Wnt1 Inducible Signaling Pathway Protein-3 Regulation and Microsatellite Structure in Arthritis

HYEONJOO CHEON, DAVID L. BOYLE, and GARY S. FIRESTEIN

ABSTRACT. ***Objective.*** Rheumatoid arthritis (RA) synovial tissue expresses several embryonic gene families, including wingless (*wnt*) and their receptors, frizzled (*fz*). The Wnt proteins, including Wnt-1, activate the Wnt inducible signaling pathway proteins (WISP), which are members of the CCN family that regulate cell growth and differentiation. WISP3 is of particular interest because it contains a microsatellite region in its coding region that is susceptible to frameshift mutations and leads to a truncated protein. To investigate the contribution of WISP3 to synovial inflammation, we evaluated its expression and regulation in arthritis.

Methods. mRNA and protein expression of WISP3 were determined by quantitative real-time polymerase chain reaction (PCR) and Western blot analysis, respectively. For mutation analysis, PCR product amplified from genomic DNA of synovial tissue and cultured fibroblast-like synoviocytes (FLS) was subcloned and sequenced.

Results. WISP3 mRNA is expressed in synovial tissue, but is 11-fold higher in RA than osteoarthritis (OA) or normal samples. Surprisingly, WISP3 protein levels are similar in RA, OA, and normal synovium samples. Immunohistochemistry of synovial tissue reveals that WISP3 protein is located primarily in the synovial intimal lining. WISP3 mRNA expression is also 6-fold higher in RA FLS compared with OA FLS and 50-fold higher in RA than in normal FLS. When RA FLS are stimulated with interleukin 1 or tumor necrosis factor-α, WISP3 mRNA is significantly increased. The cytokines also increase WISP3 mRNA in OA FLS, but the maximal level in stimulated OA FLS is still less than medium-treated RA FLS. Mutation analysis in the coding region microsatellite of the WISP3 gene in RA and OA synovium and FLS shows a limited number of insertion and deletion mutations.

Conclusion. WISP3 gene expression is higher in RA synovium and FLS compared with OA and normal synovial tissue and is further induced by proinflammatory cytokines *in vitro*. Protein levels are not increased, indicating discoordinate regulation of WISP3 protein and mRNA. Although functionally relevant mutations were observed in genomic DNA, they were noted in both OA and RA samples. (J Rheumatol 2004;31:2106–14)

Key Indexing Terms:
STROMAL CELLS HUMAN RHEUMATOID ARTHRITIS
CYTOKINES INFLAMMATION

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by invasion of synovial tissue into cartilage and bone[1]. Fibroblast-like synoviocytes (FLS) contribute to this process and exhibit some features of transformed cells, including anchorage-independent growth *in vitro*, loss of contact inhibition, and expression of several oncogenes[2-4]. In addition, expression of embryonic gene-like members of the *wnt* family in rheumatoid arthritis (RA) synoviocytes suggests that the cells dedifferentiate in the context of chronic synovial inflammation[5]. Although the mechanisms of autonomous behavior and dedifferentiation are not fully understood, they might include permanent genetic changes, including somatic mutations in genes such as *p53*, *hprt*, and *H-ras*[6-11]. A mutagenic environment and/or deficient DNA repair mechanisms can also cause insertion or deletion mutations in microsatellites, which are mononucleotide and dinucleotide repeat sequences. Recent studies described microsatellite mutations (also called microsatellite instability, or MSI) in RA synovium compared with osteoarthritis (OA) tissue[12].

The dedifferentiation process in RA is reflected by increased expression of several embryonic gene families in RA synovium, including wingless (*wnt*) and frizzled (*fz*, which is a Wnt receptor) families[5]. These genes play a key role in embryonic cell proliferation, adhesion, and polarity as well as the establishment of bone marrow progenitors and limb bud mesenchyme[13,14]. The *wnt* and *fz* signaling

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pathways are activated in RA fibroblasts and induce expression of proinflammatory cytokines and fibronectin[15]. Normal fibroblasts transfected with *wnt5* display some features of the RA FLS phenotype, including increased cytokine expression.

Because RA FLS express *wnt* genes and exhibit some features of dedifferentiation, we investigated signaling molecules involved in this pathway. Of these, the Wnt-1 inducible signaling pathway proteins (WISP) are especially intriguing because they can contribute to the transformed phenotype in *wnt-1*-transduced mouse epithelial cells[16]. The WISP proteins (WISP1, 2, and 3) are members of the CCN family, which encode growth regulators with highly conserved cysteine residues[16-18]. WISP3 was identified as a homologous protein of unknown function when the expressed sequence tag databases were screened with the WISP1 sequence[17]. WISP3 gene contains a mononucleotide microsatellite region with 9 adenines in the coding region that is susceptible to frameshift mutation. Thorstensen, *et al* found frameshift mutations in this microsatellite in 31% of colorectal carcinomas, and 11% of gastric carcinoma from patients with DNA repair defects[18,19]. Perhaps more relevant to rheumatic diseases, WISP3 mutations cause progressive pseudorheumatoid dysplasia, suggesting that WISP3 plays a role in skeletal homeostasis and could be expressed by synovium[20].

Based on these data, we evaluated WISP3 expression and regulation in RA synovium. These studies showed that the WISP3 mRNA is overexpressed in the rheumatoid joint and could be further induced in RA synoviocytes by proinflammatory cytokines. Protein expression, however, was similar in RA, OA, and normal synovium, indicating that WISP3 protein and gene regulation are discoordinate. Further, a limited number of microsatellite frameshift mutations were found in RA and OA synovium and synoviocytes. This is the first study describing the regulation of WISP3 in non-neoplastic disease.

MATERIALS AND METHODS

Synovial tissue samples. Synovial tissue samples were obtained from patients with RA and OA at the time of joint replacement surgery. The diagnosis of RA conformed to the American College of Rheumatology 1987 revised criteria[21]. The samples were either processed for cell culture or snap-frozen or embedded in TissueTek OCT compound (Miles Diagnostics, Elkhart, IN, USA) by immersion in liquid nitrogen and stored at –80°C until used. Normal synovium was obtained at the time of autopsy from individuals without evidence of arthritis. The studies were approved by the University of California, San Diego Human Subjects Research Protection Program. Separate patient samples were processed for WISP3 expression studies and microsatellite analysis.

