Protein Phosphatase PP5 Controls Bone Mass and the Negative Effects of Rosiglitazone on Bone through Reciprocal Regulation of PPARγ ( Peroxisome Proliferator-activated Receptor γ) and RUNX2 ( Runt-related Transcription Factor 2)*

Received for publication, August 8, 2016, and in revised form, September 28, 2016. Published, JBC Papers in Press, September 29, 2016, DOI 10.1074/jbc.M116.752493

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Edited by Roger Colbran

WITHTDRAWN
May 3, 2018

This article has been withdrawn by the authors following concerns raised by the Journal. Specifically, the image for total ERK in Fig. 1A was incorrectly used in Ge et al. (Ge, C., Wang, Z., Zhao, G., Li, B., Liao, J., Sun, H., and Franceschi, R. T. (2016) Discoidin receptor 2 controls bone formation and marrow adipogenesis. J. Bone Miner. Res. 31, 2193-2203). Also, the Journal had concerns about the data integrity related to immunoblots in Fig. 2A. The authors believe that the responsible course of action is to withdraw the article to maintain their publication standards and those of the Journal. However, the authors stand by the overall conclusions of Fig. 2A that, in the authors’ opinion, has been verified using a different cell line in Fig. 4E of the same article and subsequently and independently verified in Fig. 4L of Wang et al. (Wang, J., Cao, Y., Qiu, B., Du, J., Wang, T., Wang, C., Deng, R., Shi, X., Gao, K., Xie, Z., and Yong, W. (2018) Ablation of protein phosphatase 5 (PP5) leads to enhanced both bone and cartilage development in mice. Cell Death Dis. 9, 214). The withdrawn manuscript is available from the authors upon request.

* This work was supported, in whole or in part, by National Institutes of Health Grants DK105825 (to B. L.-C.), DE11723 (to R. T. F.), and DK70127 (to E. R. S.). This work was also supported by the American Diabetes Association Grant 7-13-085 (to B. L.-C.). The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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‡ The abbreviations used are: MSC, mesenchymal stem cell; PPARγ, peroxisome proliferator-activated receptor γ; RUNX2, runt-related transcription factor 2; TMD, tissue mineralized density; BFR, bone formation rate; BMD, bone mineral density; mCT, micro-computed tomography; PTM, posttranscriptional modification.
The ability of PP5 protein phosphatase to dephosphorylate Ser-319pRUNX2 increased in the presence of MEK1 but decreased in the presence of PP5, and MEK1-induced phosphorylation of Ser-319 was reduced in the presence of PP5 (Fig. 1B). As expected, substitution of serine 319 by alanine in RUNX2 (SA mutant) led to loss of reactivity with anti-Ser-319 pRUNX2 antibody. MEK1 is a threonine/tyrosine kinase that phosphorylates and activates MAPK via the extracellular signal-regulated kinases (ERK) pathway. Thus, the levels pERK serve as a control of MEK1 activity. As shown in Fig. 1, A and B, transfection of MEK1 increased pERK levels regardless of the presence of PP5 or functional phosphorylation sites in PPARγ or RUNX2. This indicates that MEK1 activity is not affected by PP5 and that Ser-112 and Ser-319 are direct targets for PP5 phosphatase activity.

Next, we tested how Ser-112 and Ser-319 PTMs correlate with transcriptional activities of both factors. Increased phosphorylation at Ser-112 by MEK1 resulted in decreased PPRE-mediated transcriptional activity of PPARγ (Fig. 1C). Overexpression of PP5 activated PPARγ; however, in the presence of MEK1 this activity returned to control level. The basal activity of the construct carrying S112A mutation, S112A (SA), was increased by 50% compared with the activity of the S112E (SE) mutation (SE), which mimics phosphorylation of Ser-112. We did not observe either SA nor SE contributing to transcriptional activity of Ser-112 inhibition on transcriptional activity, a response that is consistent with previous studies (12).

