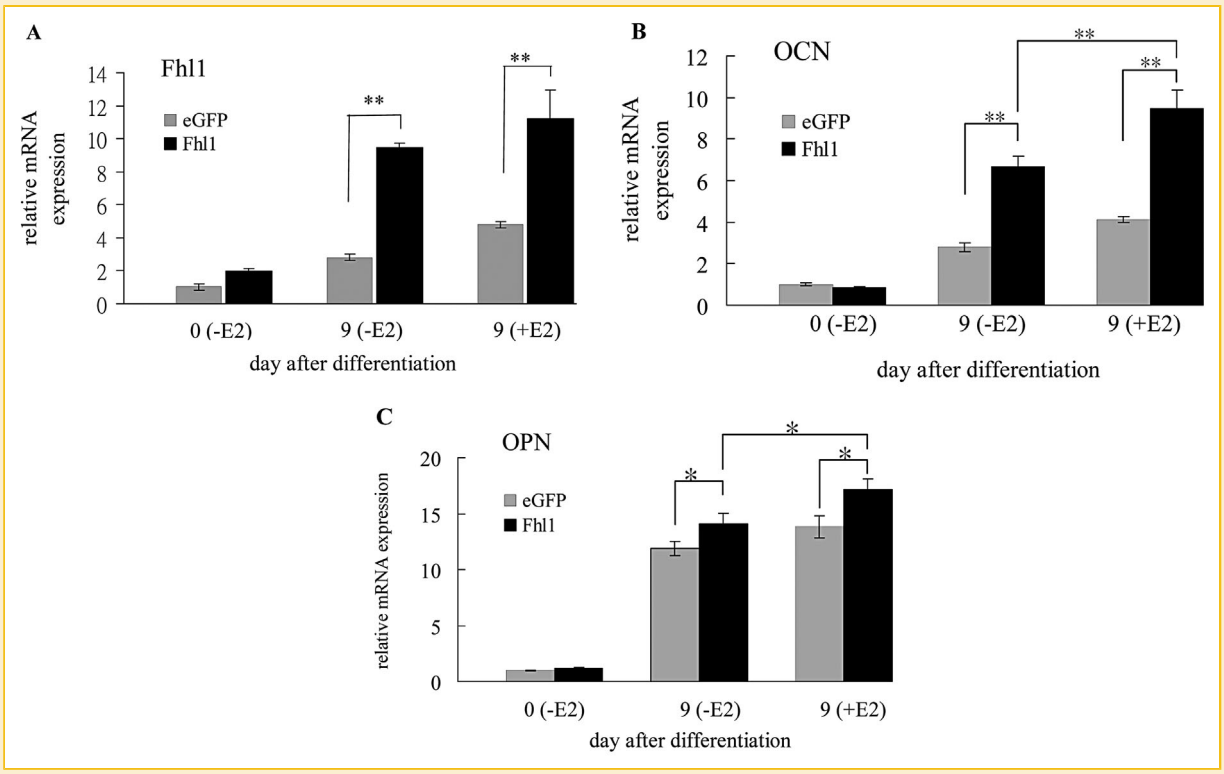

Fig. 6. Estrogen increased the osteogenic gene expression upon *Fhl1* overexpression. MC3T3-E1 cells were infected by lentivirus carrying either *Fhl1* or *eGFP*, and were cultured in the differentiation medium for 9 days in the presence or absence of 10 nM 17β-estradiol (E2). Differentiation status was then evaluated by marker gene expression. QRT-PCR revealed that (A) total *Fh1l* expression was insignificantly altered on Day 9 in the presence of E2. However, E2 significantly facilitates the stimulatory effect of *Fhl1* on (B) *OCN* and (C) *OPN* expression. The Student's *t*-test was used; $^{*}P < 0.05$ and $^{**}P < 0.01$.

