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# TGF-β1 and WISP-1/CCN-4 Can Regulate Each Other's Activity to Cooperatively Control Osteoblast Function

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**Abstract** Wnt-induced secreted protein-1 (WISP-1), like other members of the CCN family, is expressed in skeletal tissues. Its mechanism of action remains unknown. Expression of WISP-1 was analyzed in human bone marrow stroma cells (hBMSC) by RT-PCR. We identified two major transcripts corresponding to those of full-length WISP-1, and of the splice variant WISP-1va which lacks a putative BMP/TGF-β binding site. To investigate the function of WISP-1 in bone, hBMSC cultures were treated with recombinant human (rh)WISP-1 and analyzed for proliferation and osteogenic differentiation. WISP-1 treatment increased both BrdU incorporation and alkaline phosphatase (AP) activity. Considering the known functional synergy found between the TGF-β super-family and members of the CCN family, we next tested the effect of WISP-1 on TGF-β1 activity. We found that rhWISP-1 could reduce rhTGF-β1 induced BrdU incorporation. Similarly, rhTGF-β1 inhibited rhWISP-1 induction of AP activity. To explore functional differences between the WISP-1 variants, WISP-1 or WISP-1va were transfected into hBMSC. Both variants could strongly induce BrdU incorporation. However, there were no effects of either variant on AP activity without an additional osteogenic stimulus such as TGF-β1. Taken together our results suggest a functional relationship between WISP-1 and TGF-β1. To further define this relationship we analyzed the effect of WISP-1 on TGF-β signaling. rhWISP-1 significantly reduced TGF-β1 induced phosphorylation of Smad-2. Our data indicates that full-length WISP-1 and its variant WISP-1va are modulators of proliferation and osteogenic differentiation, and may be novel regulators of TGF-β1 signaling in osteoblast-like cells. J. Cell. Biochem. 104: 1865–1878, 2008. Published 2008 Wiley-Liss, Inc.†

**Key words:** WISP-1; CCN; TGF-β1; BMP; wnt; osteoblast; proliferation; differentiation; Smad-2; VWF-IIc; chordin

Wnt-induced secreted proteins (WISPs 1–3) are members of the CCN family (**C**ysteine rich angiogenic protein 61, Cyr61; **C**onnective tissue growth factor, CTGF; and **N**ephroblastoma over-expressed gene, NOV), implicated in the pathogenesis of cancerous and fibrotic disorders. Members of this multifunctional protein family can act as either matrix components that regulate adhesion and migration, or as growth factors that modulate cell proliferation and differentiation [Rachfal and Brigstock, 2005]. The overlapping developmental and pathogenic roles of CCNs, particularly for CTGF, are very well established. However, emerging evidence suggests that CCNs also have tissue-specific functions under normal physiological conditions, chiefly in the skeleton. In addition to their developmental functions in the angiogenesis and chondrogenesis processes [Wong et al., 1997; Babic et al., 1999; Chen et al., 2001; Yu et al., 2003; Lafont et al., 2005; Schutze et al.,

Abbreviations used: WISP-1, wnt-induced secreted protein-1; WISP-1va, splice variant of WISP-1 lacking exon 3; CCN, **C**YR61, **C**TGF and **N**OV; CYR61, cysteine-rich angiogenic protein 61; CTGF, connective tissue growth factor; NOV, nephroblastoma overexpressed; BMSC, bone marrow stromal cells; AP, alkaline phosphatase; TGF, transforming growth factor; VWFcII, von Willebrand factor type cII; BrdU, 5-bromo-2-deoxyuridine; BMP, bone morphogenic protein; OPN, osteopontin; BSP, bone sialoprotein; GAPDH, glyceraldehyde-3-phosphate; CR, cysteine rich; CT, C-terminal.

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2005], CCN proteins are involved in the maintenance and remodeling of the adult skeleton [Safadi et al., 2003; Omoto et al., 2004; Lafont et al., 2005; Schutze et al., 2005; Si et al., 2006]. In skeletal tissues, the expression of CCN proteins is induced by wnts, BMPs, and TGF-βs, all of which can modulate bone development and remodeling [Eguchi et al., 2002; Luo et al., 2004; Moritani et al., 2005; Parisi et al., 2006; Si et al., 2006]. CTGF, Cyr61 and Nov have also been implicated as modulators of these same bone-regulating pathways. Specifically, CTGF and NOV directly bind to, and affect the activity of BMPs and TGF-βs [Abreu et al., 2002; Rydziel et al., 2007]. Wnt signaling, now known to be an essential regulator of bone mass, can also be modulated by Cyr61and CTGF through binding to low-density lipoprotein (LDL) receptor related protein (LRP) proteins such as LRP5/6, the co-receptors of the canonical wnt-signaling pathway [Gao and Brigstock, 2003; Latinkic et al., 2003; Mercurio et al., 2004]. In addition to their functions in fibrosis and cancer, CCN members are associated with musculoskeletal disorders such as rheumatoid and osteoarthritis [Omoto et al., 2004]. Moreover, mutations in the WISP-3 gene cause a rare form of progressive pseudorheumatoid dysplasia [Hurvitz et al., 1999; Kumar et al., 1999; Cheon et al., 2004; Omoto et al., 2004; Kutz et al., 2005]. These studies suggest that CCN proteins could be crucial factors influencing the activity of signaling pathways that control postnatal bone development and maintenance.

The CCN family is highly conserved and its members share significant sequence homology, at both the protein and nucleotide level. Each CCN protein is encoded by a separate homologous gene composed of 5 exons, that when transcribed gives rise to protein products comprised of four distinct functional domains [Rachfal and Brigstock, 2005]. These domains also bear significant homology and functional similarity to other proteins and growth factors. Domain I contains an IGFBP-like sequence which has proven in some CCNs to have limited IGF-binding activity [Kim et al., 1997]. Domain II has significant similarity to the core domain of the von Willebrand factor type c II (VWFcII) protein and contains the consensus-binding motif for the BMP antagonist, chordin [Abreu et al., 2002], as well as numerous integrin-binding motifs [Leu et al., 2002, 2003]. Domain III bears homology to thrombospondin-I domain. The fourth and final, C Terminal (CT) domain, contains more integrin-binding motifs and a cysteine knot region similar to those found in BMP/TGF-βs, and BMP/TGF-β binding proteins such as noggin [Groppe et al., 2002; Avsian-Kretchmer and Hsueh, 2004]. Hence much of the knowledge of how this protein family functions has been derived from structural analogies to these conserved domains. Furthermore, the identification and analysis of CCN splice variants and cleaved extra-cellular products has been key in elucidating the role of CCN proteins [Brigstock et al., 1997; Ball et al., 1998; Kumar et al., 1999; Ball et al., 2003; Grotendorst and Duncan, 2005].