On transcriptional activity of RUNX2 is not affected by PP5 (1D). MEK-dependent phosphorylation of Ser-319 was associated with decreased transcriptional activity, which was reduced to 50% in the presence of PP5. Serine to alanine mutations of the Ser-301 and Ser-319 sites also reduced MAPK-dependent transcriptional activity by 50%, and this activity was not reduced further by PP5. Because PP5 reduced MAPK-dependent Ser-319 phosphorylation to basal levels, we conclude that other non-PP5-sensitive sites also participate in MAPK regulation of RUNX2. As expected, the basal transcriptional activity of combined phosphomimetic mutant S301E/S319E (SE) was high and was not affected by any of the tested conditions.

The reciprocal regulation of PPARγ and RUNX2 activities was also observed in conditions of PP5 partial deficiency. Lentiviral expression of the shRNA to PP5 in bone marrow-derived bipotential U-33/γ2 cells, which reduced PP5 protein levels by 40%, resulted in a 40–50% increase in the levels of Ser-112 pPPARγ and Ser-319 pRUNX2 proteins (Fig. 2A). These correlated with decreased expression of PPARγ-regulated gene markers with and with either increased expression of RUNX2-induced markers or with increased suppression of Axin2, which is negatively regulated by RUNX2 (Fig. 2B) (12).

In conclusion, PP5 phosphatase increased PPARγ transcriptional activity by dephosphorylating Ser-112 and simultaneously suppressed RUNX2 activities by dephosphorylating Ser-319.

PP5 Deficiency Increased Bone Mass and Decreased Volume of Marrow Fat—Mice, males and females, with global deficiency in PP5 (PP5KO) are characterized by high bone mass in

### TABLE 1

| Variable                  | WT        | PP5 KO     |
|---------------------------|-----------|------------|
| Proximal tibia (%)        | 4.5 ± 0.2 | 7.9 ± 2.6* |
| Tb.N (1/mm)               | 3.756 ± 0.144 | 4.301 ± 0.479* |
| Tb.Th (mm)                | 0.030 ± 0.002 | 0.030 ± 0.005 |
| Tb.Sp (mm)                | 0.271 ± 0.011 | 0.230 ± 0.013* |
| Conn.D (1/mm³)            | 188.4 ± 48.8 | 453.2 ± 161.1* |
| L4 vertebra (%)           | 8.9 ± 1.0 | 13.1 ± 1.8* |
| Tb.N (1/mm)               | 5.077 ± 0.239 | 5.689 ± 0.153* |
| Tb.Th (mm)                | 0.030 ± 0.001 | 0.035 ± 0.004* |
| Tb.Sp (mm)                | 0.197 ± 0.009 | 0.174 ± 0.004* |
| Conn.D (1/mm³)            | 216.3 ± 34.2 | 312.7 ± 35.1* |

*p > 0.05 versus WT.
To analyze an effect of Ser-112 phosphorylation on PPAR activity, the increased mineralization of the mid-density (TMD) in tibia midshaft, indicating a higher degree of mineralized bone diameter and bone area but had no effect on cortical thickness in PP5KO mice (Fig. 3B). Similarly, to analyze an effect of Ser-301/Ser-319 phosphorylation on RUNX2 activity, Cos7 cells were co-transfected with PPRE-luc reporter plasmid and either empty vector LacZ (C) or constitutively active MEK1 (SP) or PP5 or both (SP + PP5). Western blots, A, PP5 effect on PPARγ phosphorylation (Ser-112pPPARγ) or against total PPARγ (T-PPARγ), and B, PP5 effect on RUNX2 phosphorylation (Ser-319pRUNX2) or antibodies specific to phosphorylated Ser-319 of RUNX2 (Ser-319pRUNX2) or antibodies specific to total RUNX2 (T-RUNX2) analysis as in A (n = 3–6). C, analysis of PP5 on PPARγ and RUNX2 activities. The same samples of Cos7 cells as in A expressing either C, SP, PP5, or SP + PP5 were co-transfected with PPRE-luc reporter plasmid and either expression vectors for WT or PPARγ phosphorylation mutants S112A (SA) or S112E (SE), PPARγ activity was measured after 48 h. Firefly luciferase activities were normalized to Renilla pyriformis luciferase activities. D, analysis of transcriptional activity of RUNX2. Samples of Cos7 cells as in B expressing either C, SP, PP5, or SP + PP5 were co-transfected with GOS2-luc reporter plasmid and either expression vectors for WT or RUNX2 phosphorylation mutants S301A/S319A (SA), and S301E/S319E (SE) and analyzed as in C.* p < 0.05; **, p < 0.01; *** p < 0.001.

