0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(5):2415–2423
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doi: 10.1210/en.2004-1050

Secreted Frizzled-Related Protein 1 Modulates Glucocorticoid Attenuation of Osteogenic Activities and Bone Mass

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Prolonged glucocorticoid treatment is known to cause osteoporosis or aseptic necrosis. Secreted frizzled-related proteins 1 (SFRP1) and low-density lipoprotein-related protein 5 (LRP5), a Wnt protein antagonist and a coreceptor, have been found to regulate skeletogenesis. Whereas recent studies have reported that excess glucocorticoid promotes bone loss, the biological role of SFRP1 and LRP5 in regulating glucocorticoid attenuation of bone formation is not fully understood. We showed that a supraphysiological level of glucocorticoid enhanced SFRP1 but not LRP5 expression of primary mesenchymal cell cultures *in vitro* and osteoblasts at metaphyseal trabecular endosteum and chondrocytes at calcified cartilage *in vivo*. Glucocorticoid augmentation of SFRP1 expression was transcriptionally mediated. The inhibitory action of glucocorticoid on osteogenic differentiation appeared to be regulated by SFRP1 mediation of β-catenin destabilization because knocking down SFRP1 by RNA interference abrogated the supraphysiological level of glucocorticoid attenuation of osteogenesis. Recombinant human SFRP1 reduced the promoting effect of physiological level of glucocorticoid on cytosolic β-catenin accumulation, runt-related transcription factor-2 activation, and osteogenic activities. Glucocorticoid and recombinant human SFRP1 significantly increased osteochondral cell apoptosis associated with reduced mineral density, biomechanical properties, trabecular bone volume, and midshaft cortical bone areas in rat femurs. These findings suggest that SFRP1 modulates glucocorticoid-induced bone loss. Regulation of Wnt/SFRP signal transduction can be used in the future as an alternative strategy for the prevention of glucocorticoid-induced osteoporosis. (*Endocrinology* 146: 2415–2423, 2005)

PROLONGED GLUCOCORTICOID treatment has been found to induce osteoporosis or aseptic necrosis (1). Previous studies have demonstrated that excess glucocorticoid treatment promotes regional trabecular bone matrix protein degeneration and osteoblast apoptosis and reduces osteogenic factor gene transcription (2–4). However, the molecular mechanisms by which glucocorticoid attenuates osteogenesis of mesenchymal stem cells and bone microenvironment have not been fully elucidated.

The Wnt family consists of cysteine-rich secreted glycoproteins that initiate β-catenin, glycogen synthase kinase-3β, and T-cell factor pathways to activate the Wnt-responsive gene upon binding to the frizzled family receptors and their low-density lipoprotein-related protein (LRP) coreceptors (5). Wnt proteins have been found to regulate tissue morphogenesis (6), long bone development (7), and osteocalcin expression of osteogenic precursor cells (8). Knockout of LRP5 gene results in osteopenia and persistent eye vascularization (9). LRP5 transgenic mice display increased mineralized bone surface and reduced osteoblast apoptosis associated with high bone mass (10).

Secreted frizzled-related proteins (SFRPs) have been found to act as competitive inhibitors of Wnt signaling by competing with membrane-bound frizzled proteins for Wnt binding (11). SFRP proteins are important in bone formation, cartilage development, and skeletal disorders. Deletion of the SFRP1 gene results in increased trabecular bone mineral density and up-regulated osteoblast proliferation and differentiation (12). Intensive SFRP2 expression and SFRP3 gene mutation have been associated with chondrocyte apoptosis of osteoarthritic articular cartilage (13) and hip osteoarthritis in women (14), respectively. Recent studies demonstrated that excess glucocorticoid treatment inhibits glycogen synthase kinase-3β-regulated cell proliferation of osteoblasts, suppresses osteogenic differentiation of mouse mesenchymal stem cells, and retards osteoblastogenesis in the skeleton (15–17). These findings imply that SFRP or LRP5 signaling in osteogenic cells may be altered by glucocorticoid treatment, which is known to inhibit bone formation activities. We hypothesize that glucocorticoid treatment may inhibit osteogenic differentiation through regulating the synthesis of SFRP or LRP5 in bone marrow stromal cells.

This study investigates whether SFRP1 or LRP5 are involved in glucocorticoid regulation of osteogenic activities of mesen-

First Published Online January 27, 2005

Abbreviations: Ct, Intensity threshold; FBS, fetal bovine serum; LRP, lipoprotein-related protein; rh, recombinant human; Runx, runt-related transcription factor; SFRP, secreted frizzled-related protein; si, small interfering; TdT, terminal deoxynucleotidyl transferase; TUNEL, TdT-mediated deoxyuridine triphosphate-biotin nick end-labeling.

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chymal stem cells and whether exogenous SFRP1 treatment can regulate bone mass and biomechanical properties of rat femurs.

Materials and Methods

Primary bone marrow stromal cells

All studies and protocols were approved by the Institutional Animal Care and Use Committee of Chang Gung Memorial Hospital. Three-month-old male Sprague Dawley rats (National Experimental Animals Production Center, Taiwan) were anesthetized with an overdose of pentobarbital sodium. Bone marrow stromal cells in tibiae and femurs were harvested as previously described (18). Briefly, nucleated cells were seeded in DMEM with 10% fetal bovine serum (FBS) (Life Technologies, Gaithersburg, MD) for 30 min to separate adherent macrophages from nonadherent cells containing the stromal elements; nonadherent cells were collected and plated (5×10^5 cell/well, six-well plates). Twenty-four hours later, all nonadherent cells were discarded, and the adherent stromal cells were then harvested by trypsinization. Cell number was counted using a hemacytometer after staining with 0.4% trypan blue.

Glucocorticoid treatment

Primary mesenchymal cells (5×10^5/well, 6-well plates) were cultured in DMEM containing 10% charcoal-treated FBS with or without 10^{-6} M dexamethasone, hydrocortisone, or methylprednisolone (Sigma Chemical Inc., St. Louis, MO) for 72 h. In some experiments, cells were cultured in medium containing 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M dexamethasone for 0 and 12 h and 1, 3, 6, 9, and 18 d, respectively. Cells were also pretreated with 10 μg/ml actinomycin D, 10 μg/ml cycloheximide, or 1 μM mifepristone (Tocris Cookson Ltd., Bristol, UK) and then cultured in medium containing 10^{-6} M dexamethasone.