WISP-1 (CCN-4) is expressed in the early condensing mesenchyme of the developing skeleton, and later in both pre-osteoblastic and osteoblastic cells adjacent to bone forming sites [French et al., 2004]. WISP-1 has also been associated with the fracture healing process [French et al., 2004], and is expressed in adult cartilages [Yanagita et al., 2007]. However, the mechanism(s) by which WISP-1 participates in the processes of bone development and repair are not clearly understood. WISP-1 was first identified as a β-catenin responsive oncogene that affected cell proliferation and/or apoptosis of breast, colonic and gastric cancers [Pennica et al., 1998; Xu et al., 2000]. Various functional studies demonstrated that these events were mediated by the activation of intracellular signaling via pathways such as c-myc, rac, akt, p53, and map kinases [Tanaka et al., 2001; Su et al., 2002; You et al., 2002; Soon et al., 2003]. Yet very little is currently known about WISP-1 function or mechanism of action in normal physiology. Here we present evidence that WISP-1 regulates the proliferation and differentiation of osteoblast-like cells. Moreover, our analyses of WISP-1 expression in bone marrow stromal cells has identified a splice variant encoding a protein completely lacking one of the functional domains retained in other CCN proteins. Further characterization of this splice variant showed it can regulate osteoblastic activity. Similar to the pathogenic synergy found between CTGF and TGF-β1 in fibrotic disorders, we have uncovered a functional relationship between WISP-1 and TGF-β1 in the process of osteogenesis. Recent advances have pointed to a link between the wnt and BMP/TGF-β super-family signaling pathways in the maintenance of skeletal tissues. We

propose that WISP-1, and its alternatively spliced variants, may be molecules that link these two important pathways.

## MATERIALS AND METHODS

### Cell Culture

Human bone marrow stromal cells were isolated and cultured as previously described [Kuznetsov and Gehron Robey, 1996; Kuznetsov et al., 1997]. All specimens were used in accordance with the NIH regulations governing the use of human subjects under protocol D-0188. Briefly, minced fragments of trabecular bone and marrow were placed in α-modified Minimum Essential Medium (αMEM, Invitrogen, Grand Island, NY). The resulting preparations were pipetted repeatedly to release the marrow cells and then passed consecutively through 16 and 20 gauge needles to break up cell aggregates and obtain bone marrow single cell suspensions. Finally, the cell suspensions were filtered through a 2350 nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ) to remove remaining cell aggregates. To generate polyclonal strains of bone marrow stromal cells, bone marrow single cell suspensions were plated at $0.3–1.0 \times 10^7$ nucleated cells per 75 $cm^2$ flask (Becton Dickinson, Lincoln Park, NJ). Growth medium consisted of α-MEM, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin sulfate (Invitrogen, Carlsbad, CA), and 20% fetal bovine serum (FBS, Equitech-Bio, Kerrville, TX). Cells were maintained at 37°C in a humidified mixture of 5% $CO_2$/95% air. with medium replacement on day 1, and twice a week thereafter. The cultures were first passaged upon approaching confluence, usually on day 11–14, with two consecutive applications of trypsin-EDTA (Invitrogen, Carlsbad, CA) for 5–10 min each at room temperature. The cells were replated at $2–3 \times 10^6$ per 75 $cm^2$ flask with subsequent passages performed in the same manner when BMSCs neared confluency. In all experiments, cells of passage 5 or less were used.

### Semi-Quantitative RT-PCR

Total RNA was extracted from cultures using TRIzol reagent (Invitrogen, Carlsbad, CA) according to manufacturer's instructions, and quality/concentration determined using the NanoDrop® ND-100 (Wilmington, DE) spectrophotometer. Synthesis of cDNA by reverse transcriptase (RT) using random hexamer primers was carried out with 1 μg of total RNA. Forward and reverse PCR primers were designed using the primer design program Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi), for bone sialoprotein (*BSP*), osteopontin (*OPN*), and for glyceraldehyde-3-phosphate (*GAPDH*). Primer sequences for *WISP-1* were obtained from a previous report [Tanaka et al., 2001] (see Table I for sequences of these and other oligonucleotides used for quantitative RT-PCR). Hot start PCR was carried out using a program of 5 min denaturation at 95°C, followed by 35 cycles at 95°C (60 s), 57°C (30 s), 72°C (45 s), and then a final 7 min extension at 72°C. PCR products were separated on 6% acrylamide/TBE gels (Invitrogen, Carlsbad, CA), stained with SYBR Safe (Invitrogen, Carlsbad, CA), and visualized using a UV light box. For sequence analysis, PCR bands were isolated from 1% agarose/TBE gels, and purified using QIAquick gel purification kit (QIAGEN, Valencia, CA). Sequencing was carried out with the same primers used in the PCR reaction at the NIDCR central sequencing core facility.

### Antibody Production

A rabbit polyclonal antibody was produced from a synthetic peptide (CRNPNDIFADLESYPDFEEIAN) corresponding to amino acid residues 346–367 located in the CT domain of

**TABLE I. Genes Are Listed on the Left Followed by Accession Number to Their Right**

| Gene | Accession | Forward | Reverse |
|---|---|---|---|
| *WISP-1* | AF100779 | 5′TCGGTCGATGCCTGTGCCACTG3′ | 5′TCCACCTCACCAACAGCATGTGC3′ |
| *BSP* | NM_004967 | 5′ATGGCCTGTGCTTTCTCAAT3′ | 5′TCCTCTCCATAGCCCAGTGT3′ |
| *OPN a* | NM_000582 | 5′CATCACCTGTGCCATACCAG3′ | 5′GGGGACAACTGGAGTGAAAA3′ |
| *OPN b* | NM_000582 | 5′CTGTGTTGGTGGAGGATGTCTGC3′ | 5GTCGGCGTTTGGCTGAGAAGG3′ |
| *AP* | NM_000478 | 5′GCACCGCCACCGCCTACC3′ | 5′CCACAGATTTCCCAGCGTCCTTG3′ |
| *S29* | BC032813 | 5′TCTCGCTCTTGTCGTGTCTGTTC3′ | 5′ACACTGGCGGCACATATTGAGG3′ |
| *GAPDH* | NM_02046 | 5′CGACCACTTTGTCAAGCTCA3′ | 5′AGGGGTCTACATGGCAACTG3′ |

Forward primer corresponds to the sense and reverse to the antisense primers respectively. *WISP-1*, *BSP*, *OPN and GAPDH* were used for semi-quantitative PCR *and OPN b*, *AP and s29* were used for real-time PCR.

human WISP-1 isoform-1 (Accession Number NP-003873). The peptide was conjugated through the cysteine to maleimide-activated keyhole limpet hemocyanin (Pierce, Rockford, IL) and injected ($4 \times 1$ mg into multiple sites at ~2 weeks intervals) into New Zealand White rabbits at an AAALAC-approved facility (Covance, Denver, PA). The antiserum produced was named LF-187 and worked in direct ELISA on microtiter plates, immunocytochemistry, and in Western blot (see below).