ESTROGEN FAILS TO ENHANCE OSTEOGENESIS UPON REDUCED EXPRESSION OF FHL1

As described, even though estrogen induced *Fhl1* expression and osteoblast differentiation, fulvestrant blocked this effect on Day 14. Thus, upregulated expression of *Fhl1* should be necessary for osteogenesis. Moreover, this developmental process may rely on an interaction between ER-dependent signaling and Fhl1. Therefore, we considered whether the sufficient expression of Fhl1 is necessary to transduce estrogen activity. We knocked down the *Fhl1* mRNA expression by *shRNA* in MC3T3-E1, and cells were cultured in a differentiation medium for 14 days in the presence or absence of estrogen. Results of QRT-PCR show that *Fh1l* mRNA in the differentiating MC3T3-E1 was induced by approximately 2-fold after estrogen treatment; however, *shFhl1* transduction greatly reduced estrogen induction (Fig. 7A). Consequently, although the expressions of OCN (Fig. 7B) and OPN (Fig. 7C) were also induced by estrogen, indicating an increase of osteoblast differentiation, this increase was maintained only if *Fhl1* expression was undiminished by *shFhl1*. Similar results were also observed from the ARS (Fig. 7D) and CPC assays (Fig. 7E), in which estrogen enhanced osteoblast mineralization; however, the effect of estrogen was nearly abolished when *Fhl1* expression was blocked. These results implied that the osteoblast differentiation was induced mainly by Wnt signaling through Fhl1 activity and facilitated by estrogen signaling.

DISCUSSION

ER signaling has been reported to be necessary for strain-mediated Wnt/β-catenin activation in osteoblasts to stimulate osteogenic proliferation and differentiation. Mechanically induced Wnt/β-catenin activation is reducible by treatment with fulvestrant [Armstrong et al., 2007; Bhukhai et al., 2012; Sunters et al., 2010], suggesting that ER inhibits a negative regulator of Wnt signaling, in turn activating Wnt/β-catenin. The expression of Wnt antagonist *Sost* is downregulated by strain and estradiol through ER signaling [Galea et al., 2013]. In the current study, we propose that a novel Wnt downstream molecule, Fhl1, might be involved in ER signaling to regulate osteoblast behavior. We previously reported that the promoter of *Fhl1* contains four consensus TCF/LEF binding sites that can be stimulated by β-*catenin* or by treatment with LiCl to induce muscle hypertrophy [Lee et al., 2012], and to repress chondrogenesis [Lee et al., 2013]. In this study, we further demonstrated that enhanced Wnt signaling mimicked by LiCl treatment stimulates *Fhl1* gene expression in preosteogenic cells, and subsequently promotes osteogenesis. Supporting data were acquired using gene knock-down, during which the reduction of osteogenesis corresponded with the repressive activity of *shFhl1*. Among four lineages from mesenchymal origin, osteoblasts and myocytes, but not chondrocytes and adipocytes, are required to