Fibroblast-like synoviocytes. Synovial tissues were minced and incubated with 1 mg/ml collagenase in serum-free Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Grand Island, NY, USA) for 2 h at 37°C, filtered through nylon mesh, extensively washed, and cultured in DMEM supplemented with 10% fetal calf serum (FCS, endotoxin content < 0.006 ng/ml; Life Technologies), penicillin, streptomycin, and L-glutamine in a humidified 5% CO_2 atmosphere. After overnight culture, nonadherent cells were removed. Adherent cells were later trypsinized, split at a 1:3 ratio, and cultured in medium. Synoviocytes were used from passages 3 through 8 in these experiments when they were a homogenous population of FLS (< 1% CD11b, < 1% phagocytic, and < 1% FcγRII positive)[22].

Immunohistochemistry. Frozen tissues embedded in OCT compound were cut into 5 µm sections and fixed in acetone for 10 min[23]. After blocking with 1% bovine serum albumin in phosphate buffered saline (PBS), 5% horse serum in PBS, and 5% human serum in PBS, C-terminal-specific polyclonal goat anti-WISP3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was incubated with the sections at 4 mg/ml in PBS/0.1% Triton X-100, 2% horse serum. After 1 h at room temperature, sections were washed with PBS/0.1% Triton X-100. Biotinylated horse anti-goat IgG (Vector Laboratories, Burlingame, CA, USA) was applied at 1:3000 dilution in PBS/2% horse serum, incubated for 30 min, and washed with PBS/0.1% Triton X-100. After endogenous peroxidase was depleted by quenching with 0.3% H_2O_2 for 20 min, the sections were incubated with ABC peroxidase reagent (Vector) for 30 min. The slides were then washed with PBS, developed with DAB, and counterstained with hematoxylin.

Real-time polymerase chain reaction (PCR). Real-time reverse transcriptase-PCR was performed using the GeneAmp 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). Total RNA was isolated from RA, OA, and normal synovial tissues and FLS using RNA STAT-60 (Tel-Test, Friendswood, TX, USA). Extracted RNA was reverse-transcribed into cDNA with random hexamer and Moloney murine leukemia virus reverse transcriptase according to the manufacturer's protocol (TaqMan reverse transcription reagent; Perkin-Elmer). PCR was performed in a SYBRgreen universal PCR Master Mix (Perkin-Elmer) with WISP3 and GAPDH primers (forward for WISP3: 5'-CAG CAG CTT TCA ACA AGC TAC A-3'; reverse for WISP3: 5'-TTC CCA TCC CAC ATG TTC TG-3') using the following protocol: initial activation at 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. cDNA for standard curves was prepared from FLS treated with tumor necrosis factor-α (TNF-α) for 48 h after 48 h synchronizing in DMEM containing 0.5% FCS. Aliquots of standard cDNA were included in each PCR run, and standard curves for WISP3 and GAPDH were generated by linear regression using log [Ct] versus log (cell number). Sample Ct values were then used to calculate the cell equivalent (CE) number for WISP3 and GAPDH in unknown samples. Data were expressed as the relative expression (RE), which is the ratio of WISP3 CE to GAPDH CE, as described[24]. Each PCR run also included nontemplate controls containing all reagents except cDNA. These controls generated a Ct greater than 40 in all experiments. WISP3 primers produced a single band by standard PCR using an ethidium stained gel, and the specificity was confirmed by sequencing the amplified product. Only WISP3 was identified in either the amplified PCR product or individual subclones of the PCR product.

Western blot analysis. Cultured FLS or pulverized snap-frozen synovial tissue aliquots were suspended in cold TE/150 mM sodium deoxycholate, 1% Nonidet P-40 for 30 min on ice. Protein concentrations were measured with the DC protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Protein samples from FLS lysates (40–80 µg) or synovial tissue lysates (100 µg) were separated by electrophoresis using a sodium dodecyl sulfate-12% polyacrylamide gel, and transferred onto a nitrocellulose membrane at 140 mA in 25 mM Tris Cl (pH 8.3), 200 mM glycine, and 20% methanol. Membranes were blocked with 5% nonfat dry milk in Tris buffered saline-0.1% Tween 20 (TBS-T) for 3 h, followed by overnight incubation at 4°C with goat polyclonal anti-C-terminal WISP3 antibody, anti-actin antibody, or control goat IgG. In some experiments, a goat-anti-N-terminal WISP3 (Santa Cruz Biotechnology) was used. Blots were washed with TBS-T and incubated 1 h at room temperature with horseradish peroxidase-conjugated secondary anti-goat IgG (Santa Cruz Biotechnology). The proteins were visualized using ECL solution as a substrate (Amersham, Buckinghamshire, UK) with Kodak X-OMAT AR film (Eastman Kodak, Rochester, NY, USA). To quantify the amount of WISP3 proteins, each band density was normalized to actin and digitized using US National Institutes of Health image analyzer software version 1.61.

Microsatellite mutation analysis. Genomic DNA was isolated from RA and OA synovial tissues and FLS using DNA STAT-60 (Tel-Test). Exon 4 of the WISP3 gene was amplified from genomic DNA by PCR with primers flanking the microsatellite $(A)_9$ repeats (forward: 5'-CTC CTA AGA GAC TGG ACT GTA ATA CTT GAT-3'; reverse DNA: 5'-TTC TCA TTT CAC AGT TGC TGT TTT C-3'). The PCR mixture consisted of 5 U of Amplitaq Gold (Perkin-Elmer), 1× PCR buffer, 0.8 mM dNTP mix, 1 µM forward and reverse primers, and DNA samples in a total volume of 100 µl. For each PCR, DNA was amplified for 35 cycles of 94°C/30 s, 54°C/45 s, and 72°C/50 s, with a hot start at 94°C for 5 min and a final extension at 72°C for 10 min. Amplified PCR products were resolved by electrophoresis on 1.8% agarose gel containing ethidium bromide. The bands were excised from the gels with a sterile blade and purified using a QIAquick gel extraction kit (Qiagen, Valencia, CA, USA). Purified PCR products were subcloned using the TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) and sequenced at the Center for AIDS Research Molecular Biology Core at UCSD.

Statistical analysis. Data are expressed as means ± SEM. Statistical analysis was performed using the t-test. P values less than 0.05 were considered significant.

