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WISP-3 functions as a ligand and promotes superoxide dismutase activity

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Abstract

WISP-3 (Wnt1 inducible secreted protein-3) mutations have been linked to the connective tissue diseases progressive pseudorheumatoid dysplasia and polyarticular juvenile idiopathic arthritis, both of which are accompanied by disorders in cartilage maintenance/homeostasis. The molecular mechanism of WISP-3 mediated effects in the sustenance of cartilage has not been described in detail. Our previous study illustrates the potential role of WISP-3 in regulating the expression of cartilage-specific molecules that sustain chondrocyte growth and cartilage integrity. The present study was conducted to investigate the mode of action of WISP-3 in greater detail. Experimental results depicted here suggest that WISP-3 can function as a ligand and signal via autocrine and/or paracrine modes upon being secreted by chondrocytes. Furthermore, apart from regulating collagen II and aggrecan expression, WISP-3 may also promote superoxide dismutase expression and activity in chondrocytes.

Keywords: Chondrocyte; Cartilage; WISP-3; CCN6; Arthritis; Dysplasia; Collagen II; Aggrecan; Superoxide-dismutase; Baculovirus

The WISP-3 (Wnt1 inducible secreted protein-3) gene product is a member of the WISP family of growth modulators (WISPs 1, 2, and 3) [1,2]. On account of sequence homology, the WISP gene products are considered to be members of the CCN (CTGF, connective tissue growth factor; Cyr61, cysteine rich 61; Nov, nephroblastoma overexpressed) family of connective tissue growth modulators [1–6]. CCN growth modulators are secreted proteins that regulate cell proliferation/survival, cell migration and adhesion, and cell differentiation in connective tissues [1–6]. The functional significance of the WISP proteins in connective tissues has not been clearly established at the molecular level.

WISP-3 maps to chromosome 6q21–22, codes for a 354-amino acid protein, and is expressed in cell types and tissues of skeletal origin [7]. Mutations in the WISP-3 gene have been reported to be associated with progressive pseudorheumatoid dysplasia (PPRD), a disease that involves cartilage growth plate defects, loss of articular cartilage, and skeletal dysplasia [7,8]. PPRD is manifested postnatally in the early years of adolescence. Recently, WISP-3 mutations have been reported in patients with polyarticular juvenile idiopathic arthritis (JIA), a different disease, but also presented with apparent defects in cartilage maintenance [9].

Radiographic assessments of WISP-3 gene mutation associated skeletal deformities suggest that these abnormalities could arise from poor maintenance and premature/untimely hypertrophy of cartilage during limb growth and maturation [7,8]. Although the mechanism of action of WISP-3 is unclear, data previously published from our laboratory [10] suggest that WISP-3 induces the expression of cartilage-specific molecules such as collagen II and aggrecan by chondrocytes, thus corroborating the concept that WISP-3 contributes to preserving the integrity of cartilage. Whether or not, however, WISP-3 is secreted by chondrocytes and functions independently as a ligand has not been investigated in these studies. Knowledge in this regard will not only be crucial for our understanding of the mechanism of action of WISP-3, but will also help address important questions about the potentially harmful

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effects of specific disease associated WISP-3 mutations. Thus, we confirmed that WISP-3 is a secreted protein in chondrocyte lines, and investigated the effect of pure recombinant WISP-3 protein as a ligand.

Quite interestingly, the expression pattern of WISP-3 we previously observed in fetal cartilage growth plates [10] is quite similar to the pattern of superoxide dismutase (SOD) activity as determined in cartilage growth plates by independent studies [11]. Superoxide dismutases are known to sustain tissue homeostasis under conditions of cellular hypertrophy and stress [12–14]. Perturbations in the regulation of cellular SOD activity during growth and development could thus be involved with premature hypertrophy and tissue damage/dysplasia. Accordingly, we evaluated the potential of recombinant WISP-3 to regulate SOD production and activity in chondrocytes.

In the current study, we report that (i) WISP-3 is secreted by chondrocyte lines and (ii) WISP-3 functions as a ligand not only in promoting collagen II/aggrecan/production, but also in enhancing SOD production and activity.

Materials and methods

Cell cultures. The immortalized chondrocyte cell line C-28I2 was derived from human juvenile costal cartilage and generated by infection with a replication defective retroviral vector expressing SV40 large T antigen [15]. Culture of C-28I2 cells was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 5% CO_2 incubator at 37 °C and passaged at subconfluency every 5–6 days.

