WISP3–IGF1 interaction regulates chondrocyte hypertrophy

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Summary

WISP3 (Wnt induced secreted protein 3) is a multi-domain protein of mesenchymal origin. Mutations in several domains of WISP3 cause PPRD (progressive pseudo rheumatoid dysplasia), which is associated with cartilage loss and restricted skeletal development. Despite several studies focusing on the functional characterization of WISP3, the molecular details underlying the course of PPRD remain unresolved. We are interested in analyzing the function of WISP3 in the context of cartilage integrity. The current study demonstrates that WISP3 binds to insulin-like growth factor 1 (IGF1) and inhibits IGF1 secretion. Additionally, WISP3 curbs IGF1-mediated collagen X expression, accumulation of reactive oxygen species (ROS) and alkaline phosphatase activity, all of which are associated with the induction of chondrocyte hypertrophy. Interestingly, both IGF1 and ROS in turn trigger an increase in WISP3 expression. Together, our results are indicative of an operational WISP3–IGF1 regulatory loop whereby WISP3 preserves cartilage integrity by restricting IGF1-mediated hypertrophic changes in chondrocytes, at least partly, upon interaction with IGF1.

Key words: Hypertrophy, IGF1, WISP3

Introduction

WISP3 (Wnt induced secreted protein 3) or CCN6, along with WISP1 (CCN4) and WISP2 (CCN5) is a member of the CCN (CTGF, Cyr61, NOV) family proteins. CCN proteins are connective tissue growth modulators that have been reported to be involved in cell growth/differentiation during different phases of development (Moussad and Brigstock, 2000; Perbal, 2001; Katsube et al., 2009). Contemporary literature suggests that most of these proteins are involved in the regulation of osteo/chondrogenesis and angiogenesis during development through a complex interplay with BMP/Notch/Wnt signaling pathways (Takigawa et al., 2003; Holbourn et al., 2008; Katsuki et al., 2008; Katsube et al., 2009; Ono et al., 2011). Like all CCN family members, WISP3 possesses a signal sequence followed by several domains that portend to have specific functions founded on specific sequence homologies. The first domain of WISP3 – the IGFBP (insulin-like growth factor binding protein) domain has the potential to bind to IGF1, based on its sequence homology to the IGF1 binding domain of IGFBPs. The second domain of WISP3, a von Willbrand factor type C repeat domain could likewise, potentially bind to peptides. The third domain, a Thrombospondin type I domain, similarly has the potential to bind to sulfated glycosaminoglycans, and the last Cysteine Knot domain could be involved in dimerization and receptor binding (McDonald and Hendrickson, 1993; Hurvitz et al., 1999). Although there is yet no clear biochemical documentation of specific proteins interacting with the designated domains of WISP3, the domain organization of WISP3 suggests that it may act as a chaperone/scaffold protein, regulating the functions of other proteins through specific interactions.

WISP3 is expressed in cells and tissues of mesenchymal origin (Schutze et al., 2005). Mutations within the WISP3 gene are linked mostly to progressive pseudorheumatoid dysplasia (PPRD), an autosomal recessive skeletal disorder, and rarely to juvenile idiopathic arthritis (JIA) with a polyarticular course (Hurvitz et al., 1999; Delague et al., 2005; Lamb et al., 2005). Reported clinical and radiographic findings connected with disease pathogenesis mostly include joint stiffness, narrowing of joint space, loss of joint cartilage and widening of epiphyses. The disease manifestations linked to WISP3 mutations suggest that WISP3 is required for proper skeletal growth and maintenance of cartilage integrity (Hurvitz et al., 1999; Delague et al., 2005). Although the detailed molecular mechanism of the action of WISP3 is not resolved yet, existing functional studies justifying a role of WISP3 in skeletal development and cartilage maintenance are in accordance with the clinical and radiographic picture associated with WISP3 mutations (Hurvitz et al., 1999; Sen et al., 2004; Delague et al., 2005; Lamb et al., 2005; Davis et al., 2006; Miller and Sen, 2007; Nakamura et al., 2007; Baker et al., 2012; Garcia Segarra et al., 2012).

Prior investigations revealed that WISP3 promotes expression of the cartilage specific proteins collagen-II and aggrecan, controls the accumulation of reactive oxygen species (ROS) and regulates metalloprotease expression, corroborating that WISP3 sustains cartilage differentiation in vivo (Sen et al., 2004; Davis et al., 2006; Miller and Sen, 2007; Baker et al., 2012). Other studies have reported that WISP3 interacts with BMP and components of the Wnt1 signaling pathway including LRPl and Fz8 (Nakamura et al., 2007). In light of the fact that WISP3 harbors a potential IGF1 binding motif and IGF1 is required for cartilage/bone growth (McQuillan et al., 1986; Ohlsson et al., 1992; Wang et al., 1999; Wu et al., 2008), it is
important to decipher if WISP3 also physically interacts with IGF1 and controls its mode of actions. Although, WISP3 has been demonstrated to suppress IGF1 signaling in breast cancer as well as chondrocyte cell lines (Kleer et al., 2004; Cui et al., 2007; Lorenzatti et al., 2011), WISP3–IGF1 interaction and its potential influence on chondrocyte hypertrophy has not been documented. Furthermore, the mechanism of regulation of WISP3 expression also remains unclear.