### Western Blot Analysis

To induce cells to an osteoblast-like phenotype, cells grown in T25 flasks were supplemented with 100 μg/ml L-ascorbic acid (Wako, Japan), 5 mM β-glycerophosphate and 10 nM dexamethasone (Sigma, St. Louis, MO) and protein and RNA samples taken at 3-day intervals. Cultures of BMSC were incubated with M-PER mammalian protein extraction reagent (Pierce), supplemented with a protease inhibitor cocktail (1 μg/ml) (Roche, Indianapolis, IN), for 10 min on ice before cell scraping. Following centrifugation at 10,000 rpm for 10 min at 4°C to remove cell debris, the protein content of the cell lysate was determined using a BCA assay kit (Pierce). Lysates were added to NuPage loading buffer (Invitrogen, Carlsbad, CA) with added β-mercaptoethanol (0.5%) and heated at 100°C for 10 min. Equal amounts of total protein were separated on 10% Bis-Tris pre-cast polyacrylamide gels (NuPage, Invitrogen, Carlsbad, CA) by electrophoresis at 100 V for 1 h in MOPS (for WISP-1) or MES (for all other proteins) buffer, followed by transfer of separated proteins onto Hybond-P (GE Healthcare, Piscataway, NJ) PVDF membranes (200 V for 2–3 h with cooling). Membranes were then washed with Tris-buffered saline with 0.05% Tween-20 (TBS-T) and blocked using 4% dried milk prepared in TBS-T for 1 h at room temperature with gentle agitation. Antibodies to pSmad-2 (#3108), Smad2/3 (#3102) were purchased from Cell Signaling Technologies (Danvers, MA) and HSP-90 from Santa Cruz Inc. (Santa Cruz, CA, # sc-33755). WISP-1 (LF-187, 1:2,000), pSmad-2 (1:1,000), Smad-2/3 (1:1,000), HSP-90 (1:2,000) antibodies were diluted in blocking solution and incubated overnight at 4°C. Membranes were probed with a human serum-absorbed horse radish peroxidase (HRP)-conjugated goat anti-rabbit antibody (Kirkegaard and Perry Laboratories, Gaithersburg, MD, # 074–1516) diluted 1:50,000 in blocking solution for 1 h at room temperature. After washing with TBS-T, membranes were incubated with SuperSignal® West Pico chemiluminescent reagent (Pierce), exposed on BIOMAX light imaging film (KODAK, Rochester, NY) for 1–2 min before developing. Blots were stripped of antibodies using BlotFresh membrane stripping reagent (SignaGen Laboratories, Gaithersburg, MD), and re-probed to determine relative loading efficiency using the antibody to HSP-90. To examine WISP-1 protein expression goat anti-rabbit IRDye 680 was used for WISP-1 detection and goat anti-mouse IRDye 800CW for HSP-90 detection following recommendations from the Odyssey Infrared Imaging System manufacturer (LI-COR, Lincoln, NE).

### DNA Cloning

Full-length human WISP-1 and WISP-1va clones were a kind gift from Arnold Levine [Su et al., 2002] and Shinji Tanaka [Tanaka et al., 2003], respectively. WISP-1 cDNA was purified from a pBabe-Puro retroviral vector by restriction digestion with *Bam*HI and *Eco*RI and subcloned directionally into the pcDNA3.1+ mammalian expression vector (Invitrogen, Carlsbad, CA). Full-length WISP-1va was retrieved from a PCR3.1 TA cloning vector by PCR using primers to the T7 promoter and bovine growth hormone reverse priming site flanking the cDNA. Isolated and purified PCR product was then cloned into pcDNA3.1+ using restriction sites for *Hin*dIII and *Xba*I. Large-scale synthesis and purification of endotoxin-free plasmids was carried out using standard techniques and the Qiagen- endo-free maxi preparation purification kit (QIAGEN, Gaithersburg, MD). The integrity of the sub-cloned DNA was confirmed by sequencing.

### Transfection

Transient transfection of hBMSC was carried out using the Nucleofector system (AMAXA Inc., Gaithersburg, MD) and a protocol modified for transfecting mesenchymal stem cells (MSC). Briefly, cells were trypsinized and $4–5 \times 10^5$ cells per reaction were centrifuged at 1,000 rpm for 10 min. After re-suspension in the provided transfection solution, 2 μg total DNA per $5 \times 10^5$ cells were subjected to electroporation using the provided cuvettes and the C-17 program. Cells were recovered from cuvettes by the addition of

300–600 μl of pre-warmed, low calcium RPMI culture media (without serum), and placed at 37°C immediately for a 15-min recovery period. Recovered cells were re-plated in culture plates containing standard culture media. Optimal transfection conditions were first determined using a construct containing GFP, such that approximately 60–80% transfection efficiency for at least 72 h after transfection was obtained (data not shown). Using the optimized conditions for transfection WISP-1 or WISP-1va were tested for effects on proliferation and differentiation as described below.

### Measurement of Proliferation

Proliferation rates were determined using 5-bromo-2-deoxyuridine (BrdU) incorporation. hBMSC were plated at approximately $1 \times 10^4$ cell/well in 96-well plates, either immediately after transfection or the day prior to treatment with exogenously added WISP-1 or TGF-β1. Recombinant human WISP-1 (rhWISP-1, prepared in *E. coli*) was purchased from PeproTech (Rocky Hill, NJ) and recombinant human TGF-β1 (rhTGF-β1) was purchased from R&D Systems (Minneapolis, MN). Cells were grown in standard culture medium with reduced serum conditions (0.5%) for 4 h prior to growth factor treatments. Increasing concentrations of rhWISP-1 (50–500 ng/ml), or rhTGF-β1 (2 ng/ml) were prepared in reduced serum media and cells were treated with growth factors or vehicle control for 24 h to 5 days (media replacement every 2 days for longer experiments, all experiments carried out in the absence of any further osteogenic stimulus). For the proliferation assay, cells were first treated for 24 h and then incubated with 10 μM BrdU solution for 6 h before assay of BrdU incorporation using the chemiluminescent BrdU incorporation ELISA assay kit (Roche).