The gene expression profile of osteoblasts freshly isolated from the endosteal surface of femora confirmed their increased activity in the absence of PP5. The expression of all three osteoblast-specific transcription factors (RUNX2, DLX5, and Osterix) and Wnt10b ligand were increased (Fig. 3F). Consequently, the trabecular bone fractions in male proximal tibiae are 70 and 40% higher, respectively, than wild type (WT) controls. Trabecular bone in vertebrae, L4 (Fig. 3A) and L3 (Fig. 3C), are 70 and 40% higher, respectively, than wild type (WT) controls. Trabecular bone in vertebrae, L4 (Fig. 3A) and L3 (Fig. 3C), are 70 and 40% higher, respectively, than wild type (WT) controls. Trabecular bone in vertebrae, L4 (Fig. 3A) and L3 (Fig. 3C), are 70 and 40% higher, respectively, than wild type (WT) controls.
**TABLE 2**

Cortical parameters of midshaft tibia analyzed with mCT

| Variable | WT       | PP5 KO   |
|----------|----------|----------|
| T.Ar (mm²) | 0.083 ± 0.005 | 0.099 ± 0.010* |
| B.Ar (mm²) | 0.029 ± 0.001 | 0.032 ± 0.002* |
| M.Ar (mm²) | 0.054 ± 0.004 | 0.067 ± 0.009* |
| Cl.Th (mm) | 0.181 ± 0.007 | 0.188 ± 0.006 |
| pMOI (mm²) | 0.145 ± 0.014 | 0.186 ± 0.030* |
| Imax/Cmax (mm³) | 0.125 ± 0.010 | 0.154 ± 0.017* |
| Imin/Cmin (mm³) | 0.107 ± 0.004 | 0.124 ± 0.012* |
| TMD (mg HA/cm³) | 903.9 ± 13.2 | 924.7 ± 11.1* |

* p > 0.05 versus WT.

**FIGURE 2.** PP5 knockdown increased PPARγ and RUNX2 activities leading to increased PPARγ and RUNX2 activities.

A. To achieve stable knockdown of PP5 in AD2 pre-adipocytic cells, U-33/H9253 cells were infected with lentiviruses carrying PP5-specific (KD) or PP5-specific (KD) shRNA. Whole cell extracts were analyzed by Western blotting with antibodies against total PPARγ, RUNX2, and PP5, respectively. Graphs represent the means ± S.E. of three experiments (n = 6). Signals from bands representing protein expression were normalized to signals from total protein levels. B. Silencing of PP5 in U-33/γ2 cells on expression of gene markers (CD36, Dio2, Scf, Sost, Cx43, Tcf21, Dmp1, Runx2) and RUNX2 transcriptional activities (n = 6). Graphs represent the means ± S.E. of three experiments (n = 6). Signals from bands representing protein expression were normalized to signals from total protein levels. All transcripts expression was normalized to 18S ribosomal RNA. *p < 0.05; **p < 0.01; ***p < 0.001.