Cell transfection. Lipofectamine (Invitrogen, CA) was used for transfecting C-28I2 cells with either WISP-3 expression vector or empty vector (pcDNA). The construction of WISP-3 expression vector has been explained in our previous work [10]. Briefly, cells (~2 times 10^5/ml) were plated 1 day before transfection in 6-well tissue culture plates (2 ml/well) and incubated at 37 °C in 5% CO_2. A complex of plasmid DNA (~1 μg) with 6 μl Lipofectamine in 200 μl of serum-free, antibiotic-free medium was prepared in a sterile microfuge tube for 30 min, after which 800 μl of medium containing ~5% FBS was added. A similar complex was prepared for each well of the 6-well plate. The cells in each plate were washed with sterile PBS and then incubated with 1 ml of the transfection mixture for 7–12 h, after which 1 ml of culture medium with ~5% FBS was added to each well. After 24 h, the transfection mixture was replaced with fresh culture medium containing 10% FBS. The incubation was continued for an additional 24–26 h, and the cells were harvested for either RNA or protein extraction.

Generation of pure recombinant WISP-3 protein and treatment of cell cultures with pure protein. A DNA fragment encoding a TEV (protease) site—FLAG-poly histidine fusion tag was engineered at the C-terminus of WISP-3 cDNA by PCR, and the entire DNA fragment was inserted into the baculovirus transfer vector pFastBac (Invitrogen CA). For creating the fusion tag, the primers 5′-GAAGAAGATATCGGCGCTGAGAA TCTTTATTTTCAGGGAAGCGGCGCTGACTACAAGGACGACGA TGACAAAAATTCAGATTATAAAGATGAT-3′ and 5′-TCAAAGCT CGAGTCAGCCTGGGTGATGGTGATGGTGATGGCTGCTTTTG TCATCATCATCTTTATAATCTGAATTTTT-3′ were first mixed, heated for a min at 95 °C, and annealed at 65 °C. Subsequently, PCR amplification of the tag was performed for 25 cycles with the primers 5′-GAAGAAGATATCGGCGCTGAGA-3′ and 5′-TCAAAGCTCGA GTCAGCCTGG-3′. The PCR product was gel purified, digested with *Eco*RV and *Xho*I, and inserted into the *Eco*RV and *Xho*I sites of the WISP-3-pcDNA expression vector [10]. The DNA fragment with the entire WISP-3-fusion tag was then excised out with *Eco*RI/*Xba*I and subcloned into the *Eco*RI and *Xba*I sites of pFastBac transfer vector. Competent DH10Bac *Escherichia coli* cells were transformed with WISP-3-pFastBac transfer vector. A single white colony was isolated from the plate and recombinant bacmid DNA was prepared. A monolayer of SF9 insect cells was transfected with the bacmid DNA and supernatant collected from the transfected cells was used to infect fresh SF9 cells. 72 h post-infection cells were harvested and the pellet was used to test the expression of WISP-3 by immunoblot. Infection of fresh cells with virus containing supernatant was carried on for several cycles in order to generate large amounts of viral particles. WISP-3 baculovirus was used at a multiplicity of infection (MOI) of 5 in culture flasks at a cell density of 2×10^6/ml. The cells were incubated with gentle stirring and harvested 72 h post-infection. Infected cells were centrifuged at 4 °C at 1200*g*. Both media and cell pellet were saved for WISP-3 purification.

Three grams of infected cells were resuspended in 50 ml lysis buffer M (20 mM Tris–HCl, pH 7.5, 10 μg/ml TPCK, 10 μg/ml TLCK, 5 μg/ml E64, 5 μg/ml leupeptin, and 7 μg/ml PMSF) supplemented with 150 mM NaCl. The cell suspension was sonicated on ice for 30 s in pulsed mode with 50% duty cycle. The extract was incubated on ice for 30 min and then centrifuged at 10,000*g* at 4 °C for 45 min. The supernatant was collected and was loaded on a 2 ml Ni^{2+} affinity column. The column was extensively washed in 20 mM Tris–HCl, pH 7.5, 150 mM NaCl buffer supplemented with 10 mM imidazole. Protein was eluted with 250 mM imidazole in 20 mM Tris–HCl, pH 7.4, 150 mM NaCl buffer. Imidazole was removed by buffer exchange with phosphate-buffered saline (PBS). The protein yield was about 500 μg/3 g of infected cells. The media collected from the infected cells was concentrated by precipitation with ammonium sulfate (70% saturation). The precipitate was dissolved in 20 mM Tris, pH 7.5, and 150 mM NaCl, dialyzed against the same buffer, and loaded onto a 1 ml Ni^{2+} column. After loading, the column was washed exhaustively and WISP-3 was eluted with 250 mM imidazole. Imidazole was removed by buffer exchange with PBS. About 5% of total WISP-3 protein expressed was secreted to media and therefore, amount of WISP-3 obtained by single step purification was low (~100 μg/L media). Because of this problem further purification could not be done to remove other minor impurities. Recombinant WISP-3 obtained from soluble cell extract and supernatant was used on fresh cultured cells (60–80% confluence) at assay concentrations of 1 μg/ml and 400 ng/ml, respectively. Cells were harvested for assays 24–26 h after protein addition.