Cell proliferation

Cell growth was measured using a colorimetric cell proliferation kit (Roche Molecular Biochemicals GmbH, Mannheim, Germany) according to the manufacturer's instructions. Briefly, primary cells (2×10^4 cells/well, 96-well) with or without 10^{-6} M dexamethasone were cultured for 72 h before adding 10 μl/well 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyl tetrazolium bromide for an additional 4-h culture. Formazan synthesis in each well was resolved by 10% sodium dodecyl sulfate and 0.01 M HCl and spectrophotometrically measured at 550 nm.

Bone formation activities

Primary cells (1×10^5 cells/well, 24-well plate) were cultured in osteogenic medium containing DMEM, 10% charcoal-treated FBS, 50 μg/ml L-ascorbic acid, and 10^{-2} M β-glycerophosphate in the presence or absence of 10^{-8} or 10^{-6} M dexamethasone for 21 d in a 5% CO_2, 37 C incubator. The medium was changed every 3 d. In some experiments, mesenchymal cells were cocultured with osteogenic medium, 10^{-8} M dexamethasone, and recombinant human (rh) SFRP1 (0, 5, 25, and 50 ng/ml; R&D Systems Inc., Minneapolis, MN). Cell cultures were subjected to assessment of bone alkaline phosphatase activity and von Kossa staining as previously described (19). The number of bone nodules greater than 2 mm^2 showing positive von Kossa staining was counted under an inverted microscope. Osteocalcin levels in cultured supernatants were measured using rat osteocalcin ELISA kits (Biomedical Technologies Inc., Stoughton, MA). Three days after culture, trypsinized and floating cells were pooled, spun (1×10^4 cells) onto glass slides, and fixed in 70% methanol for investigating cell apoptosis.

RT-PCR

Total RNA was extracted and purified by the Tri reagent (Sigma) from 10^6 cells. One microgram of total RNA was reverse transcribed into cDNA, followed by PCR amplification using rat gene-specific primers: SFRP1 (sense) (5′-CAG TCG GAT ATC GGC TCCTA-3′), SFRP1 (antisense) (5′-GTG GCA GTT CTT GTT GAG CA-3′) (201 bp expected); LRP5 (sense) (5′-GGC TCG GAT GAA GCT AAC TG-3′), LRP5 (antisense) (5′-CAG GAT GAT GCC AAT GAC AG-3′) (210 bp expected); β-actin (sense) (5′-AGT ACC CCA TTG AAC ACG GC-3′), β-actin (antisense) (5′-TTT TCA CGG TTA GCC TTA GG-3′) (168 bp expected). The RT-PCR cycling parameters were set as previously described (18). The PCR products were electrophoresed on 1.5% agarose gels containing ethidium bromide and visualized by UV-induced fluorescence.

Real-time PCR

Twenty-five microliters of PCR mixture containing cDNA template equivalent to 20 ng total RNA, 2.5 μM each of forward, and reverse primer as well as $2 \times$ SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) was amplified using an ABI Prim 7700 sequence detection system with an initial melt at 95 C for 10 min followed by 40 cycles of 95 C for 15 sec, 60 C for 30 sec, and 78 C for 40 sec. The number of amplification steps required to reach an arbitrary intensity threshold (Ct) was computed. The relative gene expression were presented $2^{(-\Delta Ct)}$, where $\Delta Ct = Ct_{target} - Ct_{\beta\text{-actin}}$. Fold change for the treatment was defined as the relative expression, compared with the vehicle, and was calculated as $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{vehicle}$.

Inhibition of SFRP1 expression by RNA interference

Endogenous SFRP1 mRNA expression was knocked down using RNA interference as previously described (20). Briefly, *SFRP1* small interfering (si)RNA was synthesized and purified using a Silence siRNA kit (Ambion, Austin, TX). The sequences of the 21-mer *SFRP1* siRNA oligonucleotides were 5′-AAC TTC TTG GGG ACA ATC TTC-3′ and 5′-AAG AAG ATT GTC CCC AAG AAG-3′. Cell cultures were transfected with double-strand *SFRP1* siRNA (10 nM) or scrambled siRNA (Silencer negative control) using siPORT amine transfection agent according to the manufacturer's instructions.

Immunoblotting

Cytosolic and nuclear fractions of cell lysates were prepared as previously described (21). Nuclear extracts were immunoprecipitated with runt-related transcription factor (Runx)2 antibodies and protein A agarose (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Immunoprecipitates, cytosolic extracts, lysophilized cultured supernatants, and sera were subjected to Western blotting. The designated proteins on the blots were probed by β-catenin (Upstate Biotechnology, Lake Placid, NY) or SFRP1 or LRP5 (Santa Cruz Biotechnology Inc.) or Runx2 antibodies, followed by horseradish peroxidase-conjugated IgG secondary antibodies, and visualized with chemiluminescence agents. After stripping the membrane, phosphorylated Runx2 bands on the blot were recognized by reprobing with mouse antiphosphotyrosine 4G10 antibodies using a similar procedure.

Glucocorticoid and rhSFRP1 treatment in vivo

Forty 3-month-old male rats were randomly divided into four groups. Rats in each group were sc given 0.5 mg/kg·d dexamethasone (n = 10) or vehicle (n = 10; 100 μl of a mixture containing 95% corn oil and 5% ethanol) or 1 μg/kg·d rhSFRP1 (n = 10) or vehicle (BSA, n = 10) for 30 consecutive days. At d 30, rats were killed using an overdose of pentobarbital sodium. Rat blood was harvested via intracardiac needle to assess serum calcium, phosphorus, and PTH using respective assay kits (Randox Laboratory Ltd., Antrim, UK) according to the manufacture's instructions. After bone mineral density assay, the left femurs were dissected for biomechanical assessment. The right femurs were harvested for histomorphometry.

Bone mineral density

Mineral density of the proximal, midshaft, and distal femur was measured using a dual-energy x-ray absorptiometry (QDR 2000 Plus; Hologic Inc., Bedford, MA) and an ultrahigh resolution software program. The evaluation of each scan was based on the exact positioning and placement of the region of interest on the baseline scan using comparative technique. Bone mineral density coefficient of variation was calculated by repeated scanning six times with and without sample repositioning between scans.

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Biomechanical assay

Femurs were three point bended to failure using a Material Testing System QT-10 (MTS Corp., Minneapolis, MN) with a jig span of 30 mm at a displacement rate of 2 mm/min. Load-displacement curves were plotted, and failure load and stiffness were calculated.

