

Original

Expression of WISP-1 (ccn4), WISP-2 (ccn5) and WISP-3 (ccn6) in Rheumatoid Arthritic Synovium Evaluated by DNA Microarrays

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(Accepted for publication, September 10, 2006)

Abstract : Rheumatoid disease (RA) is one of the complex diseases that showed multiple progression stages. At first inflammatory response is occurred by self antibodies, and then the proliferation of synoviocytes is abnormally promoted. These synoviocytes invade into articular structures and finally articular cartilage is destroyed. In order to analyze growth-promoting factors in synovial cells from articular tissues of RA, we performed DNA microarray analysis and compared the expression patterns of their mRNA to that of synoviocytes from osteoarthritis (OA). The results showed that the expression of many genes was up-regulated in RA synoviocytes comparing with OA synoviocytes. Among these genes, RT-PCR analysis revealed that Wnt-1-induced secreted protein 2 (WISP-2, CCN5) and protein 3 (WISP-3, CCN6) which are belong to the novel gene family, CCN, were highly up-regulated in RA synoviocytes. In addition, one truncated isoform of WISP-3 which may be related to the ongoing of some type of RA, was detected in RA synoviocytes. Furthermore, immunohistochemical analysis showed that the products of both CCN genes were highly distributed in active synoviocytes of articular tissues of RA. These results indicate that both CCN genes and their products play some roles in the progression of RA.

Key words: Rheumatoid arthritis, Synovial cells, Microarray, WISP gene, CCN family

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease characterized by inflamed synovial hyperplasia with excessive inflammatory cell infiltration, leading to erosion of articular cartilage and marginal bone, with subsequent joint destruction. Although an explosion of information over the past two decades contributed to the better understanding of the mechanism of inflammation, the pathogenesis of synovitis has not been fully elucidated¹⁾. Abnormal proliferation of synoviocytes is promoted in RA, and these synoviocytes invade into articular structures and finally destroy articular cartilage. Molecular basis of these phenomena are poorly understood. Recently, contribution of some cytokines, receptors and transcription factors to this disease was suggested²⁻⁶⁾. In addition, some genetical background that

contribute to the generation of this disease was revealed⁷⁾.

Recent progress in genome science made it possible to analyze the expression of thousands of genes at one time. This microarray (DNA chip) technology⁸⁾ reveals the difference of gene expression between different types of cells or cells in different conditions. Although it still has some technical problem, the use of this technique has been widely developing. This technique also contributes the detection of genetic mutation occurred in tumor cells and detection of genetic polymorphisms (SNPs)⁹⁾. These results contribute to develop novel therapy ie. ordermade medicine.

The CCN family of genes¹⁰⁾ which stands for Cyr61 (cysteine rich protein)¹¹⁾, Ctgf (connective tissue growth factor)¹²⁾, and Nov (nephroblastoma overexpressed gene)¹³⁾ consists of six distinct members. CTGF and Cyr61 are thought to be positive regulators of cell growth, while Nov and resent three new members, WISP-1 (Wnt-1-induced secreted protein 1) /Elm-1¹⁴⁾, WISP-2 (Wnt-1-induced secreted protein 2)/rCop1/CTGF-3¹⁵⁾ and WISP-3 (Wnt-

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1-induced secreted protein 3¹⁶⁾ are thought to be negative regulators. In addition, these proteins play fundamentally important processes in cell differentiation, attachment, migration and are also involved in pathological process, such as fibrosis, tumorigenesis and tumor invasion¹⁷⁾. Recently defined nomenclature designated Cyr 61 as CCN1, CTGF as CCN2, NOV as CCN3, and WISP-1-3 as CCN4-6. WISP-2 is also designated as CTGF-L¹⁸⁾ that lacks characteristic C-terminal domain of CCN family protein. These Wisp factors identified as Wnt-inducible secreted protein indicating that these factors are important for morphogenesis and development.