RNA extraction and mRNA analysis. C-28I2 cells, either transfected with WISP-3/pcDNA or treated with recombinant WISP-3 protein or control buffer, were used for RNA extraction for m-RNA analysis. Total RNA was extracted using TRIzol (Invitrogen). Primers specific for type II collagen (COL2A1), aggrecan, SOD1 (Cu/Zn-SOD), SOD2 (Mn-SOD), and fibronectin were used for estimating the levels of expression of the corresponding m-RNA. Reverse transcriptase-PCR (RT-PCR) was performed using a reverse transcription kit following instructions provided by the manufacturer (Invitrogen). During cDNA synthesis, ~1 μg RNA was used for each specimen and 28 cycles of PCR were carried out. Table 1 summarizes the primer pairs and experimental conditions used for RT-PCR analysis.

Preparation of whole cell protein lysates, immunoblotting, and collection and concentration of cell supernatant. To prepare whole cell lysates for immunoblot analysis, cells were lightly scraped off 6-well plates with a cell scraper, harvested to sufficient density, and disrupted with lysis buffer (20 mM Tris–HCl, pH 7.5, 500 mM NaCl, 1% Triton X-100, 1 mM EDTA, 50 mM dithiothreitol, and 2 mM phenylmethylsulfonyl fluoride) in a microfuge tube. The lysate was spun down, cleared of debris by centrifugation, and assayed for total protein concentration using Bradford reagent (Bio-Rad, Richmond, CA). About 20 μg of each lysate was used for immunoblotting. Supernatant was collected before preparing the cells for lysis and concentrated at least 4-fold with centricon-30 (Millipore, USA). About 40 μg of concentrated supernatant was used for immunoblotting.

Polyclonal anti-human aggrecan antibody (R&D Systems, CA) was used as the primary antibody to detect aggrecan, polyclonal anti-human SOD1 antibody (Chemicon International, USA) was used as the primary antibody to detect SOD1, monoclonal anti-human actin antibody (Santa

Table 1
Primer pairs and experimental conditions

Gene product	Forward and reverse primers (5′–3′)	PCR conditions	Product size (bp)
COL2A1	GAAACCATCAATGGTGGCTTCC&CGATAACAGTCTTGCCCCACTT	28 cycles, 62 °C annealing	301
Aggrecan	TGAGGAGGGCTGGAACAAGTACC&GGAGGTGGTAATTGCAGGGAACA	28 cycles, 62 °C annealing	349
Fibronectin	GTGTGACCCTCATGAGGCAAC&TACTCTCGGGAATCTTCTCTGT	28 cycles, 62 °C annealing	299
SOD1	CCAGTGCAGGTCCTCACTTTA&TATTGGGCGATCCCAATTACAC	28 cycles, 62 °C annealing	288
SOD2	TGTTGGTGTCCAAGGCTCAG&TTTACTTTTTGCAAGCCATGTATC	28 cycles, 62 °C annealing	253
WISP-3/bgh	TGGCACAGTTCTGCTGCAGGG&AACTAGAAGGCACAGTCGAGG	28 cycles, 62 °C annealing	1000

Cruz, CA) was used as a primary antibody to detect actin, and polyclonal anti-human WISP-3 antibody (Santa Cruz) was used as primary antibody to detect WISP-3. The secondary antibody for each of the above-mentioned antibodies was either HRP-conjugated anti-goat, anti-rabbit, or anti-mouse IgG (Santa Cruz, CA). Secondary antibody incubation of protein blots was followed by visualization with a chemiluminescence system (Amersham Pharmacia Biotech, Piscataway. NJ).