Experimental results obtained from our current investigations reveal that WISP3 not only colocalizes and binds with IGF1 but also blocks IGF1 secretion. Additionally, we demonstrate that WISP3 inhibits IGF1 induced collagen X induction, ROS accumulation and alkaline phosphatase activity, all of which are associated with the induction of chondrocyte hypertrophy (Böhme et al., 1992; Wang et al., 1999; Kronenberg, 2003; Zheng et al., 2003; Morita et al., 2007; Mueller and Tuan, 2008; Wu et al., 2008; Kim et al., 2010). Moreover, both IGF1 and ROS in turn stimulate WISP3 expression. In all, our findings suggest that WISP3 operates in conjunction with IGF1 thereby constraining IGF1 induced cellular hypertrophy. The observed modulation of IGF1 functions by WISP3 may be attributed at least partly to its ability to bind to IGF1 and block IGF1 secretion.

Results
WISP3 binds to IGF1
IGF1 is known to be important for cartilage growth and differentiation (McQuillan et al., 1986; Ohlsson et al., 1992; Wang et al., 1999; Wu et al., 2008). Since WISP3 harbors an IGFBP like motif, we were interested in evaluating the IGF1 binding potential of WISP3. Co-transfection of HEK-293 cells with IGF1-FLAG and WISP3-His expression vectors and subsequent immunoprecipitation of IGF1-FLAG from the co-transfected cell lysates revealed that WISP3 immunoprecipitates along with IGF1 (Fig. 1A). WISP3 could also be immunoprecipitated by IGF1 from lysates of the chondrocyte line C-28/2, thus validating interaction between endogenous WISP3 and IGF1 (Fig. 1B). The authenticity of the antibody used to probe endogenous WISP3 was checked using WISP3-His as a reference (supplementary material Fig. S1). In order to ascertain if direct interaction between WISP3 and IGF1 is possible, immunoprecipitation of IGF1 was conducted separately on a mixture of recombinant IGF1 and WISP3 proteins. Similar to previous results, recombinant WISP3 (rWISP3) was found to co-immunoprecipitate with rIGF1 (Fig. 1C). IGF1–WISP3 interaction was also reflected in the co-localization of IGF1 and WISP3 within seemingly membranous aggregates in the IGF1/WISP3 co-transfected chondrocyte line C-28/2 by confocal microscopy (Fig. 1D). Additionally, colocalization of endogenous WISP3 and IGF1 was validated in two-dimensional as well as three-dimensional alginate cultures of the C-28/2 cells (Fig. 2A–C, the orange-yellow color in merge panel demonstrating colocalization) thus supporting WISP3–IGF1 interaction. Similar to some of the other CCN proteins (Kim

![Fig. 1. WISP3 interacts with IGF1.](image-url)

(A) Immunoblot documenting WISP3-His immunoprecipitated by anti-FLAG antibody and probed with anti-His antibody from lysate of HEK-293 cells co-transfected with Wisp3-His and IGF1-FLAG (lane 1). Heavy chain (~50 kDa) and light chain (~26 kDa) from immunoprecipitating antibody are apparent. Lane 2 depicts the negative control for immunoprecipitation using cells co-transfected with IGF1-FLAG and empty vector (pcDNA). Lanes 3 and 4 represent WISP3-His (~40 kDa) and IGF-FLAG (~17 kDa), respectively, from the corresponding input cell lysates. Arrowheads represent the presence of WISP3 in immunoprecipitate (lane 1) and in input cell lysate (lane 3). (B) Immunoblot depicting endogenous WISP3 immunoprecipitated by anti-IGF1 (H-70) antibody and probed with anti-WISP3 (N-18) antibody (shown with arrowhead, lane 1). 

H2O2-mediated increase in WISP3 expression in C-28/2 cells facilitated detection of WISP3–IGF1 interaction (explained in Fig. 5). Rabbit IgG was used as isotype control for immunoprecipitation (lane 2). (C) Immunoblot demonstrating rWISP3 immunoprecipitated with rIGF1 and probed with anti-WISP3 antibody (lane 1). Lane 2 represents the negative control for immunoprecipitation, using just the diluent for rWISP3 and rIGF1. The corresponding input proteins, rWISP3 (36 kDa) and rIGF1 (7 kDa) are shown in lanes 3 and 4, respectively. Arrowheads represent the presence of rWISP3 in immunoprecipitate (lane 1) and in input (lane 3). (D) Immunofluorescence demonstrates that WISP3-His (green) colocalizes with IGF1-FLAG (red), producing orange-yellow fluorescence, as revealed in the merged panel, in C28/2 chondrocyte lines co-transfected with WISP3-His and IGF1-FLAG. Cell nuclei are shown with DAPI (blue). Scale bar: 10 μm.
et al., 1997; Burren et al., 1999; Grotendorst et al., 2000), WISP3 was found to bind to IGF1 with a rather weak affinity ($K_d \sim 30 \mu M$) (supplementary material Fig. S2; Table S1), several thousand-fold lower than that of IGFBP3, a documented high affinity binder of IGF1 (Mireuta et al., 2011). Nonetheless, the weak interaction between WISP3 and IGF1 could be correlated with outcomes of considerable biological significance.