Immunohistochemistry

Femurs were fixed in 4% PBS-buffered paraformaldehyde, decalcified, embedded in paraffin, and then cut longitudinally into 5-μm-thick sections. SFRP1 or LRP5 antibodies were used for immunostaining. Immunoreactivity in sections was demonstrated using a horseradish peroxidase-conjugated 3′-3′-diaminobenzidine immunohistochemical staining kit (R&D Systems), followed by counterstaining with hematoxylin, dehydration, and mounting. Those with mouse IgG were enrolled as negative controls for the immunostaining.

Terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate-biotin nick end-labeling (TUNEL)

Apoptotic cells were detected using *in situ* cell death detection kits (Roche Diagnostics GmbH) according to the manufacturer's instructions. Specimens pretreated with 50 U/ml DNase I (Sigma) or incubated in reaction buffer without TdT were used as positive or negative controls. TUNEL-stained cells were recognized using fast red (cell cultures) or 5-bromo-4-chloro-3-indoyl-phosphate/4-nitro blue tetrazolium chloride (bone specimens) as substrates.

Histomorphometry

Three random images of 0.75 mm^2 from each area (3 mm^2) were taken and counted using a Cool charge-coupled device camera and an Image-Pro Plus image-analysis software (Media Cybernetics, Sliver Spring, MD). Five areas within proximal femurs from three sections obtained from eight rats were randomly selected. Trabecular bone volume (percent, square millimeters) in the proximal femoral metaphysis and cross-sectional midshaft femoral cortical bone volume (square millimeters) were microscopically measured under ×100 magnifications. Mean number of positively SFRP1, LRP5, or TUNEL immunostained cells per high-power field (0.75 mm^2) in each section was calculated (22). Cell apoptosis was obtained by calculating TUNEL-stained cells per total cell ratios from five random fields of three sections under ×200 magnification. Two professional pathologists blinded to the treatment regimen performed measurement on all sections.

Statistical analysis

All values were expressed as means ± SE calculated from six paired triplicate experiments. Wilcoxon test was used to evaluate differences between the sample of interest and its respective control. For analysis of concentration effect and time course, a multiple range of ANOVA and *post hoc* tests were used. $P < 0.05$ was considered significant.

Results

Glucocorticoid treatments increased SFRP1 but not LRP5 expression

Compared with the vehicle group, glucocorticoids significantly increased SFRP1 but not LRP5 mRNA (Fig. 1A) and protein levels (Fig. 1B) 72 h after treatment. Of the glucocorticoids, dexamethasone promoted SFRP1 levels highest. Dexamethasone was used in the succeeding experiments.

Real-time PCR results showed that 10^{-9} and 10^{-8} M dexamethasone did not significantly alter SFRP1 levels; 10^{-5} M dexamethasone had a maximum effect on SFPR1 expression (Fig. 2A). The EC_{50} of dexamethasone for inducing SFRP1 mRNA was 4.2×10^{-7} M. Treatment with 10^{-6} M dexamethasone significantly increased SFRP1 levels in 12 h (Fig. 2B) and reduced cell proliferation in 48 h (Fig. 2C).

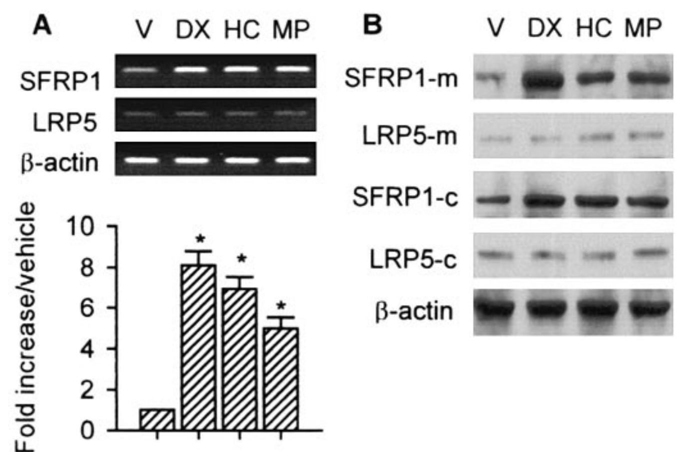

FIG. 1. Glucocorticoids promoted SFRP1 but not LRP5 mRNA (A) and protein expression (B). Mesenchymal cells were treated with or without 10^{-6} M dexamethasone or hydrocortisone or methylprednisolone for 72 h. The results graphed represent relative abundance of the SFRP1 gene by real-time PCR normalized to housekeeping gene β-actin. Each primary culture from each of 12 rats was assessed independently and then combined for statistical analysis using a Wilcoxon test (*, Difference from vehicle group; $P < 0.05$). SFRP-1 and LRP5 levels in cultured supernatants and cell lysates were assessed by Western blot. Immunoblotting of β-actin showed equal loading and transfer for all lanes. All blots are representative of at least three different experiments, with comparable results. V, Vehicle; DX, dexamethasone; HC, hydrocortisone; MP, methylprednisolone. SFRP1-m, LRP5-m, SFRP1-c, LRP5-c, SFRP1, and LRP5 from cultured supernatants and cytosolic extracts of cell cultures, respectively.

Glucocorticoid augmentation of SFRP1 expression is transcription controlled

Blockade of transcription by actinomycin D decreased baseline and glucocorticoid-stimulated SFRP1 mRNA expression (Fig. 3). Inhibition of translation by cycloheximide did not affect baseline or glucocorticoid-augmented SFRP1 mRNA expression (Fig. 3). Cell culture concomitant treatment with mifepristone significantly reduced the promoting effect of 10^{-6} M dexamethasone on SFRP1 levels, suggesting that glucocorticoid augmentation of SFRP1 expression occurs through glucocorticoid receptor-dependent transcriptional regulation.

Glucocorticoid treatment increased SFRP1 expression in bone microenvironments

We elucidated SFRP1 and LRP5 expression in bone microenvironment after glucocorticoid treatment. No immunostaining of SFRP1 or LRP5 was visible in IgG group. Cells positive for SFRP1 or LRP5 expression exhibited brown immunostaining in the cell periphery and cytoplasm. In the vehicle group, osteoblasts and bone marrow stromal cells lined at metaphyseal trabecular endosteum displayed weak SFRP1 and LRP5 expression. Chondrocytes at the growth plate displayed slight SFRP1 expression (Fig. 4). In the glucocorticoid-treated group, osteoblasts and bone marrow stromal cells adjacent to trabecular endosteum and chondrocytes at cartilage expressed strong SFRP1 immunoreac-

