Research Article

Dysfunction of Collagen Synthesis and Secretion in Chondrocytes Induced by Wisp3 Mutation

Min Wang,1,2 Xiao-Fei Man,1 Ya-Qing Liu,1 Er-Yuan Liao,1 Zhi-Feng Shen,1 Xiang-Hang Luo,1 Li-Juan Guo,1,2 Xian-Ping Wu,1 and Hou-De Zhou1

1 Institute of Endocrinology and Metabolism, The Second Xiang-Ya Hospital of Central South University, Changsha, Hunan 410011, China
2 Department of Endocrinology and Metabolism, Xiang-Ya Hospital of Central South University, Changsha, Hunan 410008, China

Correspondence should be addressed to Hou-De Zhou; houdezhou@xysm.net

Received 23 December 2012; Revised 21 January 2013; Accepted 28 January 2013

Academic Editor: Peng-Fei Shan

Copyright © 2013 Min Wang et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Wisp3 gene mutation was shown to cause spondyloepiphyseal dysplasia tarda with progressive arthropathy (SRDT-PA), but the underlying mechanism is not clear. To clarify this mechanism, we constructed the wild and mutated Wisp3 expression vectors and transfected into human chondrocytes lines C-20/A4; Wisp3 proteins subcellular localization, cell proliferation, cell apoptosis, and Wisp3-mediated gene expression were determined, and dynamic secretion of collagen in transfected chondrocytes was analyzed by 14C-proline incorporation experiment. Mutated Wisp3 protein increased proliferation activity, decreased apoptosis of C-20/A4 cells, and aggregated abnormally in cytoplasm. Expression of collagen II was also downregulated in C-20/A4 cells transfected with mutated Wisp3. Wild type Wisp3 transfection increased intracellular collagen content and extracellular collagen secretion, but the mutated Wisp3 lost this function, and the peak phase of collagen secretion was delayed in mutated Wisp3 transfected cells. Thus abnormal protein distribution, cell proliferation, collagen synthesis, and secretion in Wisp3 mutated chondrocytes might contribute to the pathogenesis of SEDT-PA.

1. Introduction

Wnt-1-induced secreted protein 3 (Wisp3/CCN6) is a cysteine-rich protein that belongs to the cysteine-rich 61-connective tissue growth factor, nephroblastoma overexpressed CCN family members, maps to chromosome 6q21-22, and encodes a 354 amino acid secreted protein [1]. Wisp3 proteins are characterized by an N-terminal secretory signal followed by four structural domains with partial sequence identity to insulin-like growth factor binding protein (IGFBP) (GCGCCXXXC); Von Willebrand factor type C like motif, thrombospondin type 1 module, and a C-terminal cysteine knot-like domain (CK) putatively involved in dimerization [1, 2], and IGFBP can be upregulated by implementation of exercise [3]. The members of CCN family are multifunctional in which they are involved in regulation of cell adhesion, migration, proliferation, growth arrest, survival, apoptosis, differentiation, endochondral ossification, and extracellular matrix production [4–6].

Wisp3 mutations have been demonstrated in most patients of an autosomal recessive hereditary cartilage metabolic disorder, spondyloepiphyseal dysplasia tarda with progressive arthropathy (SEDT-PA), or progressive pseudorheumatoid dysplasia (PPD), which characterized by deformation and limitation of most large and small joints clinically, and continuous degeneration and loss of articular cartilage pathologically [7–11]. In our previous work, we found a novel compound mutation (840delT/T1000C) of Wisp3 in Chinese PPD kindred [12, 13]; the two probands carried a substitution mutation (1000T→C, Ser334Pro) in paternal allele, and a deletion (840delT) mutation in maternal allele that caused a truncated Wisp3 protein to miss 43 residues in C-terminus [14], and we also discovered the biological behavior changes of the articular chondrocytes (ACs) separated from the patients [15]. Wisp3 also had growth-, invasion-, and angiogenesis-inhibitory functions in inflammatory breast cancer (IBC) in vitro and in vivo [16] and was a key genetic determinant of the IBC phenotype [17]. However,
the precise action of Wisp3 in cartilage maintenance and metabolism and the mechanisms of SEDT-PA/PPD caused by Wisp3 mutations have not been elucidated.

