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Disruption of Four-and-a-Half LIM 2 Decreases Bone Mineral Content and Bone Mineral Density in Femur and Tibia Bones of Female Mice

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Abstract

Four-and-a-half LIM 2 (FHL2) is a member of a family of LIM domain proteins which mediate protein-protein interactions. FHL2 acts as a coactivator and binds to important regulators of bone formation such as insulin-like growth factor binding protein (IGFBP)-5, androgen receptor, and β -catenin. We hypothesized that FHL2 is an important regulator of bone formation. We evaluated growth and skeletal parameters in FHL2 knockout (KO) and wild-type (WT) mice at 4, 8, and 12 weeks of age. At 4 weeks of age, lack of FHL2 reduced femur, tibia, and total bone mineral content (BMC) and body weight in all mice. A gender-by-treatment interaction ($P \leq 0.05$) was observed for several parameters due to a greater reduction in females. Specifically, femur BMC was reduced 11–27% at 8 and 12 weeks of age and BMD was reduced 7–13% at all ages in female KO mice ($P < 0.05$). A similar reduction was observed in the tibias at 8 weeks of age. A 6% reduction ($P = 0.07$) in femur cortical thickness was observed at 12 weeks of age in female KO mice. Interestingly, a gender-specific reduction in IGFBP-5 expression was observed in the femurs of female KO mice. During differentiation of bone marrow stromal cells into osteoblasts, expression of osteocalcin, alkaline phosphatase, and bone sialoprotein was reduced 47–96% in FHL2 KO cells ($P < 0.001$). In conclusion, FHL2 is an important regulator of peak bone mass, lack of FHL2 produces gender- and site-specific effects on bone accretion and IGFBP-5 expression, and FHL2 is important for optimal osteoblast differentiation *in vitro*.

Keywords

Bone density; Female mice; FHL2; Osteoblast differentiation

Four-and-a-half LIM 2 (FHL2) is a LIM-only protein which consists of four and a half LIM domains and is a member of a family of LIM domain proteins that includes FHL 1–4 and activator of CREM in testis (ACT) [1,2]. The lim domain proteins are characterized by a zinc finger motif [3], mediate protein-protein interactions, and bind to several proteins [4]. Specifically, FHL2 functions as a coactivator by binding to proteins such as the androgen receptor (AR), insulin-like growth factor binding protein (IGFBP)-5, cyclic adenosine

monophosphate (cAMP) response element binding protein (CREB), cAMP responsive element modulator (CREM) activator protein (AP)-1, β -catenin, CREB binding protein (CBP)/p300, breast cancer 1 (BRCA1), extracellular signal-regulated kinase 2 (ERK2), and α 7 β 1 [5–12] and is a corepressor of mitogen/extracellular-regulated kinase (MEK1)-ERK1/2 signaling, by binding to ERK2, and of Hand1/E-protein transcriptional coactivation [13,14]. FHL2 may also be important in tissue development by binding to factors such as CREB and CREM, which are involved in the regulation of proliferation and differentiation [10]. FHL2 binds to several factors which are important to the regulation of bone formation. Specifically, it increases the transcriptional activity of the AR [12], which is important in the processes of bone formation and resorption, as demonstrated in the AR knockout (KO) mouse model [15,16]. In addition, we recently determined, using a yeast two-hybrid system, that FHL2 binds to IGFBP-5 and the two proteins translocate into the nucleus [11]. IGFBP-5 is the most abundant IGFBP stored in bone, and several *in vivo* and *in vitro* experiments have confirmed its important role in regulating bone formation [17–20]. FHL2 has also been implicated as a modulator of the Wnt signaling pathway, thereby promoting differentiation of mouse myoblasts via its interaction with β -catenin and CBP/p300 [6,8,9]. Based on these findings and that FHL2 interacts with a number of key signaling molecules that regulate proliferation, differentiation, and apoptosis of bone-forming osteoblasts, we hypothesized that FHL2 is an important regulator of bone formation *in vivo*. To test this hypothesis, we evaluated growth and skeletal parameters in FHL2 KO mice.

