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ARTICLES

The Wnt Antagonist Secreted Frizzled-Related Protein-1 Controls Osteoblast and Osteocyte Apoptosis

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Abstract Mechanisms controlling human bone formation remain to be fully elucidated. We have used differential display-polymerase chain reaction analysis to characterize osteogenic pathways in conditionally immortalized human osteoblasts (HOBs) representing distinct stages of differentiation. We identified 82 differentially expressed messages and found that the Wnt antagonist secreted frizzled-related protein (sFRP)-1 was the most highly regulated of these. Transient transfection of HOBs with sFRP-1 suppressed canonical Wnt signaling by 70% confirming its antagonistic function in these cells. Basal sFRP-1 mRNA levels increased 24-fold during HOB differentiation from pre-osteoblasts to pre-osteocytes, and then declined in mature osteocytes. This expression pattern correlated with levels of cellular viability such that the pre-osteocytes, which had the highest levels of sFRP-1 mRNA, also had the highest rate of cell death. Basal sFRP-1 mRNA levels also increased 29-fold when primary human mesenchymal stem cells were differentiated to osteoblasts supporting the developmental regulation of the gene. Expression of sFRP-1 mRNA was induced 38-fold following prostaglandin E_2 (PGE_2) treatment of pre-osteoblasts and mature osteoblasts that had low basal message levels. In contrast, sFRP-1 expression was down-regulated by as much as 80% following transforming growth factor (TGF)-β1 treatment of pre-osteocytes that had high basal mRNA levels. Consistent with this, treatment of pre-osteoblasts and mature osteoblasts with PGE_2 increased apoptosis threefold, while treatment of pre-osteocytes with TGF-β1 decreased cell death by 50%. Likewise, over-expression of sFRP-1 in HOBs accelerated the rate of cell death threefold. These results establish sFRP-1 as an important negative regulator of human osteoblast and osteocyte survival. J. Cell. Biochem. 96: 1212–1230, 2005.
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Osteoblasts (OBs) are bone-forming cells that arise from mesenchymal precursors located in bone marrow [Bodine and Komm, 2002; Lian et al., 2003]. These cells undergo a somewhat defined progression to mature-OBs that includes commitment to and proliferation of osteoprogenitors, differentiation to and maturation of pre-OBs, and finally bone matrix production. After mature-OBs have finished synthesizing the bone matrix, they have one of three fates: (i) they can become organized as quiescent lining cells that guard the bone matrix; (ii) they can differentiate into osteocytes (OCYs), which serve as mechanosensors upon entrapment within the mineralized matrix; or (iii) they can die [Bodine and Komm, 2002; Lian et al., 2003]. It has been estimated that as many as 50%–80% of cells recruited to the bone-forming surface will undergo programmed cell death (PCD) [Manolagas, 2000; Boyce et al., 2002]. Although it is thought that drugs such as estrogens and parathyroid hormone (PTH) may act in part by blocking this process [Manolagas, 2000; Boyce et al., 2002], the mechanisms by which OBs are directed towards apoptosis are not entirely clear. However, recent studies have shown the importance of canonical Wnt signaling in the control of OB and OCY PCD [Babij et al., 2003].

Some of the well-characterized molecular markers of the OB lineage are the bone-specific transcription factor Runx2, the enzyme

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alkaline phosphatase (ALP), and the bone matrix proteins type I collagen and osteocalcin [Bodine and Komm, 2002; Komori, 2002; Lian and Stein, 2003; Lian et al., 2003]. In addition, transcription factors like Twist [Lee et al., 1999], Msx-2 [Bidder et al., 1998], and Osterix [Nakashima et al., 2002] are also known to play important roles in regulating osteogenesis [Marie, 2001; Yang and Karsenty, 2002]. Many studies have documented the differential expression of these and other genes during OB differentiation using a variety of in vitro models [Harris and Harris, 2002; Stains and Civitelli, 2003]. Although these studies have been very informative, they do not provide insight into the extracellular signals that lead to transcription of target genes in bone. Furthermore, many studies have focused on rodent cells and less is known regarding the molecular events of human OB differentiation.

We have developed a collection of conditionally immortalized adult human OBs or HOB cell lines that contains representatives of several stages of differentiation including a pre-OB cell line, many mature-OB cell lines, and the first human OCY cell lines [Bodine and Komm, 2002]. These cells were immortalized with a temperature-sensitive simian virus (SV) 40 large T-antigen, and they exhibit a transformed phenotype at the permissive temperature (34°C) when the T-antigen mutant is active. However, in contrast to osteosarcoma cells [Stein and Lian, 1993], the HOB cell lines are faithful to the proliferation/differentiation relationship at the non-permissive temperature (≥37°C) when the T-antigen mutant is inactivated [Bodine and Komm, 2002]. Recently, we have used this collection of cell lines to characterize the osteogenic process at the molecular level with the goal of creating a more in depth understanding of how the human OB differentiates and synthesizes bone [Billiard et al., 2003]. In these studies, microarray technology was used to elucidate the differential transcription profiles of distinct stages of human OB development, and we identified 47 genes whose expression was found to change threefold or more between the pre-OB and pre-OCY stages. Although microarray utilizes small amounts of RNA, is rapid and quantitative, it is limited by RNA abundance in samples and prior gene sequence knowledge. In contrast, techniques like differential-display polymerase chain reaction (DD-PCR) analysis can identify low abundant and previously unknown mRNAs [Chang et al., 2005]. In addition, DD-PCR produces a cDNA fragment that can serve as the starting point for gene isolation.

In the current study, we have also utilized the HOB cells as models to study the molecular events associated with human OB differentiation. To identify genes associated with this process, we used known bone-forming agents and a high-throughput robotic form of DD-PCR analysis known as RADE, or rapid analysis of differential expression [Shiue, 1997]. We identified 82 differentially expressed genes, one of which was the Wnt antagonist, secreted frizzled-related protein (sFRP)-1 [Finch et al., 1997; Rattner et al., 1997] or secreted apoptosis-related protein (SARP)-2 [Melkonyan et al., 1997]. We previously reported that deletion of sFRP-1 in mice leads to increased trabecular bone formation [Bodine et al., 2004]. Here, we describe the characterization of sFRP-1 in vitro and demonstrate that it is an important regulator of human OB and OCY apoptosis.

MATERIALS AND METHODS

Materials

Except where noted, tissue culture reagents were purchased from Invitrogen Corporation (Carlsbad, CA), and other reagents and chemicals were purchased from either Invitrogen or Sigma-Aldrich Chemical Company (St. Louis, MO). Primary human mesenchymal stem cells (hMSCs) were purchased from BioWhittaker, Inc. (San Diego, CA). Ascorbate-2-phosphate was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Bovine serum albumin (BSA) was purchased from Serologicals Proteins, Inc. (Kankakee, IL). Synthetic human PTH 1-34 was purchased from BACHEM Biosciences, Inc. (King of Prussia, PA). Recombinant human transforming growth factor (TGF)-β1, interleukin (IL)-1β, and insulin-like growth factor (IGF)-1 were purchased from R&D Systems (Minneapolis, MN). Forskolin, phorbol-12-myristate-13-acetate (TPA), 1α, 25-dihydroxyvitamin D_3 (vitamin D_3), and caspase inhibitor I were purchased from Calbiochem (San Diego, CA). Dexamethasone (Dex), 17β-estradiol, recombinant human bone morphogenetic protein (BMP)-2, and a glycogen synthase kinase (GSK)-3 inhibitor were obtained from Wyeth Research. Sense (5′-GGCATGGGCATCGGGCGC-3′) and

anti-sense (5′-GCGCCCGATGCCCATGCC-3′) initiation-site directed phosphorothioate oligonucleotides to human sFRP-1 were synthesized and purified by the Wyeth Research Women's Health Research Institute DNA Technology Core facility. Anti-peptide rabbit anti-serum generated to amino acids 217–231 of human sFRP-1 was purchased from Covance Research Products, Inc. (Richmond, CA). Expression vectors for murine Wnt-3 and human TCF-1, as well as pUSEamp and the TOPflash and FOPflash reporter plasmids, were purchased from Upstate Cell Signaling Solutions (Waltham, MA). CMV promoter-driven β-galactosidase reporter gene (pCMVβ) was purchased from BD Biosciences Clonetech (Palo Alto, CA).

Cell Culture

The HOB cell lines were established and characterized as previously described [Bodine et al., 1996a,b, 1997; Bodine and Komm, 2002; Billiard et al., 2003]. The cells were cultured with vented flasks at 34°C in 5% CO_2/95% humidified air incubators (Forma Scientific, Marietta, OH) using either phenol red-containing (HOB-03-C5 and HOB-01-C1) or phenol red-free (HOB-03-CE6 and HOB-05-T1 cells) D-MEM/F-12 medium with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) penicillin/streptomycin, and 2 mM GlutaMax-1 (HOB growth medium).

Explant cultures of normal human OBs (hOBs) were established from cancellous bone chips and cultured with vented flasks in 5% CO_2/95% humidified air incubators at 37°C using HOB growth medium as previously described [Robey and Termine, 1985; Bodine et al., 1995, 1996b].

Primary cultures of fetal rat calvarial-derived OBs (ROBs) were established and cultured as previously described [Bodine et al., 1998]. The animals were housed at the University of Massachusetts Medical School and used in accordance with the Animal Welfare Act and the NIH Guide for the Care and Use of Laboratory Animals.

HMSCs were cultured with vented flasks at 37°C in 5% CO_2/95% humidified air incubators using phenol red-free D-MEM medium containing 10% (v/v) heat-inactivated FBS and 1% (v/v) penicillin/streptomycin (hMSC growth medium).

The HOB-01-09 cell line was established as a sub-clone of the HOB-01-C1 pre-OCYs [Bodine et al., 1996a]. To obtain the HOB-01-09 sFRP-1 clone #1 stably over-expressing cells, HOB-01-09 cells were transfected with full-length human sFRP-1 (cloned into pcDNA3.1, Invitrogen) or with empty vector (pcDNA3.1) by electroporation and seeded into HOB growth medium [Bodine et al., 1996a] supplemented with 125 μg/ml Geneticin (Invitrogen). The cells were then cultured at 34°C in 5% CO_2/95% air humidified incubators to allow for the formation of isolated colonies.