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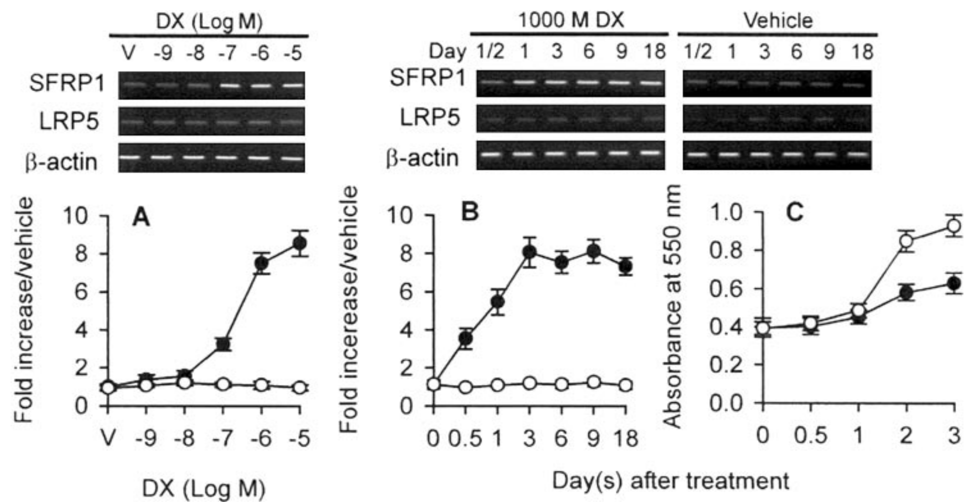

FIG. 2. Dexamethasone elicited a dose-dependent effect on SFRP1 mRNA expression (A). Dexamethasone treatment greater than 10^{-7} M significantly increased SFRP1 levels. Dexamethasone (10^{-6} M) significantly increased SFRP1 levels in 12 h (B) and reduced cell proliferation in 48 h (C). Dexamethasone did not alter LRP5 mRNA expression. cDNA was electrophoresed after amplifying by reverse transcription and PCR. Relative abundance of the SFRP1 gene was quantified by real-time PCR normalized to β-actin. Each primary culture from each of 12 rats (mRNA expression) and six rats (cell proliferation) was assessed independently and then combined for statistical analysis using ANOVA and Bonferroni *post hoc* tests. ●, Dexamethasone-treated group; ○, vehicle group.

tivities. Glucocorticoid treatment did not alter LRP5 immunoreactivity of osteoblasts and chondral cells (Fig. 4).

SFRP1 modulated glucocorticoid regulation of osteogenesis

In comparison with the vehicle group, 10^{-8} M dexamethasone significantly increased alkaline phosphatase activity (Fig. 5A), osteocalcin level (Fig. 5B), and bone nodule formation (Fig. 5C). Dexamethasone (10^{-6} M) reversed the osteogenic activities of cell cultures to the baseline. Synthetic *SFRP1* siRNA was used to reduce 10^{-6} M dexamethasone promotion of SFRP1 mRNA (Fig. 6A) and protein expression (Fig. 6B). The β-actin levels were not affected by the treatment, indicating that knocking down SFRP1 by siRNA was not due to general suppression of gene expression. Knocking down SFRP1 alleviated the inhibitory effect of 10^{-6} M dexamethasone on bone formation activities (Fig. 5). Moreover, 25 and 50 ng/ml rhSFRP1 significantly reduced the stimulatory effect of 10^{-8} M dexamethasone on osteogenesis (Fig. 7).

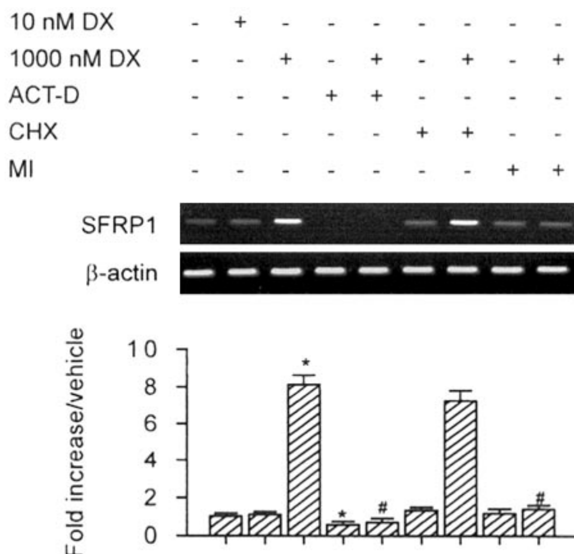

FIG. 3. Dexamethasone promoted SFRP1 mRNA expression via glucocorticoid receptor-dependent transcriptional regulation. Cells were pretreated with actinomycin D or cycloheximide or cocultured with mifepristone in the presence or absence of 10^{-6} M dexamethasone. The results graphed represent relative abundance of SFRP1 gene by real time PCR normalized to β-actin Each primary culture from each of 12 rats was assessed independently and then combined for statistical analysis using Wilcoxon test (*, #, and +, Respective difference from vehicle, 10 nM DX, and 1000 nM DX group, $P < 0.05$). DX, Dexamethasone, ACT-D, actinomycin D; CHX, cycloheximide; MI, mifepristone.

β-catenin and Runx2 immunoblotting and TUNEL staining

Immunoblotting showed that 10^{-8} M dexamethasone increased cytosolic β-catenin accumulation and nuclear Runx2 activation, and 10^{-6} M dexamethasone reversed the expression of cytosolic β-catenin and phospho-Runx2 to the baseline (Fig. 8). Knocking down SFRP1 abrogated the suppressing effect of 10^{-6} M dexamethasone on β-catenin stabilization and Runx2 phosphorylation. rhSFRP1 attenuated the promoting effect of 10^{-8} M dexamethasone on cytosolic β-catenin accumulation (Fig. 9A) and Runx2 activation (Fig. 9B).

We investigated whether SFRP1 was involved in glucocorticoid-induced cell apoptosis. In the absence of TdT, no TUNEL staining was observed. Cells positive for TUNEL exhibited red staining in the nucleus. Dexamethasone (10^{-8} M) did not significantly affect cell apoptosis, whereas cells treated with 10^{-6} M dexamethasone or cocultured with rhSFRP1 and 10^{-8} M dexamethasone significantly increased apoptosis. Knocking down SFRP1 reduced the promoting effect of 10^{-6} M dexamethasone on cell apoptosis (Fig. 9, C and D).