During the preparation of our report, two studies were published that examined the consequence of FHL2 disruption on the skeleton. Gunther et al. [21] demonstrated that loss of FHL2 resulted in reduced bone formation rate and osteopenia, by histomorphometric analysis. They also demonstrated that FHL2 modulates transcription of Runx2, a key osteoblast differentiation factor. In contrast, Lai et al. [22] did not observe a significant difference in whole-body BMD in male and female mice; however, when females were ovariectomized, a greater reduction in total-body BMD was observed in the FHL2 KO mice. In the current study, we demonstrate that disruption of FHL2 reduced body weight and femur and tibia bone mineral density (BMD) in female, but not male, mice. In addition, expression of IGFBP-5 was reduced in the femurs of female, but not male, mice, suggesting a role for FHL2 in mediating the effects of estrogen.

Materials and Methods

Animals

FHL2 KO mice were generated as previously described [13] in a Swiss black/129-Sv/J and C57BL/6 background. Breeding pairs heterozygous for deletion of the FHL2 gene were maintained and used to generate homozygous KO and wild-type (WT) littermates for experiments. Mice were weaned and genotyped at 3 weeks of age. Genotypes were determined using polymerase chain reaction (PCR) as previously described [13], with the following primer pairs: neo1, 5'-GGATCGGCCATTGAACAAGATG-3'; neo2, 5'-GAGCAAGGTGAGATGACAGGAG-3'; FHL2-P2, 5'-AGCATGACTGAACGCTTTGACT-3'; FHL2-P3, 5'-GGTAACCAGAACAGGGAGAGTG-3'. The experimental procedures were in compliance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals* and approved by the Animal Studies Subcommittee at the Jerry L. Pettis Memorial Veterans Affairs Medical Center. Bone densitometry, bone mineral content (BMC), and areal BMD (aBMD) were measured by dual X-ray absorptiometry (DXA) at 4, 8, and 12 weeks of age, using the PIXImus instrument (Lunar, Madison, WI). The precision for the BMC and BMD measurements was $\pm 1\%$ for repeat measurements of the same bones several times [23,24]. Animals were anesthetized by a ketamine/xylazine (50/5 mg/kg body weight) injection prior to measurement. Following DXA analysis at 12 weeks of age, mice were killed by CO₂

inhalation and decapitation. The femur and tibia from one leg were removed for volumetric BMD (vBMD) analysis, and the femur and tibia from the other leg (marrow was flushed from bones), liver, muscle, and kidney were collected, immediately frozen in liquid nitrogen, and stored at -70°C until RNA was extracted.

vBMD and Geometric Parameters

vBMD and geometric parameters at the mid-diaphysis of the femur of bones isolated at 12 weeks of age were determined by peripheral quantitative computed tomography (pQCT; Norland, Medical Systems, Inc., WI). Scans were analyzed using the manufacturer-supplied software program (Bone Density Software, version 5.40, Stratec Medzizintechnik, GmbH, Germany). Total vBMD and geometric parameters were estimated with loop analysis. The threshold was set at 230–630 mg/cm^3 . For femur analysis, nine scans per bone were measured, and the data are presented as the average of the fourth, fifth, and sixth scans (mid-diaphysis region). The coefficients of variation for total vBMD, periosteal circumference, and endosteal circumference for repeat measurements of four mouse femurs (two to five measurements) were <3%, <1%, and 2%, respectively [23–25]. The longitudinal lengths of the femurs were measured with a caliper.

Osteoblast Differentiation

To determine if lack of FHL2 altered osteoblast differentiation, bone marrow stromal cells were isolated from KO and WT mice and plated in α -minimal essential medium (α -MEM, containing 10% calf serum, 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin, 50 $\mu\text{g}/\text{mL}$ ascorbic acid, and 10mM β -glycerol phosphate) to induce differentiation into osteoblast cells, as previously described [26]. At days 0, 6, and 18 after differentiation, medium was added and cells were washed with phosphate-buffered saline, extracted with 1 mL Tri-Reagent (Molecular Research Center, Cincinnati, OH), and stored at -70°C until RNA extraction was performed.

RNA Extraction

RNA was extracted from the tissues using the Lipid Tissue Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. RNA was extracted from cells using TriReagent and the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Following extraction, residual DNA was removed from up to 10 μg of RNA with a DNA-free kit (Ambion, Austin, TX). RNA quality was determined using a 2100 Bioanalyzer (Agilent, Palo Alto, CA), and RNA was quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE).