Rapid Analysis of Differential Expression (RADE)

sFRP-1 was identified using RADE technology as described by Shiue [1997]. HOB cell lines were seeded into 150 mm dishes at ~40,000 cells/cm^2 with HOB growth medium and incubated overnight at 34°C. The next day, the medium was removed, the cells were rinsed with phosphate-buffered saline (PBS), serum-free medium was added to the dishes [phenol red-free D-MEM/F-12 containing 0.25% (w/v) BSA, 1% (v/v) penicillin–streptomycin, 2 mM GlutaMAX-1, 50 μM ascorbate-2-phosphate, and 10 nM menadione sodium bisulfite (vitamin K3)], and the dishes were incubated at 39°C for 24 h. The following day, the medium was removed and the cells were treated at 39°C for an additional 24 h with fresh serum-free medium containing 0.1% (v/v) dimethylsulfoxide (DMSO) vehicle (control), 8 nM human PTH 1-34, 100 nM prostaglandin E_2 (PGE_2), or 0.1 nM human TGF-β1. After the treatment period, the dishes were rinsed with PBS, and total cellular RNA was isolated from the cells using TRIzol (Invitrogen).

The RNA samples were treated with DNAse I (Qiagen, Valencia, CA) and subjected to reverse transcription (RT) reaction for 1 h at 37°C. Each RT reaction contained 6 μg of total RNA, 6,000 U Superscript II RT (Invitrogen), 20 μM dNTPs and 0.2 μM anchor primer (containing the sequence $T_{11}A$, $T_{11}C$, or $T_{11}G$) in a final volume of 630 μl. For differential display, each cDNA sample was subjected, in duplicate, to 80 PCR reactions using the appropriate anchor primer and one of 80 arbitrary primers (HAPs 1-80; Genhunter, Nashville, TN). Two microliters aliquots of this reaction were then used for the second strand synthesis by PCR. Each PCR reaction, performed on Genesis II robot using RADE procedures licensed from Millenium Pharmaceuticals Inc. (Cambridge, MA),

contained a 2 μl aliquot of the RT reaction, 2 μM dNTPs, 15 nM [α-^{33}P]dATP (2,000 Ci/mmol, PerkinElmer, Wellesley, MA), 1 U AmpliTaq DNA polymerase (PerkinElmer) and 1 μM primers in a final volume of 20 μl. The PCR conditions were as follows: 1 cycle of 92°C for 2 min, 40 cycles of 92°C for 15 s, 40°C for 2 min, 72°C for 30 s, and 1 cycle of 72°C for 5 min. The PCR products were separated by electrophoresis on a 6% denaturing polyacrylamide gel at 2,000 V for 3 h. The differentially expressed cDNAs were identified by transferring the gels to filter paper and exposing them to X-ray film (Kodak, Rochester, NY). These cDNAs were excised from dried gel and re-amplified using primers and PCR conditions of the original reaction. The products were then ligated into pCR2.1 (Invitrogen), cloned and sequenced. A BLAST (basic local alignment search tool) search of the public databases was then performed. Sequence alignment indicated that one of the cDNAs, a 276 base pair (bp) fragment, shared 77% sequence identity to the 3′-end of mouse sFRP-1 gene (GeneBank™ Accession #U88566 [Rattner et al., 1997]). It also exhibited significant identity (87%) to the 3′-end of a related bovine cDNA called frizzled-related protein A (FrzA, GeneBank™ Accession #U85945 [Xu et al., 1998; Duplaa et al., 1999]). Additionally, this cDNA was highly homologous to at least three expressed sequence tags (ESTs), human clone TM010 (GeneBank™ Accession #U54715), human CA11 tumor suppresser (GeneBank™ Accession #U69122) and a human infant brain EST (GeneBank™ Accession #H16753, H16861). The human sFRP-1 276 bp cDNA was identified by RADE using the following primer pair: 5′-AAGCTTTTTTTTTTA-3′ (HT11A) 3′ end (reverse primer) and 5′-AAGCTTGATTGCC-3′ (H-AP1) 5′ end (forward primer).

Cloning of Full-Length Human sFRP-1 cDNA

Since the sFRP-1 276 bp cDNA identified from the HOB cells by RADE was highly homologous to several human ESTs, an analysis of the EST database was performed in order to assemble the full-length cDNA. This analysis indicated that the 276 bp cDNA was in fact from the known human gene, FRP-1 (GeneBank™ Accession #AF001900; [Finch et al., 1997]) or SARP-2 (GeneBank™ Accession #AF017986-AF017989; [Melkonyan et al., 1997]). Based on this analysis, an RT-PCR-based strategy was designed to obtain the full-length (coding region) 1.1 kb sFRP-1 cDNA from both human placenta RNA and PGE_2-treated HOB-03-CE6 cell RNA. RT-PCR was performed using 1 μg of total RNA, primers that spanned the coding region of hFRP-1/SARP-2 (forward primer: 5′-GCTGGGGACTGCGCCTTTTGT-3′; reverse primer: 5′-CCTGCCCCCGGGAGAATCACTTA-3′), 35 cycles of PCR, and the Advantage-GC PCR kit (BD Biosciences Clonetech). In order to detect expression of the mRNA, a Southern hybridization analysis was performed with the RT-PCR products using a ^{32}P-oligonucleotide probe, which specifically hybridized to bases 501–530 of the FRP-1/SARP-2 coding region [Bodine et al., 1997]. Likewise, RT-PCR of total RNA isolated from HOB-03-C5 cells treated with PGE_2 identified a 2.2 kb cDNA that spanned from the 5′-region of the FRP-1/SARP-2 cDNA to the 276 bp HOB sFRP-1 RADE fragment at the 3′-end. These cDNA fragments were cloned into either the pcDNA3.1 (Invitrogen) mammalian expression vector (1.1 kb cDNA) or the TA (Invitrogen) cloning vector (2.2 kb cDNA) and sequenced. Sequence analysis of the HOB sFRP-1 1.1 and 2.2 kb cDNAs enabled the assembly of a 2.6 kb cDNA that included the transcription start site at the 5′-end and the RADE fragment at the 3′-end. A BLAST search of the public databases using the 1.1 kb cDNA indicated that it was essentially identical to FRP-1/SARP-2. The translated sequence for the HOB sFRP-1 cDNA contains one amino acid difference from the published sequence for SARP-2: alanine 174 instead of proline at this position [Melkonyan et al., 1997].

Cloning of Full-Length Rat and Mouse sFRP cDNAs

Using PCR primers derived from the human and mouse sequences, a full-length rat sFRP-1 cDNA was amplified from heart RNA and cloned into pcDNA3.1 (Invitrogen). Sequence analysis indicated that the rat cDNA shared 92% identity to the human sequence at the DNA level and 95% identity at the amino acid level. Full-length mouse sFRP-1 cDNA was also amplified from heart RNA and cloned into pcDNA3.1; sequence analysis indicated that the cDNA was identical to the published sequence [Rattner et al., 1997].

RNA and Protein Analysis

HOB cell lines and normal hOB cells were seeded into 150 mm dishes at ~40,000 cells/cm^2 with HOB growth medium and incubated overnight at 34°C (HOB cell lines) or 37°C (primary hOB cells). The next day, the medium was removed, the cells were rinsed with PBS, serum-free medium was added to the dishes, and they were incubated at 39°C (HOB cell lines) or 37°C (primary hOB cells) for 24 h. The following day, the medium was removed and the cells were treated at 39°C or 37°C for an additional 24 h with fresh serum-free medium containing 0.1% DMSO (vehicle), PGE_2, TGF-β1, forskolin, TPA, IL-1β, vitamin D_3, Dex, IGF-1, or BMP-2. Osteoblastic differentiation of ROBs was performed in medium supplemented with 50 μg/ml of ascorbic acid and 10 mM β-glycerol phosphate as previously described [Bodine et al., 1998], and the cells were treated with PGE_2 for 24 h. After the treatment period, the dishes were rinsed with PBS, and total cellular RNA was isolated using TRIzol reagent (Invitrogen). The poly (A)+ RNA fraction was also obtained using an Oligotex mRNA Maxi kit (Qiagen). Northern hybridization analysis was performed as previously described [Bodine et al., 1996b] using either the excised 276 bp HOB sFRP-1 RADE cDNA, the cloned HOB sFRP-1 276 bp cDNA, the cloned full-length HOB sFRP-1 1.1 kb cDNA or full-length rat sFRP-1 cDNA ^{32}P-labeled probes. The hybridizations were quantified using a Molecular Dynamics Phosphoimager SI (Sunnyvale, CA) and expression of sFRP-1 mRNA was normalized to either glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA or β-actin mRNA using the corresponding ^{32}P-DNA probes.

Quantification of human sFRP-1 mRNA was also performed with total RNA using real-time quantitative RT-PCR on an ABT PRISM 7700-sequence detection system as described by the manufacturer (Applied Biosystems, Foster City, CA). The following Taqman primers and probe sets were used: forward 5′-CGGTGTGTCCTCCCTGTGA-3′, reverse 5′-CTTTTATTTTCATCCTCAGTGCAAAC-3′, probe 6-FAM-5′-TGTTCAATGATGGCCTCAGATTTCAACTCG-TAMRA-3′). The RT step was performed at 48°C for 30 min, and the cDNA was amplified for 40 cycles at the following conditions: 95°C for 15 s and 60°C for 1 min. The sFRP-1 results were normalized to 18S ribosomal RNA levels using VIC-probe reagents from PE Applied Biosystems (part # 4308329), and mRNA levels were calculated using the Standard Curve Method as described by Applied Biosystems in User Bulletin #2.

Total cellular protein was extracted, and Western blot analysis for β-catenin was performed using a monoclonal antibody (Transduction Laboratories, Lexington, KY) as previously described [Bodine et al., 1996a, 1997; Melkonyan et al., 1997; Bodine and Komm, 2002].

Human Mesenchymal Stem Cell (hMSC) Differentiation and Analysis

hMSCs were seeded at 3,100/cm^2 into 6-well dishes, and 24 h later (day 0) the medium was replaced with fresh hMSC growth medium or hMSC osteogenic medium containing 0.1 μM Dex, 50 μM ascorbic acid, and 10 mM β-glycerophosphate and incubated for 21 days. Every 7 days, the total cellular RNA was isolated using RNeasy mini kit (Qiagen) and subjected to the real-time quantitative RT-PCR analysis for sFRP1 mRNA expression. For ALP activity and osteocalcin secretion, hMSC cells were seeded at 3,100/cm^2 into 96-well plates and incubated with growth medium or osteogenic medium for 21 days as for RNA analysis. ALP activity and osteocalcin secretion were then assessed every 3 days as previously described [Bodine et al., 1996b].