**WISP3 inhibits IGF1 secretion**

Although WISP3 is secreted, a substantial portion of it is also cell associated (Davis et al., 2006; Miller and Sen, 2007). Since IGF1 is usually known to be functional as a secreted protein (Ohlsson et al., 1992; Wang et al., 1999; Wu et al., 2008), it was important to investigate if the potential of WISP3 to bind to IGF1 influences IGF1 secretion from the cell. Assessment of supernatant/lysate ratio of IGF1 revealed that in cells co-transfected with WISP3 and IGF1, WISP3 indeed inhibited IGF1 secretion by about 50% (Fig. 3A,B). Moreover, secretion of endogenous IGF1 from C-28/I2 cells increased by about 40% when endogenous WISP3 expression was knocked down by siRNA transfection (Fig. 3C,D).

Taken together, our experimental findings reveal the interactive potential of WISP3 and IGF1 both inside and outside the cell and suggest that WISP3 may be able to dictate the biological activity of IGF1 through colocalization and physical interaction. WISP3–IGF1 interaction may in fact influence the processing of mature IGF1 from its precursor polypeptides within the cell prior to its secretion (Philippou et al., 2007).

**The IGFBP domain of WISP3 is not sufficient for IGF1–WISP3 interaction**

In light of the observation that WISP3 binds to IGF1, it was of interest to evaluate the contribution of the IGFBP domain of WISP3 in IGF1–WISP3 interaction. The results of similar immunoprecipitation experiments as described before indicated that the IGFBP domain of WISP3 does not bind to IGF1 as well as the full length WISP3 (Fig. 4A). The level of secreted IGF1 in the presence of the IGFBP domain of WISP3 was almost the same as in the presence of the empty vector control in the corresponding co-transfected cells. Moreover, the effect of the IGFBP domain on secretion of IGF1 was distinctly less than that of wild-type WISP3 (Fig. 4B,C,D). Deletion of the conserved GCGCC segment of the IGFBP domain (Fig. 4E) from full-length WISP3 also did not abolish binding to IGF1 (Fig. 4F). Deletion mutagenesis within the conserved segment GLYC resulted in poor expression probably on account of misfolding. Thus this deletion mutant was not tested for binding to IGF1. Although it is not clear at this stage exactly how the IGFBP domain of WISP3 influences its binding to IGF1, these findings definitely indicate that regions of the WISP3 protein other than the IGFBP domain are essential for setting the optimum conformation that is required for efficient binding to IGF1. As demonstrated by immunoprecipitation experiments in supplementary material Fig. S3, addition of the VWC and TSP domains to the IGFBP domain restores much of the binding potential of full length wild-type WISP3.

**WISP3 inhibits IGF1-mediated ROS accumulation and is in turn regulated by both IGF1 and ROS**

The involvement of both reactive oxygen species (ROS) and IGF1 during chondrocyte differentiation and hypertrophy is well documented (Wang et al., 1999; Morita et al., 2007; Wu et al., 2008; Kim et al., 2010). However, although IGF1 induced ROS accumulation has been reported in smooth muscle cells (Meng et al., 2007), there is no document of ROS accumulation in chondrocytes by IGF1. In the current study, we demonstrated that ROS accumulation in the chondrocyte line C-28/I2 increased
with increasing concentrations of recombinant IGF1 (Fig. 5A). With overexpression of WISP3, increments in ROS mediated by rIGF1 were lower, implying that WISP3 modulated IGF1 induced ROS accumulation (Fig. 5B). Both IGF1 and ROS (H₂O₂) in turn stimulated increase in WISP3 expression (Fig. 5C–E). As demonstrated in supplementary material Fig. S4, neither IGF1 addition nor WISP3 expression caused significant change in cell numbers under the prevalent experimental conditions.

**Collagen X induction by IGF1 is inhibited in the presence of WISP3**

The production of Collagen X is known to increase as chondrocytes differentiate into a hypertrophic state during bone formation (Stephens et al., 1992; Wang et al., 1999; Zheng et al., 2003; Wu et al., 2008). The involvement of IGF1 during this process has been documented (Wang et al., 1999). As depicted in Fig. 6A, we accordingly demonstrated that both collagen X mRNA (Fig. 6Aii) and protein (Fig. 6Aiii) increase in response to increasing concentrations of recombinant (r) IGF1, in the human chondrocyte line C-28/I2 (the number on top of each lane representing relative band intensity). However, when rIGF1 was added in the presence of increasing concentrations of rWISP3, rIGF1-mediated increase in collagen X expression subsided (Fig. 6B). Similar regulation of collagen X level by WISP3 was also observed in chondrogenic ATDC5 cells, used as a positive control for collagen X detection under hypertrophic conditions (supplementary material Fig. S5A,B). Since endogenous IGF1 could act on cells locally to enhance collage X expression, we tested collagen X expression in IGF1/WISP3 co-transfected C-28/I2 cells. Fig. 6C demonstrates that with increasing amount of WISP3 expressing plasmid, collagen X expression driven by a fixed amount of IGF1 expressing plasmid decreased. Moreover, the effect of recombinant IGF1 on collagen X protein expression was more pronounced in cells with siRNA-directed reduction in WISP3 expression (Fig. 6D). Overexpression of WISP3 in C-28/I2 cells accordingly opposed the effect of rIGF1 on collagen X protein expression. As demonstrated in Fig. 6E,F, the effect of rIGF1 (50 ng/ml and 100 ng/ml) was more pronounced on empty vector (E) transfected cells as compared to WISP3 (W) transfected cells. These findings are in accordance with the observed downregulation of IGF1 mediated activation of the hypertrophy inducing transcription factor Runx2 in the same cells by WISP3 (supplementary material Fig. S6) (Fujita et al., 2004; Qiao et al., 2004).