Gene Expression Analysis

Quantitative real-time reverse transcriptase (RT)-PCR analysis was used to determine the expression levels of FHL1 (F, 5'-GGCGATAAAGCTCCCCTGAGAC-3'; R, 5'-AGAAGGCGAGAAGGGAGTTGGAG-3'), FHL2 (F, 5'-AGCATGACTGAACGCTTTGACT-3'; R, 5'-TTGGCATAGAGTTCCTCAAAGC-3'), FHL3 (F, 5'-CACCATGAGCGAGGCATTTGACT-3'; R, 5'-GGTGTGGCGAAGGTGTTGTCAT-3'), IGFBP-5 (F, 5'-ATACAACCCAGAACGCCAGCT-3'; R, 5'-ACCTGGGCTATGCACTTGATG-3'), osteocalcin (F, 5'-CTCTCTCTGCTCACTCTGCT-3'; R, 5'-TTTGTAGGCGGTCTTCAAGC-3'), alkaline phosphatase (ALP; F, 5'-ATGGTAACGGCCTGGCTACA-3'; R, 5'-AGTTCTGCTCATGGACGCCGT-3'), bone sialoprotein (BSP; F, 5'-AACGGGTTTCAGCAGACAACC-3'; R, 5'-TAAGTCTCGGTAAGTGTGCCA-3'), and peptidylprolyl isomerase A (PPIA) (endogenous control; F, 5'-TCCTGGACCCAAAACGCTCC-3'; R, 5'-CCATGGCAAATGCTGGACCA-3'), as previously described [26,27]. Primers were

validated as previously described [26]. Delta C_T values (comparative threshold) were determined (C_T value for gene of interest minus C_T value for control gene), and comparisons of C_T values were used for relative quantification of gene expression [28].

Statistical Analysis

Bone parameters analyzed by DXA at 4, 8, and 12 weeks of age were analyzed by repeated measures analysis of variance (ANOVA). vBMD and geometric parameters determined by pQCT, gene expression at 12 weeks of age, and cell culture data were analyzed by ANOVA. Post hoc analysis was performed using the Newman-Keuls test. All data were analyzed using Statistica 6 software (StatSoft, Tulsa, OK) and presented as mean \pm standard error. A significant difference was determined at $P \leq 0.05$.

Results

Growth and Skeletal Parameters

Growth and skeletal parameters measured by DXA are listed in Table 1. For all FHL2 KO mice, 11% and 4% reductions ($P < 0.01$) in body weight were observed at 4 and 8 weeks of age, respectively; however, no difference in body length was observed compared with WT mice. Total-body BMD was not different between WT and KO mice ($P \geq 0.15$) at all time points (data not shown). Reductions of 14% and 19% ($P < 0.05$) in femur and tibia BMC, respectively, were observed at 4 weeks of age by comparing mean BMC in the combined male and female KO mice versus control mice. Interestingly, a significant gender-by-treatment interaction ($P \leq 0.05$) was observed for several parameters, and through further analysis we determined that lack of FHL2 had a greater effect on bone parameters in female mice when analyzed separately from males. Specifically, in female FHL2 KO mice, 27% and 11% reductions ($P < 0.05$) in femur BMC were observed at 8 and 12 weeks, respectively. Femur BMD was reduced 8%, 13%, and 7% ($P < 0.05$) at 4, 8, and 12 weeks, respectively. Tibia BMC and BMD were reduced 16% and 10% ($P < 0.05$), respectively, at 8 weeks of age. Although there was an 8% reduction in body weight in FHL2 KO female mice, the above parameters were still significant ($P \leq 0.05$) when adjusted for body weight.

Bone Size and vBMD

Since differences in body weight can influence aBMD, we evaluated vBMD and bone size in the femurs of all mice at 12 weeks of age (Table 2). Although femur vBMD was reduced by 5% in female FHL2 KO mice, this difference was not statistically significant. Interestingly, a tendency ($P = 0.09$) for a treatment-by-gender interaction was observed for cortical thickness. Analysis of females demonstrated a 6% reduction ($P = 0.07$) in cortical thickness in the femurs of FHL2 KO mice.

mRNA Expression of FHL Genes and IGFBP-5

We confirmed the disruption of FHL2 expression in tissues of female and male FHL2 KO mice compared with WT mice by real-time RT-PCR (data not shown). To determine if expression of other FHL genes was altered by lack of FHL2, we determined their expression in bones of FHL2 KO and WT female and male mice. Accordingly, a difference in expression of FHL1 and -3 was not observed in male and female mice (Fig. 1). Interestingly, expression of IGFBP-5 was reduced 28% in the bones of female FHL2 KO mice, but no change was observed in FHL2 KO male mice compared with WT mice (Fig. 1). When the percent of control data was analyzed, a significant difference ($P = 0.01$) was observed between male and female mice for IGFBP-5 expression (Fig. 1). No difference in expression of FHL1, FHL3, and IGFBP-5 was observed in the muscle of FHL2 KO and WT mice (data not shown).