Glucocorticoid and SFRP1 treatments attenuated bone mass and strength

Glucocorticoid treatment between 2.5 and 9 mg/kg·d has been reported to successfully induce osteopenia in rats (23–25). In the current study, dexamethasone (0.5 mg/kg)- and rhSFRP1-treated rats remained healthy and gained body weight, even though rats treated with dexamethasone gained less weight than the vehicles (Fig. 10). The discrepancy between end-of-study weights between dexamethasone and vehicle groups might theoretically exaggerate the negative effect of glucocorticoid on weight-sensitive bone parameters (24) but will equally underplay the effect of SFPR1 on bone mass. Dexamethasone and rhSFRP1 increased serum SFRP1 levels. rhSFRP1 did not significantly alter serum calcium (9.3 ± 0.1 *vs.* 9.2 ± 0.2% mg), phosphorus (6.2 ± 0.3 *vs.* 6.4 ±

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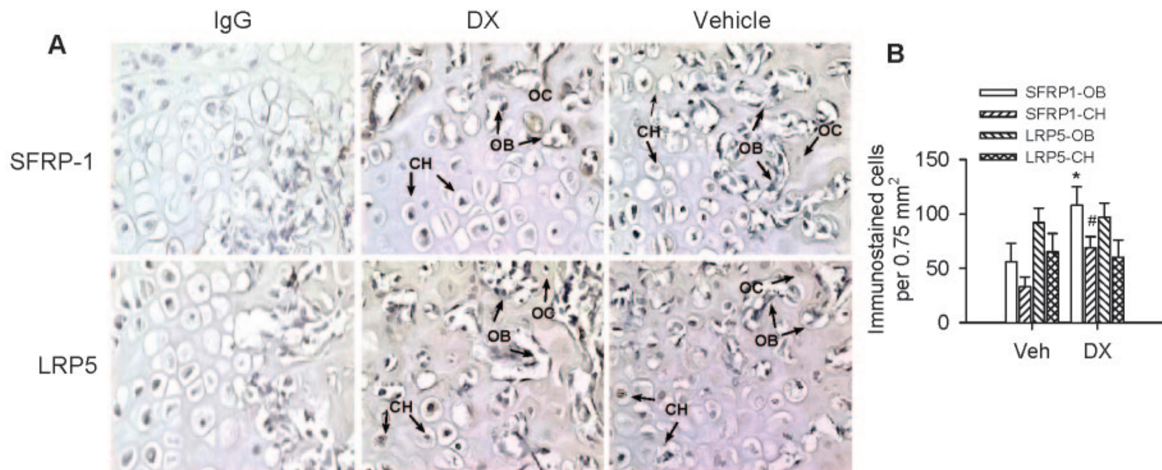

FIG. 4. Immunohistochemical photographs of rat proximal femurs. A, In the vehicle group, mesenchymal cells and osteoblasts at the junction of calcified cartilage and chondrocytes at growth plate expressed weak SFRP1. Osteoblasts and bone marrow stromal cells adjacent to trabecular endosteum and osteocytes at the trabecular bone expressed intense SFRP1 after dexamethasone treatment. The positive SFRP1 and LRP5 immunostained cells showed *brown color*. B, Dexamethasone significantly increased SFRP1 but not LRP5 expression of osteoblasts and chondrocytes, respectively. Magnification, ×400. * and #, Differences from dexamethasone-treated group and vehicle group ($P < 0.05$). DX, Dexamethasone; OB, osteoblasts; CH, chondrocytes; OC, osteocytes; Veh, vehicle.

0.2% mg), and PTH (3.6 ± 0.4 *vs.* 3.2 ± 0.3 pg/ml) levels. Dexamethasone and rhSFRP1 significantly reduced mineral density, biomechanical property, trabecular and cortical bone mass (Fig. 10 and supplemental data) associated with increased apoptosis of osteoblasts, osteocytes and chondrocytes located at trabecular endosteum, trabecular bone, and cartilage (Fig. 11).

Discussion

In this study, the elevation of SFRP1 levels in mesenchymal cells was found to follow glucocorticoid treatment. This Wnt antagonist plays a critical role in regulating cytosolic β-catenin accumulation and osteogenic activities. Whereas a number of reports have implicated that glucocorticoid stimulates osteoblast (osteocyte) apoptosis (26) and osteoclastic resorption (27) and inhibits osteoblast attachment to bone extracellular matrix (28), little research has been done to define the role of Wnt signaling molecules in glucocorticoid promotion of bone loss. Our findings provide the first indication that SFRP1 mediated glucocorticoid promotion of cell apoptosis and attenuation of osteogenic activities in mesenchymal cells, thus providing a clear molecular explanation

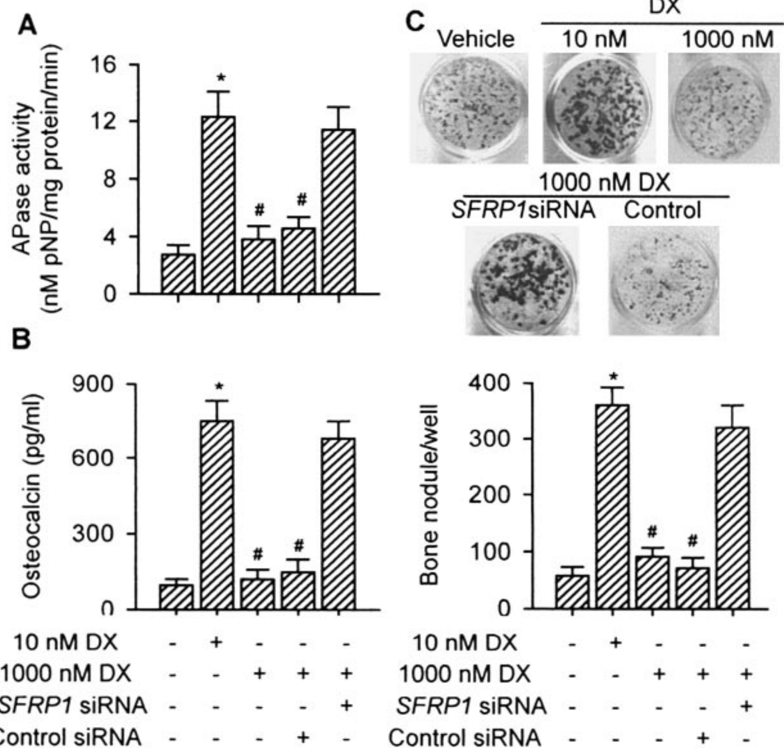

FIG. 5. Biphasic effect of dexamethasone on alkaline phosphatase activity in 12 d (A), osteocalcin levels in 18 d (B), and bone nodule formation in 21 d (C). Dexamethasone (DX; 10^{-8} M) stimulated osteogenesis, whereas 10^{-6} M dexamethasone reversed to the baseline. Knocking down SFRP1 abrogated the suppressing effect of 10^{-6} M dexamethasone on osteogenic activities. Mesenchymal cells were cultured in osteogenic medium in the presence or absence of 10^{-8} or 10^{-6} M dexamethasone for 21 d. siRNA-transfected cells were cultured in osteogenic medium and 10^{-6} M dexamethasone. Each primary culture from each of nine rats was assessed independently and then combined for statistical analysis using Wilcoxon test (* and #, difference from vehicle and 10 nM DX group, $P < 0.05$).