**FIGURE 3.** PP5 regulates PPARγ and RUNX2 activities in response to the PPARγ agonist Rosiglitazone.

Because rosiglitazone induces adipocyte and suppressing osteoblast phenotypes in narrow MSCs, we examined the role of PP5 in this process. As shown in Fig. 3A and B, rosiglitazone treatment causes simultaneous PP5 recruitment to the PPARγ and RUNX2 complexes. Interestingly, regardless of their activation...
status, both factors are already in complexes with HSP90 chaperone, which facilitates PP5 binding and activation via its TPR domain (11). Complex formation with either PPARγ or RUNX2 is associated with a partial redistribution of PP5 protein from the perinuclear space to the nucleus where it co-localizes with both transcription factors (Fig. 5, C and D) and correlates with their dephosphorylation (Fig. 5E). Silencing of PP5 using lentiviral expression of PP5-specific shRNA blocks rosiglitazone-induced dephosphorylation of PPARγ Ser-112 and RUNX2 Ser-319 (Fig. 5E). Consequently, PP5KD makes PPARγ resistant to rosiglitazone activation and RUNX2 resistant to rosiglitazone inhibition at their respective promoters (Fig. 5F) and at endogenous genes (Fig. 5G).

PP5 Protects from Rosiglitazone-induced Bone Loss—The above results suggested that PP5KO mice may be resistant to the negative effects of rosiglitazone on bone. To test this, WT and PP5KO mice received rosiglitazone at the dose of 11 mg/day/kg of body weight for 8 weeks. Bone mineral density (BMD) measured at the beginning and the end of the experiment showed that PP5KO mice are refractory to the decrease in

FIGURE 3. PP5 deficiency increased bone mass and bone formation and reduced lipid content in the marrow. A, mCT-generated coronal section images of trabecular bone in proximal tibia of 5-month-old male WT and PP5-deficient (PP5KO) mice. B, the same tibiae as in A stained for fat with osmium tetroxide. C, mCT renderings of trabecular bone in L4 vertebra. Graphs on panels A–C represent measurements of trabecular bone mass (BV/TV) and fat volume (FV/TV). FV, fat volume; TV, tissue volume. Bar, 1 mm. D, double calcein labeling of trabecular bone surface (magnification, ×40; bar, 0.02 mm). E, static and dynamic histomorphometry of trabecular bone in the proximal tibia of 5-month-old male mice. MAR, mineral apposition rate; N.Ob/B.Pm, osteoblast number/bone surface; O.N/Oc.N, ratio of osteoblast to osteoclast. F, gene expression analysis of osteoblasts freshly isolated from endosteal surface of femur. G, gene expression analysis of osteocytes freshly isolated from femur cortical bone. n = 4–6 animals per group. *, p < 0.05; **, p < 0.01 versus age-matched WT control.
BMD caused by rosiglitazone observed in WT mice (Fig. 6A). Consistent with our previous results, rosiglitazone increased body weight in WT animals, but the weight of PP5KO mice remained the same as control animals (Fig. 6B). Analysis of trabecular bone in proximal tibia confirmed bone loss in WT mice receiving rosiglitazone. Bone mass was decreased by 50% due to a decrease in trabeculae number (Fig. 6C). None of these changes were observed in the bone of PP5KO mice. Both trabecular bone mass and structure in tibia (Fig. 6C) and vertebra (Fig. 6D) were preserved in PP5KO animals receiving rosiglitazone. Moreover, rosiglitazone had a very modest effect (1.8-fold increase) on fat accumulation in the marrow cavity of PP5KO, whereas such accumulation was very robust (40-fold increase) in the bone of WT animals (Fig. 6C). In contrast to WT, rosiglitazone did not affect the number of osteoblasts and osteoclasts and did not affect BFR in PP5KO mice (Fig. 6E). Consistent with
these measurements, osteoblasts isolated from the endosteal surface of femora of rosiglitazone-treated PP5KO mice maintained high expression of all three osteoblast-specific transcription factors (Runx2, Dlx5, and Osx), whereas the expression of Dlx5 and Osx was decreased in rosiglitazone-treated WT mice (Fig. 5F). Interestingly, osteocytes isolated from rosiglitazone-treated WT mice had increased expression of Sost, Dkk1, and Rankl, suggesting an osteocyte contribution to the negative effect on bone formation and the increase in bone resorption observed in WT animals (Fig. 6G). This osteocyte response to rosiglitazone was not observed in PP5KO mice (Fig. 6G). These results indicate that PP5 mediates rosiglitazone-induced bone loss.
Phosphorylation is the most common PTM regulating protein activities. We previously identified ERK/MAP kinases as responsible for reciprocal regulation of PPAR\(\gamma\)/H9253 and RUNX2 activities at the level of serine phosphorylation (2–4). Using this mechanism, MAPK may control the ratio of osteogenesis to marrow adipogenesis in a variety of physiological conditions, including skeletal loading, where Ser-319pRUNX2 is increased (15, 16). However, little is known about mechanisms opposing bone anabolic signals and stimulating marrow adipogenesis, such as the response to the TZD, rosiglitazone. Here we show for the first time that PP5 phosphatase is a unique regulator of both PPAR\(\gamma\)/H9253 and RUNX2 activities associated with serine dephosphorylation. PP5-mediated dephosphorylation of PPAR\(\gamma\)/H9253 at Ser-112 and RUNX2 at Ser-319 stimulates adipogenesis and suppresses osteoblastogenesis from marrow MSC. Thus, PP5 phosphatase provides a missing link for understanding mechanisms controlling MSC differentiation to adipocytes at the expense of osteoblasts.