Reduced Expression of Markers of Osteoblast Differentiation in FHL2 KO Cells

To determine if the reduced BMD was due to reduced differentiation of osteoblast cells, we looked at expression of markers of osteoblast differentiation during the differentiation of bone marrow stromal cells into osteoblasts in FHL2 KO and WT mice. Accordingly, expression of osteocalcin, ALP, and BSP was reduced 47–96% at days 6 and 18 of culture in FHL2 KO cells compared with WT cells ($P < 0.001$, Fig. 2). Expression of osteocalcin and ALP was similar between FHL2 KO and WT cells at day 0 of cell culture ($P \geq 0.25$). Since bone marrow stromal cells were pooled from male and female mice for our *in vitro* studies, we could not assess the functions of osteoblasts in male versus female KO mice.

Discussion

FHL2 is an important regulator of growth and bone formation, as demonstrated by our novel findings that body weight and femur and tibia aBMD were reduced in female KO mice. This is not surprising since we know that FHL2 binds several factors which are important regulators of osteoblast proliferation and differentiation [6,9,11,20]. In addition, our current findings are similar to those of a recent study in which mice deficient in FHL2 displayed characteristics of osteopenia [21]. Those authors also demonstrated that overexpression of FHL2 increased bone mass, as evaluated by histomorphometric analysis [21]. The degree of reduced bone formation and density observed between the two studies may be explained by the different methods employed (histological analysis vs. DXA and pQCT), later time points (1, 3, 6, and 9 vs. 1, 2, and 3 months of age), and different genetic backgrounds (C57BL/6 vs. Swiss black/129-SV/J and C57BL/6). Importantly, both studies demonstrate an important role for FHL2 in regulating bone formation.

During preparation of the current report, other results were published in which ovariectomized bone loss was accelerated in the absence of FHL2 [22]. Interestingly, those authors did not detect a reduction in total-body aBMD in control female mice between 1 and 4 months of age. Similarly, we did not observe a difference in total-body aBMD in female FHL2 KO mice (data not shown); however, we did observe a reduction in femur and tibia aBMD at 8 weeks of age in female mice, which was not reported in the previous study. Interestingly, in female mice, reductions in BMC and BMD were greater at 8 weeks than at 4 weeks of age. Puberty is a period of rapid growth, and these data suggest that FHL2 may be required for optimal bone development during this period. The deficit in BMD at some, but not other, skeletal sites in FHL2 KO mice may be explained by recent evidence that mechanisms that regulate bone accretion may vary depending on the skeletal site. Accordingly, it has been shown that the genetic loci that contribute to BMD variation are site-specific [29–34]. There are several potential explanations for the site-specific differences in the FHL2 effect: (1) FHL2 expression may vary depending on the site; (2) factors that bind to FHL2 may be expressed differently at various skeletal sites; and (3) other mechanisms could compensate for the loss of FHL2 at some, but not other, skeletal sites.

Another interesting finding in this study is the loss of FHL2-induced bone accretion in female, but not male, mice. Based on the findings that FHL2 binds to the AR, colocalizes with the AR in the nucleus of prostate epithelial cells, and increases the transcriptional activity of the AR [12], we anticipated that loss of FHL2 would affect bone accretion in male mice; however, that was not the case. In this regard, it is possible that androgen action in bone cells may not require FHL2. Further studies are needed to understand the molecular basis for gender-specific differences in FHL2 action.

FHL2 is highly homologous to FHL1 and -3, and the expression patterns of the three genes often overlap [13]. Therefore, we anticipated that lack of FHL2 would result in a compensatory

overexpression of FHL1 and/or -3. However, similar to heart tissues, a difference in FHL1 and -3 expression was not observed in the bone and muscle tissues of KO compared to WT mice.