**IGF-1 induced alkaline phosphatase activity is inhibited by WISP3**

Similar to collagen X induction, alkaline phosphatase activity is enhanced during chondrocyte hypertrophy. The requirement of IGF1 during induction of alkaline phosphatase activity is documented (Iwasaki et al., 2008). We demonstrated that addition of IGF1 to the chondrocyte line C-28/I2 almost doubled alkaline phosphatase activity compared to control. However, inclusion of WISP3 along with IGF1 curbed the observed increase in presence of IGF1 alone (Fig. 7A), indicating that WISP3 restricts the alkaline phosphatase activity induced by IGF1 during chondrocyte hypertrophy. Fig. 7B demonstrates reduced alkaline phosphatase activity in WISP3 transfected C-28/I2 cells exposed to IGF1, as compared to the empty vector transfected cells.

**Discussion**

Our current findings suggest that WISP3 curbs induction of chondrocyte hypertrophy. Shrinkage of joint space, widening of the epiphyses, cartilage loss and periarticular calcifications in PPRD patients harboring mutant WISP3 gene (Hurvitiz et al., 1999; Garcia Segarra et al., 2012) may be the result of untimely and unregulated hypertrophic changes in growth plate and articular cartilage chondrocytes due to lack of a properly functional WISP3 protein. Timely interaction with the appropriate protein partners by virtue of its domain organization could be a key feature of WISP3 mediated regulation of chondrocyte hypertrophy.

The presence of an IGFBP-like domain in WISP3 is suggestive of interaction of WISP3 with IGF1. Several reports have substantiated the importance of IGF1, a growth factor with pleiotropic functions, in cartilage growth and differentiation (McQuillan et al., 1986; Ohlsson et al., 1992; Wang et al., 1999; Wu et al., 2008). While sustaining collagen II/aggrecan expression in proliferating chondrocytes of the cartilage growth plate, IGF1 also plays a crucial role in mediating chondrocyte hypertrophy during ossification perhaps through activation of the transcription factor Runx2 (Fujita et al., 2004; Qiao et al., 2004). Although there is documentation of a positive feedback on IGF1...
Fig. 4. The IGFBP domain of WISP3 is not sufficient for IGF1–WISP3 interaction. (A) Immunoblot depicting that the IGFBP domain of WISP3 (IGFBP-His) does not significantly immunoprecipitate with IGF1-FLAG (lane 1) as compared with full-length WISP3 (lane 3), using cells co-transfected with WISP3/IGFBP-His and IGF1-FLAG. Lane 2 depicts the negative control using empty vector and IGF1-FLAG co-transfected cells. Lanes 4, 5 and 6 depict IGFBP-His, WISP3-His and IGF1-FLAG input cell lysates, respectively. Arrows indicate the presence of WISP3 in immunoprecipitate (lane 1) and in input cell lysate (lane 5). Immunoprecipitated IGFBP (lane 1) is shown in the red box. Arrowheads represent the presence of IGFBP (lane 4) and IGF1 (lane 6) in input cell lysates. (B) Immunoblot depicting that IGFBP domain of WISP3 does not significantly reduce IGF1 secretion (lane 1; sup) compared with empty vector in the corresponding transfected cells (lane 2; sup). IGF1, IGFBP (WISP3) and β-actin expression in the corresponding cell lysates is shown. (C) Transfection with IGFBP (WISP3) correlates with increased secretion of IGF1 (7 kDa) (lane 2) compared with transfection with wild-type WISP3 (lane 1). Expression of WISP3 and IGFBP domain probed with anti-His antibody and expression of cell-associated IGF1 (13 kDa) probed with anti-IGF1 antibody are shown. (D) Supernatant/lysate ratio of IGF1 in the presence of IGFBP or WISP3 from three independent experiments (*P<0.05). (E) Multiple alignment of IGFBP domain of WISP3 with IGFBP (1–6) proteins. Conserved amino acids are highlighted. The level of amino acid conservation is as follows: black > dark grey > light grey. (F) Immunoblot demonstrates that deletion of GCGCC in IGFBP domain of WISP3 (mut) does not affect its interaction with IGF1 (lane 2), as detected by immunoprecipitation. Lane 1 shows immunoprecipitated wild-type WISP3.