Superoxide dismutase activity analysis. Superoxide dismutase (SOD) activity analysis of cells either transfected with WISP-3/empty vector, or treated with recombinant WISP-3/buffer was performed using SOD activity analysis kit (Cayman Chemical, USA), following instructions provided by the manufacturer. Briefly, cells to be assayed were scraped off plates, collected by centrifugation, and homogenized in cold 20 mM Hepes buffer (pH 7.2), containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose. After centrifugation at 1500*g* for about 5 min, the supernatant was removed for SOD assay. About 12 μg of sample was used for SOD assay. SOD unit calculation of each sample was based on % dismutation of superoxide radicals generated by xanthine oxidase and hypoxanthine upon addition of the sample. A tetrazolium salt was used as the superoxide radical detector.

Image analysis and statistical analysis. All reported results of RT-PCR analyses of RNA and immunoblotting analyses of protein represent at least four separate experiments. Differences in m-RNA and protein levels were quantified using Kodak Image Analysis Software, version 2.0.2 for Macintosh. Statistical analysis was performed with Student's *t* test.

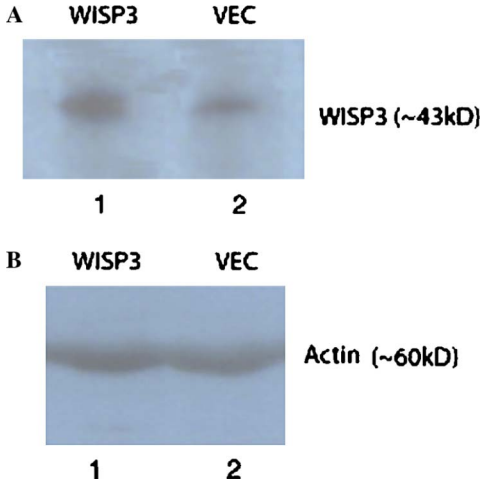

Fig. 1. Secretion of WISP-3 by C28I2 chondrocyte line. (A) Immunoblot analysis of concentrated supernatant collected from WISP-3-pcDNA and empty vector (pcDNA) transfected C28I2 cells with anti-WISP-3 antibody. (B) Immunoblot analysis of the corresponding cell extracts with anti-actin antibody. WISP-3 transfected cells secrete about 5-fold more WISP-3 protein than the corresponding empty vector transfected cells (A, lane 1 vs. lane 2), and both WISP-3 and empty vector transfected cells express similar levels of actin (B, lane 1 vs. lane 2) ($P < 0.05$).

Results

WISP-3 is secreted from chondrocytes

In order to elucidate the mechanism of WISP-3 function in chondrocytes in greater detail, we first investigated if WISP-3 functions via autocrine/paracrine modes upon being secreted. For our experimental purposes, we used the chondrocyte cell line C28I2. WISP-3 expression in C28I2 cells was promoted by transfection with a WISP-3 expression vector. In parallel, similar transfection with empty vector (pcDNA) was also performed as a control. Supernatant was collected 48 h post-transfection from both WISP-3 transfected and empty vector transfected cells separately, and the concentrated cell supernatant samples were subjected to immunoblot analysis with anti-WISP-3 antibody. We found that not only did C28I2 cells secrete WISP-3, but also WISP-3 transfected cells (overexpressing WISP-3) secreted considerably more WISP-3 protein (Fig. 1A, lane 1) than the corresponding empty vector transfected cells (Fig. 1A, lane 2). Immunoblot analysis of the corresponding cell extracts demonstrated that both WISP-3 and empty vector transfected cells express similar levels of the housekeeping protein actin (Fig. 1B). These results suggest that WISP-3 can function as a ligand upon being secreted by chondrocytes.

Purification of recombinant WISP-3

To characterize WISP-3 as a ligand, it is important to examine the effect of pure WISP-3 protein on cells. We thus generated pure recombinant WISP-3 by expressing WISP-3 in WISP-3 baculovirus infected SF9 insect cells. For detection and purification purposes, we attached a poly-histidine and FLAG fusion peptide at the C-terminus of WISP-3. Recombinant WISP-3 was purified from both the media collected from infected cells and the infected cell pellet (Figs. 2A and B) by Ni affinity chromatography. Fig. 2A depicts purity analysis of the protein prepared from both soluble cell extract (lane 2) and medium (lane 3) by Coomassie stain. Fig. 2A, lane 1 depicts the protein molecular weight marker. As demonstrated in Fig. 2B, the authenticity of the recombinant WISP-3 protein was confirmed by immunoblotting with anti-WISP-3 antibody (Santa Cruz, CA). Due to the low yield of total protein, it was difficult to remove all impurities. We are hopeful that better