FHL2 is an important regulator of osteoblast function, as demonstrated by the reduced expression of markers of osteoblast differentiation. Our findings are consistent with the recent report by Lai et al. [22] as well as previous findings that FHL2 deficiency reduces osteoblast activity [21]. In addition, FHL2 overexpression increased osteoblast proliferation and differentiation [21]. FHL2 may regulate osteoblast activity by binding to factors involved in bone formation and acting as a coactivator. For example, we previously demonstrated that FHL2 binds to IGFBP-5 and that both translocate into the nucleus [11]. IGFBP-5 is an important regulator of osteoblast proliferation and differentiation; therefore, FHL2 may mediate bone formation through IGFBP-5 [20]. In addition, FHL2 binds to β -catenin and modulates the Wnt signaling pathway, an important mediator of bone development. However, if FHL2 is the sole intracellular mediator of these factors, we would anticipate a greater deficit in BMD in FHL2 KO mice. Therefore, FHL2 does not appear to be the sole mediator of these factors. However, based on the data presented here, FHL2 is required for optimal bone formation in female mice.

To identify the mechanistic basis for the gender-specific effect, we focused on IGFBP-5 expression in FHL2 KO mice based on our recent findings that transgenic overexpression of IGFBP-5 exerts gender-specific effects on bone density and bone formation parameters [35] and findings that estrogen regulates IGFBP-5 expression in multiple cell types [36–38]. Therefore, we tested the possibility that IGFBP-5 expression may be differentially affected in male versus female FHL2 KO mice, which could in part explain the observed gender difference. Our finding that IGFBP-5 expression was reduced in FHL2 female mice suggests that FHL2 action may be important in mediating estrogen effects. While FHL2 has been shown to interact with the AR [12], no such interaction between FHL2 and the estrogen receptor has been reported. Thus, whether FHL2 interacts with the estrogen receptor or components of the estrogen receptor signaling pathway to modulate IGFBP-5 expression and bone formation remains an interesting possibility which needs to be investigated.

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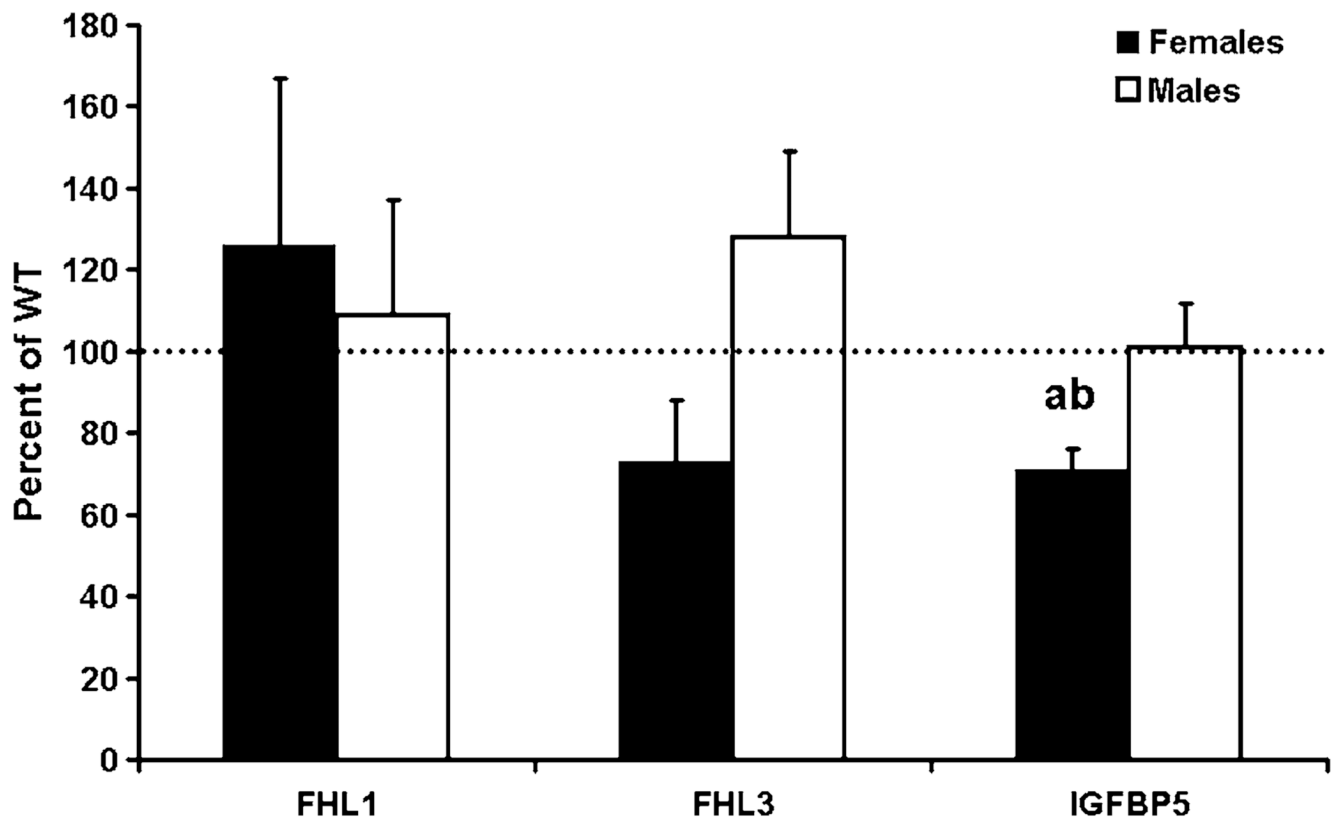