RESULTS

WISP3 gene and protein expression in synovial tissue. Initial studies were performed to determine if the WISP3 gene is expressed in synovium. Real-time PCR was performed on cDNA from whole synovial tissue extracts and compared using the standard curve method as described[24] (n = 5 for RA, OA, and normal samples). As shown in Figure 1, normalized WISP3 mRNA levels were 11-fold greater in RA synovial tissues compared with OA or normal synovial tissues ($p < 0.05$ RA vs OA or normal). WISP3 protein expression was then examined in RA, OA, and normal synovium by Western blot analysis using antibody specific for the C-terminal region of WISP3 (n = 7 for RA and OA, n = 3 for normal). Figure 2 shows that WISP3 protein was readily detected in RA, OA, and normal tissues. Although there was a trend toward higher actin-normalized protein expression in RA compared with OA, differences between the groups did not reach statistical significance. Therefore, WISP3 protein expression was not increased in RA synovium and did not correlate with high mRNA content. Immunohistochemistry confirmed the expression of WISP3 protein in synovial tissue (a representative example is shown in Figure 3; n = 3 for RA and OA). The protein was detected primarily in the synovial cells in the intimal lining region, and the distribution was similar in OA and RA samples. No staining of synovial tissue sections was observed when an irrelevant control antibody was used.

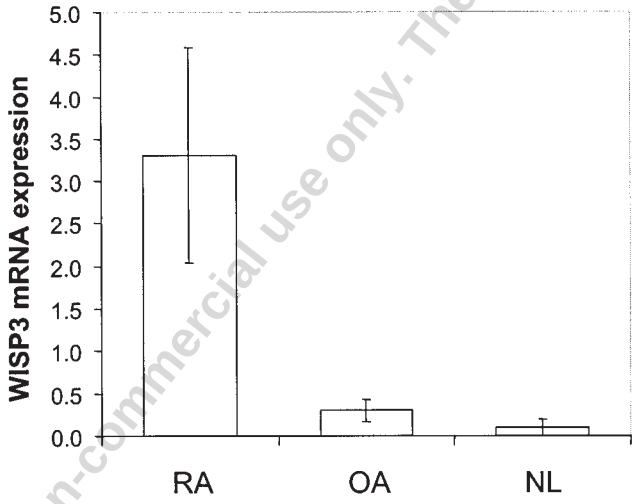

Figure 1. Expressions of WISP3 mRNA in synovial tissue. Total RNA was extracted from 5 RA, 5 OA, and 5 normal (NL) synovial tissue samples. Real-time PCR was performed using 1 ng total RNA from each sample with WISP3 primer pair. GAPDH-normalized WISP3 gene expression was significantly greater in RA than OA or normal samples ($p < 0.05$).

WISP3 gene and protein expression in RA, OA, and normal FLS. Because the *wnt* pathway is activated in RA FLS[5], we next examined WISP3 gene expression in FLS using real-time PCR. Significantly higher levels of WISP3 mRNA were observed in RA FLS compared with OA or normal samples (Figure 4; n = 6 for RA and OA, n = 5 normal). WISP3 gene expression was 6-fold greater in RA FLS than in OA samples ($p < 0.01$) and 50-fold greater than in normal samples ($p < 0.01$). WISP3 protein expression was examined in RA, OA, and normal FLS by Western blot analysis (n = 4 for RA and OA, n = 3 normal). WISP3 protein was readily detected in RA, OA, and normal FLS (Figure 5). As with synovial tissues, there were no differences between RA, OA, and normal with regard to constitutive WISP3 protein expression. The specificity of the antibody for WISP3 was indicated by observations that (1) some synoviocyte cell lines were negative (Figure 5C); (2) the band detected by the anti-WISP3 antibody was a single band at the appropriate molecular weight (39 kDa); (3) a control tumor cell line known to express WISP3 had the same 39 kDa band (Figure 5C); (4) the 39 kDa band was not present when a control antibody was used (Figure 5C); and (5) similar bands could be detected with an antibody that binds the N-terminal of the molecule rather than the C-terminal (data not shown).

Regulation of WISP3 gene expression by proinflammatory cytokines. Although Wnt-1 is one established mechanism that induces WISP, high expression in FLS and synovium suggests that proinflammatory cytokines might also contribute. Therefore, WISP3 gene expression was examined in TNF-α and interleukin 1ß (IL-1ß)-stimulated FLS using real-time PCR (Figure 6; n = 6 for RA and OA, n = 5 normal). WISP3 expression was increased 2.6-fold ($p < 0.03$) after 24 h of IL-1ß stimulation, and 3.5-fold after 48 h ($p < 0.03$). IL-1ß also increased WISP3 mRNA expression by 2-fold in OA and normal samples after 24 h ($p < 0.03$). However, the maximum IL-1-induced WISP3 mRNA levels in OA and normal FLS were still significantly less than medium-treated RA FLS ($p < 0.05$). WISP3 mRNA expression was also increased by 2.2-fold after 48 h of TNF-α stimulation ($p < 0.01$). TNF-α significantly increased WISP3 expression in OA and normal FLS after 48 h (2-fold

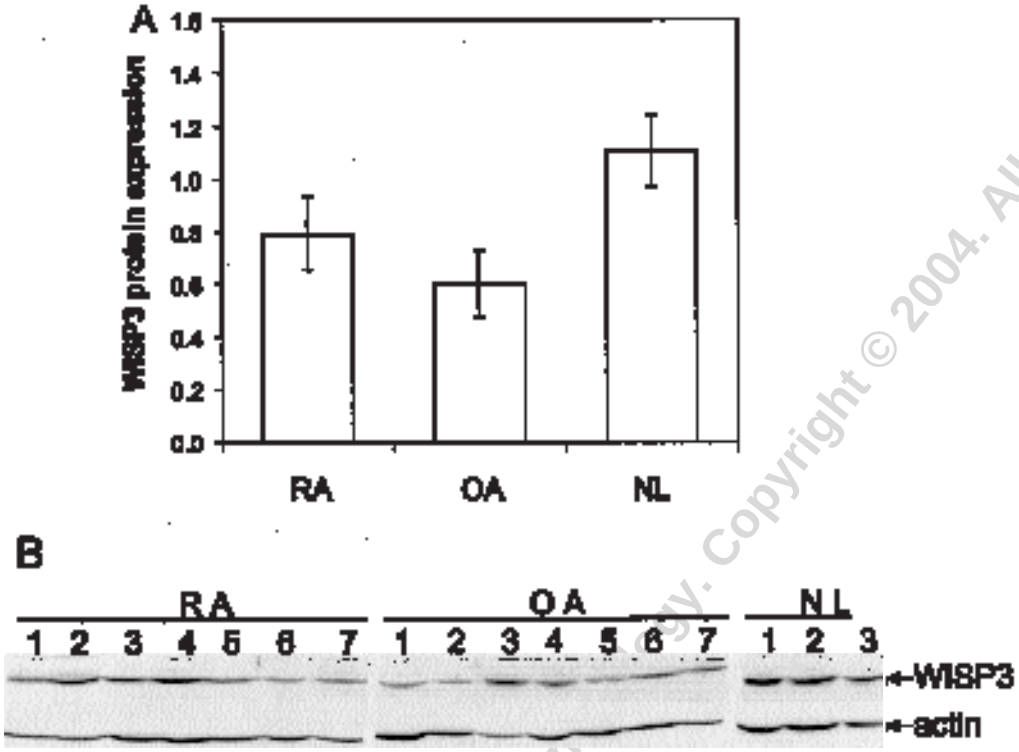

Figure 2. Expressions of WISP3 protein in synovial tissue; extracts from 7 RA, 7 OA, and 3 normal (NL) synovial tissue samples were evaluated by Western blot. (A) Actin-normalized WISP3 protein expression was similar in RA, OA, and normal samples. (B) Representative Western blots show the 39 kDa bands detected by WISP3 C-terminal antibody.