Fig. 5. WISP3 inhibits IGF1-induced ROS accumulation and is in turn regulated by both IGF1 and ROS. (A) In C-28/I2 cells, rIGF1 increases accumulation of ROS in a dose-dependent manner, as measured by DCF-DA fluorescence; ‘0’ depicts the control with PBS. (B) WISP3-transfected cells accumulate less ROS than the cells transfected with empty vector in the presence of different concentrations of rIGF1. (C) RT-PCR analysis with WISP3-specific primers indicates significant induction of endogenous WISP3 expression in the chondrocyte cell line exposed to H2O2 (ROS) or rIGF1. (D–E) Estimated induction of WISP3 expression by H2O2 (D) or rIGF1 (E) as normalized with GAPDH from three independent experiments. *P<0.05, **P<0.005, ***P<0.001.
expression in chondrocytes exposed to exogenous IGF1 (Nixon et al., 2001), it is not clear how IGF1 secretion and its autocrine/paracrine actions on chondrocytes are regulated locally during cartilage growth and differentiation. Studies on inflammatory breast cancer cell lines and chondrocyte lines have suggested that IGF1 mediated signaling is inhibited by WISP3 (Kleer et al., 2004; Cui et al., 2007). However, the molecular details of WISP3–IGF1 synergism and its functional significance in the context of chondrocyte hypertrophy have not been resolved.

Our results from immunoprecipitation experiments document that WISP3 interacts with IGF1 at both the endogenous level and upon overexpression. As a corollary, while overexpression of WISP3 blocks IGF1 secretion, suppression of endogenous WISP3 expression enhances it. Comparative analyses of constructs expressing either a full length wild-type WISP3, or specific domain deletion mutants of WISP3 reveal that not just the IGFBP domain alone but all domains of WISP3 act jointly to interact with IGF1. In this context, it is to be noted that the Cys78Arg mutant (IGFBP domain mutant of WISP3, present in PPRD patients) displays the same binding intensity to IGF1 as wild-type WISP3 in immunoprecipitation assays, upon overexpression (supplementary material Fig. S7). It is unclear at this stage exactly how the Cys78Arg WISP3 mutation leads to the development of disease phenotype. WISP3–IGF1 interaction may facilitate the assembly of other signaling proteins such as integrins, involved in chondrocyte growth/differentiation and maintenance of cartilage integrity (Hirsch et al., 1997; Aszodi et al., 2003; Aszodi et al., 2007).
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Materials and Methods

Cell culture

The immortalized chondrocyte cell line C-28/22 was derived from human juvenile costal cartilage and generated by infection with a replication defective retroviral vector expressing SV40 large T antigen (Goldring et al., 1994). Cultures of C-28/I2 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 mM CaCl2 solution using 22 gauge needle. The alginate beads containing cells were prepared with the addition of 33% alginate using 150 mM NaCl solution (Seki et al., 2003). Mouse chondrogenic ATDC5 cells were maintained with DMEM/F12 (Human Embryonic Kidney) cells were maintained similarly in DMEM (low-glucose). Mouse chondrogenic ATDC5 cells were incubated in DMEM/F12 media containing 1x ITS (insulin, transferrin and selenium) (Invitrogen) (Atsumi et al., 1990; Sen et al., 2004; Davis et al., 2004; Miller and Sen, 2007). About 2 μg of total RNA was used for cDNA synthesis. Primers specific for human collagen X (forward 5’-GCAAACAG- CATTATGCCCCAACGAGG-3’ and reverse 5’-CTCAGTTGGAGCCACT-AAG- AA), human WISP3 (forward 5’-ACCAAAGCTGACGTGAGCGTCTC-3’ and reverse 5’-CTCAGTTGGAGCCACT-AAG- AA), and GAPDH (forward 5’-ACCAACAGCGTGGCAATCATCTG-3’ and reverse 5’-TCCACAC CCTGTTGCTGTA-3’) were used as a control for the expression of the corresponding mRNAs by RT-PCR (PCR: Qiagen, Biobharati, Kol, India). Expression of transfected genes were analyzed similarly using gene and vector specific primers – WISP3 (forward 5’- TGGCAAGGTTCGCTGAGG-3’ and reverse 5’-AACATAGAGGACATCTG- GAGG-3’), IGF1 (forward 5’-TGAAGACACACCATGTCCTC-3’ and reverse (bgh primer). Taq polymerase was from Biobharati, Kolkata, India.

Transfection

C-28/22 cells were transfected with WISP3 expression vector, IGF1 expression vector or pcDNA (empty vector) using Lipofectamine LTX reagent (Invitrogen) as per manufacturer instructions. Briefly, cells were plated 24 hours before transfection in 6-well tissue culture plates at a density of ~5x10^5 cells/ml. For transfection, a single well of cells, 500 μl of Opti-MEM (Invitrogen) was taken in two microcentrifuge tubes. In one tube plasmid DNA (1–3 μg) and 3 μl of plus reagent was added and in another tube 6 μl of Lipofectamine LTX was added subsequent to which the contents of both tubes were mixed and incubated for 30 minutes at room temperature. After the incubation, the total mixture (1 ml) was added to one well of the 6-well plate and incubated under normal tissue culture conditions for 7–8 hours before the addition of 1 ml Opti-MEM. The same procedure was followed for all other wells. 24 hours after transfection, fresh medium (containing 10% FBS) or charcoal stripped serum medium containing recombinant protein was added depending on the assay being performed. C-28/22 cells were separately transfected using RNAmax (Invitrogen) with WISP3 siRNA or control siRNA (50 nmol) (Dharmacon) following instructions provided by the manufacturer. 48–50 hours after transfection cells were treated with IGF1 (50 ng/ ml) or PBS for 18 hours, and then analyzed for collagen X expression. Calcium chloride transfection method was followed for transfecting HEK-293 cells.