Fig. 1. IGFBP-5 mRNA expression is reduced in bones of FHL2 KO female mice at 12 weeks of age. mRNA expression was determined by real-time RT-PCR. Data are presented as mean \pm standard error ($n = 7$). A significant difference between WT and KO mice was not observed for FHL1 and -3 ($P \geq 0.4139$). Expression of FHL2 was not detectable in KO mice. ^aSignificant difference from WT mice at $P < 0.05$. ^bSignificant difference from male mice at $P = 0.01$ when analyzed for percent control values.

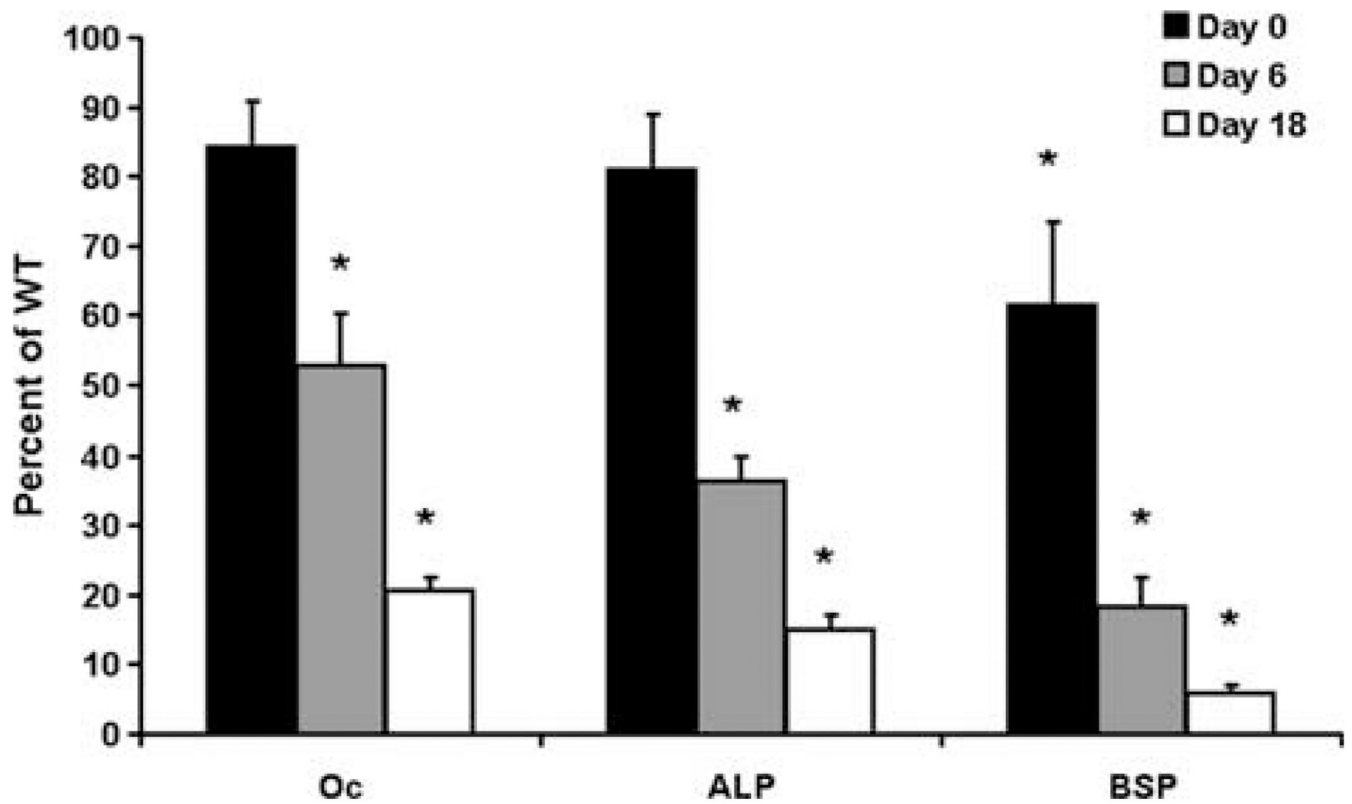


Fig. 2. mRNA expression of markers of osteoblast differentiation in FHL2-deficient cells. mRNA expression was determined by real-time RT-PCR. Oc, osteocalcin. Data are presented as mean \pm standard error ($n = 6/\text{treatment}/\text{day}$). * Significant difference from WT ($P < 0.001$).

Table 1

Growth and skeletal parameters at 4, 8, and 12 weeks of age

Parameter	Age (weeks)					
	4		8		12	
	WT	FHL2 KO	WT	FHL2 KO	WT	FHL2 KO
Body weight (g)						
Female	12.82±0.71	11.80±0.55*, **	19.28±0.37**	17.76±0.28*, **, ****	22.12±0.50**	20.90±0.56**
Male	15.28±0.43	13.17±0.82*	23.96±0.39	23.23±0.62*	27.80±0.91	27.25±0.93
Body length (mm)						
Female	73.40±2.54	73.33±1.26	85.8±1.20**	84.83±1.22**	89.20±1.36**	89.00±1.29**
Male	77.60±1.21	73.67±1.52	93.20±0.37	90.83±1.78	95.00±0.63	95.00±1.75
Femur BMC (mg)						