**Discussion**

Phosphorylation is the most common PTM regulating protein activities. We previously identified ERK/MAP kinases as responsible for reciprocal regulation of PPAR\(\gamma\) and RUNX2 activities at the level of serine phosphorylation (2–4). Using this mechanism, MAPK may control the ratio of osteogenesis to marrow adipogenesis in a variety of physiological conditions, including skeletal loading, where Ser-319pRUNX2 is increased (15, 16). However, little is known about mechanisms opposing bone anabolic
The role of PP5 in regulation of MSC lineage commitment is reflected in the high bone mass of mice deficient in this phosphatase and their protection from the negative effect of rosiglitazone. Thus, this single phosphatase is a major contributor to the negative effect of rosiglitazone on bone mass and osteoblast differentiation. Mice deficient in PP5 have a high number of active endosteal osteoblasts, which are resistant to rosiglitazone. Similarly, primary MSCs derived from PP5KO mice are neutral to the anti-osteoblastic and pro-adipocytic effects of rosiglitazone. This provides definitive evidence that PP5 is a unique phosphatase reciprocally regulating RUNX2 and PPARγ activities.

PP5 belongs to the family of tetratricopeptide repeat proteins, which can bind HSP90 chaperone in order to modulate nuclear receptor activity (11). We previously demonstrated that PP5 phosphatase mediates cellular lipid metabolism through reciprocal control of glucocorticoid receptor-α (GRα) and PPARγ (10). Here, we demonstrate that a similar reciprocal mechanism between PPARγ and RUNX2 regulates MSC differentiation to osteoblasts and adipocytes. Moreover, we showed that, like PPARγ, RUNX2 forms a complex with HSP90 and, upon activation of adipogenesis, PP5 binds to this complex and dephosphorylates RUNX2 at Ser-319. This finding demonstrates that PP5 can modulate the activity of transcription factors other than nuclear receptors and that the mechanism by which PP5 regulates client activity is common and involved in the formation of a complex with HSP90.

Although PP5 overexpression was able to partially reverse MAPK-dependent phosphorylation of both RUNX2 (Ser-319) close to unstimulated control values in PP5totally reversed MAPK-dependent suppression of PPARγ transcription, we also showed that PP5 may also affect phosphorylation of Ser-273 in PPARγ which is regulated by the activity of the negative regulator, glucocorticoid receptor. Although it remains to be clarified what precise mechanisms cause translocation of PP5 to the nucleus and induce its binding to PPARγ and RUNX2, the phenomenon is highly similar to the hormone-induced recruitment of another TPR-containing chaperone, FKBP52, to nuclear receptors. In the latter case, binding of cognate hormone leads to interaction of FKBP52 with both the glucocorticoid and mineralocorticoid receptors and movement of the receptors from cytoplasm to nucleus with involvement of dynein-dynactin motor complexes (19–21).

With respect to the physiological role of PP5, it is important to note that rosiglitazone and other TZD agonists are not endogenous activators of PPARγ. That role is performed by various polyunsaturated fatty acids, which also activate PP5 (22–24). We, therefore, propose that the major function of PP5 in the regulation of osteogenesis is to act as a fatty acid controlled rheostat maintaining the balance between PPARγ and RUNX2 activities.