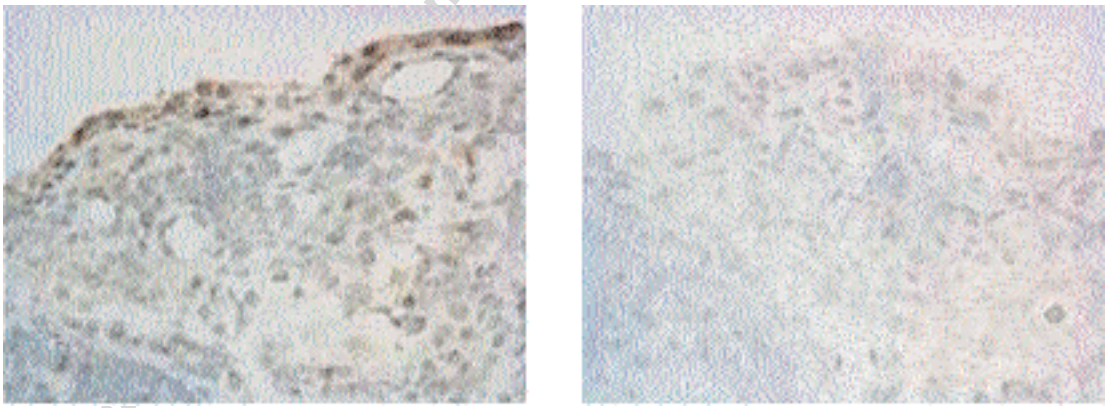

Figure 3. WISP3 protein localization in synovial tissue was examined by immunohistochemistry using antibody against WISP3 C-terminal portion (left) or control antibody (right). A representative RA tissue section is shown. Note that expression was primarily localized to the synovial intimal lining (brown color). The intensity and location of staining was similar in OA synovium (data not shown).

in OA, $p \leq 0.05$; 5-fold in normal samples, $p < 0.01$), but expression levels still remained well below RA FLS.

Lack of WISP3 protein induction by proinflammatory cytokines. Because discoordinate expression of WISP3 mRNA and protein was observed in other experiments, the effect of cytokines on protein expression was also determined in FLS. Synoviocytes were stimulated with medium, IL-1ß or TNF-α for up to 72 h (Figure 7; n = 2 for RA). Neither IL-1ß nor TNF-α significantly increased WISP3 protein expression. The cytokines also had no effect on WISP3 protein expression in OA FLS (n = 4 for OA, data not shown).

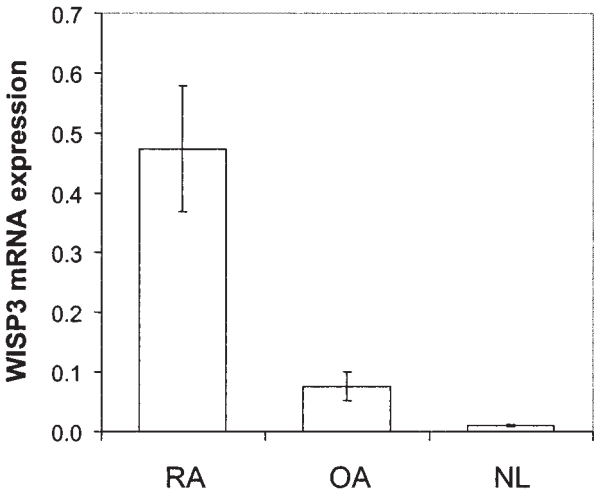

Figure 4. Expression of WISP3 mRNA in fibroblast-like synoviocytes (FLS). Total RNA was extracted from 6 RA, 6 OA, and 5 normal (NL) FLS and evaluated by real-time PCR. GAPDH-normalized WISP3 expression was significantly greater in RA compared with OA or normal samples ($p < 0.05$).

Mutation analysis in the microsatellite region of WISP3 gene. One reason we initiated studies of WISP3 expression in RA is the presence of a coding region containing a microsatellite in exon 4. A deletion or insertion mutation in this repeat leads a premature stop codon and a truncated protein. Hence WISP3 gene is a potential target gene in RA that might be susceptible to mutations due to microsatellite instability. We examined the mononucleotide repeat and the flanking sequences of the WISP3 gene by analyzing synovial tissue and FLS genomic DNA from patients with RA and OA (n = 3 for RA and OA). Nine to 12 subclones from each sample were sequenced. Mutation analysis in the coding region microsatellite of WISP3 gene in RA and OA synovium showed that most of the samples contained the wild-type sequence. A low incidence of functionally relevant frameshift mutations that produce a truncated form of the protein was observed, including in one RA and one OA synovium sample (Table 1). Similarly, a small number of insertions and deletions were observed in RA and OA FLS lines. The specificity of the mutations for the mononucleotide repeat was confirmed by the finding that no mutations occurred in the microsatellite flanking sequences. A normal synovial tissue was also analyzed. One subclone out of 10 analyzed contained a substitution mutation (G to A) in the first base of the microsatellite. This mutation does not produce the truncated protein as encoded by the mutations in the arthritis samples. Figure 8 shows examples of microsatellite mutations in the WISP3 coding region.

DISCUSSION

RA is polyarticular arthritis that is marked by synovial lining hyperplasia and degradation of the extracellular matrix. Cells in the synovial intimal lining, especially fibroblast-like synoviocytes, display evidence of autonomous activation and dedifferentiation that could contribute to their invasive expression. For instance, embryonic genes of the *wnt* and *fz* signaling pathways are activated in RA fibroblasts and are associated with increased expression of fibronectin and cytokines like IL-15[15]. These studies suggested that the *wnt* pathway might participate in the pathogenesis of RA and led us to explore downstream Wnt signal pathways. Wnt/frizzled signaling stabilizes beta-catenin, which interacts with transcription factor TCF/Lef1[25] and forms a complex that migrates to the nucleus. The WISP proteins are especially interesting because they are defined by their activation in cells that have been exposed to Wnt-1.

