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Potential role of WISP3 (CCN6) in regulating the accumulation of reactive oxygen species

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Abstract

Several mutations and atypical splice variants of WISP3 (CCN6) have been linked to connective tissue disorders and different forms of malignancies. Functional studies have suggested that WISP3 contributes to tissue maintenance/homeostasis. The precise molecular mechanism of WISP3 function in different cell types, however, remains unresolved. The present study was conducted to investigate the potential impact of WISP3 on the accumulation of reactive oxygen species (ROS) and oxidative stress, which are central to cell/tissue maintenance. Our experimental results suggest that WISP3 regulates the accumulation of cellular ROS, and mutations in WISP3 or loss of expression of WISP3 compromise this function.

Keywords: WISP3; CCN6; Connective tissue; Skeletal dysplasia; ROS; Nitration; Carbonylation

WISP3 (Wnt induced secreted protein 3)/CCN6 is a member of the CCN (CTGF, CYR61, and NOV) family of connective tissue growth factors. The CCNs are secreted proteins that regulate cell proliferation/survival, cell migration, and cell differentiation [1–3]. WISP3 is expressed in most cells of mesenchymal and endothelial lineage. As in the other CCNs, each domain of WISP3 is potentially functional [4]. It is thus not surprising that disease associated mutations of WISP3 spread across all of its potentially functional domains [4–6].

Several mutations in the WISP3 coding sequence are associated with tissue dysplasia and hypertrophy as are evident in progressive pseudorheumatoid dysplasia (PPRD) and juvenile idiopathic arthritis (JIA) [4–6]. In recent years, loss of WISP3 expression and incidences of WISP3 truncations and/or atypical splice variants have been reported in several cancers, such as inflammatory breast cancer and colon cancer [7–9]. Some functional studies have accordingly suggested that WISP3 contributes to tissue maintenance/homeostasis [10,11].

WISP3 promotes cartilage specific gene expression and superoxide dismutase (SOD) like activity among other functions [10,11]. SODs sustain tissue homeostasis under conditions of stress. Perturbation in the regulation of SOD activity correlates with the accumulation of reactive oxygen species (ROS) [12–16]. Accumulated ROS react with proteins, DNA, lipids, and carbohydrates, potentially altering/disrupting normal cellular physiology in different tissues [16,17]. Since cellular hypertrophy/inflammation and skeletal malformations could be promoted by unregulated accumulation of ROS, we have further investigated (i) if WISP3 contributes to the regulation of cellular ROS levels, and (ii) if disease associated WISP3 mutations or loss of WISP3 expression compromise this function.

Materials and methods

Cell cultures. The C28I2 chondrocyte line [10] was maintained in DMEM (Invitrogen, CA) supplemented with 10% FBS in a 5% CO_2 incubator at 37 °C and passaged at subconfluency. Human umbilical vein

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endothelial cells (HUVEC) were obtained from Cambrex (MD), maintained in culture medium provided by Cambrex, and split at subconfluency for ~6 passages.

Expression constructs and transfection. C28I2 cells were transfected with either wild-type/mutant WISP3 expression vectors (WISP3, trp331stop, and cys78Arg), or WISP3 siRNA. The empty vector pcDNA 3.1 or scramble RNA was used as corresponding controls. The construction of the WISP3 and Cys78Arg expression vectors has been explained [10]. Trp331stop was constructed by PCR mutagenesis using wild-type WISP3 as a template. The primers used were 5′-GGAATGA ATTCGTCCCAGCGACATGCAGGG-3′ (forward) and 5′-GCAATG ATATCTCACAGCATCTTCCATTTAAATGAC-3′ (reverse), with *Eco*R1 and *Eco*RV sites, respectively, engineered into the flanking ends. The amplified segment was subcloned into the *Eco*R1 and *Eco*RV sites of the pcDNA 3.1 plasmid. For protein expression studies, a Flag-poly His tag [16] was introduced in frame with either the wild-type WISP3 or the Trp331stop mutant.