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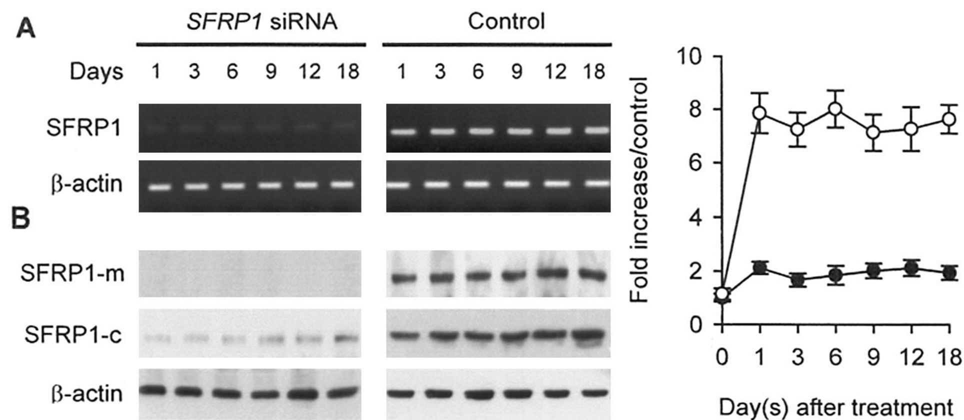

FIG. 6. Inhibition of endogenous SFRP1 by siRNA reduced SFRP1 mRNA (A) and protein expression (B). Cell cultures were transfected with siRNA molecules complementary to the *SFRP-1* coding sequence or control and then cultured in osteogenic medium and 10^{-6} M dexamethasone for 21 d. Each primary culture harvested from each of six rats was assessed independently and then combined for statistical analysis using Wilcoxon test. SFRP1-m and SFRP1-c were from cultured supernatants and cytosolic extracts of cell cultures, respectively. Immunoblotting of β-actin showed equal loading and transfer for all lanes. All blots are representative of at least three different experiments, with comparable results. ●, *SFRP1* siRNA group; ○, control group.

for glucocorticoid-induced bone loss. We propose that it is the increased SFRP1 expression of mesenchymal cells that brings about the pathogenesis of bone microenvironment in glucocorticoid-induced osteoporosis.

Glucocorticoids at physiological levels are required for developing trabecular bone architecture and initiating osteogenesis of osteogenic precursors (29, 30). In the current study, basal osteogenic activities were observed in primary mesenchymal cells cultured without glucocorticoid. Dexamethasone (10^{-8} M) markedly increased osteogenic differentiation, indicating that two pools of osteoprogenitors exist in rat bone marrow stromal cells. Few osteogenic cells differentiate into bone nodules in the absence of exogenously added glucocorticoid. Most osteoprogenitor cells require glucocorticoid stimulation to form mineralized nodules (31). We showed that 10^{-6} M dexamethasone promoted cell apoptosis and inhibited bone formation activities. These findings agree with those of previous studies, which have demonstrated that excess glucocorticoid reduces osteogenic colony and bone nodule formation of osteogenic cells (32, 33). Our findings suggest that glucocorticoid elicited a biphasic effect on mesenchymal cells proliferation and apoptosis.

Wnt signaling molecules have recently been implicated in skeletal disorders such as multiple myeloma-induced osteolytic lesion (34) and rheumatoid arthritis (35). However, the biological role of Wnt signal transduction in regulating glucocorticoid-induced bone loss remained unclear. In this study, cytosolic β-catenin accumulation coincided with 10^{-8}

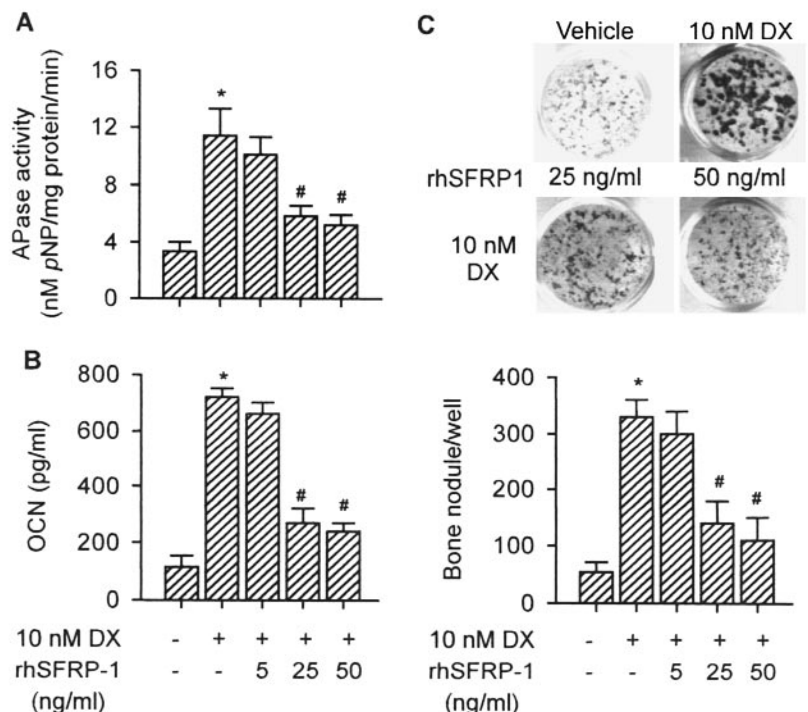

FIG. 7. The rhSFRP1 reduced alkaline phosphatase activity in 12 d (A), osteocalcin (OCN) in 18 d (B), and bone nodule formation (C). Mesenchymal cells were cocultured for 21 d in osteogenic medium and 10^{-8} M dexamethasone (DX) in the presence or absence of 0–50 ng/ml rhSFRP1. Each primary culture from each of eight rats was assessed independently and then combined for statistical analysis using Wilcoxon test (* and #, difference from vehicle and 10 nM DX group, $P < 0.05$).