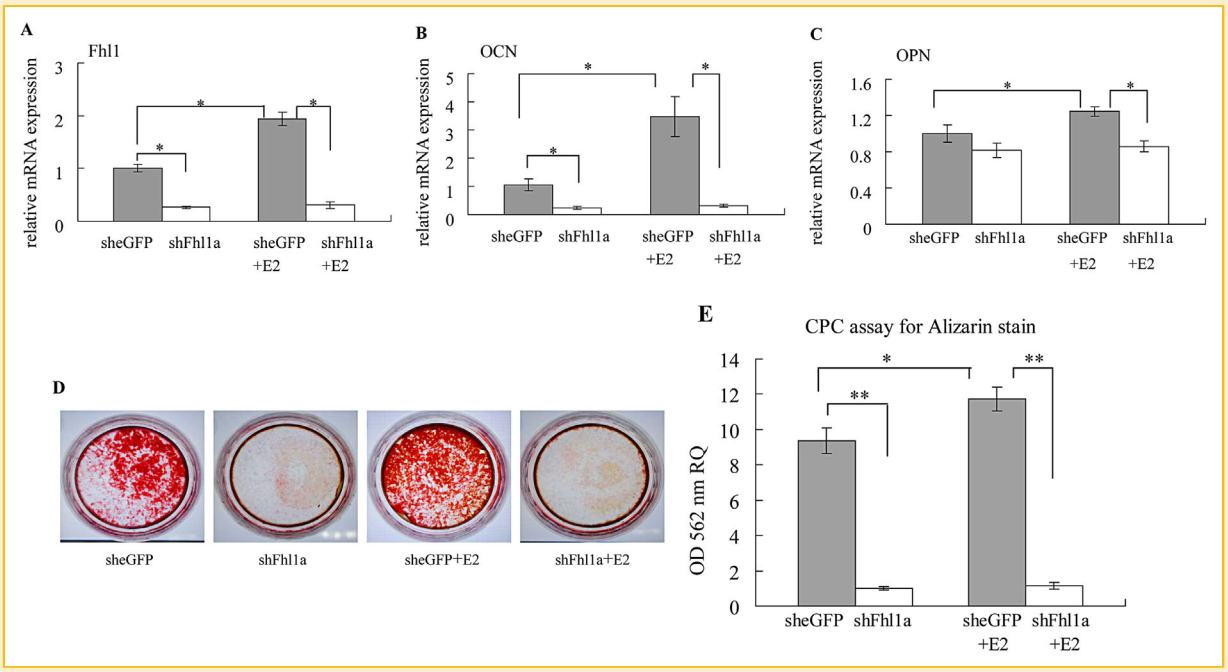

Fig. 7. Estrogen failed to enhance osteogenesis upon decreased expression of *Fhl1*. MC3T3-E1 cells were infected by lentivirus carrying either *shFhl1a* or *sheGFP* and were cultured in the differentiation medium for 14 days in the presence or absence of 10 nM 17β-estradiol (E2). Differentiation status was then evaluated by marker gene expression and mineralization assay. QRT-PCR revealed that (A) *Fh1l*, (B) *OCN*, and (C) *OPN* mRNAs were enhanced by estrogen in *sheGFP* control cell, whereas the enhanced effect of estrogen was blocked in *shFhl1a* cells. (D) ARS and (E) CPC assay showed that estrogen enhanced osteoblast mineralization; however, rare calcified deposits were observed when *Fhl1* expression was largely diminished. Student's *t*-test was used; $^{*}P < 0.05$ and $^{**}P < 0.01$.

virtually respond to mechanical stimuli. Consequently, the body can be supported and moved by bones and muscles. To reflect the mechanical stimuli, signaling is transmitted through the cytoskeleton, regulating cellular behaviors such as cell proliferation and adhesion [Wang et al., 1993; Provenzano and Keely, 2011]. Because Fhl1 regulates the assembly of cytoskeletal proteins through interactions with myosin-binding protein C [McGrath et al., 2006], this regulation may be necessary for osteoblasts and myocytes in response to mechanical strain. Accordingly, Fhl1 is a positive modulator in osteogenesis and myogenesis, but negatively regulates chondrogenesis and adipogenesis (data not shown).

The regulation of Fhl1 and estrogen at a transcriptional level or in a protein–protein interaction has been described elsewhere. FHL1 protein interacts with ERα and AKT, and then represses the translation and transcription of estrogen receptor-responsive genes in cancer cell lines [Ding et al., 2011; Lin et al., 2009; Zhang et al., 2012]. Another study demonstrated that the binding between receptor interacting protein of 140kDa (RIP140) and Fhl1 is necessary in the presence of estrogen to interact with ERα. This interaction results in the repression of ERE-dependent gene expression in human breast cancer cells [Lin et al., 2009]. Thus, Fhl1 functions as a repressor of ER in this situation through the mechanism of protein interaction. The downregulation of *Fhl1* expression by estrogen receptor signaling was also reported. The *Fhl1* promoter of rat L6GNR4 muscle cells can be bound by ER; however, this binding represses *Fhl1* expression at the transcriptional level [Wang et al., 2010]. By contrast, we found that ER-mediated signaling in MC3T3-E1 upregulates *Fhl1* expression, which was stimulated by ER signaling and verified by fulvestrant, the ER inhibitor that blocks the *Fhl1* expression in a dose-dependent manner. A previous study revealed that fulvestrant was unable to block mineralization in MC3T3-E1 [Brennan et al., 2012]. We observed the same results using either 1 or 10 μM fulvestrant. Nevertheless, when cells were treated with more than 20 μM fulvestrant (data not shown), mineralization was inhibited. In this study, we show that treating cells with 100 μM fulvestrant in differentiation medium caused a marked decrease in osteoblast differentiation. Because fulvestrant is an ER-specific inhibitor, insufficient inhibition to mineralization in MC3T3-E1 with low dose treatments may be attributed to the abundant ER in MC3T3-E1. By contrast, a high fulvestrant dose, such as 100 μM, sufficiently blocks ER signaling, inhibiting osteogenesis and repressing *Fhl1* gene expression. This verifies that ER signaling is positive for *Fhl1* expression in MC3T3-E1. Endogenous *Fhl1* expression was elevated 2- to 4-fold for differentiated cells induced by estrogen treatment, which was less than the exogenous *Fhl1* expression in cells after lentivirus-mediated gene transduction. Thus, we found that over-expressed *Fhl1* by viral transduction induced mineralization more efficiently than estrogen did.