Cell Death Analysis

HOB cells were seeded into 6-well dishes at ~20,000 cells/cm^2 with HOB growth medium and incubated overnight at 34°C. The next day, one set of dishes was rinsed with PBS to remove non-adherent cells, trypsinized, and baseline (day 0) cell number was determined using a Beckman Coulter Multisizer IIe (Beckman Coulter, Inc., Fullerton, CA) as previously described [Bodine et al., 1996a,b, 1997]. The other set of dishes was rinsed with PBS and incubated at 39°C in either HOB growth medium or serum-free medium containing vehicle, PGE_2, TGF-β1 or Dex, and cell number was determined at 1–6 day intervals (the medium was changed on day 3 for 6 day incubations).

Cell number was also quantified by DNA content. For these experiments, HOB-01-09 empty vector or sFRP-1 (clone #1) over-expressing cells were seeded at ~15,000 cells/cm^2 per

well into 96-well plates using HOB growth medium. After 6 h incubation at 34°C, the wells were rinsed with PBS, and the cells were incubated in serum-free medium at 39°C for 3 days. At the end of the incubation, the wells were rinsed again with PBS, and the cells were assayed for DNA content using the CyQuant DNA fluorescence assay (Molecular Probes, Eugene, OR).

To measure apoptosis, HOB-03-C5 cells were seeded at ~20,000 cells/cm^2 into 6-well dishes with HOB growth medium and incubated overnight at 34°C. The next day, the dishes were rinsed with PBS and incubated at 39°C in serum-free medium containing vehicle, PGE_2 or Dex for 6 days (the medium was changed after 3 days). At the end of the incubation, the dishes were rinsed with PBS. The cells were then trypsinized, incubated with FITC-conjugated annexin V (BD Biosciences Clonetech) and propidium iodide, washed with PBS, and subjected to a flow cytometry analysis using a FACSORT flow cytometer (BD Biosciences Clonetech). The cells that bound the annexin V stain, but did not incorporate propidium iodide, were considered early apoptotic, and their number was normalized to the total number of cells in each sample. Late apoptotic cells were those that bound the annexin V stain and incorporated propidium iodide.

Apoptosis was also measured by nucleosome fragmentation. For these experiments, HOB-01-09 sFRP-1 (clone #1) over-expressing cells were seeded at ~19,000 cells/cm^2 into 12-well plates using HOB growth medium. After 24 h incubation at 34°C, the wells were rinsed with phenol red-free and calcium/magnesium-free hanks balanced salt solution (HBSS), and the cells were incubated in serum-free medium containing either 0.1% DMSO (vehicle) or inhibitors of caspases 1, 3, 4, and 7 (caspase inhibitor I) or GSK-3 [Coghlan et al., 2000] at 39°C for 3 days. At the end of the incubation, the wells were rinsed again with HBSS, and the cells were assayed for internucleosomal DNA fragmentation using the Cell Death Detection ELISA^{PLUS} assay (Roche, Penzberg, Germany).

Reporter Gene Assay

HOB-03-CE6 cells were seeded at 75,000 cells/cm^2 into 24-well plates with HOB growth medium and incubated overnight at 34°C. The next day, the medium was changed, and the cells were transfected with plasmids in OPTI-MEM 1 (Invitrogen) with Lipofectamine 2000 transfection reagent as described by the manufacturer (Invitrogen). Combinations of the following cDNA-containing plasmids were used for these experiments (the total DNA was adjusted to 1,525 ng per well with pcDNA3.1): 1,120 ng of TOPflash or FOPflash reporter; 35 ng of human/mouse chimeric TCF-1; 70 ng of human/mouse chimeric Wnt-3 or empty pUSEamp; 280 ng of human, rat or mouse sFRP-1 or pcDNA3.1; and 20 ng of pCMVβ. The cells were incubated at 37°C for 24 h, rinsed with HBSS, and lysed at 25°C for 25 min on a shaker with 50 μl/well of cell culture lysis reagent (Promega, Madison, WI). Cell extracts were then assayed for luciferase activity using Luciferase Assay Reagent (Promega) and for β-galactosidase activity using Galacto-Light Reagent (Applied Biosystems) as described by the manufacturers. Light emission was measured with a MicroLumat LB 96P luminometer (EG & G Berthold, Bandoora, Australia) by integration over 10 s for luciferase and 5 s for β-galactosidase. The light emission values obtained for luciferase were normalized to those for β-galactosidase.

Statistical Analysis

Data were analyzed for statistical significance by one-way ANOVA using the Dunnett's test with the JMP software (SAS Institute, Cary, NC).

RESULTS

Differential Expression of sFRP-1 During Osteoblast Differentiation and Regulation by Osteogenic Agents

In order to characterize the molecular events associated with human OB differentiation and to identify new and important genes and pathways associated with the osteogenic process, we employed a collection of HOB cell lines [Bodine et al., 1996a, 1997; Bodine and Komm, 2002] and a high-throughput robotic version of DD-PCR analysis known as RADE [Shiue, 1997; Chang et al., 2005]. For these experiments, we treated three of the HOB cell lines (HOB-03-C5, HOB-03-CE6, and HOB-01-C1), representing three stages of differentiation (pre-OBs, mature-Obs, and pre-OCYs, respectively) [Bodine and Komm, 2002], for 24 h with three osteogenic agents (PTH 1-34, PGE_2, and TGF-β1) [McCarthy et al., 2000; Vrotsos et al., 2003;

Qin et al., 2004; Quattrocchi and Kourlas, 2004; Rosen, 2004]. We then isolated total cellular RNA and preformed RADE. From these experiments, we identified 82 differentially expressed genes. Of these, only six of the genes were regulated by more than one of the ostegenic agents in more than one stage of OB differentiation. The top candidate of these was sFRP-1 (Fig. 1), which was identified as a 276 bp cDNA fragment whose expression was completely induced by PGE_2 in the pre-OBs (HOB-03-C5 cells), strongly up-regulated by PGE_2 in the mature-OBs (HOB-03-CE6 cells), but was down-regulated by TGF-β1 in the pre-OCYs (HOB-01-C1 cells). Furthermore, basal expression of this gene dramatically increased with advancing stage of differentiation such that the pre-OCYs expressed the highest levels of this message. In contrast to the results achieved with PGE_2 and TGF-β1, treatment of the HOB cells with PTH did not alter sFRP-1 gene expression.

Cloning and sequence analysis of this gene indicated that it was human sFRP-1 [Rattner et al., 1997], which is also known as FRP-1 [Finch et al., 1997], FrzA [Xu et al., 1998; Duplaa et al., 1999], or SARP-2 [Melkonyan et al., 1997]. sFRP-1 is a secreted Wnt antagonist that has a cysteine rich domain or CRD that binds Wnt proteins [Jones and Jomary, 2002; Kawano and Kypta, 2003]. Wnts are a large family of 19 growth factors that mediate fundamental biological processes like embryogenesis, organogenesis, and tumorigenesis [Wodarz and Nusse, 1998; Miller, 2002; Logan and Nusse, 2004; Moon et al., 2004], and these proteins have previously been reported to be involved in skeletal development [Church and Francis-West, 2002] and more recently in postnatal bone formation [Johnson et al., 2004; Westendorf et al., 2004].

In order to confirm the RADE findings, we performed a Northern hybridization analysis with poly (A)+ RNA isolated from either the pre-OB HOB-03-C5 cells (Fig. 2A) or the pre-OCY HOB-01-C1 cells (Fig. 2B). Treatment of the pre-OBs with 100 nM PGE_2 for 24 h strongly induced steady-state levels of the 4.4 kb sFRP-1 mRNA. In contrast, treatment of the pre-OCYs, which expressed high basal levels of the 4.4 kb message, with 0.1 nM TGF-β1 for 24 h down-regulated sFRP-1 mRNA expression by 80%. The observation that basal expression of sFRP-1 was dramatically increased in the pre-OCY cells suggests that it is associated with the differentiation process.

Treatment of the HOB-03-C5 pre-OBs for 24 h with increasing concentrations of PGE_2 induced sFRP-1 steady-state mRNA levels in a dose-dependent manner with an EC_{50} of 8 nM (Fig. 2C). In addition, time-course experiments of cells treated with 100 nM PGE_2 indicated that sFRP-1 message was up-regulated in a time-dependent manner; a significant increase in gene expression was observed after 4 h of treatment, and the steady-state mRNA levels continued to rise up to 24 h after the addition of PGE_2 to the cell culture medium (Fig. 2D). These results suggest that sFRP-1 is a late-response gene for PGE_2 action in the HOB cells,

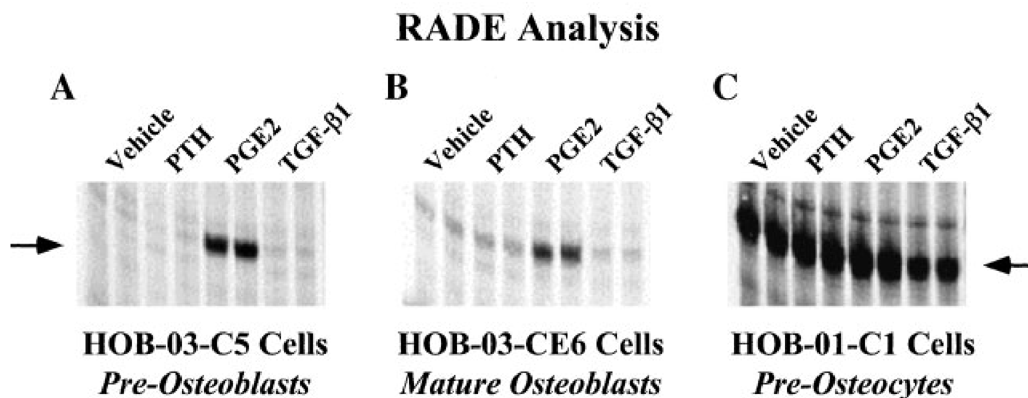

Fig. 1. Differential expression of sFRP-1 mRNA in human osteoblast cell lines. **A–C**: Autoradiograms of DD-PCR gels from RADE experiments that were performed in duplicate with pre-OB HOB-03-C5 cells (A), mature-OB HOB-03-CE6 cells (B), and pre-OCY HOB-01-C1 cells (C), respectively. The cells were treated for 24 h in serum-free medium at the non-permissive temperature of 39°C with 0.1% DMSO (vehicle control), 8 nM PTH 1-34, 100 nM PGE_2, or 0.1 nM TGF-β1 prior to RNA isolation. The arrow points to the 276 bp sFRP-1 cDNA fragment.