Three WISP genes have been identified, although their function and regulation are not fully defined. Of these, WISP3 attracted our attention because it is associated with microsatellite instability[19], a feature that has also been observed in RA[12]. The gene is located in chromosome 6q21–22 and encodes for a 354 amino acid 39 kDa protein in the CCN family. This heterogeneous family includes connective tissue growth factor (CTGF), Cyr61, Nov, WISP1, and WISP2[17,18,26-31]. The typical CCN protein is composed of 4 distinct modules, including an insulin-like growth factor binding protein-like domain, a Von Willebrand factor type C sequence, a thrombospondin type I motif, and a cysteine knot region[17].

CCN proteins participate in normal growth, differentiation, and cartilage morphogenesis[28,29]. Recently, CCN proteins have also been implicated in tumorigenesis, although the effects are highly variable[17-19,32-36]. WISP1 suppresses development of metastatic disease in murine melanomas, but its expression is increased in most human gastrointestinal carcinomas[18,25]. A human WISP1 variant lacking the Von Willebrand factor type C repeats occurs in gastric scirrhous carcinomas and can induce transformation and rapid growth[36]. The WISP3 gene is also often overexpressed in human colon carcinomas, while it is suppressed in inflammatory breast cancer[32]. Transduction with full-length WISP3 cDNA suppresses *in vivo* tumor growth in the nude mouse, indicating possible tumor-suppressor activity. Recently, WISP-3 expression has been described in chondrocytes and appears to regulate production of matrix proteins like type II collagen[37].

To investigate a potential role for WISP3 in an inflammatory disease such as RA, we initially assessed RNA transcripts and protein levels in synovium. WISP3 mRNA expression was investigated in RA, OA, and normal synovial tissue by real-time PCR, and significantly higher levels in RA samples were noted. Surprisingly, this difference was not observed in protein expression as determined by Western blot analysis, and provides evidence of discoordinate regulation of WISP3. Because immunohistochemistry confirmed WISP3 protein in the intimal lining, we then examined its expression in cultured FLS. As with synovial

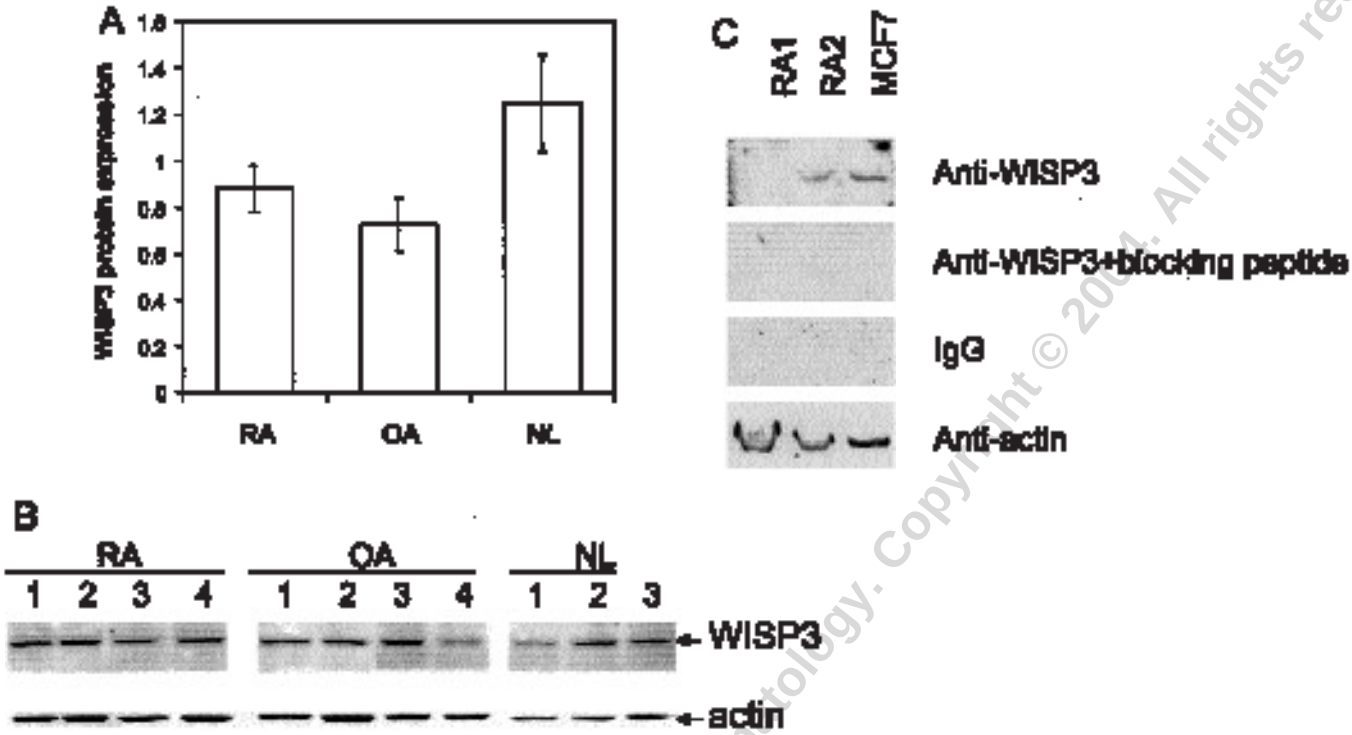

Figure 5. Expression of WISP3 protein in fibroblast-like synoviocytes. Extracts from 4 RA, 4 OA, and 3 normal (NL) synovial tissue samples were evaluated by Western blot. (A) Actin-normalized WISP3 protein expression was similar in RA, OA, and normal samples. (B) Representative Western blots show the 39 kDa bands detected by WISP3 C-terminal antibody. (C) Example of a Western blot showing the 39 kDa band in one RA FLS line and a breast tumor cell line known to express WISP3 (MCF7). Note that a second cell line (RA1) was negative for WISP3. The blot was stripped and reprobed with anti-WISP-3 antibody in the presence of excess of WISP3 C-14 blocking peptide, which is the epitope recognized by the antibody. Anti-WISP3 antibody binding was completely inhibited, confirming specificity for the 39 kDa WISP3 protein. The same blot was negative using a control goat IgG instead of anti-WISP3. Actin expression is shown as a positive control.

tissue, markedly higher levels of mRNA expression but not protein production were observed in RA cells compared with OA or normal synoviocytes. The proinflammatory cytokines IL-1 and TNF-α increased WISP3 expression in FLS, and this represents the first evidence that the WISP genes are regulated by cytokines. Because basal expression of WISP3 is also greater in cultured FLS, other signals likely contribute to *wnt* activation and WISP3 induction in RA. Significant increases in WISP3 mRNA were observed for RA as well as non-RA samples, although the level of expression in OA and normal FLS remained well below basal expression in RA. Protein studies, however, did not show induction of WISP3 in disease-derived or normal synoviocytes.