Cell transfections were based on previously published procedures [10,11]. Approximately 24 h of post transfection, cells were maintained in 1–2% serum medium for another 24 h, after which they were harvested for either RNA/protein extraction or NBT assay. C28I2 cells were transfected with either 100 nM WISP3 siRNA or scramble RNA (Dharmacon, CO) following a similar procedure.

RNA extraction and mRNA analysis. Total RNA from transfected cells was extracted using TRIzol (Invitrogen, CA). Primers specific for wild-type or mutant WISP3, β-gal, and β-actin were used for estimating the levels of expression of the corresponding mRNAs by RT-PCR (Invitrogen). Table 1 summarizes the experimental conditions.

Preparation of whole cell protein lysates, immunoblot/oxyblot analysis and nitroblue tetrazolium chloride (NBT) assay. Cells transfected with either WISP3 expression vectors/empty vector or WISP3 siRNA/scramble RNA were disrupted with lysis buffer [10,11]. Fifteen to twenty micrograms of each lysate was used for immunoblotting with anti-WISP3 antibody (Santa Cruz, CA), anti-β-actin and anti-Flag antibodies (Sigma, MO) and anti-nitrotyrosine antibody (Chemicon, CA). For oxyblot analysis, ~15 μg of each cell lysate was treated with dinitrophenyl hydrazine (DNPH) and analyzed with anti-DNPH antibody using the Oxyblot Kit (Chemicon). For the nitrotyrosine immunoblot analyses, antinitrotyrosine antibody preincubated with nitrotyrosine (10 mM) was used as a negative control and nitrotyrosine BSA (~100 ng/ml) was used as a positive control. For oxyblot analysis, each protein lysate was treated with a control solution provided in the kit, as a negative control, and oxidized BSA (Oxyblot kit manual) was used as a positive control.

NBT assay was performed following published procedures [18,19] with some modifications. About 48 h post transfection, or 24 h post WISP3 protein addition, 0.1% NBT (Sigma) in PBS was added to transfected cells either pretreated for 15–20 min with 0.4 μM diphenylene iodonium (DPI) (Sigma) or untreated, and incubation was carried on for ~50 min in a tissue culture incubator. Subsequently, the culture medium was aspirated, cells from each well were scraped off in PBS into a microfuge tube and centrifuged at 2000–3000 rpm. Finally, each blue/purple pellet was mixed in 200 μl of 65% acetic acid by sonication and the absorbance at 560 nm was determined. A_{560} reflected the extent of formazan production/NBT reduction and was used as a rough estimate of ROS.

Generation of pure recombinant WISP3 protein and treatment of cell cultures with pure protein. The generation of baculovurus WISP3 construct and recombinant WISP3 protein has been described previously [11]. Competent *Escherichia coli* cells were transformed with WISP3-pFastBac transfer vector to isolate Bacmid DNA, with which SF9 cells were infected to generate virus. The media collected from the virus-infected cells were saved for WISP3 purification. Recombinant WISP3 protein was added to fresh cultured cells (60–80% confluence) at an assay concentration of 400 ng/ml, and incubation continued for 24 h in a tissue culture incubator before harvesting cells for assays. A low serum concentration (1–2%) was maintained during WISP3 protein addition to C28I2. HUVEC was maintained in defined medium provided by Cambrex.

Image and statistical analyses. All documented results represent at least four separate experiments. Differences in mRNA and protein levels were quantified using Image Quant TL v2003.02 (Amersham Biosciences), respectively. Statistical analysis was performed with Student's *t* test.