PP5 activity is not restricted to regulation of MSC lineage commitment. We found that mice deficient in PP5 also have higher osteoclast numbers, which remained unchanged upon rosiglitazone treatment. Although this finding is not a focus of this report, in separate studies we recently demonstrated that PPARγ regulation of osteoclast differentiation involves phosphorylation of Ser-273, the same serine that is also responsible for insulin sensitization (25). Thus, the role of PP5 phosphatase in the maintenance of bone homeostasis may go beyond regulation of adipocyte and osteoblast differentiation and may include hematopoietic differentiation and regulation of energy metabolism. Mechanisms of PP5 activity are currently under investigation.

PP5-deficient osteocytes show increased endosteal osteoblasts and, as we showed previously (17–18), the phenomenon is highly similar to RUNX2 and PPARγ activities. PP5 unifies mechanisms controlling white adipocyte and osteoblast differentiation and may include hematopoietic differentiation and regulation of energy metabolism. These aspects of PP5 activity are currently under investigation.

In conclusion, PP5 is the first identified phosphatase reciprocally regulating PPARγ and RUNX2 activities. PP5 unifies...
activities of these transcription factors at the level of MSC lineage commitment and bone response to rosiglitazone.

**Experimental Procedures**

**Animals**

PP5−/− (PP5KO) mice were described previously (26). The colony of PP5KO and WT mice were maintained at the University of Toledo Health Science Campus. All animals were housed in a 12-h dark-light cycle and had free access to standard chow (Harlan Teklad 2016; Haslett, MI). The animal treatment and care protocols conformed to National Institutes of Health Guidelines and were performed using a University of Toledo Health Science Campus Institutional Animal Care and Utilization Committee protocol.

All presented experiments were performed on 5-month-old skeletally mature males, the age when marrow adipocyte accumulation is detectable. There are no differences in skeletal characteristics between males and females of PP5KO mice (not shown). To test the skeletal effect of rosiglitazone administration, WT and PP5KO mice were divided into two groups (n = 3–6 per group) and fed for 8 weeks with either non-supplemented chow or chow supplemented with rosiglitazone at the dose 20 mg/kg/day. During the experiment, food and water intake per cage were monitored, and the average intake of rosiglitazone per mouse was calculated at the end of experiment. There were no differences between groups within each mouse strain in daily food intake and water intake. Calculated intake per cage were monitored, and the average intake of rosiglitazone per mouse was calculated at the end of experiment. There were no differences between groups with respect to mouse strain in daily food intake and water intake. The dose of effective drug intake in WT mice was 19.1 mg/kg/day.

**Bone Analysis**

Microcomputed tomography of trabecular bone was performed using an XtremeCT micro XCT 800 (Scanco Medical, Bassersdorf, Switzerland). Briefly, scans were performed at 40 kVp with a 133-μA intensity, with a 289 μm voxel. Images of trabecular bone were processed with a 289 threshold value using the per mille scale. The measurements of bone microstructure conformed to recommended guidelines (28).

For lipid evaluation, decalcified bone specimens were stained for 1 h in solution containing 2% osmium tetroxide prepared in 0.1 m sodium cacodylate buffer, pH 7.4, according to the protocol (27). Staining was carried out in an exhaust hood and away from light due to osmium tetroxide toxicity and light sensitivity. Images of lipid depositions were acquired at 70-kVp and 113-μA settings and 12-μm nominal resolution. Image segmentation was done under global threshold conditions by applying a gray scale threshold of 480–1000 using the per mille scale with the three-dimensional noise filter set to sigma 1.2 and support 2.0. Lipid volumes were calculated directly from individual voxel volumes in three-dimensional reconstructions. Global BMD was measured by dual-energy X-ray absorptiometry (DXA) using a PIXI-plus Small Animal Densitometer (manufactured by LUNAR, Madison, WI).