Preparation of whole cell protein lysates and immunoblotting

To prepare whole cell lysates, cells were rinsed once with PBS, and disrupted with cell 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] with added protease inhibitor cocktail (Sigma) 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, USA). For immunoblot analysis, protein samples were boiled in 1x SDS buffer, separated by SDS-PAGE and transferred on to a PVDF membrane. The membrane was blocked with 0.2% 1-BLOCK (Applied Biosystems, USA) for 2 hours at room temperature and incubated with the appropriate primary antibody overnight at 4°C. After washing with tris buffered saline (containing 0.1% Tween 20), each blot was incubated with the corresponding HRP conjugated secondary antibody (Sigma) and the immunoblot signal was visualized by chemiluminescence reagents (Pierce).

Primary antibodies used in this study were anti human type X collagen, anti-β-actin, anti-FLAG (rabbit IgG1, anti-His-FLAG M2 antibody (Sigma), anti-His and anti-Alpha Anti-Biotin Life Science, Kolkata, India; Clontech, USA), anti-IGF-1/β, anti-WISP3-C14-14, anti-WISP3-N-18 antibody (Santa-Cruz) and anti-IGF-1 antibody (R&D Systems).

Co-immunoprecipitation

For co-immunoprecipitation studies HEK-293 cells were separately co-transfected with the appropriate expression constructs. 48 hours after transfection, cell lysates were made using cell lysis buffer. Protein G beads (Santa Cruz Biotechnology,
Santa Cruz, CA) (10 μl volume) were washed with cell lysis buffer and incubated with anti-FLAG M2 antibody for 2 hours. After the incubation co-transfected cell lysates (250 μg) were added to the bead slurry and the whole mixture was agitated at 6–10°C for 18 hours. The beads were subsequently collected by mild centrifugation, and washed with lysis buffer 3–4 times. At the final wash only the beads were collected for checking the presence of co-immunoprecipitated WISP3 or IGFBP domain-WISP3 or mutant WISP3 by immunoblot procedures using anti-His antibody. To check endogenous binding of WISP3 and IGF1, protein G beads were washed as mentioned before and incubated with either anti IGF1(H-70) antibody or the isotype control antibody (rabbit IgG) for 4 hours. C-28/I2 cell lysate was then incubated with the antibody bound beads for 18 hours at 6–10°C. The beads were further processed as described before and checked for WISP3 with anti-WISP3 (N-18) antibody. For further confirming the interaction of IGF1 with WISP3, recombinant proteins were used. About 30 prolines of each recombinant protein were mixed in a buffer (10 mM NaCl, 25 mM Tris pH 7.5, 0.5 mM DTT, 1% Triton-X) and incubated on ice for 1 hour subsequent to which the recombinant WISP3 was immunoprecipitated with anti-FLAG antibody and probed with anti-WISP3-C antibody. HEK-293 cells were co-transfected with His-tagged deletion constructs of WISP3 and FLAG-tagged IGF1. WISP3 deletion mutants were immunoprecipitated from the cell lysates with anti-His antibody and the immunoprecipitate was probed with anti-FLAG antibody to check the presence of IGF1.

Immunofluorescence

C-28/I2 cells were grown on tissue culture confocal dishes and co-transfected with WISP3 and IGF-1. 48 hours after transfection, cells were washed with phosphate buffered saline and fixed with 4% formaldehyde for 5 minutes. Fixed cells were blocked with 0.1% FcR block (Miltenyi Biotec) for 2 hours at 4°C and subsequently incubated with anti-His (1:200) antibody (sc-208, Santa Cruz, CA) (10 μg/ml) for 4 hours. After the incubation, cells were washed with phosphate buffered saline (containing 1% TWEEN 20) and incubated with Alexa-Fluor 488 and 546 anti-goat and anti-rabbit secondary antibodies (Molecular Probes) respectively, at 1:2000 for 2 hours at 4°C in the dark. DAPI stain was used to detect the nucleus. In a similar manner, WISP3 (N-18) (goat) and IGF1 (H-70) (rabbit) antibodies were used for evaluating colocalization of endogenous WISP3 and IGF1 in two-dimensional as well as three-dimensional cultures of C-28/I2 cells (Lee et al., 2007; Chand et al., 2012). Immunofluorescence was visualized using Nikon A1R confocal imaging system (Fig. 1D) and Andor Revolution XD Spinning Disk Microscope with Andor ixon 897 EMCCD camera (Fig. 2).