The present study was undertaken to investigate the subcellular localization and function of mutant Wisp3 in chondrocytes. The results suggest that mutated Wisp3 protein aggregated abnormally in cytoplasm, and mutated Wisp3 failed to inhibit cell proliferation and modulate the expression of type II collagen in chondrocytes, which may be an important molecule mechanism involved in the pathogenesis of SEDT-PA/PPD.

2. Materials and Methods

2.1. Cell Cultures. The immortalized human chondrocytes cell lines C-20/A4 were derived from human juvenile costal cartilage and generated by infection with a replication defective retroviral vector expressing SV40 large T antigen. Cultures of C-20/A4 cells were maintained in DMEM/Ham's F-12(1:1,v/v) supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, USA) in a 5%CO₂ incubator at 37°C and passaged at subconfluence every 5-6 days.

2.2. Wild Type and Mutant Wisp3 Expression Construct. Human Wisp3 was cloned from ACs cDNA by PCR amplification with Wisp3-specific primers, bearing Hind III and BamH I restriction enzyme sites at their flanking ends, for the purpose of subcloning into the expression vector pcDNA3.1(+). The primers used for cloning Wisp3 into pcDNA3.1 were 5′-GTAAGCTTATGGGACATTCACTCTCCTTT-3′ (forward) and 5′-GCGGATCCATGGGACATTCACTCTCCTTT-3′ (reverse), and pEGFP-C2 were 5′-TCAAGCTTACGCTACGGGAGGCTCCTTCTTT-3′ (reverse), and pEGFP-C2 were 5′-TCAAGCTTACGCTACGGGAGGCTCCTTCTTT-3′ (forward, exclude start codon) and 5′-GCGGATCCATGGGACATTCACTCTCCTTT-3′ (reverse). The amplified Wisp3 gene products (∼1.1kb) were ligated to pEGM-T easy (Promega, Madison, WI, USA), and the products were used as templates in the PCR reaction of the site-directed mutagenesis (SDM).

The mutants Wisp3 (MUT\textsuperscript{1000/C} and MUT\textsuperscript{840delT}) were constructed using SDM separately. The mutant primers for MUT\textsuperscript{1000/C} had the sequences 5′-CCTGATGGTGTGTCAGAGAAA-3′ (forward) and 5′-CCTGATGGTGTGTCAGAGAACCTTATGG-3′ (reverse), and for MUT\textsuperscript{840delT} had the sequences 5′-AATTGCTTTTCTGATGCTCA-3′ (forward) and 5′-AAGGTGAGAGGTGTTCAGACTTT-3′ (reverse). After confirmed by restriction endonuclease analysis and sequencing, the target fragments (WT-Wisp3, MUT\textsuperscript{1000/C}, and MUT\textsuperscript{840delT}) were subcloned to Hind III and BamH I sites of expression vector pcDNA3.1(+) and pEGFP-C2. The recombinant expression plasmids with pcDNA3.1(+) were used for all functional studies of wild and mutant Wisp3, and those with pEGFP-C2 were used for subcellular localization of wild and mutant Wisp3 proteins.

2.3. Cell Transfection. Lipofectamine was used for transfecting C-20/A4 chondrocyte cell lines with the recombinant plasmids and empty vector constructs. Briefly, cells (2.5-5 × 10⁵/mL) were plated 1 day before transfection in 6-well tissue culture plates (2 mL/well) and incubated at 37°C in 5%CO₂. A complex of the plasmid DNA (<1μg) with 6 μL PLUS reagent in 100 μL of serum-free, antibiotic-free medium was prepared in a sterile microfuge tube for 15 minutes; dilute 4 μL of Lipofectamine into 100 μL of serum-free medium and added to each reaction mixture, and incubated at room temperature for additional 15-30 min. A similar complex was prepared for each well of a 6-well plate. The cells in each well of the plate were washed with sterile PBS and then added 800 μL serum-free medium and the transfection mixture drop wise to each plate, and incubated for 4 h, after which 1 mL of culture medium with 5% FBS was added to each well. After 24 hours, the transfection mixture was replaced with fresh culture medium containing 10% FBS. The incubation was continued for an additional 24–26 hours, and the cells were used for observation by laser scanning confocal microscopy (LSCM) and harvested for either RNA or protein extraction. After 24 hours of transfection, the cells were placed in 25 cm² flasks for stable transfection with selection by G418 (400 μL/mL).