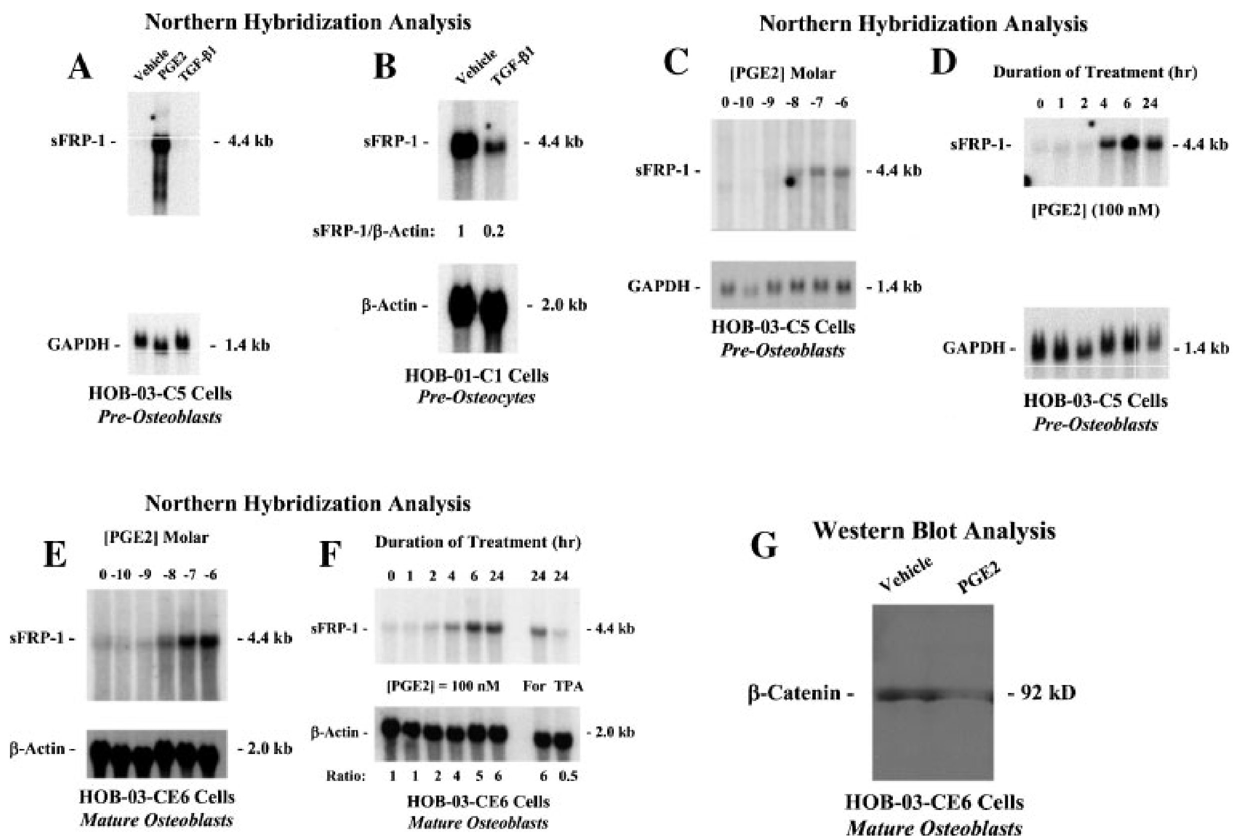

Fig. 2. Confirmation of sFRP-1 gene expression by HOB cell lines. **A–F**: Autoradiograms of Northern hybridization experiments with poly A+ RNA isolated from pre-OB HOB-03-C5 cells (A, C, and D), pre-OCY HOB-01-C1 cells (B), or mature-OB HOB-03-CE6 cells (E, F). **G**: An autoradiogram of a Western blot analysis for β-catenin (92 kDa) with whole cell extracts isolated from HOB-03-CE6 cells. The cells were treated for 24 h in serum-free medium at the non-permissive temperature of 39°C with 0.1% DMSO (vehicle control), 100 nM PGE_2, 0.1 nM TGF-β1, 1 μM forskolin (For), or 100 nM TPA prior to RNA or protein isolation (A, B, F, and G). Likewise, the cells were treated for 24 h with DMSO or 0.1–1,000 nM PGE_2 (C, E), or they were treated for 0–24 h with 100 nM PGE_2 prior to RNA isolation (D, F). The following ^{32}P-probes were used for the Northern blots: a cloned 276 bp sFRP-1 cDNA fragment and a cloned full-length GAPDH cDNA (A, C); a cloned full-length sFRP-1 cDNA and a cloned full-length β-Actin cDNA or the GAPDH cDNA (B, D, E and F). The sFRP-1 message is shown as a 4.4 kb mRNA. GAPDH and β-actin are shown as 1.4 and 2.0 kb messages, respectfully. A Molecular Dynamics Phosphoimager was used to quantify the changes in sFRP-1 gene expression after normalization to GAPDH or β-actin mRNA levels.

and imply that it is under the control of another gene product. PGE_2 treatment of the HOB-03-C5 cells also suppressed ALP activity by 57% in a dose-dependent manner with an IC_{50} of 0.7 nM (data not shown). This observation, together with induction of sFRP-1 expression, is consistent with promotion of cellular differentiation since the HOB-01-C1 pre-OCYs express high levels of sFRP-1 message and low amounts of ALP [Bodine et al., 1996a, 1997; Bodine and Komm, 2002]. Treatment of mature-OB HOB-03-CE6 cells with PGE_2 also up-regulated sFRP-1 steady-state mRNA levels in a dose- and time-dependent manner (Fig. 2E,F), and this effect was mimicked by treatment of the cells with 1 μM forskolin but not by 100 nM TPA indicating that activation of protein kinase A and not protein kinase C is involved in this process (Fig. 2F). PGE_2 treatment of the mature-OB cells also suppressed cytosolic β-catenin levels, consistent with blockage of canonical Wnt signaling by elevated expression of sFRP-1 (Fig. 2G). Finally, treatment of the HOB-01-C1 cells for 24 h with increasing concentrations of TGF-β1 suppressed sFRP-1 message levels by 64% in a dose-dependent manner with an IC_{50} of 0.004 nM as measured by real-time quantitative RT-PCR (data not shown).

We also wanted to confirm the expression and regulation of sFRP-1 in some non-immortalized OB models. Like the HOB cell lines, treatment

of primary cultures of normal hOBs with 100 nM PGE_2 for 24 h up-regulated steady-state levels of sFRP-1 mRNA by 11-fold as determined by real-time quantitative RT-PCR analysis (Fig. 3A). Similarly, treatment of primary cultures of ROBs at three different stages of differentiation with 100 nM PGE_2 for 24 h induced sFRP-1 steady-state message levels 15- to 52-fold as determined by Northern hybridization analysis (Fig. 3B). Moreover, as seen with the HOB cells, basal expression of sFRP-1 mRNA increased with increasing ROB differentiation.

In addition to PGE_2 and TGF-β1, we evaluated the regulation of sFRP-1 steady-state mRNA levels by additional agents that are known to modulate bone remodeling. For these experiments, we also used real-time quantitative RT-PCR analysis of total RNA to quantify the levels of sFRP-1 message in the HOB-03-C5, HOB-03-CE6, and HOB-01-C1 cells following 24 h treatments with various hormones, growth factors, and cytokines. As shown before, basal levels of sFRP-1 mRNA increased with increasing differentiation such that the pre-OCYs expressed over 20-times more message than the pre-OBs (Fig. 4). Treatment with 100 nM PGE_2 again up-regulated sFRP-1 message levels almost 38-fold in the pre-OBs and 14-fold in mature-OBs, but it had no effect on sFRP-1 mRNA in the pre-OCYs where basal expression was high. In addition, treatment of the mature-OBs with 1 nM IL-1β increased sFRP-1 mRNA levels about fivefold, although it had little effect on sFRP-1 expression in pre-OBs. In contrast, treatment of pre-OBs and mature-OBs with either 100 nM Dex or 100 nM vitamin D_3 down-regulated sFRP-1 message levels by as much as 70%. Moreover, treatment of the pre-OCYs with either 40 nM IGF-1, 0.1 nM TGF-β1, or 20 nM BMP-2 suppressed sFRP-1 mRNA expression by as much as 70%. Thus, sFRP-1 gene expression in OBs and OCYs is controlled by many hormones and growth factors that are known to affect bone formation and resorption. In contrast to these results, treatment of the HOB cells with 17β-estradiol did not alter sFRP-1 gene expression (data not shown).

We next addressed changes in sFRP-1 expression in relation to development of primary hMSCs to OBs following incubation for 21 days with ascorbate-2-phosphate, β-glycerol phosphate, and Dex (Fig. 5). Total cellular RNA was isolated at weekly intervals and real-time