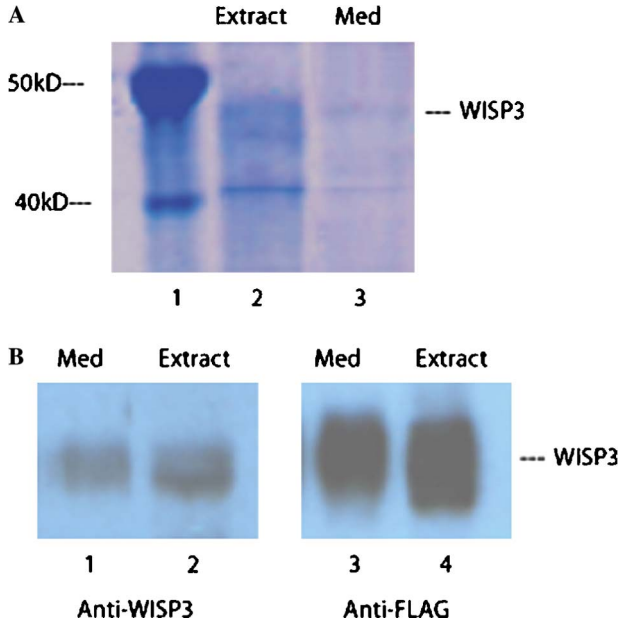

Fig. 2. Purification of recombinant WISP-3 by Ni affinity chromatography. (A) Coomassie-stained gel demonstrating purity of recombinant WISP-3 (~43 kDa) prepared from both soluble cell extract of WISP-3-baculovirus infected SF9 cells (lane 2) and the supernatant (medium) collected from similarly infected cells (lane 3). Lane 1 depicts the protein molecular weight marker. (B) Immunoblot of purified protein with anti-WISP-3 antibody (lanes 1 and 2) and anti-FLAG antibody (lanes 3 and 4).

optimization of cell infection, growth, and purification procedures will increase protein yield.

Effect of recombinant WISP-3 on C28I2 chondrocyte cells

We previously demonstrated WISP-3-induced expression of cartilage integrity-specific factors such as collagen II and aggrecan in WISP-3 expressing C28I2 cells. In order to examine the potential of WISP-3 as a ligand, we thus tested the effect of pure recombinant WISP-3 on collagen and aggrecan production in the C28I2 chondrocyte line. As demonstrated in Fig. 3, recombinant WISP-3 protein, when added to C28I2 cells at an assay concentration of 1 μg/ml, increased both collagen II and aggrecan production considerably. WISP-3 (E) treated cells expressed about 4-fold more aggrecan and collagen II (COL2A1) mRNA (Fig. 3A, lanes 2 and 4, respectively) than the corresponding PBS (C) treated cells (lanes 3 and 5, respectively). In contrast, fibronectin mRNA level did not vary significantly between WISP-3 protein and PBS treated cells (Fig. 3A, lanes 6 and 7), indicating that the effect of WISP-3 is mostly on chondrocyte/cartilage specific molecules expressed by the C28I2 cells. Fig. 3B shows that WISP-3 treated cells express at least 3-fold more aggrecan protein (lane 1) than PBS treated cells (lane 2). Both WISP-3 treated and PBS treated cells expressed similar levels of the housekeeping protein actin (Fig. 3C, lanes 1 and 2, respectively). Thus, recombinant WISP-3 protein-induced effects were comparable to those induced by WISP-3 transfection, as seen previously [10]. Because the yield of pure recombinant protein

