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TGF- β 1 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 (BMSC) 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, BMSC 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- β 1 activity. We found that rhWISP-1 could reduce rhTGF- β 1 induced BrdU incorporation. Similarly, rhTGF- β 1 inhibited rhWISP-1 induction of AP activity. To explore functional differences between the WISP-1 variants, WISP-1 or WISP-1va were transfected into BMSC. 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- β 1. Taken together our results suggest a functional relationship between WISP-1 and TGF- β 1. To further define this relationship we analyzed the effect of WISP-1 on TGF- β signaling. rhWISP-1 significantly reduced TGF- β 1 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 a novel regulators of TGF- β 1 signaling in osteoblast-like cells.

Keywords

WISP-1; CCN; TGF- β 1; BMP; wnt; osteoblast; proliferation; differentiation; smad-2; VWF-IIc; chordin

Article Text

Wnt-induced secreted protein's (WISP's 1–3) are members of the CCN family (Cysteine rich angiogenic protein 61 - Cyr61, Connective tissue growth factor - CTGF and Nephroblastoma 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 involved in adhesion and migration, or as growth factors that modulate cell proliferation and differentiation [Rachfal and Brigstock, 2005]. The overlapping developmental and pathogenic

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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 [Babic et al., 1999; Chen et al., 2001; Lafont et al., 2005; Schutze et al., 2005; Wong et al., 1997; Yu et al., 2003], CCN proteins are involved in the maintenance and remodeling of the adult skeleton [Lafont et al., 2005; Omoto et al., 2004; Safadi et al., 2003; Schutze et al., 2005; Si et al., 2006]. In skeletal tissues, the expression of CCN proteins is induced by wnts, BMPs, and TGF- β s, 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 signaling through these same bone-regulating pathways. Specifically, CTGF and NOV directly bind to and affect the activity of BMPs and TGF- β s [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 Cyr61 and 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 have also been 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 [Cheon et al., 2004; Hurvitz et al., 1999; Kumar et al., 1999; Kutz et al., 2005; Omoto et al., 2004]. 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 4 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; Leu et al., 2003]. Domain III bears homology to the thrombospondin III domain. The forth and final, C Terminal (CT) domain, contains more integrin-binding motifs and a cysteine knot region similar to those found in BMP/TGF- β s, and BMP/TGF- β binding proteins such as noggin [Avsian-Kretchmer and Hsueh, 2004; Groppe et al., 2002]. 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 [Ball et al., 2003; Ball et al., 1998; Brigstock et al., 1997; Grotendorst and Duncan, 2005; Kumar et al., 1999]. 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 [Soon et al., 2003; Su et al., 2002; Tanaka et al., 2001; You et al., 2002]. 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- β 1 in fibrotic disorders, we have uncovered a functional relationship between WISP-1 and TGF- β 1 in the process of osteogenesis. Recent advances have pointed to a link between the wnt and BMP/TGF- β superfamily 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 (α MEM, 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 cm² flask (Becton Dickinson, Lincoln Park, NJ). Growth medium consisted of α MEM, 2 mM glutamine, 100 U/ml penicillin, 100 μ g/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% CO₂/95% air, with medium replacement on day 1, and twice a week thereafter. The cultures were first passaged upon approaching confluence, usually on day 11 to 14, with two consecutive applications of 1x 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 cm² 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 μ g of total RNA. Forward (F) and reverse (R) 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 1 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 sec), 57°C (30 sec), 72°C (45 sec), 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 an amino-acid residues 346–367 located in the CT domain of 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 (4×1 mg into multiple sites at ~ 2 week intervals) into New Zealand White rabbits at an AAALAC-approved facility (Covance, Denver, PA). The antiserum produced was named LF-187 worked in direct ELISA on microtiter plates, immunocytochemistry, and in Western blot (see below) and cross-reacted with mouse WISP-1 made by mouse bone marrow stromal cells.

Western blot analysis

To induce cells to an osteoblast-like phenotype, cells grown in T25 flasks were supplemented with 100 μ g/ml L-ascorbic acid (Wako, Japan), 5mM β -glycerophosphate and 10nM 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, Rockford, IL), supplemented with a protease inhibitor cocktail (1 μ g/ml) (Roche, Indianapolis, IN), for 10 minutes on ice before cell scraping. Following centrifugation at 10,000rpm 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, Rockford, IL). Lysates were added to NuPage loading buffer (Invitrogen, Carlsbad, CA) with added β -mercaptoethanol (0.5%) and heated at 100°C for 10 minutes. Equal amounts of total protein were separated on 10% Bis-Tris pre-cast polyacrylamide gels (NuPage, Invitrogen, Carlsbad, CA) by electrophoresis at 100V for 1 hr 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 (200V for 2–3hrs 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 1hr at room temperature with gentle agitation. Antibodies to pSmad-2 (#3108), Smad2/3 (#3102) were purchased from Cell Signaling Technologies (Danvers, MA), HSP-90 from Santa Cruz Inc. (Santa Cruz, CA, # sc-33755). WISP-1 (LF-187, 1:2000), pSmad-2 (1:1000), Smad-2/3 (1:1000), HSP-90 (1:2000) 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 hr 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 1–2minutes before developing. Blots were stripped of antibodies using BlotFresh membrane stripping reagent (SignaGen Laboratories, Gaithersburg), 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, Nebraska).