Results

Reduction in endogenous WISP3 protein expression increases the levels of cellular reactive oxygen species (ROS)

Previous studies on the functional significance of WISP3 suggest that WISP3 contributes to connective tissue homeostasis. Thus we investigated the role of WISP3 in the regulation of oxidative stress, a central feature of tissue maintenance/homeostasis [13–17,20–22]. Accordingly, we determined whether inhibition of endogenous WISP3 expression had any effect on cellular ROS levels. Expression of WISP3 was inhibited in the chondrocyte line C28I2 by siRNA transfection. Subsequently, the extent of NBT reduction and the levels of total protein carbonyl and nitrotyrosine were assessed in WISP3 siRNA and scramble (control) RNA transfected cells, as a measure of cellular ROS levels [18–22].

Immunoblotting with anti WISP3 antibody confirmed WISP3 siRNA mediated disruption in WISP3 protein expression. WISP3 siRNA transfected cells expressed significantly less WISP3 protein than the control cells (Fig. 1A, lanes 1 vs. 2), but similar level of the housekeeping protein β-actin (lanes 3 vs. 4).

Reduction in WISP3 expression was accompanied by an increase in both total protein carbonylation and tyrosine–nitration, as determined by oxyblot analysis and immunoblot analysis with anti-nitrotyrosine antibody (Fig. 1B, lanes 1 vs. 2 and 5 vs. 6, respectively). The authenticity of the antibodies used to assess total protein carbonylation and tyrosine–nitration was verified by appropriate negative and positive controls (oxyblot: lanes 3 vs. 4 and -NO2-tyr blot: lanes 7 vs. 8, respectively). In addition, NBT assay (Fig. 1C) demonstrated that WISP3 siRNA transfected cells reduced ~33% more NBT than the corresponding control cells. The extent of NBT reduction was similar to that induced by ionomycin treatment, and inhibited by DPI, an inhibitor of flavoprotein enzymes such as

Table 1
Primer pairs and experimental conditions

Gene product	Forward and reverse primers (5′–3′)	PCR conditions
β-Gal (Fig. 3)	GATCTGCCATTGTCAGACATGTA and ACACCAGACCAACTGGTAATGG	28 cycles, 62° annealing
Wsp3/bgh (Fig. 3)	AGCAACTGTGAAATGAGAAAAGAG and AACTAGAAGGCACAGTCGAGG	28 cycles, 62° annealing