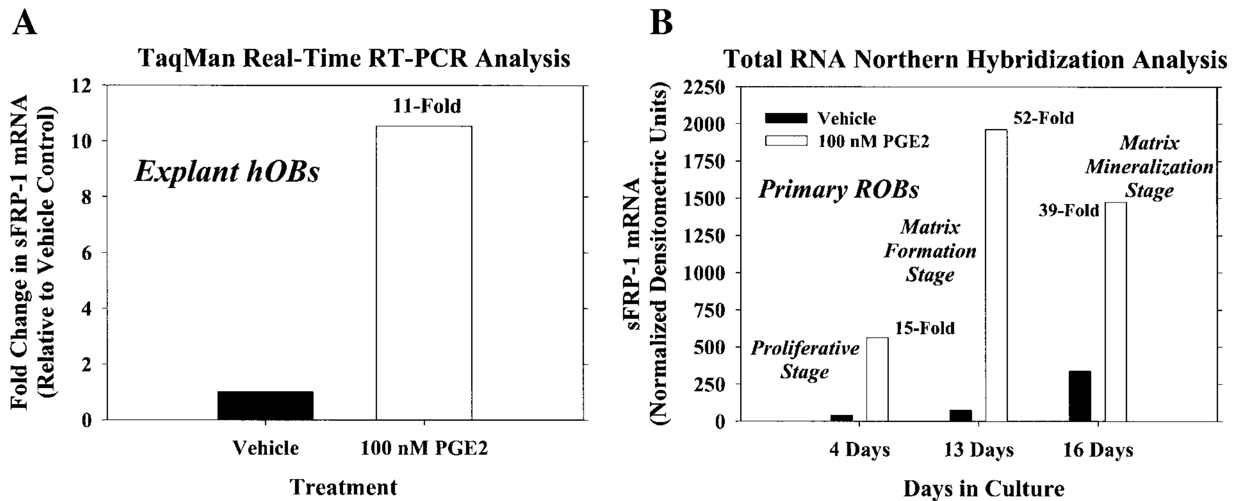

Fig. 3. PGE_2 treatment increases sFRP-1 mRNA expression in primary human and rat osteoblasts. **A**: Explant cultures of normal hOB cells were treated for 24 h in serum-free medium at 37°C with 0.1% DMSO (vehicle control) or 100 nM PGE_2, and RNA was isolated for real-time quantitative RT-PCR analysis of sFRP-1 mRNA levels. The results are reported as the fold change from the vehicle control after normalization to 18S ribosomal RNA levels, and are presented as means of triplicate samples. **B**: Quantification of autoradiograms of Northern hybridization experiments with total RNA isolated from primary fetal rat OBs that were treated for 24 h in serum-free medium at 37°C with 0.1% DMSO (vehicle control) or 100 nM PGE_2 at the indicated days during osteoblastic differentiation. The cloned full-length rat sFRP-1 cDNA and a cloned 18S ribosomal RNA cDNA fragment were used as probes. Scanning laser densitometry was used to quantify the changes in sFRP-1 gene expression after normalization to 18S rRNA levels. Differentiation of the cultures was performed in medium supplemented with 50 μg/ml of ascorbic acid and 10 mM β-glycerol phosphate, and was monitored by Northern hybridizations that quantified changes in histone H4, ALP and osteocalcin mRNA levels (data not shown).

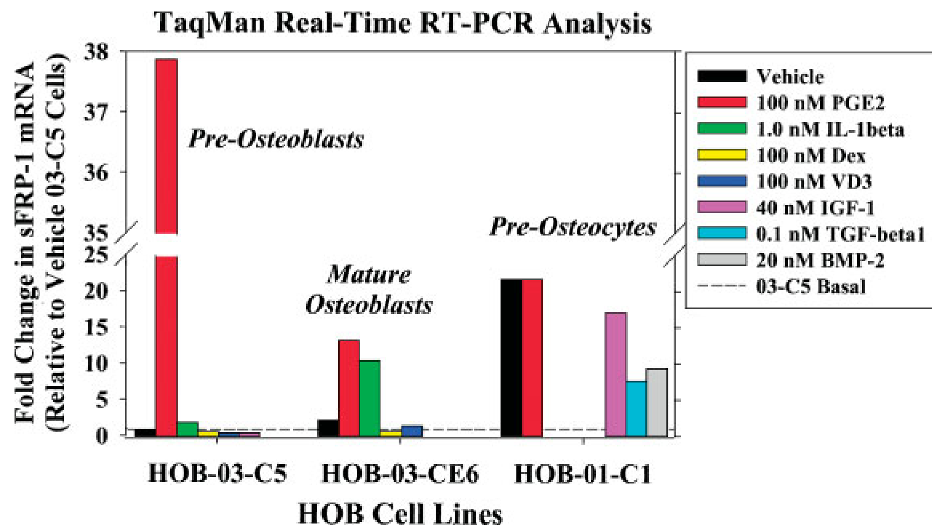

Fig. 4. Differential regulation of sFRP-1 gene expression in HOB cells by hormones, growth factors, and cytokines. Pre-OB HOB-03-C5 cells, mature-OB HOB-03-CE6 cells, and pre-OCY HOB-01-C1 cells were treated for 24 h in serum-free medium at the non-permissive temperature of 39°C with 0.1% DMSO (vehicle control), 100 nM PGE_2, 1 nM IL-1β, 100 nM Dex, 100 nM vitamin D_3 (VD3), 40 nM IGF-1, 0.1 nM TGF-β1, or 20 nM BMP-2 prior to RNA isolation. Real-time quantitative RT-PCR analysis was used to quantify the levels of sFRP-1 mRNA. The results are reported as the fold change from the vehicle-treated HOB-03-C5 cells (03-C5 basal, dotted line) after normalization to 18S ribosomal RNA levels, and are presented as mean of triplicate samples.

quantitative RT-PCR was performed to quantify sFRP-1 steady-state mRNA levels. Consistent with results from the HOB cell lines, sFRP-1 mRNA levels increased 29-fold from day 0 to day 14 and then declined to about 20-fold up-regulation by day 21.

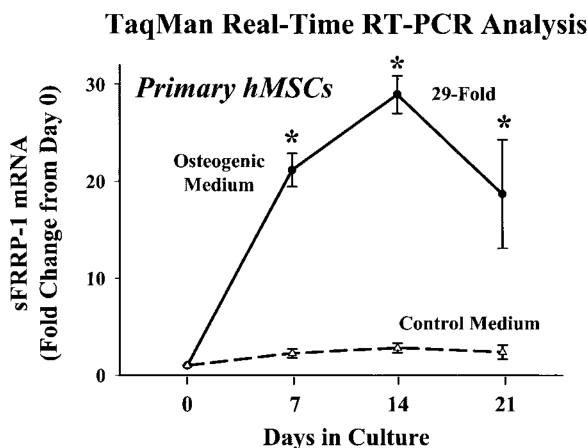

Fig. 5. Expression of sFRP-1 mRNA increases during osteogenic differentiation of primary human MSCs. Human MSCs were incubated for 21 days at 37°C with control (growth medium) or osteogenic medium, and RNA was isolated at the indicated days for real-time quantitative RT-PCR analysis of sFRP-1 mRNA levels. Assay of the cultures for ALP activity indicated that the levels of this enzyme increased 50-fold from day 0 to day 21 (data not shown). The results are reported as the fold change from day 0 after normalization to 18S ribosomal RNA levels, and are presented as mean ± SEM, n = 3, $^*P < 0.05$ versus control cells.

Control of Osteoblast and Osteocyte Apoptosis by sFRP-1

Melkonyan et al. [1997] discovered sFRP-1, -2, and -5 as genes that regulate cellular viability and referred to them as SARPs. For example, these authors reported that over-expression of sFRP-2 (SARP-1) in MCF-7 human breast cancer cells blunted the ability of tumor necrosis factor (TNF)-α to promote PCD, while over-expression of sFRP-1 (SARP-2) had the opposite effect and potentiated apoptosis. In order to determine the role of sFRP-1 in OB survival, we compared the basal message levels of this gene in the HOB cell lines to their viability in culture. Using real-time quantitative RT-PCR analysis of total cellular RNA, steady-state levels of sFRP-1 mRNA were again shown to change with increasing cellular differentiation (Fig. 6A). When compared to the HOB-03-C5 pre-OBs, basal sFRP-1 message levels increased about 4-fold in the HOB-03-CE6 mature-OBs and over 24-fold in the HOB-01-C1 pre-OCYs. Surprisingly, sFRP-1 mRNA levels declined to about 0.5-fold in the HOB-05-T1 mature-OCYs. Thus, of the cells in the OB lineage, the pre-OCY appears to express the highest levels of sFRP-1.

The HOB-03-C5 pre-OBs, which are in the proliferative-stage of differentiation [Bodine

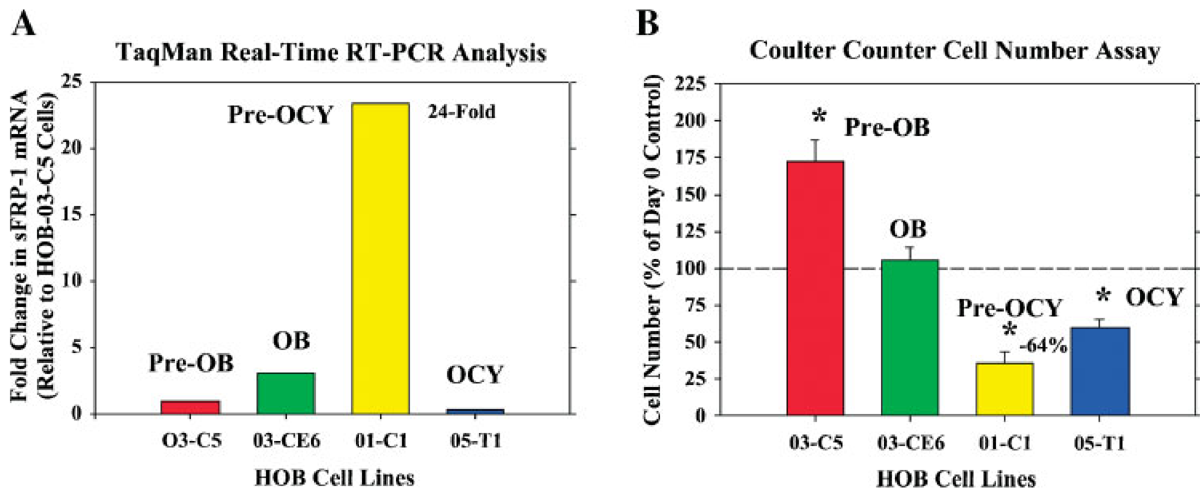

Fig. 6. Correlation of basal sFRP-1 mRNA expression with HOB cell viability. **A**: Pre-OB HOB-03-C5 cells, mature-OB HOB-03-CE6 cells, pre-OCY HOB-01-C1 cells, and mature-OCY HOB-05-T1 cells were incubated in serum-free medium at the non-permissive temperature of 39°C for 2 days prior to RNA isolation. Real-time quantitative RT-PCR analysis was used to quantify the levels of sFRP-1 mRNA. The results are reported as the fold change relative to the HOB-03-C5 cells after normalization to 18S ribosomal RNA levels, and are presented as mean of triplicate samples. **B**: The HOB cell lines were incubated in growth medium at 39°C for 6 days, and cell number was determined using a Beckman Coulter Multisizer. The results were normalized to the day 0 cell number (1 day after seeding when the cells were transferred to 39°C) and are presented as mean ± SD, n = 3–6, $*P < 0.05$ versus the day 0 control cells.

et al., 1996a, 1997; Bodine and Komm, 2002], divided slowly in FBS-containing medium at the non-permissive temperature of 39°C, and cell number increased by about 75% after 6 days in culture (Fig. 6B); this rate of cell division was similar to that of explant cultures of hOBs [Bodine et al., 1996a, 1997; Bodine and Komm, 2002]. The mature-OB HOB-03-CE6 cells did not proliferate at the non-permissive temperature, and cell number remained constant over the 6-day incubation. In contrast, the pre-OCY HOB-01-C1 cells died at 39°C such that fewer than 40% of the cells remained alive after 6 days. The mature-OCY HOB-05-T1 cells also died at the non-permissive temperature, although the level of cell death was not as great as that observed for the pre-OCYs. Therefore, these results suggest that sFRP-1 mRNA levels in human OBs correlate with cellular viability.