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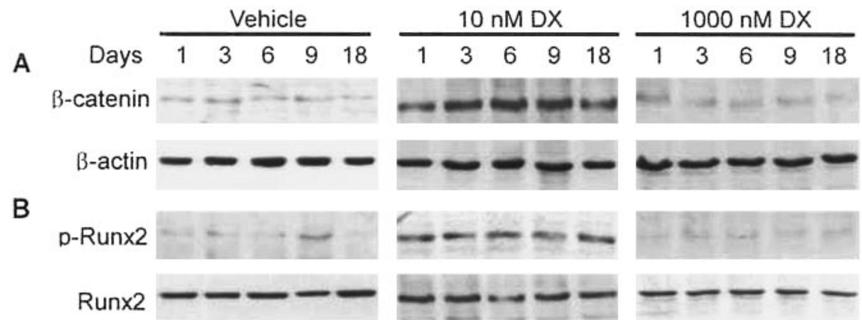

FIG. 8. Biphasic effect of dexamethasone (DX) on cytosolic β-catenin accumulation (A) and nuclear Runx2 activation (B). Dexamethasone (10^{-8} M) increased cytosolic β-catenin expression and nuclear Runx2 activation, whereas 10^{-6} M dexamethasone reversed cytosolic β-catenin expression and nuclear Runx2 activation to baseline. Immunoblotting of β-actin showed equal loading and transfer for all lanes. All blots are representative of at least three different experiments, with comparable results.

M dexamethasone-induced osteogenesis. Glucocorticoid (10^{-6} M) reduced β-catenin stabilization and Runx2 activation. Our data agree with previous studies reporting the inhibitory effect of excess glucocorticoid on Runx2 expression of rat osteoblasts (36). We suggest that Wnt signal transduction is involved in regulating osteogenic activities of mesenchymal cells. Wnt signaling molecules through stabilization of β-catenin and cooperation with lymphoid enhancer binding factor/T-cell transcription factors are found to increase osteogenesis of mesenchymal stem cells (37, 38). Our findings indicate that osteogenic cells respond to high concentrations of glucocorticoid by regulating Wnt signaling molecules to raise osteogenesis-inhibitory or attenuate osteogenesis-stimulatory mechanisms resulting in bone loss. We have shown that 10^{-6} M dexamethasone increased SFRP1 expression followed by increased cell apoptosis and reduced osteogenic activities of mesenchymal cells. Knocking down SFRP1 by siRNA alleviated cell apoptosis and restored osteogenic differentiation of mesenchymal cells. Moreover, rhSFRP1 reduced osteogenesis of bone marrow stromal cells *in vitro*, suggesting that SFRP1 acts as a negative regulator of bone formation. The results of our study also support those of previous studies that report that increased bone mass is observed in SFRP1 knockout mice (12). In contrast to previous studies demonstrating glucocorticoid treatment reduced LRP5 mRNA expression of primary human osteoblasts (39), we showed that glucocorticoid did not significantly affect LRP5 expression of primary rat bone marrow stromal cells. Excess glucocorticoid attenuation of bone formation activities in the current study can be attributed to elevation of SFRP1 but not LRP5. We speculate that the regulatory effect of glucocorticoid on bone formation may depend on the model system used, glucocorticoid concentration, and developmental potential of osteogenic cells.

Glucocorticoid control of SFRP1 expression in mesenchymal cells has not, to our knowledge, been previously reported. We found that glucocorticoid-augmented SFRP1 mRNA expression to be independent of protein synthesis and dependent on transcription. Suppression of glucocorticoid receptor by mifepristone reduced the promoting effect of glucocorticoid on SFRP1 expression, suggesting that glucocorticoid through genomic pathways augments SFRP1 levels.

Very few previous studies have focused on the effect of glucocorticoid treatment on SFRP1 expression in bone microenvironment *in vivo*. Our current study provides the first immunohistochemical evidence that osteoblasts and bone marrow cells in the areas of trabecular endosteum and chondral cells at growth plate and calcified cartilage expressed intensive SFRP1 immunoreactivity and TUNEL staining, suggesting that osteogenic and chondral cells actively re-

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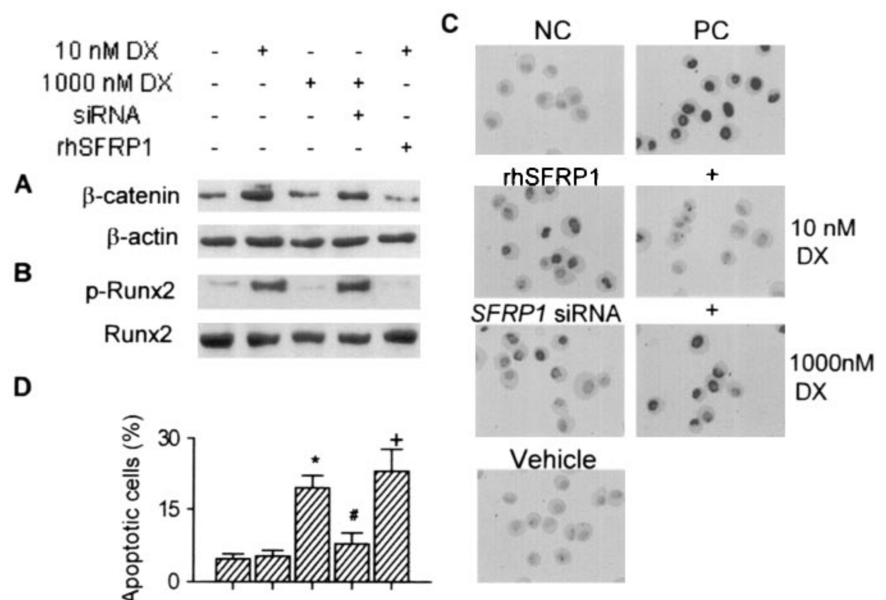

FIG. 9. Knocking down SFRP1 rescued 10^{-6} M dexamethasone attenuation of cytosolic β-catenin accumulation (A), nuclear Runx2 activation in 1 d (B), and cell apoptosis in 3 d (C and D). rhSFRP1 (25 ng/ml) increased cell apoptosis and reduced osteogenesis. All blots are representative of at least three different experiments, with comparable results. Cell apoptosis was demonstrated by TUNEL staining. Each primary culture from each of five rats was assessed independently and then combined for statistical analysis using Wilcoxon test (*, #, and +, difference from vehicle, 10 nM, and 1000 nM DX group, $P < 0.05$). NC, Negative control, cells stained without TdT; PC, positive control, cells pretreated with DNase I; DX, dexamethasone.