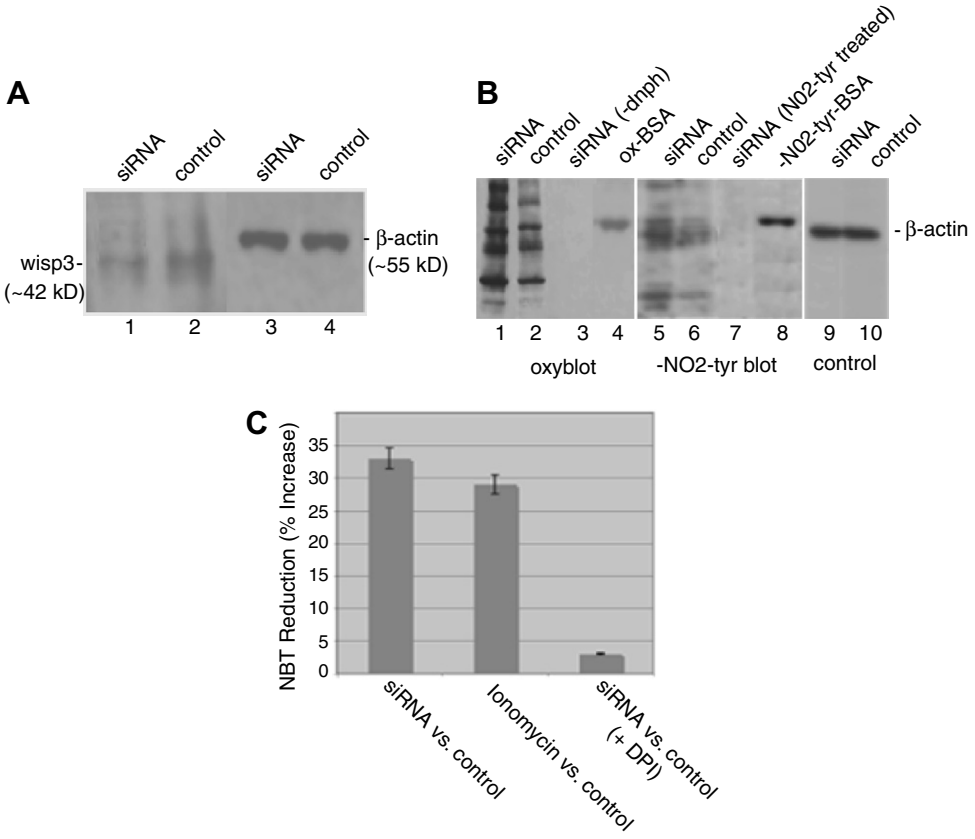

Fig. 1. siRNA mediated disruption of WISP3 expression and assessment of ROS level by oxyblot/immunoblot analysis and NBT assay. (A) Immunoblot analysis demonstrates ~3-fold reduction in WISP3 protein (lanes 1 vs. 2), but similar levels of β-actin (lanes 3 vs. 4), in siRNA transfected vs. control cells ($P < 0.05$). (B) Oxyblot analysis with anti-DNPH antibody demonstrates 2.5- to 3-fold more protein carbonylation in WISP3 siRNA transfected cells compared to control cells (lanes 1 vs. 2) ($P < 0.05$). The anti DNPH antibody does not interact with DNPH untreated cell lysate (lane 3, negative control), but binds to DNPH treated oxidized BSA (lane 4, positive control). Immunoblot analysis with anti-nitrotyrosine antibody demonstrates similar difference in total protein tyrosine nitration between siRNA and control (scramble) RNA transfected cells (lanes 5 and 6) ($P < 0.05$). No bands are detected when the antinitrotyrosine antibody is preincubated with nitrotyrosine substrate (lane 7, negative control), The antibody binds to nitrotyrosine–BSA (lane 8, positive control). Similar levels of β-actin are present in both siRNA and control RNA transfected cells (lanes 9 and 10) ($P < 0.05$). (C) NBT dye assay demonstrating ~33% increase in NBT reduction by WISP3 siRNA transfected cells over control cells. The increase is similar to that induced by ionomycin (0.2 μM) treatment of C28I2 cells and is largely inhibited by diphenylene iodonium chloride (DPI, 0.4 μM) ($P < 0.05$).

NAD(P)H oxidase [23]. These experimental findings corroborate the concept that WISP3 contributes to limiting physiological levels of cellular ROS.

Application of recombinant WISP3 protein reduces cellular ROS

Earlier observations have suggested that WISP3 can function as a ligand [11]. Thus we were interested in investigating if administration of recombinant WISP3 protein attenuates the accumulation of ROS that occurs naturally in cells under in vitro culture conditions. Accordingly, recombinant WISP3 protein was purified from the culture medium of WISP3-baculovirus infected insect cells, and NBT assays were performed on both WISP3 protein treated and corresponding PBS (control) treated cells in order to compare cellular ROS levels. Both C28I2 chondrocytes and HUVECs were included in this analysis. Treatment

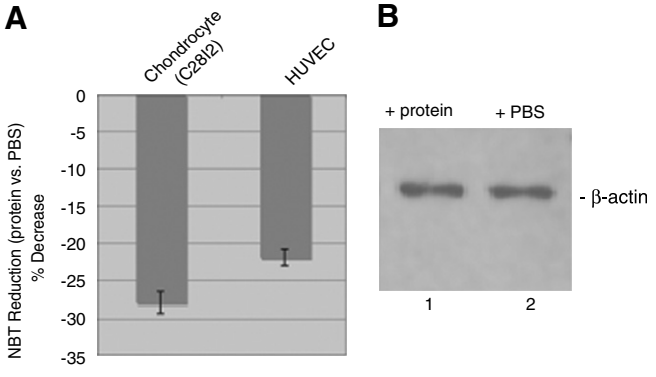

Fig. 2. Reduction in cellular ROS upon application of recombinant WISP3 protein. (A) NBT assay on both protein and PBS (control) treated C28I2 cells and HUVEC (passage 6), demonstrating about 28% and 22% decrease respectively, in NBT reduction, upon protein treatment when compared to PBS treatment ($P < 0.05$). (B) Immunoblot analysis showing similar levels of β-actin in both WISP3 protein and PBS (control) treated cells ($P < 0.05$).