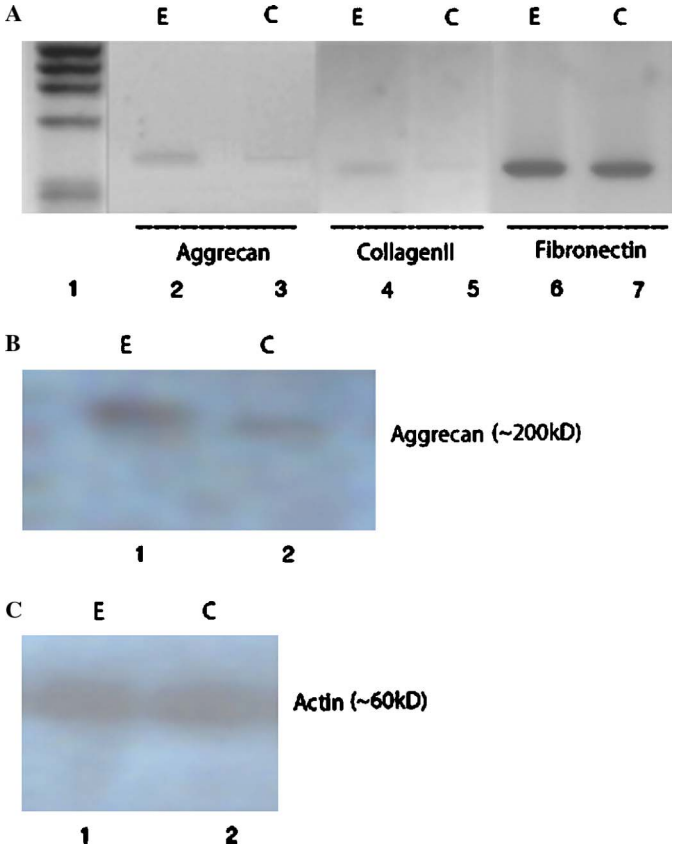

Fig. 3. WISP-3 protein mediated upregulation of collagen II (COL2A1) and aggrecan synthesis in C28I2 cells. (A) Semiquantitative RT-PCR analysis demonstrating that WISP-3 protein treated cells (E) expressed ~4-fold more COL2A1 and aggrecan m-RNA (lanes 2 and 4, respectively) than the corresponding PBS treated cells (C) (lanes 3 and 5, respectively). Fibronectin m-RNA levels did not vary significantly between WISP-3 protein and PBS treated cells (lanes 6 and 7, respectively) ($P < 0.05$). Lane 1 depicts the PhiX174 DNA standard. (B) Immunoblot analysis with anti-aggrecan antibody demonstrating that WISP-3 protein treated cells expressed ~3-fold more aggrecan protein (lane 1) than the corresponding PBS treated cells (lane 2) ($P < 0.05$). (C) Immunoblot analysis depicting similar levels of actin protein in the WISP-3 protein and PBS treated cells (lanes 1 and 2, respectively).

from cell supernatant was low, all the assays represented here involving collagen II and aggrecan expression were performed with pure WISP-3 protein obtained from the soluble pellet of infected cells. Changes in the mRNA levels of collagen II and aggrecan by cell supernatant purified WISP-3 (assay concentration, 450 ng/ml) were quite similar to those produced by soluble extract purified WISP-3 (data not shown).

WISP-3 promotes SOD production and SOD activity in chondrocytes

The question whether WISP-3 promotes SOD expression/activity was not addressed in our previous study. In order to assess if WISP-3 promoted SOD production and activity, we examined SOD expression and activity in C28I2 cells treated with recombinant WISP-3 protein. We focused on the SOD isoforms SOD1 and SOD2, both of

which are prevalent in cartilage and chondrocytes. The results obtained from recombinant protein treated cells were similar to those obtained with cells transfected with WISP-3 expression vector. Although less robust than the effect on collagen II and aggrecan, there was clearly an effect of WISP-3 protein on upregulation of SOD expression. As demonstrated in Fig. 4A, addition of recombinant WISP-3 (soluble extract purified: E) at an assay concentration of 1 μg/ml to C28I2 cells promoted both SOD1 and SOD2 expression at the m-RNA level, when compared to PBS (C) treated cells (lanes 2 vs. 3, and lanes 4 vs. 5).