### Measurement of Differentiation

To measure differentiation, hBMSC were plated and treated as described in the previous section and alkaline phosphatase activity (AP) was measured using the colorimetric *para*-nitrophenol phosphate (PNPP) assay (Sigma 104 kit, Atlanta, GA) 5 days after transfection with or without the treatments described above. Briefly, cells were lysed with a solution of 20 mM Tris, 0.5mM magnesium chloride ($MgCl_2$), 0.1 mM zinc chloride ($ZnCl_2$), containing 0.1% Triton X-100. 10μl of cell lysate distributed to 96-well plates were incubated with 90 μl of 1 mg/ml Sigma 104 *para*-nitrophenol phosphate substrate solution in a buffer of 0.02 M sodium bicarbonate ($NaHCO_3$) with 3 mM $MgCl_2$ for 10 min at room temperature. The reaction was terminated using 0.5 M NaOH and the color development measured using a Victor 3® Wallac spectrophotometer plate reader (Perkin Elmer, Waltham, MA) at 405 nm absorbance. The total protein content within the transfected cell lysates was determined using the BCA assay kit described above and AP measurements were normalized to μg of protein per ml of sample. To determine relative mRNA levels for *AP* and *OPN* real time quantitative PCR was performed. For this analysis hBMSC were plated at approximately $1.5 \times 10^5$ cell/well in 6-well plates with normal medium (20% serum). After 24 h, culture medium was changed to the reduced serum condition medium (0.5%) for 4 h prior to growth factor treatments. rhWISP-1 (50 ng/ml; PreproTech, Rocky Hill, NJ) was prepared in reduced serum media in the absence of any further osteogenic stimulus and cells were treated with growth factors or vehicle control for 5 days (media replacement every 2 days). After 5 days culture, total cellular RNA was extracted by using RNeasy (QIAGEN, Hiden, Germany) according to the manufacture's protocol. RNA samples were reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Primers for real-time PCR were designed using Beacon Designer Software (Bio-Rad). Real-time PCR was performed to quantify the expression of *AP* and *OPN* using a Bio-Rad iCycler system (Bio-Rad) with a SYBR supermix kit (Bio-Rad), and running for 40 cycles at 95°C for 20 s and 60°C for 1 min. The mRNA level of each sample for each gene was normalized to that of the *s29* mRNA. Relative mRNA level was presented as 2[(Ct/s29 – Ct/gene of interest)]).

### Statistics

For BrdU and AP assays shown in Figs. 3A,B, 4 and 5, three- and four-way comparisons were performed using an ANOVA, with Dunnett's post hoc analysis nominating the untreated or empty vector samples as controls using Systat Software Inc. (San Jose, CA) statistics program. For the real time PCR shown in Figure 3C,D an unpaired *t*-test was performed comparing treated and untreated samples using GraphPad Prism Version 4 software (San Diego, CA).

## RESULTS

### Expression of a WISP-1 Splice Variant in Human Bone Cells

Splice variants of WISP-1 have previously been identified in soft tissues. We explored the possibility that splice variants of WISP-1 are expressed in bone. mRNA isolated from cultures derived from human bone marrow stroma samples were subject to RT-PCR to test for the presence of WISP-1 variants using primers flanking exons 2 and 5 of human WISP-1 gene. In addition to full-length WISP-1, a smaller transcript was observed. Sequence analysis revealed that this transcript (Fig. 1A) corresponded to WISP-1va, a splice variant lacking exon 3 that was previously found in invasive cholangiocarcinoma [Tanaka et al., 2001]. The expression of both variants increased in abundance as cells became more osteoblast-like as judged by the expression osteopontin (*OPN*) and bone-sialoprotein (*BSP*), both markers of a more mature osteoblastic-phenotype (Fig. 1A). Two transcripts for *OPN* were observed that are products of alternative splicing and were described in a previous report [Young et al., 1990].

To analyze WISP-1 at the protein level we generated a specific rabbit polyclonal antibody for human WISP-1 (LF-187). 5ng standard human recombinant WISP-1 (rhWISP-1) ran at approximately 37 kDa which is similar to the predicted size of full-length WISP-1 (Fig. 1B). Analysis of whole cell lysates from hBMSC revealed expression of an ~45–48 kDa major band (Fig. 1B) a slightly larger molecular weight than predicted, and larger than that of the recombinant *E. coli* produced WISP-1, presumably as a result of post-translation modification. Minor larger and smaller molecular weight bands were observed using LF-187, some of which could represent splice variants or other levels of post-translational modification. However these bands were less abundant than the full-length protein and significantly less than the relative mRNA for WISP-1va. Positive controls using adenoviruses expressing WISP-1 or WISP-1va showed that LF-187 recognizes both variants (not shown).

Previously published assessments of CCN protein character and homology did not include the newly discovered WISP members therefore we performed gene and protein alignment of the WISP-1 and WISP-1va variants with the other CCN family members using the web based Clustal W (1.82) program. Our alignment showed that WISP-1va is lacking the Von Willebrand factor type C–like domain (VWFcII) [Tanaka et al., 2001]. This variable domain contains a cysteine rich (CR) consensus motif found in chordin-like molecules, and also has the putative site for the BMP/TGF-β binding that has been found in CTGF [Abreu et al., 2002]. WISP-1 also contains this CR motif, is 100% homologous to the cysteine residues present in CTGF, and is 80% homologous to those found in chordin (Fig. 2).

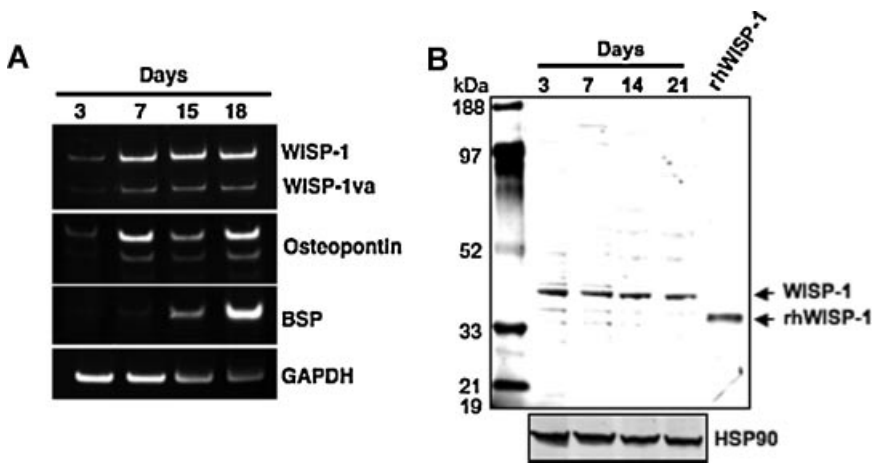


**Fig. 1.** Two variants of WISP-1 are produced in human bone marrow stromal cells. **A**: RT-PCR analysis of WISP-1 expression in hBMSC grown under osteogenic conditions to become osteoblast-like. Two different transcripts can be observed corresponding to full-length WISP-1 (**upper band**) and WISP-1va (**lower band**), expression becoming more apparent as cultures start to express osteopontin (OPN) and bone sialoprotein (BSP). **B**: Western blot analysis of WISP-1 in hBMSC grown under osteogenic conditions for 21 days in comparison to recombinant human WISP-1 (rhWISP-1). Levels of HSP-90 are shown as a loading control.