To further support the role of sFRP-1 in OB longevity, we treated the HOB cells with PGE_2 or TGF-β1, since these agents either increased or decreased sFRP-1 expression, respectively; we then measured cell number after incubation in serum-free medium at the non-permissive temperature for 3–6 days. Under these culture conditions, the cells stopped dividing and apoptosis was initiated [Melkonyan et al., 1997; Mesner and Kaufmann, 1997]. As expected, incubation of the HOB-03-C5 cells in serum-free medium caused them to die, such that after 6 days, only 30% of the cells remained alive (Fig. 7A). Treatment of the pre-OBs with 100 nM PGE_2, which up-regulated sFRP-1 expression, accelerated the rate of cell death by almost twofold. On the other hand, treatment of the HOB-01-C1 cells with TGF-β1, which down-regulated sFRP-1 expression, prolonged the life of these cells such that twice as many cells were alive after 6 days in culture (Fig. 7B). Finally, treatment of the HOB-03-CE6 mature-OBs with PGE_2, which up-regulated sFRP-1 expression, also accelerated the rate of cell death (Fig. 7C). Moreover, transfection of these cells with an anti-sense oligonucleotide to sFRP-1 reversed the ability of PGE_2 to promote cell death indicating that sFRP-1 was involved in this process (Fig. 7D). Thus, there again appeared to be a correlation between the expression of sFRP-1 mRNA and the rate of HOB cell death.

In order to demonstrate that PGE_2-induced HOB cell death was due to apoptosis, we used annexin V flow cytometry to measure PCD. Treatment of HOB-03-C5 cells with 1–100 nM PGE_2 for 6 days in serum-free medium at the non-permissive temperature increased the number of annexin +/propidium iodide—(early apoptotic cells) two to threefold in a dose-dependent manner with an EC_{50} of 6 nM

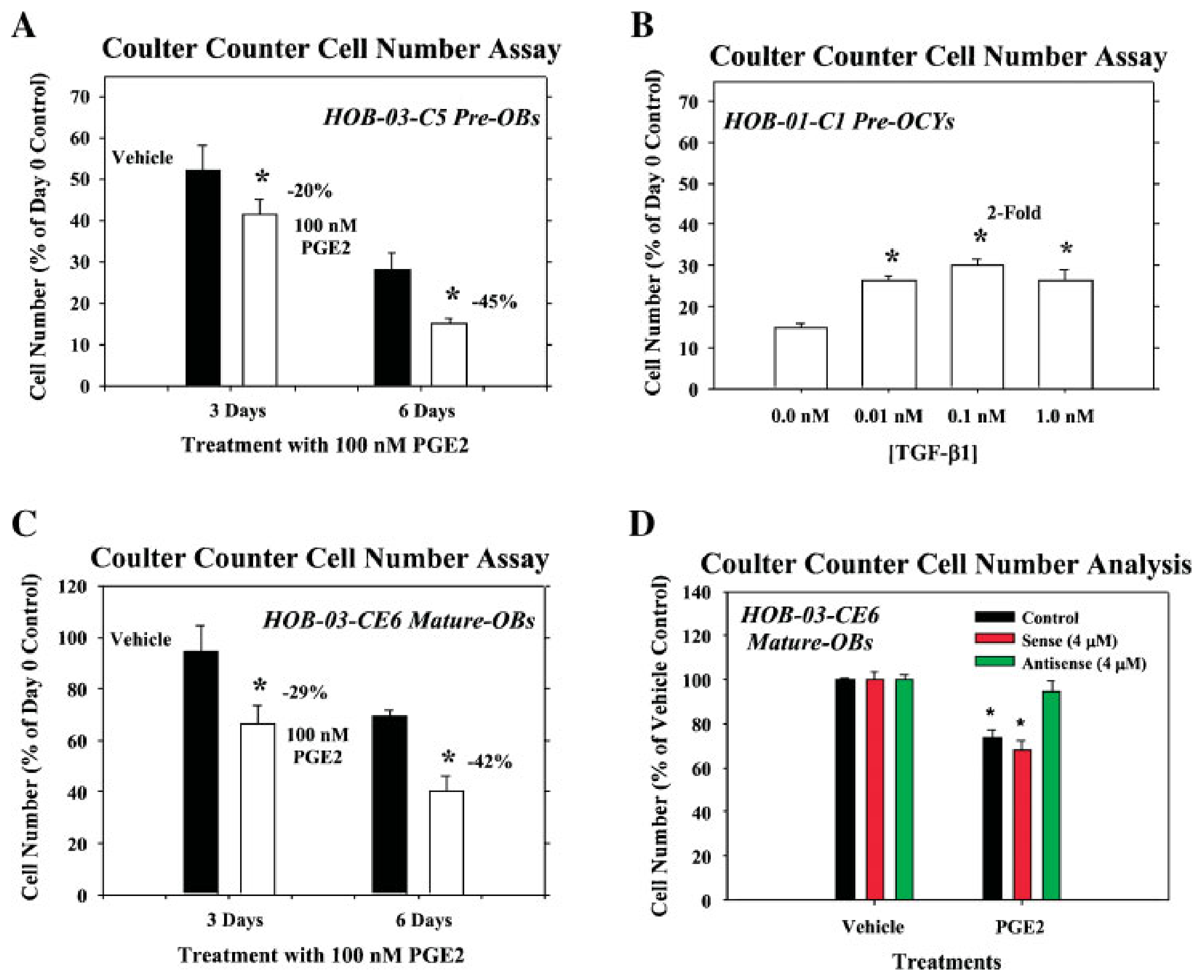

Fig. 7. PGE_2 treatment accelerates HOB cell death, while TGF-β1 treatment prolongs HOB cell life. **A**, **C**: Pre-OB HOB-03-C5 cells (A) and mature-OB HOB-03-CE6 cells (C) were treated for 3 and 6 days in serum-free medium at the non-permissive temperature of 39°C with 0.1% DMSO (vehicle control) or 100 nM PGE_2. **B**: Pre-OCY HOB-01-C1 cells were treated for 6 days in serum-free medium at 39°C with 0–1.0 nM TGF-β1. **D**: Mature-OB HOB-03-CE6 cells were treated for 6 days in serum-free medium at 39°C with 0.1% DMSO (vehicle control) or 100 nM PGE_2 in the absence (buffer control) or presence of 4 μM initiation-site directed phosphorothioate sense or antisense oligonucleotides to sFRP-1. Cell number was determined using a Beckman Coulter Multisizer. The results were normalized to the day 0 cell number (1 day after seeding when the cells were placed in serum-free medium and transferred to 39°C) and are presented as mean ± SD, n = 3–6, *$P < 0.05$ versus the day 0 control cells.

(Fig. 8A). On the other hand, treatment of the cells with 1–1,000 nM Dex, which suppressed sFRP-1 message levels, decreased apoptosis by 77% in a dose-dependent manner with an IC_{50} of 14 nM (Fig. 8B). These results further support the correlation between sFRP-1 gene expression and OB PCD.

To confirm that sFRP-1 accelerates HOB cell death, we stably over-expressed this gene in an HOB cell line that has a low basal level of sFRP-1 message. The HOB-01-09 cells are a sub-clone of the pre-OCY HOB-01-C1 cells that express about 20-times less sFRP-1 mRNA than the parental cells as determined by real-time quantitative RT-PCR (Fig. 9A). In contrast, over-expression of full-length human sFRP-1 using a CMV-promoter resulted in a clone (#1) that contained about 57-times more sFRP-1 mRNA than the empty vector (pcDNA3.1) control cells, or about 3-times more than the parental HOB-01-C1 cells. Northern hybridization analysis with poly (A)+ RNA isolated from the cell lines confirmed the over-expression of sFRP-1 mRNA relative to the empty vector cells and demonstrated that a 1.5 kb sFRP-1 message was expressed by the clone #1 cells (Fig. 9B). When the empty vector and sFRP-1 stable cell lines were incubated in serum-free medium at the non-permissive temperature, the empty vector cells died slowly with a half-life of about 120 h as

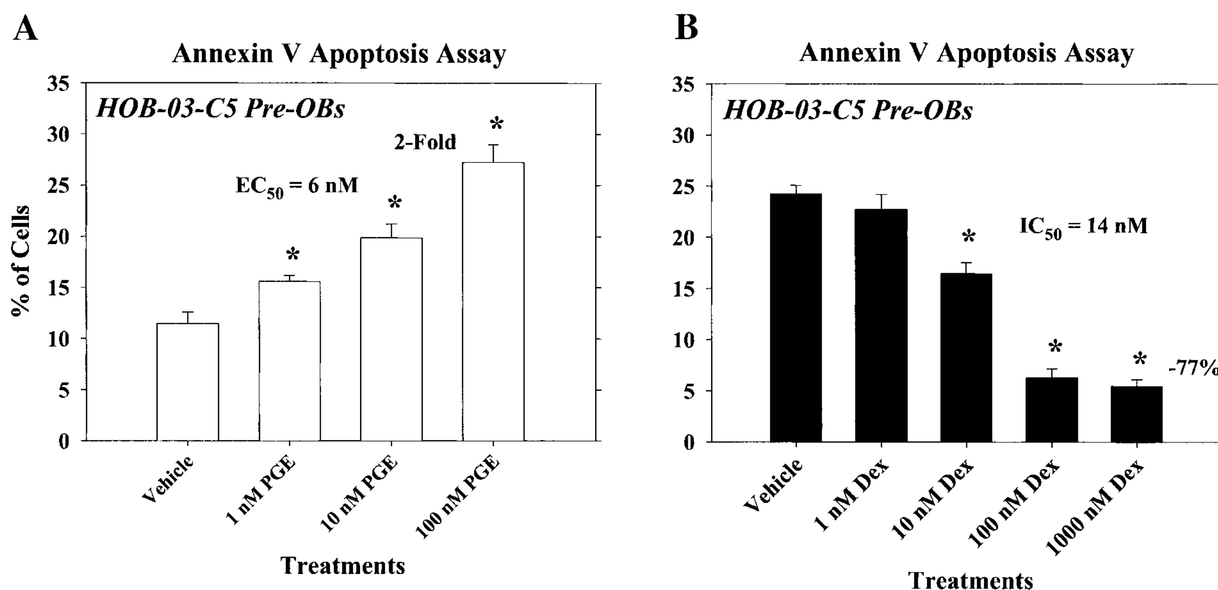

Fig. 8. PGE_2 treatment accelerates HOB cell apoptosis, while Dex treatment reduces programmed cell death. **A**: Pre-OB HOB-03-C5 cells were treated for 6 days in serum-free medium at the non-permissive temperature of 39°C with 0.1% DMSO (vehicle control) or 1–100 nM PGE_2 (PGE). **B**: The cells were also treated in a similar manner with 1–1,000 nM Dex. Apoptosis was quantified by annexin V and propidium idodide staining using flow cytometry. The cells that bound the annexin V stain, but did not incorporate propidium iodide, were considered early apoptotic (shown here), and their number was normalized to the total number of cells in each sample. Similar results were obtained when the late apoptotic cells (annexin+/propidium iodide+) were quantified (data not shown). The results are presented as mean ± SD, n = 6, $*P < 0.001$ versus the vehicle control cells.