**Bone Histomorphometry**

To permit static and dynamic bone histomorphometry, mice were intraperitoneally injected with 20 mg/kg calcine (Sigma) 8 and 2 days before sacrifice, and undecalcified tibiae were fixed in 70% ethanol and embedded in methyl methacrylate and sectioned. Pictures were taken by a Zeiss Axiovert 40 CFL Fluorescence Microscope (Carl Zeiss Microscopy, Thornwood, NY) equipped with a Micropublisher 3.3 Megapixel Cooled CCD Color Digital Camera (QImaging, Surrey, BC, Canada). Analysis of BFR was confined to the secondary spongiosa of proximal tibia and was performed using the Nikon NIS-Elements BR3.1 system. The measurements were collected with a 40× objective (numerical aperture, 0.5) at room temperature from 6 representative fields per bone sample. The terminology and units used were those recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (29).

**Immunoadsorption of PPARγ and RUNX2 Complexes**

Cells were harvested in HEMG (10 mm HEPES, 3 mm EDTA, 20 mm sodium molybdate, 1% glycerol, pH 7.4) plus protease inhibitor mixture and sonicated for 20 min followed by Dounce homogenization (800 strokes). Samples were collected after a 10-min 4 °C centrifugation at 10,000 × g and then precleared with protein A- or G-Sepharose beads, antibody for PPARγ (sc-7273) and Hsp90 (sc-8262), and approximate IgG at 4 °C under conditions recommended by the Histomorphometry Nomenclature Committee of the American Society for Bone and Mineral Research (29).

**Electrophoresis and Western Blotting**

Protein samples were resolved by SDS-polyacrylamide gel electrophoresis and electrophoretically transferred to Immobilon-FL membranes. Membranes were blocked at room temperature for 1 h in TBS (10 mm Tris-HCl, pH 7.4, 150 mm NaCl) containing 3% BSA plus phosphatase inhibitors. Incubation with primary antibody was done overnight at 4 °C. After 3 washes in TBST (TBS plus 0.1% Tween 20), membranes were incubated with infrared anti-rabbit (IRDye 800; green) or anti-mouse (IRDye 680; red) secondary antibodies (LI-COR Biosciences) at a 1:15,000 dilution in TBS at 4 °C. Immunoreactivity was visualized and quantified by infrared scanning in the Odyssey system (LI-COR Biosciences). Rabbit polyclonal antibody against PP5 was a generous gift from Michael Chinkers (University of South Alabama College of Medicine, Mobile, AL). Antibodies against PPARγ (sc-7273) and Hsp90 (sc-8262) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Runx2 (ab76956) and Ser-112 phospho-PPARγ (ab60953) were purchased from Abcam (Abcam PLC, Cambridge, MA). Ser-273 phospho-PPARγ antibody was purchased from Bios Inc. (Bios, Inc., Woburn, MA). The Ser-319 phospho-RUNX2 antibody was previously described (3).

**Immunofluorescent Imaging**

U-33/γ2 cells were seeded on laminin-coated coverslips in 6-well plates at 100,000–200,000 cells/well in DMEM contain-
PP5 Regulates PPARγ and RUNX2 Activities

Gene Expression Analysis Using Quantitative Real-time RT-PCR Analysis

One μg of total RNA was converted to cDNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific, Waltham, MA). PCR amplification of the cDNA was performed by quantitative real-time PCR using TrueAmp SYBR Green qPCR SuperMix (Smart Bioscience, Maumee, OH) and processed with StepOne Plus System (Applied Biosystems, Carlsbad, CA). The thermocycling protocol consisted of 10 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 60 °C, and 20 s at 72 °C and finished with a melting curve ranging from 60 to 95 °C to allow distinction of specific products. Relative gene expression was measured by the comparative CT method using 18S RNA levels for normalization. Primers were designed using OligoPerfect Designer (Thermo Fisher Scientific).

Statistical Analysis

Data are presented as means ± S.D. and were analyzed using statistical software (GraphPad Prism; version 5.0). p values <0.05 were considered statistically significant.

WITHDRAWN

May 3, 2018

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