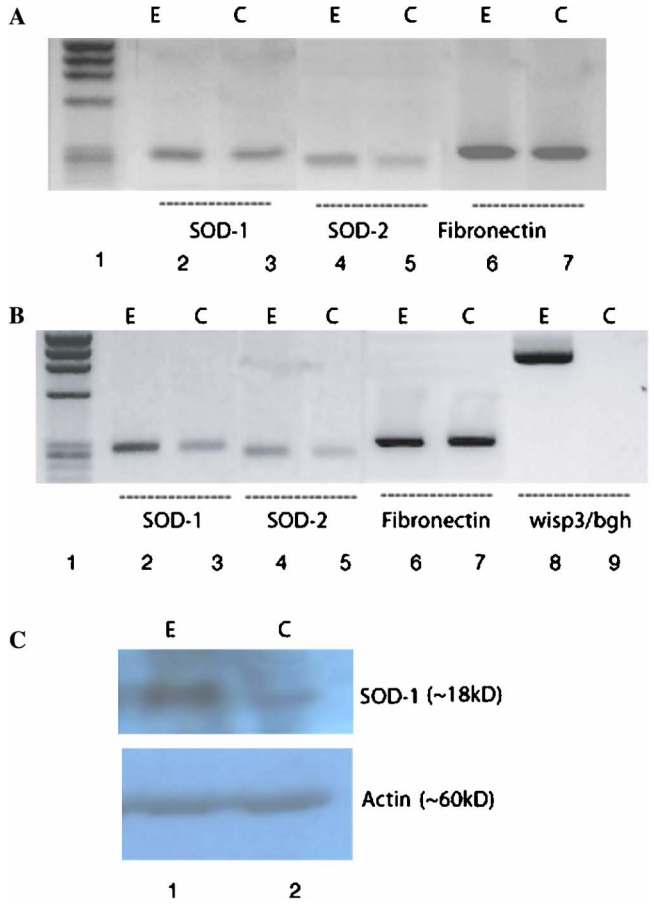

Fig. 4. WISP-3 mediated upregulation of SOD expression in C28I2 cells. (A) Semiquantitative RT-PCR analysis demonstrating ~1.6-fold more SOD-1 and ~3-fold more SOD-2 m-RNA in WISP-3 protein treated cells (lanes 2 and 4, respectively) than in the corresponding PBS treated cells (lanes 3 and 5, respectively). Fibronectin m-RNA level did not vary significantly between WISP-3 protein and PBS treated cells (lanes 6 and 7 respectively) ($P < 0.05$). (B) Similar RT-PCR analysis demonstrating ~4-fold more SOD-1 and SOD-2 m-RNA in WISP-3-pcDNA transfected cells (lanes 2 and 4, respectively) than in the corresponding empty vector (pcDNA) transfected cells (lanes 3 and 5, respectively). Both WISP-3 and empty vector transfected cells expressed similar levels of fibronectin m-RNA (lanes 6 and 7, respectively) ($P < 0.05$). Lanes 8 and 9 demonstrate that WISP-3 m-RNA transcribed off the WISP-3 expression vector is expressed in the WISP-3 transfected cells (E), but not in the empty vector transfected cells (C). (C) Immunoblot analysis demonstrating that WISP-3 protein treated cells express ~3-fold more SOD-1 protein (lane 1) than the corresponding PBS treated cells (lane 2). Both WISP-3 protein and PBS treated cells express similar levels of the housekeeping protein actin (lanes 1 and 2, respectively) ($P < 0.05$).

Fibronectin mRNA levels of WISP-3 and PBS treated cells were similar (Fig. 4A, lanes 6 and 7). Fig. 4B, likewise, demonstrates that SOD1 and 2 m-RNA are also upregulated 4- to 5-fold in WISP-3 (E) transfected cells (lanes 2 and 4, respectively) than the corresponding empty vector (C) transfected cells (lanes 3 and 5, respectively). Both WISP-3 and empty vector transfected cells expressed similar levels of fibronectin mRNA (lanes 6 and 7, respectively). Fig. 4C furthermore demonstrates that production of SOD1 protein is increased about 3-fold in cells treated with recombinant WISP-3 protein (lane 1), when compared to PBS treated cells (lane 2). Both WISP-3 and PBS treated cells expressed similar levels of the housekeeping protein actin (Fig. 4C, lanes 1 and 2, respectively).

As demonstrated in Fig. 5, both transfection of C28I2 cells with WISP-3 expression vector and addition of pure recombinant WISP-3 protein to C28I2 cell cultures upregulated SOD activity which should represent the total activity generated by all functional SOD isoforms. Soluble extract purified WISP-3 protein (1 μg/ml) and cell supernatant purified WISP-3 protein (450 ng/ml) produced 2.5- and 3.5-fold difference, respectively, in total cellular SOD activity when compared to PBS. SOD activity also increased over 3-fold upon transfection of C28I2 cells by WISP-3 expression vector. The effects of WISP-3 signaling on SOD expression and activity could either overlap or be independent of each other. SOD activity depends not just on SOD expression, but also on the intracellular availability of metal cofactors (such as Zn/Cu/Mn) and the corresponding protein chaperones (such as the Cu chaperone), which contribute to making SOD enzymes functional [16,17]. WISP-3 induced enhancement in SOD activity could thus be the result of a combined effect on both SOD expression and the availability of SOD associated cofactors/chaperones that are crucial for SOD function. The influence of WISP-3 signaling on the different components controlling SOD activity will thus depend on their