2.4. Wild and Mutant Wisp3 Proteins Subcellular localization. After 48 h of transiently transfection with WT-Wisp3/ pEGFP-C2, MUT\textsuperscript{1000/C}/pEGFP-C2, MUT\textsuperscript{840delT}/pEGFP-C2, or empty vector, cells were rinsed once with PBS after removal of culture medium, and then fixed for 15 min with freshly prepared 4% paraformaldehyde, and then incubated with 0.25% Triton X100 at 37°C for 20 min. 4′-Diamino-2-phenylindole-2HCl (DAPI; Sigma) was used at a final concentration of 100 ng/mL to stain cell nuclei. After washing three times with PBS at room temperature for 10 min, the fluorescence was observed under LSCM.

2.5. Cell Viability Assay. Cell viability was determined using a standard 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. Briefly, cells stably transfected with MUT\textsuperscript{1000/C}/pcDNA3.1(+), MUT\textsuperscript{840delT}/pcDNA3.1(+), or empty vector were seeded 10⁴ cells/well into 96-well plates. Following 24 h in culture, 5 μL MTT was added into each well and cells were incubated for an additional 3 h. The medium was discarded and the cells were solubilized in 100 μL dimethyl sulfoxide (DMSO), and then shaken for 1 min, and incubated for 5 min at room temperature, and the absorbance at 570 nm was read on Micro ELISA reader ( Molecular Devices, CA, USA).

2.6. Cell Cycle and Apoptosis Analysis. Cells stably transfected with MUT\textsuperscript{1000/C}/pcDNA3.1(+), MUT\textsuperscript{840delT}/pcDNA3.1(+), or empty vector were seeded into 25 cm² flasks at a density of 2 × 10⁵ cells/mL and cultured for 24 h. After cultured in serum-free medium for 24 h, cells were digested with 0.05% trypsin-EDTA, rinsed with PBS, fixed with 75% ethanol over night at 4°C, and stained with propidium iode. Cell cycle and apoptosis were evaluated using FAC flow cytometry (BD Biosciences). Cell proliferation index (PI) was calculated using the equation (PI) = (G2+S)/(G1+S+G2).
Apoptosis was also studied morphologically using fluorescent dyes that intercalate DNA. Acridine orange stains DNA bright green, allowing visualization of the nuclear chromatin pattern. Ethidium bromide stains DNA orange, but is excluded by viable cells. Cells stably transfected that is uniformly stained. Ethidium bromide stains DNA bright green, allowing visualization of the nuclear chromatin pattern. Apoptotic cells have condensed chromatin.

During cDNA synthesis, 2× reaction mixtures were used for reverse transcription. Primers specific for type II collagen, type I collagen, SOX9 (catalog number 84014), fibronectin, and β-actin were used for estimating the levels of expression of the corresponding mRNA. During cDNA synthesis, 2 μg of RNA was used for each specimen, and 30 cycles of PCR were carried out. The β-actin gene was used as an internal control. Table 1 summarizes the primer pairs and experimental conditions used for RT-PCR analysis.

| Gene product | Forward and reverse primers (5’-3’) | Expected product size, bp | Annealing temperature (°C) |
|--------------|-------------------------------------|--------------------------|---------------------------|
| COL2A1       | CCTAATTGAGATGCTGTCG CCAGGCAATCCAATGGTGCC | 187 bp                   | 57                        |
| COL1A1       | ATCCAGTCGACCTTCTGCGG TGAGGCAATATCTGCTC | 322 bp                   | 60                        |
| Wisp3        | GTAAGCTTAGGACATGCGAGGGCTCTCTTT GCGATCCTTTACAGATCCGAATAGTCCTGAGG | 1065 bp                  | 62                        |
| Fibronectin  | GTGGTAGACCCCTCATAGGGAAC TACCTCAGGATGCTCCTGTG | 299 bp                   | 60                        |
| SOX9         | CACACTACAGGCCCTCCTAC CCGTCTCAAGGTCGAGTGAG | 258 bp                   | 60                        |
| MMP-1        | ATGGCTGAAACCTCGAGGGTGA CAAGATTCCTCCAGGCTCCA | 305 bp                   | 60                        |
| β-actin      | TCCGTGATCCAGCAGAATCT GAAGCATTTGCGGTGGACGAT | 310 bp                   | 58                        |