of cells for about 24 h with ~400 ng/ml of soluble WISP3 protein attenuated NBT reduction by 20–28%, when compared to added PBS (Fig. 2A), implying that exogenous WISP3 can promote reduction in basal ROS levels. This experimental finding was not a reflection of reduced cell number, because no significant changes in cell number were noted under our assay conditions (data not shown). Furthermore, levels of the housekeeping protein β-actin also did not change significantly between WISP3 protein treated and PBS (control) treated cells (Fig. 2B). Difficulties in obtaining sufficient quantities of the pure WISP3 protein precluded a protein dose response study in the context of oxidative stress promoting agents.

WISP3 mutants are associated with high cellular ROS levels

Both point and truncation mutations within the WISP3 coding sequence have been linked with different pathogenic conditions in humans [4–8]. In the light of a possible link between heightened ROS accumulation and the progression of pathogenesis, it was of considerable interest to explore if some of the WISP3 mutants do in fact promote ROS accumulation in cells. Two different WISP3 mutants, WISP3 (Cys78Arg), and WISP3 (Trp331stop) were accordingly chosen and their impact on ROS accumulation compared with that of wild-type WISP3. C28I2 cells were transfected with expression vectors encoding either a mutant version of WISP3 (Cys78Arg or Trp331stop) or wild-type WISP3, and transfected cells were subsequently analyzed for assessing relative ROS levels. Since none of the mutants under investigation was documented to have a dominant phenotype, we rationalized that any potential increase in ROS accumulation in C28I2 cells expressing endogenous WISP3 protein would be contingent upon the overexpression of a gain-of-function mutation. RT-PCR analysis (Fig. 3A) demonstrated that both wild-type WISP3 and mutant WISP3 transfected cells expressed the corresponding wisp3/bgh PCR product, this being absent in the empty vector (control) transfected cells (lanes 2–5). Furthermore, all experimental and control cells were transfected with similar efficiencies (lanes 6–9) as depicted by similar levels of the β-galactosidase gene product corresponding to the pCMV-β-galactosidase plasmid that was used for cotransfection. NBT reduction assay (Fig. 3B) demonstrated that the Trp331stop truncation mutant promoted ~35% more NBT reduction than either the empty vector transfected cells or wild-type WISP3 transfected cells. This increase in NBT reduction, which was similar to that induced by 0.2 μM ionomycin in C28I2 cells, was inhibited by 0.4 μM DPI. The Cys78Arg mutant was more potent than wild-type WISP3 in NBT reduction, but much less effective than the truncation mutant. Similar results garnered from several NBT reduction assays prompted us to investigate if the Trp331stop WISP3 truncation mutant was equally effective in augmenting protein tyrosine nitration and carbonylation in cells. Accordingly, immunoblots/oxyblots of lysates prepared from cells transfected with either the truncation mutant or the empty vector were analyzed using appropriate methodologies. As depicted in Fig. 4A, the Trp331stop mutant transfected cells clearly accumulated significantly higher levels of carbonylated and tyrosine-nitrated proteins, respectively, than the corresponding empty vector transfected cells (lanes 1 vs. 2 and 3 vs. 4). There was no significant difference in cell number between the mutant and empty vector transfected cells. Moreover, both mutant and empty vector transfected cells expressed similar levels of the housekeeping protein β-actin (lanes 5 vs. 6). A Flag-tagged version of the truncated mutant was used to verify expression of the mutant protein in C28I2 cells. As demonstrated in Fig. 4B, both Flag-tagged Trp331stop mutant and Flag-tagged wild-type WISP3 transfected C28I2 cells expressed comparable levels of the wild-type and mutant proteins, respectively, and also β-actin.