### WISP-1 Can Regulate Proliferation and Differentiation of BMSC

To explore the functional role of WISP-1 in human osteoblast-like cells, we treated hBMSC with a range of exogenously added rhWISP-1 doses (50–500 ng) and analyzed their proliferation and differentiation. Analysis of proliferation by a BrdU incorporation ELISA revealed a mild dose-dependant mitogenic effect of exogenous WISP-1 treatment, with most potent effects found at 100 ng/ml after 24 h of treatment (Fig. 3A). Although some inter-experimental differences were observed, possibly due to donor variability, each experiment was performed 3 times and contained 10 replicates all giving similar results. To determine the effect of WISP-1 on differentiation, we measured alkaline phosphatase (AP) activity, an early indicator of osteoblastic differentiation. rhWISP-1 treatment also caused dose-dependant induction of AP, with peak induction of AP activity at 200 ng/ml after 5 days of treatment (Fig. 3B). Next we determined the ability of WISP-1 to increase mRNA expression of ALP, and another marker of increased osteogenic differentiation, osteopontin (OPN). ALP and OPN mRNA expression was significantly increased in BMSC after 5 days of treatment with WISP-1 (50 ng/ml) (Fig. 3C).

### Reciprocal Regulation of WISP-1 and TGF-β1

Although WISP-1 was shown to induce the proliferation of hBMSC, this response was only modest in comparison to the effect of TGF-β1 (2 ng/ml), a known stimulator of hBMSC proliferation. Considering that the structurally related CCN protein, CTGF, exerts its effect in part through TGF-β1, analysis of the effect of co-treatment with TGF-β1 and WISP-1 on BMSC proliferation was performed. 24 h of rhWISP-1 treatment impaired the mitogenic effect of TGF-β1, reducing cell numbers to the level observed for treatment with rhWISP-1 alone (Fig. 4A). Next we performed the parallel experiment and analyzed the effects on differentiation. When BMSC where co-treated with optimal concentrations rhWISP-1 (200 ng) and TGF-β1 (2 ng/ml), the AP response of BMSC to WISP-1 was abolished (Fig. 4B) by co-treatment with TGF-β1 indicating TGF-β1 could inhibit the effect of rhWISP-1 on differentiation. These results suggest a reciprocal regulation of TGF-β1 and WISP-1 activity in hBMSC where WISP-1 inhibits TGF-β1 induction of proliferation while TGF-β1 inhibits WISP-1s influence on differentiation.

### Variant Specific Effects of WISP-1

Considering the fact that WISP-1va lacks the protein domain found in CCN proteins with putative TGF/BMP regulatory capacity, we wanted to determine if WISP-1 and WISP-1va had a differential ability to affect osteoblastic function. To do this, hBMSC were transfected with constructs containing the two variants and proliferation and differentiation were measured as described before. We found that overexpression of either variant could induce proliferation of hBMSC (Fig. 5A). Treatment of WISP-1va transfected hBMSC with TGF-β1 (2 ng/ml) resulted in a diminished proliferation, mimicking the effect of exogenously added


**Fig. 2.** The WISP-1va splice variant is lacking a domain that contains a chordin-like sequence. Protein sequence homology of human CTGF (hCTGF) and human WISP-1(hWISP-1) to the CR1 domain of BMP antagonist Chordin (hChorda) showing the sequences absent in the WISP-va splice variant. Area's of complete homology are marked with an *, and area's of partial homology are highlighted in black (as are the threefold identity), areas highlighted in grey annotate change of a residue to one of similar chemical/size properties. The CR1 domain contains 10 conserved cysteine residues, 8 of which are present in both WISP-1 and CTGF (numbered). Typical chordin CR sequences contain a [**CXXCXC**] motif in the middle of the sequence and [**CCXXC**] motif at the c-terminus both of which are conserved in WISP-1 and CTGF (labeled below corresponding sequences). In addition, a glycine (G), grey dotted line at the end, or tryptophan (W) that are often conserved in between these two chordin cysteine motifs are also conserved in WISP-1 and CTGF.