2.9. 14C-Proline Incorporation Analysis. Chondrocytes were seeded into 24-well plates (5 × 10⁴ cells/cm²) in DMEM with 10% FBS for 24 hours, then cultured in 500 μl of serum-free DMEM for 4 hours. For 14C-proline incorporation, each well was added with 10 μCi of 14C-proline (100 μCi/mL) (GE) and 100 μg/mL aminopropionitrile, then incubated for 2h; cells were rinsed five times with PBS, and complete medium was added and incubated at 37°C for 0, 30, 60, 120, 180, 240, and 300 minutes; supernatant and cell lysates were collected at these time points. For the collection of cell lysates, cells were rinsed twice with PBS after the supernatant collection, two times with 5% cold trichloroacetic acid, two times with 80% ethanol and finally lysed at 37°C for 2h in 0.5 mL of 10 mM EDTA; 0.2 mL aliquots of the lysates and supernatant were dissolved in 10 mL Ecoscin H (Promab, Briare, France) separately and counted by scintillation; the quantification of intracellular collagen content and extracellular secretion was determined by the radioactivity in the cell lysate and supernatant, and the ratio of extracellular collagen secretion to intracellular collagen content was counted.
2.10. Statistical Analysis. SPSS II.0 software was used for statistical analysis and data are presented as the mean ± SD, with the exception of gene analysis data. Data were compared using one-way ANOVA or the student's t-test. All experiments were repeated at least 3 times; the representative experiments are shown.

3. Results

3.1. Abnormal Protein Localization of Cells Transfected with Wisp3 Mutants. The recombined plasmids WT-Wisp3/pEGFP-C2, MUT1000T/C/pEGFP-C2, and MUT840delT/pEGFP-C2 were transfected transiently into human chondrocytes cell line C-20/A4, and pEGFP-C2 vector was used as a control. The expression and localization of green fluorescence protein (GFP) fusion proteins were observed using LSCM after 48 hours of transfection. GFP signal was distributed throughout the cells transfected with pEGFP-C2 vector (Figure 1(a)) and uniformly in cytoplasm and cell membrane transfected with WT-Wisp3/pEGFP-C2 (Figure 1(b)); however, the fluorescence signal aggregated to speckles or agglomerates in cytoplasm transfected with MUT1000T/C/pEGFP-C2 and MUT840delT/pEGFP-C2 (Figures 1(c) and 1(d)).

3.2. Increased Cell Proliferation and Decreased Cell Apoptosis Ratio of Cells Transfected with Wisp3 Mutants. MTT assays showed cell viability in C-20/A4 cells stably transfected with MUT1000T/C/pcDNA3.1(+) and MUT840delT/pcDNA3.1(+) was obviously higher (0.38 ± 0.03 and 0.42 ± 0.04, P < 0.01) than that in cells stably transfected with pcDNA3.1(+) (0.24 ± 0.02) (Figure 2(a)).

Overproliferation of cells stably transfected with mutant wisp3 was further demonstrated by flow cytometry analysis, which indicated that the cell numbers in the G2-M plus S phases were significantly higher than that of control cells (33.6 ± 4.0%, P < 0.05, Figure 2(b)), with a proliferation index of 49.8 ± 5.0% and 53.2 ± 4.5%, respectively (Figures 2(c) and 2(d)).

The apoptosis rate of control cells was 26.1 ± 4.0% after cultured in serum-free medium for 24 h (Figure 3(a)), while that of cells stably transfected with MUT1000T/C/pcDNA3.1(+) and MUT840delT/pcDNA3.1(+) was decreased (8.5 ± 2.6% and 6.9 ± 2.4%) (Figures 3(b) and 3(c)). Acridine orange and ethidium bromide staining also suggested that the apoptosis rate was decreased dramatically in cells stably transfected with mutant Wisp3; there were no apparent apoptotic cells in them (Figures 3(d)–3(f)).