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 BamHI and EcoRI and sub-cloned 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 HindIII and XbaI. 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 Sciences, MD). The integrity of the sub-cloned DNA was confirmed by sequencing.

Transfection

Transient transfection of BMSC 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 1000 rpm for 10 minutes. After re-suspension in the provided transfection solution, 2 μ g total DNA per 5×10^5 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 μ l of pre-warmed, low calcium RPMI culture media (without serum), and placed at 37°C immediately for a 15-minute 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 hrs 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 1×10^4 cell/well in 96 well plates, either immediately after transfection or the day prior to treatment with exogenously added WISP-1 or TGF- β 1. Recombinant human WISP-1 (rhWISP-1, prepared in E-coli) was purchased from PeproTech, Rocky Hill, NJ, recombinant human TGF- β 1 (rhTGF- β 1) was purchased from R&D Systems, Minneapolis, MN. Cells were grown in standard culture medium with reduced serum conditions (0.5%) for 4 hrs prior to growth factor treatments. Increasing concentrations of rhWISP-1 (50–500ng/ml), or rhTGF- β 1 (2ng/ml) were prepared in reduced serum media and cells were treated with growth factors or vehicle control for 24hrs 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 hrs and then incubated with 10 μ M BrdU solution for 6 hrs before assay of BrdU incorporation using the chemiluminescent BrdU incorporation ELISA assay kit (Roche, Indianapolis, IN).

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 20mM Tris, 0.5mM magnesium chloride (MgCl₂), 0.1mM zinc chloride (ZnCl₂), containing 0.1% Triton X-100. 10 μ l of cell lysate distributed to 96 well plates were incubated with 90 μ l of 1mg/ml Sigma 104 para-nitrophenol phosphate substrate solution in a buffer of 0.02M sodium bicarbonate (NaHCO₃) with 3mM MgCl₂ for 10 minutes at room temperature. The reaction was terminated using 0.5M NaOH and the color development measured using a Victor 3[®] Wallac spectrophotometer plate reader (Perkin Elmer, Waltham, MA) at 405nm 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 μ g 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 1.5×10^5 cell/well in 6 well plates with normal medium (20% serum). After 24 hours, culture medium was changed to the reduced

serum condition medium (0.5%) for 4 hrs 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, Hilden, 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, Hercules, CA) Real-time PCR was performed to quantify the expression of *AP* and *OPN* using a Bio-Rad iCycler system (Bio-Rad, Hercules, CA) 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, AP and luciferase assays, groups of triplicates were compared to each other by students T-test using SigmaPlot (Systat Software Inc, San Jose, CA and GraphPad Prism Version 4.0) statistics programs. For other three way comparisons shown in Fig 4 and Fig 5 an ANOVA analysis was performed.

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 determined by the expression osteopontin (*OPN*) and bone-sialoprotein (*BSP*), a marker of a more mature osteoblastic-phenotype (Fig. 1A). Two transcripts for *OPN* were observed that are products of alternative splicing that has been previously detailed in a prior study (Young et al, 1990).

To enable the analysis of 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 ran at approximately 37kDa which is similar to the predicted size of full length WISP-1 (Figure 1B). Analysis of whole cell lysates from BMSC revealed expression of an approximately 45–48 kDa major band (Fig. 1B) slightly larger molecular weight than that of the recombinant WISP-1 presumably as a result of post-translation modification. Although some larger and smaller molecular weight bands were observed using LF-187, which may represent splice variants or other levels of post-translational modification, these bands were significantly less abundant than the full-length protein and significantly less than the relative mRNA for WISP-1va. (NEEDED?) 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 Clustalw (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).

WISP-1 can regulate proliferation and differentiation of BMSC

To explore the functional role of WISP-1 in human osteoblast-like cells, we treated BMSC with a range of exogenously added rhWISP-1 doses (50–500ng) 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 100ng/ml after 24 hrs hours of treatment (Fig. 3A). Although some intra-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 first 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 200ng/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 (50ng./ml) (Fig. 3C).