These data indicate that WISP3 expression is complex, with discoordinate regulation at the mRNA and protein levels. Proinflammatory cytokines in RA probably contribute to the higher amounts of WISP3 RNA transcripts in synovium and FLS, but this is not reflected by an alteration in protein levels. Although no previous data implicate cytokines in the regulation of WISP proteins, another member of the CNN family, cyr61, is induced by TNF-α, IL-1ß, epidermal growth factor, and basic fibroblast growth factor in osteoblasts[38]. The striking persistence of high WISP3 mRNA in cultured RA FLS is independent of exogenous cytokine stimulation and is consistent with previous observations on dedifferentiation and constitutive activation of *wnt*. This also provides additional evidence that FLS might be permanently imprinted by the rheumatoid synovial environment. However, WISP3 overexpression may not have marked functional sequelae because protein levels are unchanged.

Discoordinate regulation of WISP3 has been described in the one other CCN family member. For instance, abundant Nov protein was noted in chicken neuronal cells despite relative absence of *nov* mRNA[39]. There have been few reports on WISP3 regulation, so little information is available on its regulation in normal or tumor cell lines[21,32,40].

Microsatellite instability and suppressed levels of the DNA mismatch repair enzymes like hMSH6 have also been observed in RA synovium and cultured FLS after reactive oxygen and nitrogen stress[12]. The association of similar germline mutations in the WISP3 gene with a very rare inherited form of arthritis known as progressive

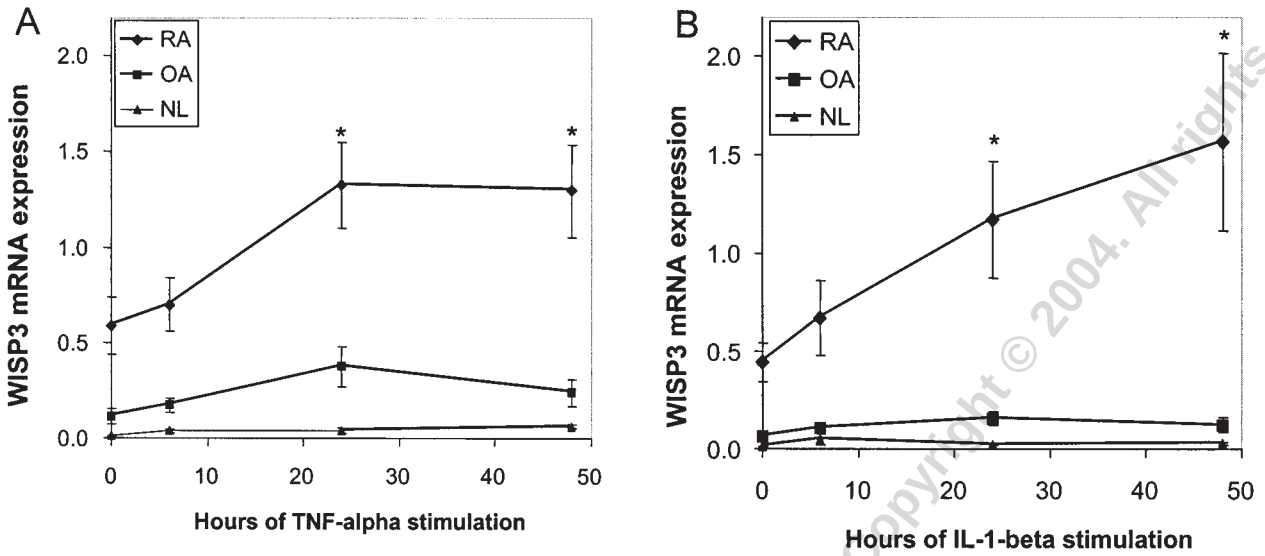

Figure 6. Regulation of WISP3 mRNA expression by IL-1ß and TNF-α in fibroblast-like synoviocytes (FLS). Expression of WISP3 gene was evaluated by real-time PCR in FLS after stimulation by TNF-α (100 ng/ml; A) or IL-1ß (2 ng/ml; B). Cytokines were added to plates of FLS at various timepoints in an individual experiment and all cells were harvested at the final timepoint; hence all cells were in culture for the same amount of time. N = 6 RA, 6 OA, and 5 normal samples (NL). *p < 0.05, medium-treated compared with cytokine-treated FLS.

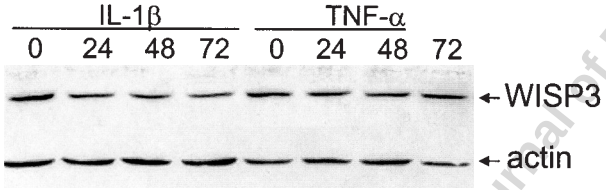

Figure 7. Regulation of WISP3 protein expression by TNF-α and IL-1ß in RA fibroblast-like synoviocytes (FLS). Cytokine regulation of 2 RA FLS lines was evaluated after TNF-α (100 ng/ml) or IL-1ß (2 ng/ml) exposure for up to 72 h. Representative Western blots show the 39 kDa bands detected by WISP3 C-terminal antibody. Unlike mRNA, no change in total WISP3 protein was observed.

pseudorheumatoid dysplasia added to our interest in this particular member of the CCN family[21]. A microsatellite in the WISP3 coding region is susceptible to mutation, especially in the presence of defective mismatch repair mechanisms. The addition or deletion of a single nucleotide leads to premature stop codons and production of a truncated protein lacking the thrombospondin and cysteine knot domains. These regions are involved in binding to sulfated glycosaminoglycans and dimerization, respectively. A high prevalence of base deletions and insertions in the microsatellite was observed in colorectal and gastric carcinoma, raising the possibility that its loss might enhance unregulated growth of certain tissues[18,19]. In this study, we identified a limited number of deletion and insertion mutations in OA and RA samples. No mutation was observed in any of the flanking regions of the microsatellite, supporting the notion that they reflect true somatic mutations rather than *in vitro* artifact. Although a single subclone with a substitution mutation was identified in a normal synovium, this type of mutation is not a primary target for mismatch repair enzyme activity and does not lead to a truncated protein.