3.3. Dysfunction of Collagen Production in Cells Transfected with Mutants. The function of Wisp3 gene in chondrocytes and the mechanism of disorders in cartilage tissue caused by Wisp3 mutation were still unclear. The C-20/A4 chondrocytes lines express very low levels of Wisp3 (Figures 4(a) and 5(a)) and cartilage specific collagens. To investigate the function of wild and mutant Wisp3 gene in chondrocytes, the mutant and control plasmids were transfected stably into chondrocyte cell line C-20/A4. The WT-Wisp3/pcDNA3.1(+) transfected C-20/A4 cells expressed 3.3-fold higher levels of COL2A1 mRNA than the cells transfected with the control vector (Figure 4(b)). Figure 5(b) demonstrated that stable transfection of C-20/A4 cells with WT-Wisp3/pcDNA3.1(+) upregulates type II collagen protein expression (P < 0.05). However, the COL2A1 expression did not change in C-20/A4 cells transfected with MUT1000T/C/pcDNA3.1(+) and MUT840delT/pcDNA3.1(+) both in mRNA and protein level, compared with the cells transfected with the empty vector.

In contrast, minimal changes were observed in the levels of mRNA of type I collagen, SOX9, and fibronectin in response to either wild or mutant Wisp3 (Figures 4(c), 4(d), and 4(e)). The mRNA expression of MMP-1, which had been found dramatically decreased in articular chondrocytes separated from SEDT-PA/PPD patient [15] (Figure 4(f)), wasn't changed in the mutant Wisp3 transfected chondrocytes.

3.4. Abnormal Intracellular Collagen Content and Secretion in Mutant Chondrocyte. By 14C-proline incorporation analysis, very low radioactivity was detected in the supernatant of and cell lysate of C-20/A4 cells transfected with control vectors, which indicated that very low collagen synthesis and secretion in this cell line (Figures 6(a) and 6(b)), and the ratio of extracellular collagen secretion to intracellular content is approximately 1. However, in wild type Wisp3 stably transfected C-20/A4 cells, high radioactivity, were detected in the culture supernatant (3000 CPM to 7000 CPM) and cell lysate (700 CPM to 1000 CPM); the peak collagen secretion and intracellular content were appeared at 120 min and 60 min separately after refreshment of the complete medium, compared to C-20/A4 cells transfected with control vector; Wisp3 increased the intracellular collagen content to about 5–10 times (P < 0.01), and especially increased the extracellular collagen secretion to 10–20 times (P < 0.01), and the ratio of extracellular collagen secretion to intracellular content is 3.5–10 (Figure 6(c)). In mutant Wisp3 (MUT840delT and MUT1000T/C) transfected cells, the peak collagen secretion and intracellular content were were backward to 120 min and 180 min separately, although the radioactivity of collagen secretion was slightly higher than that of intracellular collagen content, the extracellular collagen secretion was decreased obviously compared to the wild type Wisp3 transfected cells, and the ratio of extracellular collagen secretion to intracellular content is about 1.5.

4. Discussion

PPD was attributed to mutations of Wisp3 gene; we previously identified a novel compound heterozygous mutation (840delT/T1000C) of Wisp3 in a SEDT-PA/PPD family, and this mutation results in a dramatic decrease in the tensile strength of articular cartilage; however, the detail mechanism is not clear.

By bioinformatics analysis, we predicted that the compound heterozygous mutation formed a truncated Wisp protein and a Ser334Pro mutated proteins [14]. The 3D-conformational change of the 840delT truncated mutant
**Figure 1:** Localization of wild and mutated Wisp3 protein in C20/A4 cells by confocal microscope. Recombined plasmids WT-Wisp3/pEGFP-C2, MUT1000T/C/pEGFP-C2, and MUT840delT/pEGFP-C2 were transfected transiently into human chondrocyte cell line C20/A4, and pEGFP-C2 vector was used as a control. The cells were observed using a confocal laser scanning microscope after 48 hours of transfection at magnification 1000x. (a) EGFP; (b) WT-Wisp3; (c) MUT1000T/C; (d) MUT840delT. Green fluorescence indicates the Wisp3 EGFP fusion protein. Blue fluorescence shows cell nuclei dye by DAPI. Note the distribution of WT-Wisp3 in cytoplasm and cell membrane uniformly. In contrast, the majority of MUT1000T/C and MUT840delT were aggregated to speckles or agglomerates in cytoplasm.