determined by the Coulter cell counter (Fig. 9C). In contrast, over-expression of sFRP-1 accelerated the rate of cell death threefold in the clone #1 cells ($t_{1/2} = 39$ h), such that after 3-days in culture, over 90% of the sFRP-1 cells had died, while only 14% of the empty vectors cells had died. Moreover, treatment of the over-expressing cells with an antiserum to sFRP-1 returned the rate of cell death to that of the empty vector cells (Fig. 9D). These results confirmed that sFRP-1 expression accelerates death of HOB cells. Treatment of the sFRP-1 over-expressing cells with inhibitors of caspases 1, 3, 4, and 7 as well as GSK-3 also prevented cell death (Fig. 9E), indicating that this involves apoptosis and modulation of canonical Wnt signaling, respectively.

Antagonism of Canonical Wnt Signaling in Osteoblasts by sFRP-1

Finally, to demonstrate that sFRP-1 antagonizes canonical Wnt signaling in the HOB cells, we performed a transient transfection experiment using the TOPflash TCF-luciferase reporter gene assay. Although the HOB-03-CE6 mature-OBs express both TCF-1 and Wnt-3 mRNAs as determined by RT-PCR (data not shown), over-expression of these cDNAs was required to produce a threefold increase in TOPflash reporter gene activity (Fig. 10). However, co-transfection of TCF-1 and Wnt-3 with either human, rat or mouse sFRP-1 completely suppressed canonical Wnt signaling as measured by this reporter. Thus, these results demonstrate a correlation between antagonism of Wnt signaling by sFRP-1 and promotion of apoptosis. As a control, we also performed transfections with the FOPflash luciferase plasmid that has the TCF DNA binding sites mutated. As expected, cotransfection of TCF-1 and Wnt-3 did not activate the FOPflash reporter in the HOB cells (data not shown).

DISCUSSION

Transcription profiling of numerous OB models has identified many new genes and pathways that are involved in differentiation and osteogenesis [Harris and Harris, 2002; Stains and Civitelli, 2003]. Likewise, similar studies of OBs treated with osteogenic agents like PTH [Qin et al., 2003] and BMP-2 [Locklin et al., 2001; de Jong et al., 2002; Harris and Harris, 2002; Vaes et al., 2002; Balint et al., 2003;

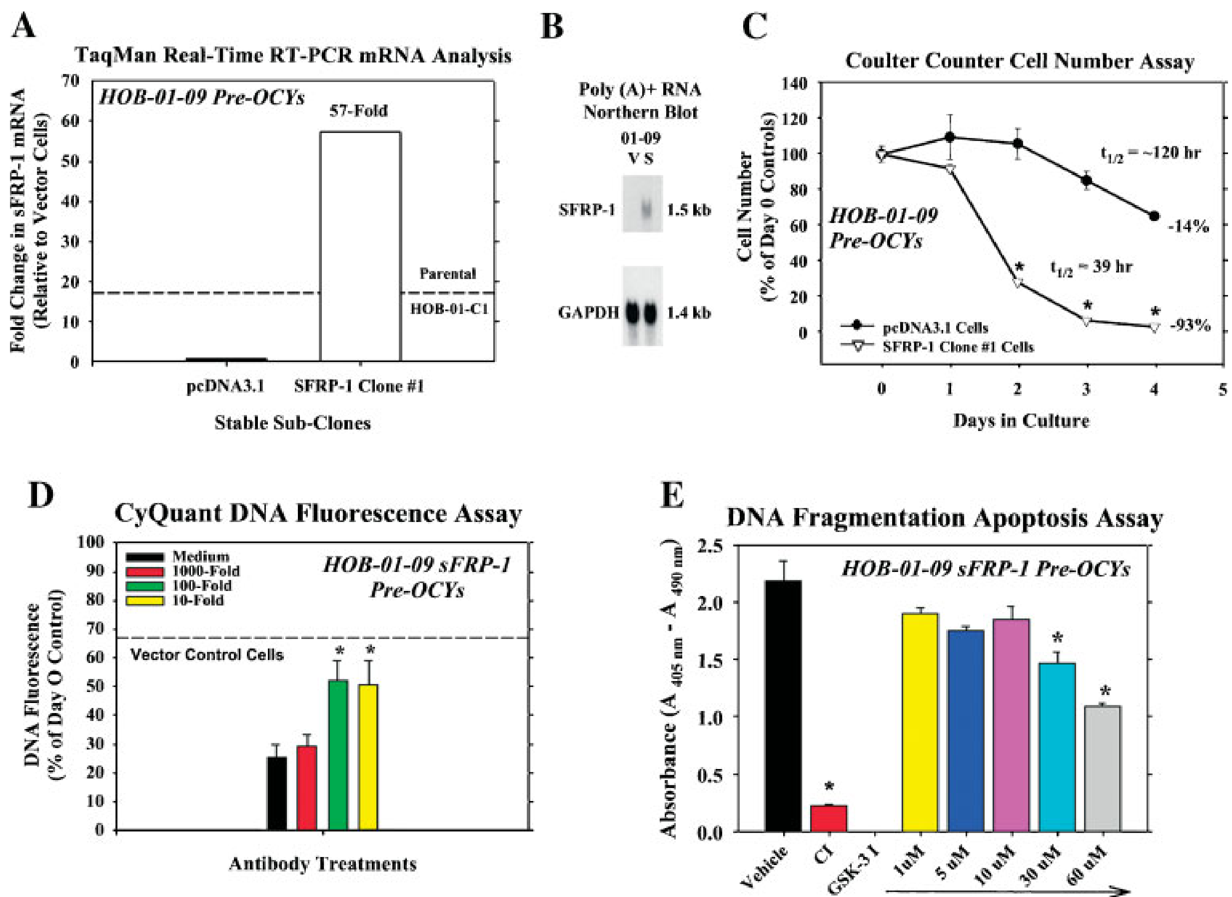

Fig. 9. Overexpression of sFRP-1 accelerates HOB cell death. **A**: Analysis of sFRP-1 mRNA levels in pre-OCY HOB-01-09 stable empty vector cells (pcDNA3.1, V) and sFRP-1 clone #1 stable over-expressing cells (S) using real-time quantitative RT-PCR analysis of total RNA. The cells were cultured in growth medium at the permissive temperature of 34°C prior to RNA isolation. For comparison, the endogenous level of sFRP-1 message in the parental HOB-01-C1 cell line as determined by real-time quantitative RT-PCR analysis is also shown (dotted line). **B**: Analysis of sFRP-1 mRNA levels in pre-OCY HOB-01-09 stable empty vector cells (V) and sFRP-1 clone #1 over-expressing cells (S) by Northern hybridization analysis of poly (A)+ RNA. The cloned full-length sFRP-1 cDNA and a cloned full-length GAPDH cDNA were used as probes for the Northern blot. The cloned full-length sFRP-1 mRNA is shown as a 1.5 kb message, while GAPDH is shown as a 1.4 kb message. **C**: Cell death analysis of HOB-01-09 empty vector cells (pcDNA3.1) and sFRP-1 clone #1 cells. The cells were incubated in serum-free medium at the non-permissive temperature of 39°C for up to 4 days, and cell number was determined using a Beckman Coulter Multisizer on the indicated days. The results were normalized to the day 0 cell number (1 day after seeding when the cells were placed in serum-free medium and transferred to 39°C) and are presented as mean ± SD, n = 6–36, *$P < 0.001$ versus the empty vector control cells. **D**: Cell death analysis of HOB-01-09 empty vector cells (dotted line) and sFRP-1 clone #1 cells. The cells were incubated in serum-free medium at 39°C for 3 days, and DNA content was quantified by the CyQuant DNA Fluorescence assay. The sFRP-1 over-expressing cells were treated with 10–1,000-fold dilutions of an sFRP-1 anti-serum. The results were normalized to the day 0 cell number and are presented as mean ± SD, n = 8, *, $P < 0.05$ versus the medium control cells. **E**: Apoptosis analysis of HOB-01-09 sFRP-1 clone #1 cells. The cells were incubated in serum-free medium at 39°C for 3 days, and apoptosis was measured by the Cell Death Detection ELISA^{PLUS} assay. The sFRP-1 over-expressing cells were treated with 0.1% DMSO (vehicle control), 100 μM caspase inhibitor I (CI), or 1–60 μM of a GSK-3 inhibitor (GSK I). The results are presented as mean ± SD, n = 3, *$P < 0.05$ versus the vehicle control cells.