Reciprocal regulation of WISP-1 and TGF- β 1

Although WISP-1 was shown to induce the proliferation of BMSC, this response was only modest in comparison to the effect of TGF- β 1 (2ng/ml), a known stimulator of BMSC proliferation. Considering that the structurally related CCN protein, CTGF, exerts its effect in part through TGF- β 1, analysis of the effect of co-treatment with TGF- β 1 and WISP-1 on BMSC proliferation was performed. 24 hrs of rhWISP-1 treatment impaired the mitogenic effect of TGF- β 1, 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 were co-treated with optimal concentrations rhWISP-1 (200ng) and TGF- β 1 (2ng/ml), the AP response of BMSC to WISP-1 was abolished (Fig. 4B) by co-treatment with TGF- β 1 indicating TGF- β 1 could inhibit the effect of rhWISP-1 on differentiation. These results indicate a reciprocal regulation of TGF- β 1 and WISP-1 activity in BMSC where WISP-1 inhibits TGF- β 1 induction of proliferation while TGF- β 1 inhibits WISP-1's 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, BMSC were transfected with constructs containing the two variants and proliferation and differentiation were measured as described before. We found that over-expression of either variant could induce proliferation of BMSC (Fig. 5, A). Treatment of WISP-1va transfected BMSC with TGF- β 1 (2ng/ml) resulted in a diminished proliferation, mimicking the effect of exogenously added rhWISP-1. In contrast, no differences were found in full length WISP-1 transfected cells, suggesting that TGF- β 1 may regulate WISP-1's 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 2ng/ml TGF- β 1 (or 100 ng/ml BMP-2, data not shown) was required to induce AP activity. Moreover, the transfection with full length WISP-1 or

WISP-1va enhanced the singular effects of TGF- β 1 or BMP-2 treatment (not shown) on AP activity.

Effect of WISP-1 in TGF- β 1 signaling

To determine if WISP-1 was exerting a direct effect upon TGF- β 1 signaling we examined the phosphorylation of Smad-2 in cells treated with TGF- β 1 for 30 min and 8 hrs by western blot analysis. After 30 min, TGF- β 1 induced phosphorylation of Smad-2 while rhWISP-1 had no significant effect upon this (Fig. 6). However after 8 hrs a significant reduction in phosphorylated Smad-2 could be observed if cells were treated with 2ng/ml TGF- β 1 and 200ng/ml WISP-1 (Fig. 6). To determine if this change in pSmad-2 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- β 1 (data not shown). Recent studies indicate that TGF- β 1 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- β 1 could work through β -catenin by immunofluorescent localization of β -catenin in BMSC treated with rhWISP-1 for 18 hours 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 paper 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- β 1 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- β 1 signalling and may indicate that WISP-1 is a novel regulator of TGF- β 1 signalling in osteoblasts.

Previous studies suggest that multiple WISP-1 variants may be present in soft tissues [Cervello et al., 2004; Tanaka et al., 2003; Tanaka et al., 2001]. In support of this the NCBI AceView web resource

(<http://www.ncbi.nlm.nih.gov/IEB/Research/AceView/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 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., 2003; Tanaka et al., 2001]. To our knowledge the present results are some of the first to report expression of and function of WISP-1 variants 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 [Parisi et al., 2006; Safadi et al., 2003; Schutze et al., 2005; Schutze et al., 1998].

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 proteins [Leu et al., 2002; Leu et al., 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., 2003; Tanaka et al., 2001], no specific differences in function between the variants in normal tissue have been reported to date.

The expression profile of 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 [Safadi et al., 2003; Schutze et al., 1998]. We therefore explored the effects of WISP-1 on these aspects of osteoblast function using BMSC 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 [Soon et al., 2003; Su et al., 2002; Tanaka et al., 2003; Tanaka et al., 2001]. 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 BMSC 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-1's role is to mediate the propagation and differentiation of progenitor cells toward an osteoblastic-like phenotype.

Considering the functional synergy found between TGF- β 1 and related CCN members, and the modest mitogenic effect that WISP-1 had in osteoblasts, we also tested the effect of TGF- β 1 on WISP-1 action. These experiments revealed an interesting relationship between TGF- β 1 and WISP-1. Not surprisingly, TGF- β 1 was a more potent mitogen than WISP-1 in these osteogenic stem cells. However, early in the proliferative phase of these cells (24hrs) WISP-1 could overpower the effect of TGF- β 1, the level of cell proliferation reverting to the low levels observed when treated only with WISP-1. A relationship between WISP-1 and TGF- β 1 was also observed when we analyzed differentiation. TGF- β 1 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- β 1, and TGF- β 1 can inhibit the osteogenic effect of WISP-1. Our results in which WISP-1 can influence