In summary, these data suggest that activation of the *wnt* pathway in RA, possibly due to cytokines, stimulates gene expression of the *wnt*-signaling protein WISP3. Overexpression of this gene persists *in vitro* and might be one manifestation of the dedifferentiation program described in this disease. However, the functional consequences are uncertain because concomitant changes in WISP3 protein

Table 1. Mutation analysis in MS region of WISP3 gene from synovial tissue and fibroblast-like synoviocyte samples.

	RA1	RA2	RA3	OA1	OA2	OA3
Synovial tissue	2/9* (1 del, 1 ins)	0/10	0/10	0/9	1/10 (1 del)	0/10
FLS	0/10	1/10 (1 del)	0/6	1/10 (1 ins)	0/9	0/9

* Mutant subclones/total subclones sequenced. del: deletion mutation; ins: insertion mutation.

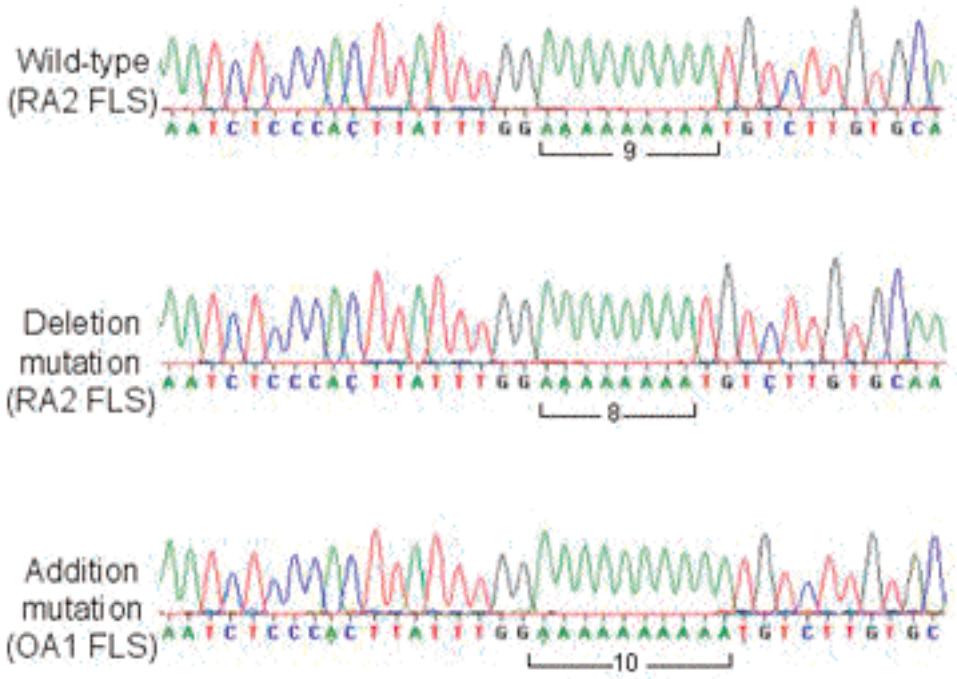

Figure 8. Frameshift mutations in WISP3 gene. Representative WISP3 microsatellite sequence tracings are shown for wild-type (9 adenines), insertion mutation (10 adenines), and deletion mutation (8 adenines) subclones obtained from RA synovial tissue genomic DNA. Both the insertion and deletion mutations lead to truncated proteins. No mutations were observed in the flanking regions in any sample.

expression were not observed. Further, somatic mutations were occasionally observed in the WISP3 gene microsatellite. Unlike p53 mutations, which are dominant-negative and alter synoviocyte function[41], WISP3 mutations are not disease-specific and have unknown downstream effects. Further elucidation of WISP regulation could provide insight into the aggressive features of synovium in patients with arthritis.

REFERENCES

1. Firestein GS. Evolving concepts of rheumatoid arthritis. Nature 2003;423:356-61.
2. Lafyatis R, Remmers EF, Roberts AB, Yocum DE, Sporn MB, Wilder RL. Anchorage-independent growth of synoviocytes from arthritic and normal joints. Stimulation by exogenous platelet-derived growth factor and inhibition by transforming growth factor-beta and retinoids. J Clin Invest 1989;83:1267-76.
3. Muller-Ladner U, Kriegsmann J, Franklin BN, et al. Synovial fibroblasts of patients with rheumatoid arthritis attach to and invade normal human cartilage when engrafted into SCID mice. Am J Pathol 1996;149:1607-15.
4. Firestein GS. Invasive fibroblast-like synoviocytes in rheumatoid arthritis. Passive responders or transformed aggressors? Arthritis Rheum 1996;39:1781-90.
5. Sen M, Lauterbach K, El-Gabalawy H, Firestein GS, Corr M, Carson DA. Expression and function of wingless and frizzled homologs in rheumatoid arthritis. Proc Natl Acad Sci USA 2000;97:2791-6.
6. Firestein GS, Echeverri F, Yeo M, Zvaifler NJ, Green DR. Somatic mutations in the p53 tumor suppressor gene in rheumatoid arthritis synovium. Proc Natl Acad Sci USA 1997;94:10895-900.
7. Inazuka M, Tahira T, Horiuchi T, et al. Analysis of p53 tumour suppressor gene somatic mutations in rheumatoid arthritis synovium. Rheumatology Oxford 2000;39:262-6.
8. Kullmann F, Judex M, Neudecker I, et al. Analysis of the p53 tumor suppressor gene in rheumatoid arthritis synovial fibroblasts. Arthritis Rheum 1999;42:1594-600.
9. Reme T, Travaglio A, Gueydon E, Adla L, Jorgensen C, Sany J. Mutations of the p53 tumour suppressor gene in erosive rheumatoid synovial tissue. Clin Exp Immunol 1998;111:353-8.
10. Cannons JL, Karsh J, Birnboim HC, Goldstein R. HPRT- mutant T cells in the peripheral blood and synovial tissue of patients with rheumatoid arthritis. Arthritis Rheum 1998;41:1772-82.
11. Roivainen A, Jalava J, Pirila L, Yli-Jama T, Tiusanen H, Toivanen P. H-ras oncogene point mutations in arthritic synovium. Arthritis Rheum 1997;40:1636-43.
12. Lee SH, Chang DK, Goel A, et al. Microsatellite instability and suppressed DNA repair enzyme expression in rheumatoid arthritis. J Immunol 2003;170:2214-20.
13. Cadigan KM, Nusse R. Wnt signaling: a common theme in animal development. Genes Dev 1997;11:3286-305.
14. Dale TC. Signal transduction by the Wnt family of ligands. Biochem J 1998;329:209-23.
15. Sen M, Reifert J, Lauterbach K, et al. Regulation of fibronectin and metalloproteinase expression by Wnt signaling in rheumatoid arthritis synoviocytes. Arthritis Rheum 2002;46:2867-77.
16. Pennica D, Swanson TA, Welsh JW, et al. WISP genes are members of the connective tissue growth factor family that are up-regulated in wnt-1-transformed cells and aberrantly expressed in human colon tumors. Proc Natl Acad Sci USA 1998;95:14717-22.
17. Perbal B. NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. Mol Pathol 2001;54:57-79.
18. Thorstensen L, Diep CB, Meling GI, et al. WNT1 inducible signaling pathway protein 3, WISP-3, a novel target gene in