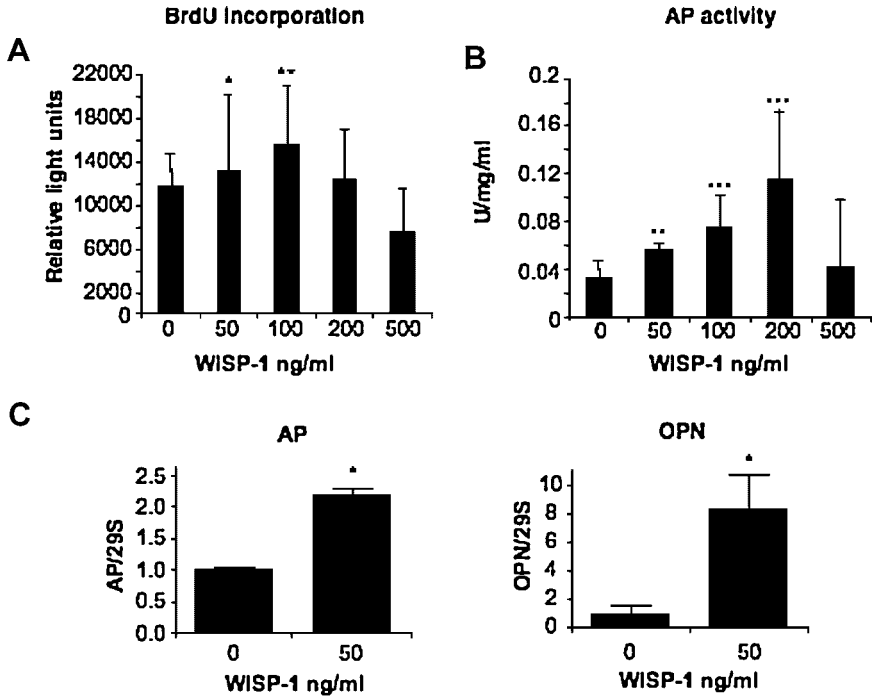


**Fig. 3.** rhWISP-1 can influence osteoblastic proliferation and differentiation. **A**: BrdU ELISA of hBMSC treated with 0–500ng/ml rhWISP-1 for 24 h. Fifty to 100 ng/ml WISP-1 treatment caused a significant increase in BrdU incorporation when compared to untreated control cells (*$P < 0.05$, **$P = < 0.01$). **B**: Assay of AP activity in hBMSC treated with 0–500 ng/ml rhWISP-1 in osteogenic media for 5 days. WISP-1 induced a concentration-dependant increase in AP activity (**$P < 0.01$, ***$P < 0.001$) up to 200 ng/ml. n = 5 per concentration, error bars represent standard deviations. **C**: mRNA expression of AP and OPN in hBMSC treated with 50 ng/ml WISP-1 revealing a significant increase in expression of both markers after 5 days of treatment (n = 3, *$P < 0.05$). Expression was normalized to 29S mRNA.

rhWISP-1. In contrast, no differences were found in full-length WISP-1 transfected cells, suggesting that TGF-β1 may regulate WISP-1 mitogenic activity in a splice variant-specific manner. The effects on AP activity were also analyzed, but cells transfected with WISP-1 or WISP-1va alone could not induce increases in AP activity (Fig. 5B,C). Under these circumstances, treatment of transfected cells with 2 ng/ml TGF-β1 (or 100 ng/ml BMP-2, data not

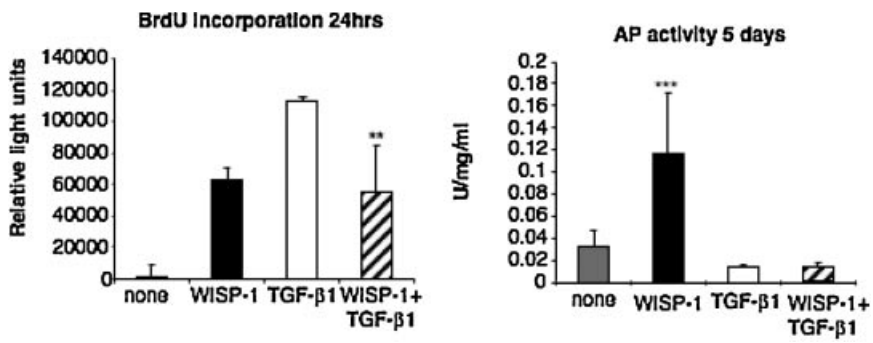


**Fig. 4.** TGF-β1 and WISP-1 have cooperative effects in regulating osteoblastic proliferation and differentiation. BrdU ELISA and AP assay of BMSC untreated (grey bars) treated with either rhWISP-1 (black bars), TGF-β1 (white bars), or co-treated with both WISP-1 and TGF-β1 (black cross-hatched) for 24 h or 5 days respectively. **A**: After 24 h of treatment WISP-1 (100 ng/ml) and TGF-β1 (2 ng/ml) significantly increased BrdU incorporation (**$P < 0.005$, ***$P < 0.001$). Co-treatment resulted in BrdU incorporation of the levels observed in treatments of WISP-1 alone. **B**: WISP-1 (200 ng/ml) significantly induced AP activity (U/mg/ml) after 5 days; TGF-β1 (2 ng/ml) had no significant effect upon AP activity. TGF-β1 inhibited the WISP-1 ability to induce AP activity. n = 10 per treatment and error bars represent standard deviations.

shown) was required to induce AP activity. Moreover, the transfection with full-length WISP-1 or WISP-1va enhanced the singular effects of TGF-β1 or BMP-2 treatment (not shown) on AP activity.

### Effect of WISP-1 in TGF-β1 Signaling

To determine if WISP-1 was exerting a direct effect upon TGF-β1 signaling we examined the phosphorylation of Smad-2 in cells treated with TGF-β1 for 30 min and 8 h by Western blot analysis. After 30 min, TGF-β1 induced phosphorylation of Smad-2 while rhWISP-1 had no significant effect (Fig. 6). However after 8 h a significant reduction in phosphorylated Smad-2 could be observed if cells were treated with 2 ng/ml TGF-β1 and 200ng/ml WISP-1 (Fig. 6). To determine if this change in Smad-2 phosphorylation had occurred through a direct or indirect mechanism, we analyzed pSmad1/5/8-BMP responsive Smads by Western blot but found no significant effect of rhWISP-1 or TGF-β1 (data not shown). Recent studies indicate that TGF-β1 may in some cases act through non-canonical pathways inducing β-catenin phosphorylation and nuclear localization [Jian et al., 2006]. We therefore tested whether WISP-1s effect on TGF-β1 could work through β-catenin by immunofluorescent localization of β-catenin in BMSC treated with rhWISP-1 for 18 h and by Western blot for phosphorylated β-catenin. No significant effect upon β-catenin could be revealed using these methods (not shown).

## DISCUSSION

The results presented in this article demonstrate the expression, regulation, and alternative function of two WISP-1 splice variants in bone cells in vitro. The data supports and builds on previous reports that WISP-1 is expressed and may act as a growth factor in bone cells. Our new data has revealed a complex relationship between WISP-1 and TGF-β1 in the regulation of osteoblast proliferation and commitment. Moreover, it is possible that this relationship is maintained by an inhibition of Smad-2 mediated TGF-β1 signaling and may indicate that WISP-1 is a novel regulator of TGF-β1 signaling in osteoblasts.

Previous studies suggest that multiple WISP-1 variants may be present in soft tissues [Tanaka et al., 2001, 2003; Cervello et al., 2004]. In support of this the NCBI AceView web resource (&http://www.ncbi.nlm.nih.gov/ieb/research/acembly/av.cgi?db=human&l=wisp1) showed there are at least 5 different transcripts of WISP-1 in 66 clones of various tissue origins. A sixth alternative transcript was described by [Yanagita et al., 2007]. The variants contained exons 1–5 (full-length, gene accession number AF100779), exons 1, 2, 4, and 5 (WISP1Va, NM_003882), exons 1, 2, and 5 [Yanagita et al., 2007] exons 1 and 2 (AY196488), exons 1, 4, and 5 (AY196487) or exons 1 and 5 (AY196486). The major transcripts that we observed in bone marrow stroma