Clancy et al., 2003] have also discovered new potential mechanisms for the bone anabolic actions of these agents. Among the important new signaling pathways that have been revealed by these studies is the Wnt pathway [de Jong et al., 2002; Doi et al., 2002; Harris and Harris, 2002; Vaes et al., 2002; Balint et al., 2003; Clancy et al., 2003; Qi et al., 2003; Roman-Roman et al., 2003; Westendorf et al., 2004]. For example, treatment of UMR-106 rat osteosarcoma cells with PTH has been reported to up-regulate sFRP-4 mRNA expression [Qin et al., 2003], and to increase Frizzled (FZD)-1 and -2 mRNA levels [Chan et al., 1992]. On the other

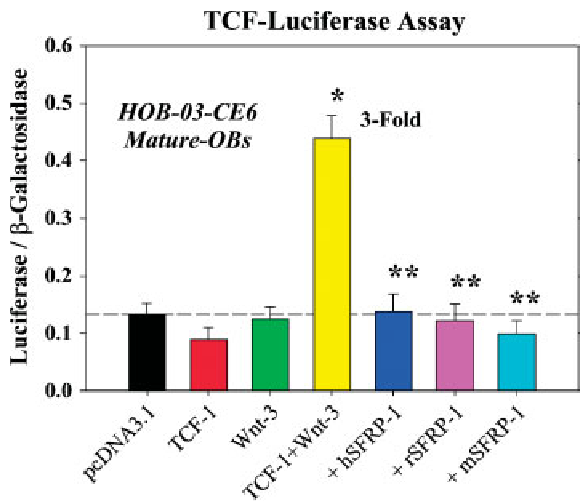

Fig. 10. sFRP-1 suppresses canonical Wnt signaling in HOB cells. Mature-OB HOB-03-CE6 cells were transfected with expression plasmids for the TOPflash reporter, TCF-1, Wnt-3, human, rat or mouse sFRP-1, and pCMVβ. The cells were incubated in growth medium at 37°C for 24 h and assayed for luciferase and β-galactosidase expression. The results are presented as mean ± SD, n = 6, $*P < 0.05$ versus the empty pcDNA3.1 control cells or ** versus the TCF-1 plus Wnt-3 cells.

hand, BMP-2 treatment of murine C2C12 premyoblastic cells has been observed to elevate Wnt inhibitory factor-1 [Vaes et al., 2002] and sFRP-2 [Vaes et al., 2002; Balint et al., 2003] messages, while treatment of mouse muscle in vivo with BMP-2 was reported to increase sFRP-1, -2, -3 and -4 as well as FZD-1 mRNAs [Clancy et al., 2003]. BMP-2 has also been observed to up-regulate Wnt-1, -3a, -5b, -7, -9b as well as FZD-1 and -9 mRNA expression in murine pluripotent mesenchymal cell lines and MC-3T3-E1 osteoblastic cells; in addition, it increases Tcf-1 and Lef-1 message levels, but suppresses low-density lipoprotein receptor-related protein (LRP)-1, LRP-5 and sFRP-2 mRNA expression in MC-3T3-E1 cells [Rawadi et al., 2003]. Finally, FZD-1 and sFRP-2 were also identified as genes whose expression changed during the differentiation of primary mouse calvarial-derived OBs cultured with ascorbic acid and β-glycerol-phosphate to promote development of the OB phenotype [Roman-Roman et al., 2003].

As noted above, Wnt antagonists like sFRPs are commonly identified in transcription profiling studies of OBs. In the current study, RADE analysis of HOB cells treated with PGE_2, TGF-β1, and PTH was used to identify 82 differentially expressed genes. Of these, the Wnt antagonist sFRP-1 was the most highly regulated gene, suggesting that it plays an important role in OB differentiation and bone formation. Increased sFRP-1 expression correlated with elevated cell death, while decreased expression correlated with decreased mortality. In addition, over-expression of sFRP-1 accelerated HOB cell death and antagonized canonical Wnt signaling. Thus, regulation of Wnt activity by sFRP-1 appears to be important for the control of OB and OCY survival. This conclusion is supported by results from the sFRP-1 knockout mice, which exhibit decreased OB and OCY apoptosis when compared to wild-type controls [Bodine et al., 2004]. Moreover, the importance of the canonical Wnt pathway in the modulation of OB and OCY longevity is underscored by the suppression of bone cell apoptosis that is observed in transgenic mice that over-express the G171V gain-of-function mutation of LRP-5 [Babij et al., 2003].

In the HOB cell lines, sFRP-1 mRNA levels increased with advancing cellular differentiation and peaked in the pre-OCY stage of development. This elevated expression correlated with the highest basal cell death rate among the HOB cell lines. These data suggest that pre-OCYs are the most susceptible of the OB lineage to apoptosis, and that this process is controlled by sFRP-1. Thus, regulation of Wnt signaling by sFRP-1 may be one of the mechanisms that modulates the number of mature-OCYs that finally become entombed in mineralized bone. The observation that apoptosis increases with advancing cellular differentiation has also been observed in ROBs and implies that PCD is a fundamental component of this process [Lynch et al., 1998; Lian and Stein, 2003].

Several hormones, growth factors, and cytokines known to modulate OB differentiation and apoptosis also controlled the expression of sFRP-1 by the HOB cells. PGE_2 treatment induced sFRP-1 mRNA levels in HOB cells representing pre-OBs (HOB-03-C5 cells) and mature-OBs (HOB-03-CE6 cells). This correlated with an increase in apoptosis and may relate to the ability of PGE_2 to promote OB differentiation [Vrotsos et al., 2003]. Increased sFRP-1 expression could also result from a feedback mechanism to dampen the osteogenic effects of PGE_2. Treatment of HOB cells representing mature-OBs with the bone-resorbing cytokine IL-1β [Mundy et al., 2003] also

strongly up-regulated sFRP-1 message levels. Although we did not examine the effect of IL-1β on HOB cell viability, this too may correspond with an increase in apoptosis. Another bone-resorbing cytokine with actions similar to IL-1β, TNF-α [McCarthy et al., 2000; Mundy et al., 2003], has also been reported to increase OB PCD in vitro [Hock et al., 2001], and both IL-1β and TNF-α activate nuclear factor (NF)-κβ signaling [Baldwin, 1996]. On the other hand, treatment of pre-OBs and mature-OBs with vitamin D_3 suppressed sFRP-1 message levels. Moreover, TGF-β1 treatment of HOB cells representing pre-OCYs (HOB-01-C1 cells), which have high basal levels of sFRP-1 message, down-regulated sFRP-1 gene expression and suppressed cell death. In addition, treatment of these cells with BMP-2 and IGF-1 also decreased sFRP-1 mRNA levels. This too may correspond to suppression of apoptosis, since these growth factors have been reported to decrease OB PCD in vitro [Boyce et al., 2002].

In contrast to published reports indicating that pharmacological concentrations of glucocorticoids increase OB and OCY apoptosis [Manolagas, 2000; Hock et al., 2001; Boyce et al., 2002], treatment of HOB cells representing pre-OBs and mature-OBs with Dex down-regulated sFRP-1 mRNA levels and inhibited PCD. Other studies have also reported that Dex treatment suppresses OB apoptosis in vitro [Zalavras et al., 2003]. A potential explanation for these discrepancies is that glucocorticoids promote OB apoptosis in vitro when cell culture conditions do not fully support differentiation (e.g., sub-confluent cultures), while they suppress in vitro PCD when OB differentiation is enhanced (e.g., confluent cultures) [Zalavras et al., 2003]. In the case of the HOB cell lines, in vitro differentiation of these cells is promoted when the cultures are incubated at non-permissive temperatures, and the temperature-sensitive T-antigen is inactivated [Bodine and Komm, 2002].

Finally, although estrogens and PTH have been reported to decrease OB and OCY apoptosis [Manolagas, 2000; Hock et al., 2001; Boyce et al., 2002], treatment of the HOB cells with these hormones did not affect sFRP-1 gene expression or cell viability.

sFRP-1 gene expression is also up-regulated by PGE_2 and IL-11 treatment of murine stromal cells and OBs; however, incubation of these cells with Dex, vitamin D_3, PTH, and IL-1, -4, -10, and -18 had no effect on mRNA levels of the Wnt antagonist [Hausler et al., 2004]. Thus, mechanisms controlling sFRP-1 expression appear to vary among species.

Wang et al. [2005] recently reported that pharmacological concentrations of Dex increased sFRP-1 expression in primary rat MSCs in vitro and in rat OBs and chondrocytes in vivo. In this study, increased sFRP-1 expression correlated with decreased cytosolic β-catenin levels, reduced osteogenesis and increased apoptosis. Moreover, the authors reported that in vivo administration of recombinant human sFRP-1 to rats decreased proximal femur bone mineral density and trabecular bone volume, which is consistent with an inhibitory effect of the protein on bone formation [Bodine et al., 2004].

In addition to suppressing PCD, deletion of sFRP-1 also increases OB proliferation, differentiation, and function [Bodine et al., 2004]. Although loss of sFRP-1 did not appear to affect bone resorption in vivo, it did result in increased osteoclastogenesis in vitro [Bodine et al., 2004]. This may be caused by increased osteoblastogenesis [Manolagas, 2000], which also occurs in the sFRP-1 knockout mice [Bodine et al., 2004]. However, another potential mechanism is the ability of sFRP-1 to bind and antagonize receptor activator of NF-κβ ligand [Chuman et al., 2004; Hausler et al., 2004], which is an important stimulator of osteoclastogenesis [Manolagas, 2000].

Other sFRPs have also been observed to affect OB physiology, mineral metabolism, and skeletal apoptosis. Using MC-3T3-E1 mouse OBs, Chung et al. [2004] recently reported that treatment of the cells with sFRP-3 decreased proliferation and increased differentiation. The authors proposed that these effects occur via regulation of β-catenin-independent pathways. Berndt et al. [2003] have observed that treatment of opossum renal epithelial cells in vitro with sFRP-4 inhibited sodium-dependent phosphate transport, while treatment of rats and mice in vivo with sFRP-4 elevated renal fractional phosphate excretion and suppressed renal phosphate re-absorption. These data indicate that sFRP-4 has phosphatonin-like properties, promoting phosphaturia and hypophosphatemia, and may therefore control bone mineralization. Finally, James et al. [2000] have reported that sFRP-4 is highly expressed in chondrocytes from patients with

osteoarthritis, while its expression is negligible in cells from normal cartilage. Moreover, the authors observed a correlation between sFRP-4 expression and chondrocyte apoptosis.

In summary, DD-PCR analysis of HOB cell lines in three stages of differentiation treated with three osteogenic agents has identified sFRP-1 as a highly differentially expressed gene. Expression of sFRP-1 is associated with OB differentiation and PCD, and it is regulated by hormones, growth factor and cytokines that are known to modulate these processes. These data lend support to other studies indicating the importance of sFRP-1 in controlling Wnt regulation of apoptosis and bone metabolism.

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