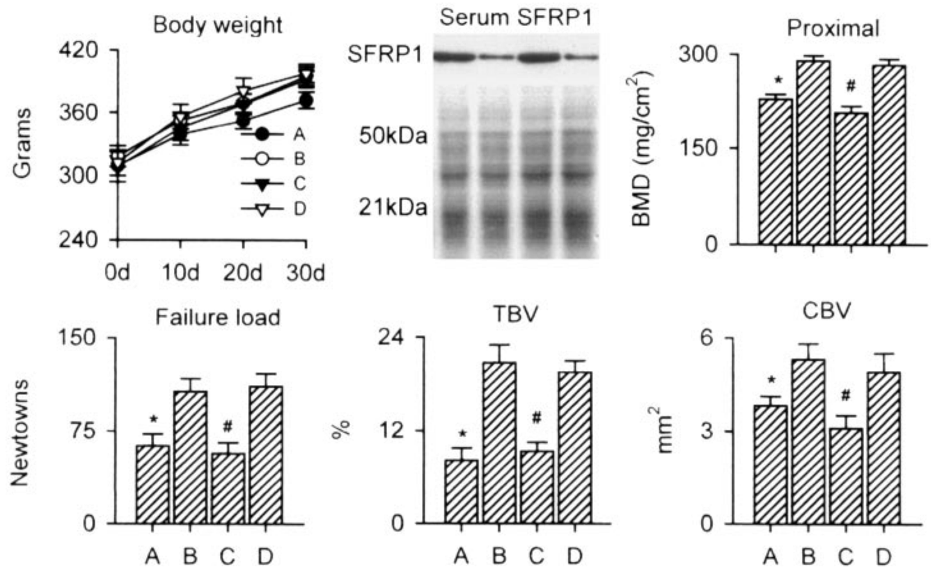

FIG. 10. Effect of dexamethasone and rhSFRP1 treatments on body weight, serum SFPR1 level, biomechanical properties, and bone mass in rats. Compared with the vehicle, rats treated with dexamethasone had less body weight. Dexamethasone and rhSFRP1 treatments increased serum SFPR1 level. Lyophilized serum was subjected to immunoblotting. Amido black staining of blot showed equal loading and transfer for all lanes. Dexamethasone and rhSFRP1 treatments significantly reduced bone mineral density (BMD) of proximal femurs, failure load, and trabecular and (TBV) cortical bone volume (CBV) of femurs. * and #, Differences from dexamethasone- and SFRP1-treated group ($P < 0.05$), respectively.

spond to glucocorticoid treatment. Previous studies have demonstrated that SFRP plays a potential role in mediating chondrocyte apoptosis (13). Misexpression of SFRP causes shortening of skeletal elements and delayed chondrocyte maturation, with consequent inhibition of marrow/bone formation during limb skeletogenesis (7). Deletion of SFRP1 is observed to inhibit osteoblastic cell apoptosis (12). Our data indicate that SFRP expression of each cell lineage in the bone microenvironment may have a distinct role in regulating bone formation. SFRP1 plays a negative regulatory role in maintaining a functional and stable phenotype that organizes the bone and cartilage tissue and supports bone microstructures. Altering the SFPR1 expression in osteoblasts and chondrocytes is proposed as a further explanation for the enhancing effects of glucocorticoid on osteoporosis and avascular necrosis.

Whereas previous studies have demonstrated that SFRP1 and SFPR3 regulates osteogenesis and osteoclastogenesis of cell cultures and that SFRP4 induces hypophosphatemia in rats (40–42), the influence of SFRP1 treatments on bone mass *in vivo* is currently unknown. In the present study, rhSFRP1 evidently increased bone cell apoptosis, coinciding with reduced bone mass and biomechanical properties of femur. This phenomenon is in line with our findings of rhSFRP1-alleviated osteogenic differentiation in cultures. We cannot exclude the possibility that other Wnt inhibitors in the bone microenvironment may be altered after glucocorticoid treatment and nonskeletal mechanisms may be involved in rhSFRP1 regulation of bone mass. We have shown that rhSFRP1 treatment did not affect serum calcium, phosphorous, and PTH levels, suggesting that SFRP1 directly increases bone cell apoptosis and bone microarchitecture deterioration. Our observations reveal that a high concentration of glucocorticoid increases SFRP1-mediated cell apoptosis and attenuates bone formation activities resulting in bone loss. This study provides further evidence that regulation of Wnt/SFRP signal transduction can be used in the future as an alternative strategy for the prevention of glucocorticoid-induced osteoporosis.

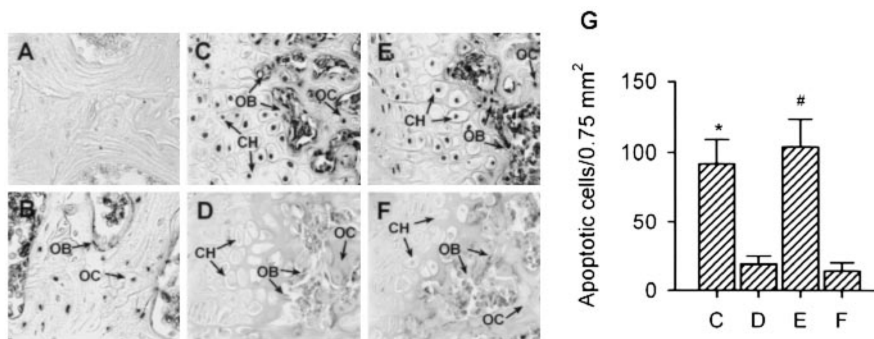

FIG. 11. Dexamethasone and rhSFRP1 treatment-induced cell apoptosis in bone microenvironments. A, Negative controls; specimens were stained without TdT. B, Positive controls; specimens were pretreated with DNAse I and then TUNEL staining. Cells with positive TUNEL staining displayed *blue color* in nucleus. Osteoblasts (OB), chondrocytes (CH), and osteocytes (OC) located at trabecular endosteum, cartilage, and cortical bone expressed intensive TUNEL staining after dexamethasone (C) or rhSFRP1 (E) treatment when compared with the vehicle (D) or BSA group (F). G, Dexamethasone and rhSFRP1 treatments increased number of apoptotic osteochondral cells in bone microenvironment. * and #, Differences from dexamethasone- and SFRP1-treated group ($P < 0.05$), respectively.

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Acknowledgments

The authors express thanks to Dr. Hsiang-Ho Chen (I-Shou University, Kaohsiung, Taiwan) for providing the material test system.

Received August 11, 2004. Accepted January 19, 2005.

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