TGF- β 1 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 BMSC. 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- β 1 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- β 1 enhanced the effect of WISP-1, and to a lesser extent that of WISP-1va. This effect is more like those observed for clonal ADTC5 cells over-expressing WISP-1 which required BMP-4 to see effects of WISP-1 [French et al., 2004]. Because this variant lacks the putative BMP/TGF binding domain it is possible that TGF- β 1 exerts its effects on WISP-1 and its variant activity through indirect protein interactions. Support of this concept comes from our observations that WISP-1's affect on TGF- β 1 signalling through Smad-2 does not occur until after 8 hours of treatment. Therefore, as WISP-1va is lacking the VWF-IIIC-like domain it is plausible that TGF- β 1 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- β 1 signalling. 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- β 1 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 cellular sorting are 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- β 1 activity, and internally added WISP-1 (transfections of the human gene) was stimulatory TGF- β 1 activity. These observations highlight the fact that WISP-1 could have different roles depending on its sub-cellular and extra-cellular localisation, and may also indicate that posttranslational modification of WISP-1 is important to its function and relationship to TGF- β 1. Taken together this data suggests a complex, reciprocal regulation between WISP-1 and TGF- β activity that may be indicative of a co-operative regulation of osteoblastic activity and eventual fate.

Because TGF- β 1 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. This type of relationship between two signalling molecules would be beneficial in a tissue which undergoes substantial turnover and repair, and may be a novel mechanism used in bone remodelling, 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 signalling 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 signalling pathways are functionally connected [Jian et al., 2006; Warner et al., 2005; Zhou et al., 2004]. As WISP-1 is a direct downstream product of the wnt signalling pathway it is plausible that WISP-1 could represent an intermediate molecule linking crosstalk between these pathways.

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ABBREVIATIONS

WISP-1, wnt-induced secreted protein-1
 WISP-1va, splice variant of WISP-1 lacking exon 3
 CCN, CYR61, CTGF and NOV
 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
 VWF α II, 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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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.

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-1va splice variant. Areas of complete homology are marked with an *, and areas of partial homology are highlighted in black (as are the 3-fold 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) or tyrosine (Y) 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 hours. 50–100ng/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 0–500ng/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 200ng/ml. $n = 5$ per concentration, error bars represent standard deviations. C) mRNA expression of AP and OPN in BMSC treated with 50ng/ml WISP-1 revealing a significant increase in expression of both markers after 5 days of treatment ($n=3$, *= $p<0.05$). Expression is normalized to 29S mRNA.

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 24hrs or 5 days respectively. A) After 24 hrs of treatment WISP-1 (100ng/ml) and TGF-β1 (2ng/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 (200ng/ml) significantly induced AP activity (U/mg/ml) after 5 days; TGF-β1 (2ng/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.

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 BMSC transfected with WISP-1, WISP-1va, or empty vector growth in regular media (black bars) or media supplemented with 2ng/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 24hrs of treatment ($** = p < 0.01$). B) Assay of AP activity in BMSC 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.

A

Fig. 6. rhWISP-1 can inhibit TGF-β1 induced smad-2 phosphorylation

A) Western blot analyses of levels of phosphorylated-Smad-2, total Smad-2 and HSP-90 loading control in hBMSC treated with 100–200ng/ml WISP-1 in the absence (first three lanes) or presence of 2ng/ml TGF-β1 (last three lanes). After 30 mins of treatment WISP-1 had no effect of TGF-β1 induced phosphorylation of Smad2 in comparison to endogenous levels of Smad-2 expression.

Table 1

Genes are listed on the left followed by accession number to their right. Forward primer corresponds to the sense and reverse to the antisense primers respectively. *WISP-1*, *BSP* and *OPN* and *GAPDH* were used for semi-quantitative PCR and *OPN b*, *AP* and *s29* were used for real-time PCR.

Gene	Accession	Forward	Reverse
<i>WISP-1</i>	AF100779	5'TCGGTCGATGCCTGTGCCACTG3'	5'TCCACCTCACCAACAGCATGTGC3'
<i>BSP</i>	NM_004967	5'ATGGCCTGTGCTTCTCAAT3'	5'TCCTCTCCATAGCCCAGTGT3'
<i>OPN a</i>	NM_000582	5'CATCACCTGTGCCATACCAG3'	5'GGGGACAACCTGGAGTGAAAA3'
<i>OPN b</i>	NM_000582	5'CTGTGTGGTGGAGGATGTCTGC3'	5 GTCGGCGTTTGGCTGAGAAGG3'
<i>AP</i>	NM_000478	5'GCACCGCCACCGCCTACC3'	5'CCACAGATTTCACAGCGTCCTTG3'
<i>s29</i>	BC032813	5'TCTCGCTCTTGTCTGTCTGTTTC3'	5'ACACTGGCGGCACATATTGAGG3'
<i>GAPDH</i>	NM_02046	5'CGACCACTTTGTCAAGCTCA3'	5'AGGGGTCTACATGGCAACTG3'