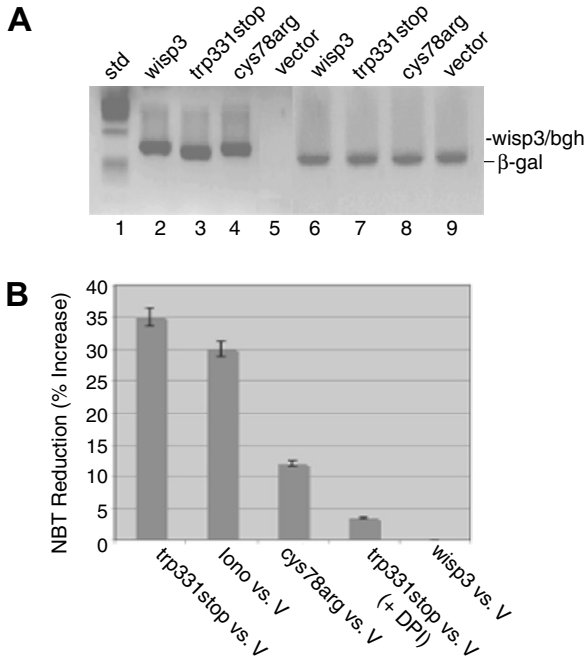

Fig. 3. NBT reduction assay on C28I2 cells transfected separately with wild type WISP3, mutant WISP3 (M) and empty vector (V). (A) RT-PCR analysis demonstrating that wild-type WISP3, trp331stop (M), and cys78arg (M) transfected cells expressed the corresponding wisp3/bgh specific PCR product (lanes 2–4, respectively), which was absent in the empty vector transfected cells (lane 5). The primers annealed to both wild type and mutant wisp3 sequences. Similar levels of the β-gal PCR product in the WISP3, M and V transfected cells, which were co-transfected with pCMV-β-galactosidase revealed similar transfection efficiency (lanes 6–9, respectively). Lane 1 demonstrates the Phi X 174 *Hae*III digest DNA standard. (B) NBT assay demonstrating ~35% increase in NBT reduction by trp331stop mutant transfected cells as compared to empty vector transfected cells. The increase was similar to that induced in C28I2 cells by 0.2 μM ionomycin (iono) and was largely inhibited by DPI (0.4 μM). NBT reduction by the cys78arg mutant was significantly less. There was no detectable increase in NBT reduction in wild type WISP3 transfected cells when compared to empty vector transfected cells ($P < 0.05$).