Female	6.54±0.49	5.83±0.31*	16.6±0.93	12.07±0.83****	19.48±0.22	17.27±0.74****
Male	7.67±0.42	6.52±0.50*	15.63±1.01	16.37±1.58	19.27±0.42	20.43±1.57
Femur BMD (mg/cm ²)						
Female	42.26±1.29	38.90±0.46	63.80±1.74**	55.53±2.26**, ****	72.02±1.03	67.12±1.49****
Male	41.20±2.15	40.17±1.28	65.03±1.95	67.18±4.56	68.72±1.92	77.02±5.24
Tibia BMC (mg)						
Female	7.60±0.49	7.50±0.43*	16.74±1.01	14.07±0.56****	18.90±0.61	16.88±1.12
Male	10.17±0.48	7.08±0.84*	16.10±0.28	17.02±1.10	18.85±0.42	19.90±1.06
Tibia BMD (mg/cm ²)						
Female	34.58±1.13	33.00±1.16	53.00±2.09	47.82±0.95****	58.10±1.91	54.57±3.11
Male	35.85±1.30	33.28±1.95	51.62±1.94	53.57±2.14	58.38±1.52	60.57±2.82

* Significant main effect for treatment at $P < 0.05$

** Significant main effect for gender at $P < 0.05$

*** Significant treatment effect for female mice at $P < 0.05$; $n = 5$ or 6 in each treatment group ($n = 23$)

Table 2

Bone size and vBMD of femurs at 12 weeks of age

	Females		Males		P		
	WT (n = 6)	KO (n = 6)	WT (n = 5)	KO (n = 7)	Treatment	Gender	Treatment × Gender
Total density (mg/cm ³)	627±15	597±11	613±8	612±18	0.3103	0.9829	0.3444
Periosteal circumference (mm)	5.02±0.02	4.96±0.06	5.21±0.07	5.28±0.08	0.9815	0.0007	0.3286
Endosteal circumference (mm)	3.22±0.05	3.26±0.04	3.41±0.06	3.42±0.06	0.6014	0.0034	0.7973
Cortical thickness (mm)	0.2866±0.0066	0.2705±0.0044	0.2874±0.0035	0.2954±0.0090	0.5570	0.0740	0.0920