Surface plasmon resonance

BLAcore 3000 system (GE Healthcare) was used for real-time binding experiments. Recombinant WISP3 (Peprotech) and IGFBP3 (R&D) were dissolved in 10 mM sodium acetate buffer (pH 4.5) and immobilized separately on CM5 sensor chip (certified grade) using amine coupling kit (Biacore). Recombinant IGF1 (R&D) was injected with running buffer (10 mM HEPES pH 7.4, 150 or 300 mM NaCl, 3 mM EDTA and 0.005% Tween 20) over the CM5 chip at various concentrations (10, 20, 30, 40, 50 μM for WISP3) and (10, 20, 30, 40, 50 μM for IGFBP3) to determine the kinetic rate constants (Mireuta et al., 2011; Pal et al., 2012).

IGF1 secretion

HEK-293 cells were co-transfected separately with WISP3 (His tag) and IGF1, IGFBP domain WISP3 (His tag) and IGF1, and IGF1 and pCDNA (empty vector). 24 hours after transfection complete medium (10% FBS) was added and incubated for another 24 hours. Subsequently, the cell supernatant was collected and analyzed for IGF1 (secreted) protein. Cell lysate was made for estimating cell associated IGF1 protein as a reference. C-28/I2 cells were transfected with WISP3 siRNA or control siRNA separately. After 48–50 hours of transfection, culture medium was replaced with serum free medium for 6 hours and incubated for another 24 hours with fresh serum free medium before assay. Secreted IGF1 was analysed from cell supernatants by ELISA. Secreted IGF1 was measured as units per milligram of protein (U/mg) (Bernstine et al., 1973).

Luciferase reporter assay

C-28/I2 chondrocyte cells were co-transfected with the 3-cranx2-LUC and renilla luciferase expression vectors at 50:1 ratio using Lipofectamine 2000 (Invitrogen). 24 hours after transfection, the culture medium was replaced with serum-free DMEM. Subsequently, cells were treated with either IGF1 (100 ng/ml) or WISP3 (250 ng/ml)/IGF1 (100 ng/ml) for 6 hours. Both firefly and renilla luciferase activity were measured using the Luciferase assay kit (Promega).

Statistical analysis

Statistical analysis was done using GraphPad Prism 4 software. Comparisons were made using unpaired Student’s t-test. Bar graphs are expressed as mean ± s.d. Statistical probability is expressed as *P<0.05, **P<0.005 and ***P<0.001; P<0.05 was considered significant. ImageJ software (NIH) was used for densitometric analysis.

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Author contributions

M.S. designed research; R.S.R., M.P. and M.S. performed research; R.S.R and M.S. analyzed data, and R.S.R. and M.S. wrote the paper.

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Supplementary material available online at


References


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cells treated with rIGF1 (100 ng/ml) either in presence or absence of WISP3 (500 ng/ ml) for 1–3 days in DMEM:F12 medium with 5% charcoal stripped serum.

Estimation of accumulation of ROS and endogenous WISP3 expression

C-28/I2 cells were treated with different concentrations of recombinant IGF1 or vehicle (PBS) for 18 hours and the accumulated ROS was analyzed using the fluorescent probe H2DCF-DA (Calbiochem, USA). Briefly, cells were washed with phosphate buffered saline and incubated with H2DCF-DA (10 μM) for 20 minutes in a CO2 tissue culture incubator. After the incubation, cells were again washed with phosphate buffered saline 3–4 times and analyzed for accumulated ROS using fluorometer (excitation at 488 nm, emission at 525 nm). In addition, ROS was determined in C-28/I2 cells either transfected with WISP3 or pCDNA, and treated with different concentrations of rIGF1 for 18 hours. In order to estimate ROS inducible WISP3 expression, C-28/I2 cells were treated with different concentrations of hydrogen peroxide (H2O2) (Merck, Germany) for 10 hours, subsequent to which cells were processed for mRNA analysis.

Alkaline phosphatase specific activity

C-28/I2 cells were treated with recombinant IGF1 (100 ng/ml) in presence or absence of recombinant WISP3 (250 ng/ml) for 18 hours. In addition, C-28/I2 cells either transfected with WISP3 or pCDNA were treated separately with 50 ng/ml or 100 ng/ml of rIGF-1 for 18 hours. Treatment with PBS (control for IGF1) or 10 mM acetic acid (ACA; control for WISP3) was used as a reference. The alkaline phosphatase activities of cell lysates were measured in DEA (diethanolamine) buffer (pH 10.0) (10 mM para-nitrophenyl phosphonate, 2 mM MgCl2, and 0.9 M diethanolamine) (McComb and Bowers, 1972; Böhme et al., 1992). Briefly, each cell lysate (40 μg) was incubated with DEA buffer at 37°C for 30 minutes and inorganic phosphate activity was measured using the Phosphoimager kit (Pompea).

Luciferase reporter assay

C-28/I2 chondrocyte cells were co-transfected with the 3-cranx2-LUC and renilla luciferase expression vectors at 50:1 ratio using Lipofectamine 2000 (Invitrogen). 24 hours after transfection, the culture medium was replaced with serum-free DMEM. Subsequently, cells were treated with either IGF1 (100 ng/ml) or WISP3 (250 ng/ml)/IGF1 (100 ng/ml) for 6 hours. Both firefly and renilla luciferase activity were measured using the Luciferase assay kit (Promega).