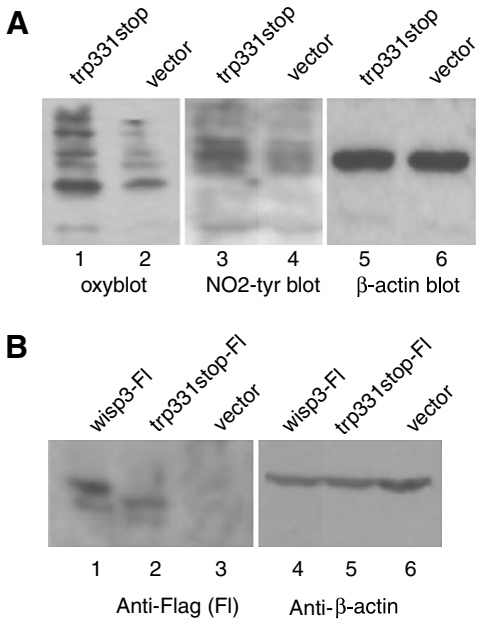

Fig. 4. Assessment of total protein carbonylation and tyrosine nitration in trp331stop mutant transfected C28I2 cells compared to empty vector transfected cells. (A) Anti-DNPH oxyblot and anti-nitrotyrosine immunoblot demonstrate that the trp331stop mutant transfected cells express ~3-fold more carbonylated protein and ~2-fold more nitrated protein, respectively, than the corresponding empty vector transfected cells (lanes 1 vs. 2 and 3 vs. 4). Anti-β-actin immunoblot demonstrates that both mutant and empty vector transfected cells express similar levels of the housekeeping protein β-actin (lanes 5 vs. 6) ($P < 0.05$). (B) Anti-Flag immunoblot demonstrates WISP3-Flag and trp331stop-Flag protein expression in the WISP3-Flag and trp331stop-Flag transfected cells, respectively, but not in the empty vector transfected cells (lanes 1–3). The truncated trp331stop mutant protein migrates lower than the wild-type. The anti-β-actin immunoblot demonstrates comparable levels of β-actin protein expression in WISP3-Flag, trp331stop-Flag and empty vector transfected cells (lanes 4–6).

A similar expression analysis was reported for the Cys78Arg mutant in our previous study [10]. It thus appears that WISP3 mutants may influence cell growth and metabolism differentially.

Discussion

Earlier observations have suggested that WISP3 regulates connective tissue specific gene expression and promotes SOD activity, among other functions [10,11]. Such interpretations of WISP3 function led us to further explore if WISP3 has any role in the regulation of ROS levels, which modulate various cellular events during growth and metabolism, and have often been presented as a central feature in connective tissue disorders and inflammation [12–16,19–22].

Experimental observations described here for the first time suggest that WISP3 contributes to the regulation of oxidative stress by limiting the accumulation of ROS in cells, and mutation(s) in WISP3 compromise this function by upregulating ROS levels. Other protein ligands such as glial growth factor (GGF) and heparin binding EGF like growth factor (HB-EGF) have been shown to inhibit cellular ROS upregulation associated with the application of cell activation stimuli [24,25]. While exogenously administered WISP3 protein mimics these cell growth modulators, there is additionally a potential requirement of WISP3 in also regulating basal cellular levels of ROS, as suggested by the heightened ROS accumulation and total cellular protein nitration/carbonylation that is concomitant with disruption of endogenous WISP3 expression. Changes in ROS reported in the current study, although modest, are consistent, and could potentially alter intracellular protein function. It is thus important to extensively investigate how WISP3 modulates ROS levels and protein carbonylation/nitration patterns in different cell types.

In the context of our previous report it is conceivable that WISP3 signaling limits ROS accumulation at least partly by promoting SOD activity [11]. However, the relative kinetics of such processes is unclear. Reduction of WISP3 expression in WISP3 siRNA transfected cells did not correlate with a significant decline in total SOD activity under our assay conditions (data not shown). Perhaps ROS accumulation caused by the loss of WISP3 expression facilitated compensatory changes in the activity of one or more SOD isoforms as part of a complex regulatory network. A detailed analysis of the influence of WISP3 signaling on the different SOD isoforms should help clarify this scenario. Potential influences of WISP3 on other important components of the cellular redox machinery such as the NAD(P)H oxidases are also worth exploring. It is quite possible that the deletion of a few C-terminal amino acids of WISP3, as in Trp331stop WISP3, or even loss of expression of WISP3, triggers heightened NAD(P)H oxidase like activity and subsequent increase in ROS levels.

In the light of contemporary literature that documents a significant influence of cellular ROS in the maintenance of cartilage integrity [13–16,26], it is conceivable that WISP3 signaling pathways mediating regulation of collagenII/aggrecan expression and ROS accumulation are interdependent or interwoven. More detailed mechanistic approaches will be necessary for carefully dissecting WISP3 mediated regulatory functions, their potential impact on the enzymatic source(s) of ROS at the sub-cellular level, and the significance of the different WISP3 domains therein.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.01.114.

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