Wisp3 protein is the single long peptide loop in the region from signal peptide to the beginning 24 amino acid residues in the first domain (IGFBP) which was subjected to folding into two smaller cross-loops accompanied with a much shorter C-terminus. It has been noted that the function of the first (IGFBP) domain of Wisp3 is involved in inhibiting the function of IGF-1 to the chondrocytes and the fourth (CK) domain is involved in disulfide-linked dimerisation and is necessary for dimer formation in the endoplasmic reticulum, an important function for the establishment and maintenance of normal 3D-conformation of Wisp3 protein [18-20]. Through GFP labeled protein localization analysis, we found that wild type Wisp3 protein did localize in cytoplasm and cell membrane of C-20/A4 cells, but the two mutated Wisp3 proteins aggregated abnormally in cytoplasm of C-20/A4 cells transfected with MUT1000T/C and MUT840delT. It needs further research to validate the hypothesis that 3D-conformational change causes localization change of mutated Wisp3 protein.

Wisp3 belongs to the CCN family of proteins, which play important roles in development during chondrogenesis and enchondromatosis and encode cysteine-rich secreted proteins with roles in cell growth and differentiation [21]. To investigate the effects of the T1000C and 840delT mutations on Wisp3 function in chondrocytes, we compared biological behaviors in C-20/A4 cells transfected separately with WT-Wisp3, MUT1000T/C, and MUT840delT. MUT1000T/C Wisp3 and MUT840delT Wisp3 increased proliferation activity as well as decreased apoptosis of C-20/A4 cells obviously, which shared the phenotype of articular chondrocytes (ACs), separated from SEDT-PA patients we described before [15]. Therefore, inhibition of cell proliferation and promotion of precursor cell differentiation are major effects of Wisp3 on chondrocytes, through which Wisp3 modulates the balance of cartilage metabolism.

We previously found that PPD cartilage had lost its flexibility, and the main matrix component of cartilage is collagen, so we detected the effect of Wisp3 gene mutation on the collagen expression in chondrocytes. The results demonstrated that both mutant Wisp3 lose the function to modulate the expression of cartilage specific matrix type II collagen when compared with wild Wisp3, which consisted of results in C-28/I2 and T/C-28a2 cells transfected with another SEDT-PA related Wisp3 mutation (Cis78-Arg) [22], but the modulation effect of Wisp3 may not be via activation of SOX9 in our study since no change of SOX expression was
found. After the collagen synthesis, it need to be secreted into the extracellular matrix, if the collagen secretion was changed by gene mutation or 3D-conformational alteration, the function of cartilage will be abnormal, and to further study the dynamic collagen synthesis and secretion, we use $^{14}$C-proline, which is the major material of collagen synthesis and a major determinant of collagen tertiary structure, to label the new synthesized collagen, through detection of the radioactivity in cell lysate and supernatant to quantify the intracellular collagen content and extracellular secretion at different time points. Compared to the wild type Wisp3, MUT$^{1000T/C}$ or MUT$^{840delT}$ Wisp3 lost the function of increasing the extracellular collagen secretion, delaying the intracellular collagen synthesis, which is one of the important mechanisms for the collagen size and density decrease in PPD cartilage described previously.

However, we could not find the difference of MMP-1 mRNA levels between the C-20/A4 cells transfected with wild and mutant Wisp3, which was dramatically decreased in ACs separated from SEDT-PA/PPD patients compared with normal ACs. The paradoxical phenomenon may be related to the following causes: (1) C-20/A4 is an immortalized cell line derived from human juvenile costal cartilage and is highly proliferative and not contact-inhibited compared with primary cells, which may have influence on the expressions of matrix and other genes at reasonable levels [23]. (2) MUT$^{1000T/C}$ or MUT$^{840delT}$ results in obviously changed biological behaviors of chondrocytes; however, that
Figure 3: Cell apoptosis analysis in C20/A4 cells transfected with mutant Wisp3. Cells stably transfected with MUT<sup>1000T/C</sup>/pcDNA3.1(+), MUT<sup>840delT</sup>/pcDNA3.1(+), or empty vector; cell apoptosis was evaluated by using FAC flow cytometry (a–c) and acridine orange/ethidium bromide staining (d–f) (apoptotic cells stained with yellow, condensed, or fragmented nuclei) analysis. (a) and (d) empty vector apoptosis rate is 27.1%; (b and e) MUT<sup>1000T/C</sup> apoptosis rate is 9.2%; (c and f) MUT<sup>840delT</sup> apoptosis rate is 7.8%. Magnification is 200 x.