colorectal carcinomas with microsatellite instability. Gastroenterology 2001;121:1275-80.
19. Thorstensen L, Holm R, Lothe RA, et al. WNT-inducible signaling pathway protein 3, WISP-3, is mutated in microsatellite unstable gastrointestinal carcinomas but not in endometrial carcinomas. Gastroenterology 2003;124:270-1.
20. Hurvitz JR, Suwairi WM, Van Hul W, et al. Mutations in the CCN gene family member WISP3 cause progressive pseudorheumatoid dysplasia. Nat Genet 1999;23:94-8.
21. Arnett FC, Edworthy SM, Bloch DA, et al. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. Arthritis Rheum 1988;31:315-24.
22. Alvaro-Gracia JM, Zvaifler NJ, Brown CB, Kaushansky K, Firestein GS. Cytokines in chronic inflammatory arthritis. VI. Analysis of the synovial cells involved in granulocyte-macrophage colony-stimulating factor production and gene expression in rheumatoid arthritis and its regulation by IL-1 and tumor necrosis factor-alpha. J Immunol 1991;146:3365-71.
23. Elices MJ, Tsai V, Strahl D, et al. Expression and functional significance of alternatively spliced CS1 fibronectin in rheumatoid arthritis microvasculature. J Clin Invest 1994;93:405-16.
24. Boyle DL, Rosengren S, Bugbee W, Kavanaugh A, Firestein GS. Quantitative biomarker analysis of synovial gene expression by real-time PCR. Arthritis Res Ther 2003;5:R352-60.
25. Tanaka S, Akiyoshi T, Mori M, Wands JR, Sugimachi K. A novel frizzled gene identified in human esophageal carcinoma mediates APC/beta-catenin signals. Proc Natl Acad Sci USA 1998;95:10164-9.
26. Babic AM, Kireeva ML, Kolesnikova TV, Lau LF. CYR61, a product of a growth factor-inducible immediate early gene, promotes angiogenesis and tumor growth. Proc Natl Acad Sci USA 1998;95:6355-60.
27. Hashimoto Y, Shindo-Okada N, Tani M, et al. Expression of the Elm1 gene, a novel gene of the CCN (connective tissue growth factor, Cyr61/Cef10, and neuroblastoma overexpressed gene) family, suppresses in vivo tumor growth and metastasis of K-1735 murine melanoma cells. J Exp Med 1998;187:289-96.
28. Kireeva ML, Mo FE, Yang GP, Lau LF. Cyr61, a product of a growth factor-inducible immediate-early gene, promotes cell proliferation, migration, and adhesion. Mol Cell Biol 1996;16:1326-34.
29. O'Brien TP, Lau LF. Expression of the growth factor-inducible immediate early gene cyr61 correlates with chondrogenesis during mouse embryonic development. Cell Growth Differ 1992;3:645-54.
30. Wong M, Kireeva ML, Kolesnikova TV, Lau LF. Cyr61, product of a growth factor-inducible immediate-early gene, regulates chondrogenesis in mouse limb bud mesenchymal cells. Dev Biol 1997;192:492-508.
31. Xu L, Corcoran RB, Welsh JW, Pennica D, Levine AJ. WISP-1 is a Wnt-1- and beta-catenin-responsive oncogene. Genes Dev 2000;14:585-95.
32. Kleer CG, Zhang Y, Pan Q, et al. WISP3 is a novel tumor suppressor gene of inflammatory breast cancer. Oncogene 2002;21:3172-80.
33. Saxena N, Banerjee S, Sengupta K, Zoubine MN, Banerjee SK. Differential expression of WISP-1 and WISP-2 genes in normal and transformed human breast cell lines. Mol Cell Biochem 2001;228:99-104.
34. Zoubine MN, Banerjee S, Saxena NK, Campbell DR, Banerjee SK. WISP-2: a serum-inducible gene differentially expressed in human normal breast epithelial cells and in MCF-7 breast tumor cells. Biochem Biophys Res Commun 2001;282:421-5.
35. Xie D, Nakachi K, Wang H, Elashoff R, Koeffler HP. Elevated levels of connective tissue growth factor, WISP-1, and CYR61 in primary breast cancers associated with more advanced features. Cancer Res 2001;61:8917-23.
36. Tanaka S, Sugimachi K, Saeki H, et al. A novel variant of WISP1 lacking a Von Willebrand type C module overexpressed in scirrhous gastric carcinoma. Oncogene 2001;20:5525-32.
37. Sen M, Cheng YH, Goldring MB, Lotz MK, Carson DA. WISP3-dependent regulation of type II collagen and aggrecan production in chondrocytes. Arthritis Rheum 2004;50:488-9.
38. Hadjiargyrou M, Ahrens W, Rubin CT. Temporal expression of the chondrogenic and angiogenic growth factor CYR61 during fracture repair. J Bone Miner Res 2000;15:1014-23.
39. Chevalier G, Yeger H, Martinerie C, et al. novH: differential expression in developing kidney and Wilm's tumors. Am J Pathol 1998;152:1563-75.
40. van Golen KL, Davies S, Wu ZF, et al. A novel putative low-affinity insulin-like growth factor-binding protein, LIBC (lost in inflammatory breast cancer), and RhoC GTPase correlate with the inflammatory breast cancer phenotype. Clin Cancer Res 1999;5:2511-9.
41. Han Z, Boyle DL, Shi Y, Green DR, Firestein GS. Dominant negative p53 mutations in rheumatoid arthritis. Arthritis Rheum 1999;42:1088-92.