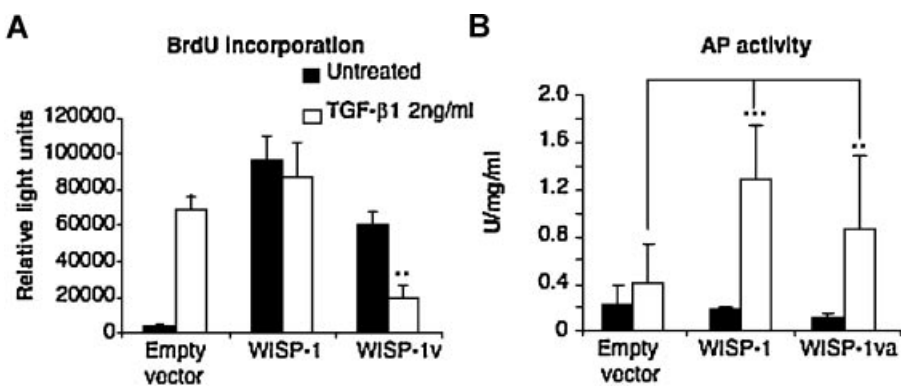


**Fig. 5.** Differential effects of WISP-1 and WISP-1va in the regulation of osteoblastic proliferation and differentiation, and response to TGF-β1. **A**: BrdU ELISA of hBMSC transfected with WISP-1, WISP-1va, or empty vector growth in regular media (black bars) or media supplemented with 2 ng/ml TGF-β1 (white bars). Both WISP-1 and WISP-1va induced a significant increase in BrdU incorporation compared to empty pcDNA 3.1+ vector (not annotated $P < 0.001$). No significant differences were observed between WISP-1 and empty vector transfected cells when treated with TGF-β1, but TGF-β1 treatment did significantly reduce BrdU incorporation in WISP-1va transfected cells after 24 h of treatment (**$P < 0.01$). **B**: Assay of AP activity in hBMSC transfected with empty vector, WISP-1 and WISP-1va grown under osteogenic conditions for 5 days. Transfection with either variant could not induce AP activity above that of empty vector controls. However, both variants could induce significant amounts AP activity if treated with TGF-β1 (**$P < 0.01$, ***$P < 0.001$). n = 9 per transfection/treatment and error bars represent standard deviations.

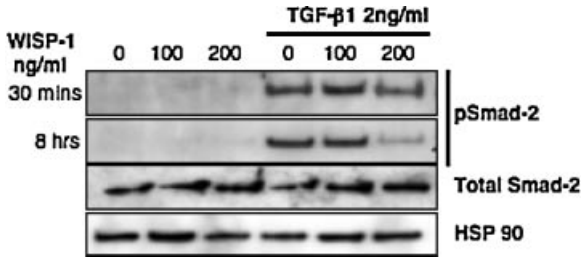


**Fig. 6.** rhWISP-1 can inhibit TGF-β1 induced smad-2 phosphorylation. Western blot analyses of levels of phosphorylated-Smad-2, total Smad-2 and HSP-90 loading control in hBMSC treated with 100–200 ng/ml WISP-1 in the absence (first three lanes) or presence of 2 ng/ml TGF-β1 (last three lanes). After 30 min of treatment WISP-1 had no effect of TGF-β1 induced phosphorylation of Smad2 in comparison to endogenous levels of Smad-2 expression. After 8 h 200 ng/ml WISP-1 reduced TGF-β1 induced Smad-2 phosphorylation with no apparent effect on the levels of total smad-2 expression.

samples were found to correspond to full-length WISP-1 and to the WISP-1va splice variant previously identified in gastrointestinal and liver cancers [Tanaka et al., 2001, 2003]. To our knowledge the present results are some of the first to report expression and function of WISP-1 and WISP-1va in tissues outside of pathological conditions. In support of our data a recent study described expression of a splice variant similar to WISP-1va and a third called WISP1vx which lacks the thrombospondin-1 like domain in chondrogenic tissue and cell lines [Yanagita et al., 2007]. We found that both WISP-1 and WISP-1va are expressed at the early, proliferative phase, and then are increased at later time points when cells are committed and express more mature markers such as OPN and BSP. This expression profile of WISP-1 and its variant in osteoblastic cultures is remarkably similar to those found for other CCNs already known to have functional roles in osteoblasts and bone [Schutze et al., 1998, 2005; Safadi et al., 2003; Parisi et al., 2006].

The regulated expression of multiple mRNA splice variants within one tissue could suggest that each variant has distinct and specific functions. We therefore examined the protein sequence of the domain missing in WISP-1va to try and determine whether this variant could have a different function. Interestingly this domain contains a BMP/TGF/chordin-like motif similar to the functionally active BMP/TGF binding sequence found in CTGF [Abreu et al., 2002]. In Cyr61 and CTGF this domain is also essential for binding to integrin receptors and mediating their roles as a 'matricellular' protein [Leu et al., 2002, 2003]. Although the WISP-1va variant appears to be a more potent mitogen than full-length WISP-1 in controlling proliferation, apoptosis and disease progression in pathological situations [Tanaka et al., 2001, 2003], no specific differences in function between the variants in normal tissue have been reported to date.

The expression profile of the two WISP-1 variants in differentiating osteoblastic cultures support the possibility of biphasic effects of WISP-1, and may be suggestive of functions in both the proliferation and differentiation of osteoblasts. Indeed, other CCN members are attributed with roles in both proliferation and differentiation of osteoblastic-like cells [Schutze et al., 1998; Safadi et al., 2003]. We therefore explored the effects of WISP-1 on these aspects of osteoblast function using hBMSC as our model system. Firstly, we analyzed the effect of full-length WISP-1 by exogenous protein treatments. A limited dose and time-dependant mitogenic activity of WISP-1 was observed. This effect was considerably less than the strong proliferative effect observed for WISP-1 in cancer [Tanaka et al., 2001, 2003; Su et al., 2002; Soon et al., 2003]. We found that the effects of exogenous WISP-1 on differentiation were much stronger. WISP-1 induced a significant increase in AP activity, comparable to that observed for established differentiation factors such as BMP-2 and BMP-7 [Cheng et al., 2001; Rawadi et al., 2003]. This is in contradiction to previous reports that ADCT5 chondrogenic cells over-expressing WISP-1 were unable to induce alkaline phosphatase activity without additional osteogenic induction by BMP-2 [French et al., 2004]. However, this may reflect differences either between primary cells and a clonal cell line in their responses to WISP-1, or differences in species and tissue origin (ATDC cells are murine and chondrogenic). It is possible that the relative changes in expression of WISP-1 in hBMSC as they become more osteoblast-like, allow this molecule to have biphasic effects. Our results are very like the effects observed with other CCNs in bone, which also display biphasic effects in osteoblast-like cells and are believed to have roles in both proliferation and in differentiation. Cyr61 for example was first identified as a vitamin 1, 25-dihydroxyvitamin D3 responsive early-immediate gene in primary human osteoblasts

[Schutze et al., 1998], but was also recently shown to be a target of wnt 3a in the induction of osteoblastic differentiation of multi-lineage potential C3H10T1/2 cells [Si et al., 2006]. Similarly, CTGF can induce the proliferation as well as the differentiation of osteoblasts [Nishida et al., 2000]. Our results suggest that WISP-1 may be a new growth factor important in osteoblasts and bone. Moreover the biphasic effect of WISP-1 in these cells could indicate that in bone WISP-1s role is to mediate the propagation and differentiation of progenitor cells toward an osteoblastic-like phenotype.