Figure 4: mRNA expression of cartilage-specific genes in C20/A4 cells transfected with wild and mutant Wisp3. Cells stably transfected with WT-Wisp3/pcDNA3.1(+), MUT<sup>1000T/C</sup>/pcDNA3.1(+), MUT<sup>840delT</sup>/pcDNA3.1(+), or empty vector. mRNA expression of cartilage-specific genes in C20/A4 cells was determined by RT-PCR. (a)–(g) represent Wisp3, COL2A1, COL1A1, SOX9, fibronectin, MMP-1, and β-actin separately.
**Figure 5:** Wisp3 and COL2A1 protein expression in C20/A4 cells transfected with wild and mutant Wisp3 analyzed by western blot.

**Figure 6:** Change of intracellular collagen content and extracellular collagen secretion in mutant chondrocytes analyzed by $^{14}$C-proline incorporation assay. (a) Time course of $^{14}$C-proline labeled collagen (detected by radioactivity) secreted to the supernatant of the cultured chondrocytes. (b) Time course of $^{14}$C-proline labeled collagen content in cultured chondrocytes. (c) Ratio of secreted collagen to intracellular collagen.
cannot fully represent the effect of compound mutation (840delT/T1000C) on the cartilage metabolism. (3) The low expression level of Wisp3 in C-20/A4 cell line may have interacted influence on the expressions of other genes.

5. Conclusions

Wisp3 mutations resulted in abnormal protein distribution and dysfunction of cell proliferation, collagen production, and dynamic secretion in chondrocytes, which may be involved in the pathogenesis of SEDT-PA.

Acknowledgments

This work was supported by Grant nos. 81070278, 30872617 from China National Natural Scientific Foundation, Natural Science Foundation from Hunan Provincial (100JJ1007), and Central South University (2010QZZD025).

References

[1] J. R. Hurvitz, W. M. Suwairi, W. Van Hul et al., "Mutations in the CCN gene family member WISP3 cause progressive pseudohematoïd dysplasia," Nature Genetics, vol. 23, no. 1, pp. 94–98, 1999.

[2] B. Perbal, C. Martinerie, R. Sainson, M. Werner, B. He, and B. Roizman, "The C-terminal domain of the regulatory protein NOVH is sufficient to promote interaction with fibulin IC: a clue for a role of NOVH in cell-adhesion signaling," Proceedings of the National Academy of Sciences of the United States of America, vol. 96, no. 3, pp. 869–874, 1999.

[3] Y. Nishida, T. Matsubara, T. Tobina et al., "Effect of low-intensity aerobic exercise on insulin-like growth factor-I and insulin-like growth factor-binding proteins in healthy men," International Journal of Endocrinology, vol. 2010, Article ID 452820, 8 pages, 2010.

[4] B. Perbal, D. R. Brigstock, and L. F. Lau, "Report on the second international workshop on the CCN family of genes," Journal of Clinical Pathology, vol. 56, no. 2, pp. 80–85, 2003.

[5] G. W. Zuo, C. D. Kohls, B. C. He et al., "The CCN proteins: important signaling mediators in stem cell differentiation and tumorigenesis," Histology and Histopathology, vol. 25, no. 6, pp. 795–806, 2010.

[6] A. Leask, "CCN6 (WISP3): a new anti-cancer therapy?" Journal of Cell Communication and Signaling, vol. 4, no. 4, pp. 199–200, 2010.