As mentioned previously, MC3T3-E1 cells have abundant ERα, and respond well to 17β-estradiol treatment [Ikegami et al., 1993; Kawate et al., 2005; Matsumoto et al., 2013]. In our current studies, whether Fhl1 binds to ERα and whether RIP140 expresses in MC3T3-E1 remains uncertain. In addition, although we found an ERE-like element near the transcriptional initiation site of a mouse *Fhl1* gene,

unlike the ER repressive effect in rat L6GNR4 muscle cells, estrogen signaling activated *Fhl1* expression in mouse MC3T3-E1 preosteoblastic cells. We attribute these differences to using different cell lineages or animal cells in these studies. Future studies may focus on whether Fhl1-ER interacts at a protein level and whether additional factors associate with ER, contributing to Fhl1 expression.

We observed that the osteogenic differentiation status of overexpressed Fhl1 cells can be mildly but substantially enhanced by 17β-estradiol, suggesting that estrogen may not merely interact with Wnt signaling to promote osteogenesis. In fact, BMP signaling was observed to cooperate with estrogen during osteogenesis [Matsumoto et al., 2013]. Furthermore, a member of the Fhl family, Fhl2 shares 48.2% of the amino acid identity of and functions similar to Fhl1. Fhl2 can interact with β-catenin, potentiating β-catenin nuclear translocation and TCF/LEF transcription [Hamidouche et al., 2008]. *Fhl2* can also be activated by Wnt3a at a transcriptional level [Brun et al., 2013], resulting in osteogenesis of bone marrow mesenchymal stem cells. Since Fhl2 acts similarly to Fhl1 during osteogenesis, they may functionally compensate each other because of evolutionary conservation. However, questions regarding both genes remain. For example, whether Fhl1interacts with β-catenin as Fhl2 does, whether estrogen activates Fhl2 expression in osteoblastic cells, and whether the two Fhl proteins functionally compensate or compete with each other during osteogenesis remains unclear.

Finally, to exclude the possibility that Fhl1, shFhl1, or E2 affects osteogenic differentiation because of altered cell viability, we examined the cell viability by using MTT assay (Supplementary Fig. S2). The cell viability of MC3T3-E1 only increased approximately 10–20% with estradiol, but was unaffected by fulvestrant treatment. In addition, cell viability only decreased approximately 8–12% with *shFhl1* or *Fhl1* transduction. Compared with the potent influence of fulvestrant treatment (approximately 70% reduced differentiation), *shFhl1* transduction (approximately 90% reduced differentiation), or *Fhl1* transduction (approximately 50% increased differentiation), the small change of cell viability probably cannot contribute much to the MC3T3-E1 differentiation.

In summary, we found that osteogenesis increases with the overexpression of *Fhl1* and decreases with reduced *Fhl1* expression. Because Fhl1 acts as a Wnt downstream molecule and ER signaling stimulates *Fhl1* expression to promote osteoblast differentiation, we conclude that Wnt signaling through Fhl1 activity plays a major role in osteogenesis, and estrogen can enhance this effect.

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