Considering the functional synergy found between TGF-β1 and related CCN members, and the modest mitogenic effect that WISP-1 had in osteoblasts, we also tested the effect of TGF-β1 on WISP-1 action. These experiments revealed an interesting relationship between TGF-β1 and WISP-1. Not surprisingly, TGF-β1 was a more potent mitogen than WISP-1 in these osteogenic stem cells. However, early in the proliferative phase of these cells (24 h) WISP-1 could overpower the effect of TGF-β1, the level of cell proliferation reverting to the low levels observed when treated only with WISP-1. A relationship between WISP-1 and TGF-β1 was also observed when we analyzed differentiation. TGF-β1 at day 5 had no effect upon differentiation. However, at this same time point WISP-1 strongly stimulated AP activity. Co-treatment on the other hand resulted in complete inhibition of osteoblast differentiation. In summary, exogenous WISP-1 can regulate the proliferative effect of exogenous TGF-β1, and TGF-β1 can inhibit the osteogenic effect of WISP-1. Our results in which WISP-1 can influence TGF-β1 action in osteoblasts, and vice versa, presents a new basis for understanding the role of TGF-β in regulating osteoblast differentiation.

Since the WISP-1va variant lacks the putative BMP/TGF binding domain we wanted to see if this would affect its capacity to induce proliferation and osteoblast differentiation of hBMSC. Because no recombinant WISP-1va protein was available, and as CCN proteins are notoriously difficult to purify we employed a transfection approach with cDNAs encoding both variants driven under a strong universal promoter. Using this method we found that alone both variants appeared to be very potent stimulators of proliferation, unlike exogenous WISP-1 treatments which only had a limited effect on proliferation. This effect is closer to those observed for WISP-1 in cancerous situations [Tanaka et al., 2003]. Intriguingly, under these circumstances TGF-β1 was only able to inhibit the proliferative effect of the WISP-1va splice variant. We also examined the effect of the WISP-1 variants on differentiation toward an osteoblast-like phenotype. Unlike exogenous WISP-1 treatment, no effect upon differentiation could be seen after transfection of either WISP-1 variant. In this circumstance TGF-β1 enhanced the effect of WISP-1, and to a lesser extent that of WISP-1va. This effect is more like that observed for clonal ADTC5 cells over-expressing WISP-1 which required BMP-4 to see any effects of WISP-1 [French et al., 2004]. Because WISP-1va lacks the putative BMP/TGF binding domain it is possible that TGF-β1 exerts its effects on WISP-1 and its variant's activity through indirect protein interactions. Support of this concept comes from our observations that WISP-1s affect on TGF-β1 signaling through Smad-2 does not occur until after 8 h of treatment. Therefore, as WISP-1va is lacking the VWF-IIC-like domain it is plausible that TGF-β1 may exert its effect on WISP-1 and vice versa through a third molecule possibly mediated via interactions with this domain. There is substantial evidence for additional modifying molecules in the regulation of the BMP and TGF-β1 signaling. For example chordin can exert differential effects upon cell fate during xenopus embryogenesis via its interacting partner tsg [Fisher and Halpern, 1999; Wijgerde et al., 2005]. This hypothesis supports our observations of co-operative functions for WISP-1 and TGF-β1 in osteogenesis, as together these molecules become potent stimulators of differentiation when usually alone these molecules would act to induce proliferation. These results also imply that post-translational processing of WISP-1 is significant in WISP-1s function. The use of *E. coli* produced recombinant protein, which will lack mammalian post-translational modifications, and had differential effects than induced WISP-1 expression, suggest that post-translational modifications and intracellular sorting could be important for WISP-1s function. Previous reports allude to the fact that more of WISP-1va variant is found in conditioned media when compared to full-length WISP-1 [Tanaka et al., 2003], and might suggest that the regulation of WISP-1 secretion is an important factor

governing WISP-1 activity. As we have already discussed WISP-1 can have dose-dependant effects, if indeed there was more WISP-1va secreted into the extra-cellular environment then differential effects may be observed for WISP-1va than for full-length WISP-1. Although both variants possess the same secretion signal peptide, it is also possible that these two variants are differently post-translationally modified and processed inside the cell. A full study on the production, storage and secretion of WISP-1 has not been carried out and would be needed to discern if these processes can affect the activity of this protein.

To reiterate, we observed differences in WISP-1 activities and function when different experimental approaches were used. Specifically, externally added WISP-1 (recombinant human WISP-1 added to cultures) was inhibitory to TGF-β1 activity, and internally added WISP-1 (transfections of the human gene) was stimulatory to TGF-β1 activity. These observations highlight the fact that WISP-1 could have different roles depending on its sub-cellular and extra-cellular localization, and indicate that post-translational modification of WISP-1 could be important to its function and relationship to TGF-β1. Taken together this data suggests a complex, reciprocal regulation between WISP-1 and TGF-β1 activity that may be indicative of a co-operative regulation of osteoblastic activity and eventual fate. Because TGF-β1 has a functional link to other CCN members such as in CTGF in fibrotic disorders it is plausible that WISP-1 may have an analogous effect in normal physiological conditions in bone. This type of relationship between two signaling molecules would be beneficial in a tissue which undergoes substantial turnover and repair, and may be a novel mechanism used in bone remodeling, or the fracture healing processes. Future studies will be needed to verify the functional role of WISP-1 in vivo during bone formation in normal and pathological conditions. A substantial overlap in expression and subsequent crosstalk exists between the wnt and BMP signaling pathways in many tissues [Labbe et al., 2000; Nishida et al., 2000; Warner et al., 2005]. It is now emerging that in bone these two key signaling pathways are functionally connected [Zhou et al., 2004; Warner et al., 2005; Jian et al., 2006]. As WISP-1 is a direct downstream product of the wnt signaling pathway it is plausible that WISP-1 could represent an intermediate molecule linking crosstalk between these pathways.

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