[7] S. A. Al-Awadi, T. I. Farag, and K. Naguib, "Spondyloepiphysial dysplasia tarda with progressive arthropathy," Journal of Medical Genetics, vol. 21, no. 3, pp. 193–196, 1984.

[8] A. Dalal, G. Bhavani SL, P. P. Togaratti et al., "Analysis of the WISP3 gene in Indian families with progressive pseudohematoïd dysplasia," American Journal of Medical Genetics A, vol. 158, pp. 2820–2828, 2012.

[9] S. Ehl, M. Uhl, R. Berner, L. Bonafé, A. Superti-Furga, and A. Kirchhoff, "Clinical, radiographic, and genetic diagnosis of progressive pseudohematoïd dysplasia in a patient with severe polyarthritis," Rheumatology International, vol. 24, no. 1, pp. 53–56, 2004.

[10] J. Ye, H. W. Zhang, W. J. Qiu et al., "Patients with progressive pseudohematoïd dysplasia: from clinical diagnosis to molecular studies," Molecular Medicine Reports, vol. 5, pp. 190–195, 2012.

[11] M. F. Kahn, "Chondrodysplasias rheumatism," British Journal of Rheumatology, vol. 37, no. 8, article 917, 1998.

[12] E. Y. Liao, Y. Q. Peng, H. D. Zhou et al., "Gene symbol: WISP3. Disease: spondyloepiphysial dysplasia tarda with progressive arthropathy," Human Genetics, vol. 115, no. 2, article 169, 2004.

[13] E. Y. Liao, Y. Q. Peng, H. D. Zhou et al., "Gene symbol: WISP3. Disease: spondyloepiphysial dysplasia tarda with progressive arthropathy," Human Genetics, vol. 115, no. 2, article 174, 2004.

[14] Y. Q. Peng, E. Y. Liao, H. M. Gu et al., "Pathology and molecular pathogenesis of spondyloepiphysial dysplasia tarda with progressive arthropathy caused by compound CCN6 heterogeneous gene mutations," Zhonghua Yi Xue Za Zhi, vol. 84, no. 21, pp. 1796–1803, 2004.

[15] H. D. Zhou, Y. H. Bu, Y. Q. Peng et al., "Cellular and molecular responses in progressive pseudohematoïd dysplasia articular cartilage associated with compound heterozygous WISP3 gene mutation," Journal of Molecular Medicine, vol. 85, no. 9, pp. 985–996, 2007.

[16] A. Pal, W. Huang, X. Li, K. A. Toy, Z. Nikolovska-Coleska, and C. G. Kleer, "CCN6 modulates BMP signaling via the Smad-independent TAK1/p38 pathway, acting to suppress metastasis of breast cancer," Cancer Research, vol. 72, pp. 4818–4828, 2012.

[17] W. Huang, M. E. Gonzalez, K. A. Toy, M. Banerjee, and C. G. Kleer, "Blockade of CCN6 (WISP3) activates growth factor-independent survival and resistance to anoikis in human mammary epithelial cells," Cancer Research, vol. 70, no. 8, pp. 3340–3350, 2010.

[18] Q. H. Liang, Y. Jiang, X. Zhu et al., "Ghrelin attenuates the osteoblastic differentiation of vascular smooth muscle cells through the ERK pathway," PLoS ONE, vol. 7, no. 4, Article ID e33126, 2012.

[19] C. G. Kleer, Y. Zhang, and S. D. Merajver, "CCN6 (WISP3) as a new regulator of the epithelial phenotype in breast cancer," Cells Tissues Organs, vol. 185, no. 1–3, pp. 95–99, 2007.

[20] A. Katsumi, E. A. Tuley, I. Bodo, and J. E. Sadler, "Localization of disulfide bonds in the cystine knot domain of human von Willebrand factor," Journal of Biological Chemistry, vol. 275, no. 33, pp. 25585–25594, 2000.

[21] W. E. Kutz, Y. Gong, and M. L. Warman, "WISP3, the gene responsible for the human skeletal disease progressive pseudohematoïd dysplasia, is not essential for skeletal function in mice," Molecular and Cellular Biology, vol. 25, no. 1, pp. 414–421, 2005.

[22] B. Perbal, "NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues," Journal of Clinical Pathology, vol. 54, no. 2, pp. 57–79, 2001.