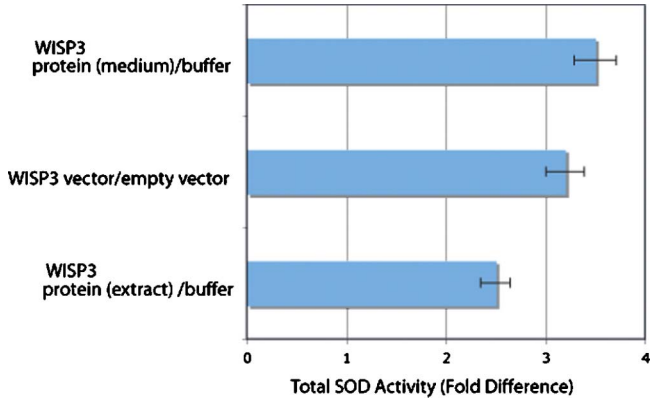

Fig. 5. WISP-3 mediated upregulation of SOD activity in C28I2 cells. SOD activity assay performed with Cayman Chemical SOD assay kit demonstrates that treatment of cells with WISP-3 protein increases total SOD activity by 2.5- to 3.5-fold, when compared to treatment with PBS, and WISP-3 transfection increases SOD activity ~3.3-fold when compared to empty vector transfection ($P < 0.05$).

relative stoichiometry, which may vary between different cell types, their various stages of differentiation, and different environmental conditions.

Discussion

WISP-3 (CCN6) is a member of the CCN family proteins. The CCN proteins are multifunctional growth factors that are important for skeletal growth and development [1–6]. CTGF (CCN1) plays an important role in the maturation and hypertrophy of growth cartilage cells [18]. Cyr61 (CCN2) has been shown to promote chondrogenesis in the mouse limb bud [19]. Experimental evidence suggests that Cyr61 and NOV (CCN3) could also promote angiogenesis [20,21]. The binding of WISP-1 (CCN4) to small leucine-rich cartilaginous proteoglycans [22] suggests that it is an important component of cartilage, but it is not clear if such interactions promote connective tissue growth and maintenance. The role of WISP-2 (CCN5) in development has not been elucidated in detail.

WISP-3 mutations have been associated with diseases such as PPRD and JIA, which involve skeletal malformations and dysfunctions [7,9]. Radiographs of the joints of PPRD patients carrying mutations in WISP-3 have depicted defects in the cartilage growth plate, enlargement of the femoral and tibial epiphysis, and narrowing of joint spaces with irregular articular surfaces [7,8]. JIA, particularly JIA with a polyarticular course clinically mimics PPRD. The N-terminal WISP-3 mutation associated with JIA could potentially block WISP-3 secretion and thus abolish its functional consequences [9].

Our previous studies demonstrating the expression of WISP-3 in the proliferating chondrocytes of fetal growth plates and the WISP-3 mediated regulation of collagen II and aggrecan corroborate the concept that WISP-3 plays a major role in cartilage growth and maintenance. Defects in WISP-3 function may thus facilitate the closure of cartilage growth plates and promote skeletal defects [23]. The precise mechanism of WISP-3 function during postnatal cartilage/bone growth and homeostasis, however, is not clear yet.

Our current study addresses the mode of action of WISP-3 and provides additional information with regard to its importance during cartilage growth and maintenance. We demonstrate that WISP-3 is secreted from chondrocyte lines. Furthermore, experimental observations depicted here using pure recombinant WISP-3 suggest that secreted WISP-3 can function as a ligand, signaling via autocrine/paracrine modes. Apart from regulating collagen II and aggrecan expression. WISP-3 also promotes SOD expression and activity in C28I2 chondrocytes. Accordingly, it is conceivable that WISP-3 contributes to cartilage growth and maintenance at least partly by regulating SOD expression and activity in chondrocytes. Thus, the different potentially useful aspects of WISP-3 mediated regulation render WISP-3 an important structural component of cartilage.

There is still more to learn about the mechanism of action of WISP-3. Based on its homology with other CCN family members, it is likely that WISP-3 can signal upon binding to integrins [3]. WISP-3 could also function synergistically with IGF-1 by virtue of its IGF-1 binding motif [7,10]. Our previous work and separate studies on breast cancer cells suggest such a scenario [10,24]. Future dose–response studies with recombinant WISP-3 protein should highlight the potency of WISP-3 as a ligand in chondrocyte gene regulation. We previously reported SOX9 as a potential transcriptional regulator of WISP-3 mediated collagen II and aggrecan production [10]. The multimodular architecture of WISP-3 suggests that it could interact with multiple proteins. Thus WISP-3 could impart its activity as a ligand also as an extracellular matrix associated protein. More detailed mechanistic approaches will yield better knowledge regarding the overlap/divergence of WISP-3 mediated signaling pathways that regulate collagen II/aggrecan/SOD production and SOD activity.

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