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Chondrocyte survival and differentiation in situ are integrin mediated. Dev. Dyn. (1994). Interleukin-1 beta-modulated gene

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Fig. S1. Evaluation of Specificity of WISP3 antibody. (A) Immunoblot (IB) represents the expression of WISP3 (His tag) protein in WISP3-His transfected HEK-293 cells with anti-WISP3 (N-18) antibody. The same lysates were separately probed with anti-His antibody, producing the same result. (B) Empty vector (pcDNA) transfected cell lysate was used as a negative control.

Fig. S2. Kinetics of WISP3-IGF1 interaction using Surface Plasmon Resonance (SPR). Sensograms indicate the response unit (RU) for different concentrations of rIGF1 flown over the flow cell of CM5 chip immobilized with either rWISP3 (3000 RU) or IGFBP3 (1500 RU). (A) rIGF1 (10, 20, 30, 40 and 50 µM) in running buffer containing 300 mM NaCl was injected for 4 mins to flow over rWISP3 (36 kDa) at 10°C. (B) rIGF1 (10, 20, 30, 40 and 50 nM) was injected for 1 min to flow over IGFBP3 (29 kDa) under same conditions. (C) rIGF1 (10, 20, 30, 40 and 50 nM) in running buffer containing 150 mM NaCl was injected for 1 min to flow over IGFBP3 (29 kDa) at 10°C. Biacore evaluation software was used to calculate the $k_{on}$, $k_{off}$ and $K_D$ ($k_{off}/k_{on}$) by fitting 1:1 Langmuir binding model to sensograms.
Fig. S3. Evaluation of interaction of different deletion versions of WISP3 with IGF1. (A) Schematic representation of deletion constructs of WISP3 protein with His-tag at C-terminus. (B) Immunoblot represents IGF1-Flag coimmunoprecipitated (IP) from cells cotransfected with IGF-Flag and WISP3 (Δ)-His, with anti-His antibody and probed with anti-Flag antibody. Expression of wild type WISP3–His, WISP3 (Δ)-His and IGF1-Flag is shown with anti-His antibody and anti-Flag antibody respectively in input cell lysates.
**Fig. S4. Estimation of cell proliferation upon IGF1 addition and WISP3 / pcDNA transfection.** (A) Bar graph representing that there is no significant proliferation of cells treated with rIGF1 as determined by MTT assay, under prevalent experimental conditions (Materials and Methods). ‘C’ is control medium without IGF1. (B) No significant change in cell number upon IGF1 treatment of WISP3 / pcDNA transfectants. PBS is control.

**Fig. S5. Evaluation of collagen X expression with rIGF1 in presence or absence of rWISP3 in ATDC5 cells.** (A) ATDC5 cells are treated with rIGF1 (100 ng/ml) in presence or absence of rWISP3 (500 ng/ml) for 1-3 days. RT-PCR analysis of collagen X indicates that rWISP3 reduces collagen X expression. (B) Immunoblot represents the expression of collagen X in presence or absence of ITS (Insulin Transferrin Selenium) for 9-12 days in ATDC5 cells. Collagen X induction in C28/I2 cells with or without rIGF-1 (100 ng/ml) is shown as reference using the same antibody.
**Fig. S6. Evaluation of influence of WISP3 on Runx2 activity.** 3x-RUNX2-LUC and renilla luciferase cotransfected C-28/I2 chondrocyte cells were incubated with indicated combinations of IGF1 (100 ng/ml) and WISP3 (250 ng/ml) to measure luciferase activity. Bars represent firefly to renilla luciferase ratio.

**Fig. S7. Evaluation of C78R-WISP3 (Mutant WISP3 found in WISP3 in PPRD patients) interaction with IGF1.** Immunoblot represents that C78R-His coimmunoprecipitates along with IGF1-Flag similar to wild type WISP3-His. Immunoprecipitation was performed with anti-Flag antibody and immunoblot probed with anti-His antibody. Presence of C78R-His, wild type WISP3-His and IGF1-Flag are demonstrated with anti-His and anti-Flag antibodies respectively in input cell lysates.

**Table S1. Kinetics of WISP3-IGF1 interaction using Surface Plasmon Resonance (SPR)**

<table>
<thead>
<tr>
<th>IGF 1</th>
<th>Temperature (°C)</th>
<th>$k_{on}$, M$^{-1}$s$^{-1}$</th>
<th>$k_{off}$, s$^{-1}$</th>
<th>$K_D$, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>WISP3 (300 mM NaCl)</td>
<td>10</td>
<td>$6 \times 10^2$</td>
<td>$20.0 \times 10^{-3}$</td>
<td>30</td>
</tr>
<tr>
<td>IGFBP3 (300 mM NaCl)</td>
<td>10</td>
<td>$5 \times 10^5$</td>
<td>$2.0 \times 10^{-3}$</td>
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<tr>
<td>IGFBP3 (150 mM NaCl)</td>
<td>10</td>
<td>$7 \times 10^5$</td>
<td>$0.6 \times 10^{-3}$</td>
<td>0.0008</td>
</tr>
</tbody>
</table>