In this paper, we compared the expression levels of about a thousand of genes between synovial cells from RA and osteoarthritis (OA) by using cDNA microarrays, and identified the characteristic genes which were up-regulated or down-regulated in RA synoviocytes. Furthermore, we analyzed the distribution of two of such genes, WISP-2 and WISP-3 which are included in the CCN gene family and thought to be candidates for future diagnosis and therapeutic targets in RA synoviocytes.

Materials and methods

Isolation and culture of synovial cells

Fresh synovial tissues were minced and digested with collagenase and DNase I. Tissue debris was removed through a cell strainer, and cells were washed twice with medium. The resultant single cells were dispensed into the wells of a 24-well microtiter plate (Costar, Cambridge, MA) at a density of 2×10^6 cells/ml in 2 ml of Dubecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD) supplemented with 10% HEPES (Life Technologies), 100 IU/ml penicillin and 100 µg/ml streptomycin. The plates were incubated at 37°C in a humidified atmosphere containing 5% CO₂. Synovial tissue cells were split weekly once primary cultures had reached confluence, and used after the third passage. These cells appeared to be homogeneous fibroblast-like cells morphologically. They were cultured at a density of 1×10^5 cells/ml in 2 ml DMEM with 10% FCS in a 24-well microtiter plate and allowed to adhere overnight. After the third passage of cell culture, cells are harvested and RNA was isolated from cells using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's recommendations.

cDNA microarray analysis

Human1.0 Microarrays (Clontech, Mountain View, CA) containing over 1,000 known human genes, were used for hybridization studies. The filters were prehybridized with 5.0 µg of denatured cot-1 DNA, 5.0 µg poly-dA, and 5.0 ml MicroHyb hybridization solution for 2h at 42°C. Total cellular RNA from primary cultures was prepared as described above. Probes were generated from reverse transcribed total RNA (1.0 µg) incubated with 2.0 µg oligo-dT, 6.0 µl of 5x first strand buffer, 1.0 ml dithiothreitol, 1.5 ml reverse transcriptase, 1.5 µl dNTP mixture

containing dATP, dGTP, dTTP at 20 mmol/l, and 10 ml ³²P-dCTP (3000Ci/mmol) for 90 min at 37°C. Labeled probes were added to the pre-hybridization solution and left to hybridize with the membranes for 18hr at 42°C. Washes were done twice at 50°C in 2xSSC with 1% sodium dodecylsulfate (SDS) for 20 min and once at room temperature in 0.5xSSC with 1% SDS for 15 min. The membranes were aligned on a Imaging screen and exposed for 16h at room temperature. The screens were scanned using a Bioimaging analyzer (Fuji Film, Tokyo, Japan) and saved as digital images. Arrayage (Fuji Film) was used for data analysis. Differential gene analysis was performed for all pairwise comparisons.

Semiquantitative reverse transcriptase-PCR (RT-PCR)

The total RNA from normal or RA synoviocytes was reverse transcribed to cDNA using oligo dT₁₆ primers with AMV-derived reverse transcriptase, and the resulted first-strand cDNA was amplified with each CCN specific primer set. The amplification condition was as follows: 95°C (1 min) - 57°C (1 min) - 72°C (2 min) for 35 cycles. The PCR products were applied on agarose gels (2%) and electrophoresed. Nucleotide sequence of the primers are as follows: cyr61 5'- GGCTTCTACAGTVGTAAGAG -3' (sense), 5'- CCTGATTTCTGTTTGCAGTTTAGAG -3' (antisense); ctgf 5'- ATAGCTGATCAGTTTTTTCACC -3' (sense), 5'- CAACTAGAAAGGTG CAAACATGTAAC -3' (antisense); wisp-1 5'- GGGCTTTGGGCAGTTGG CCAGG -3' (sense), 5'- TGCTACCAAGGGCACCCACTTCTTGG -3' (antisense); wisp-2 5'- TCCACAAAACAGTGCCTTCTAG -3' (sense), 5'- TGATATAGGCTGTGTGTGTAGGCAG -3' (antisense); wisp-3 5'-GAG AAAAGACTGTGTTACATTCAGCC -3' (sense), 5'- TTATATGACAGGA TTACTAAC -3' (antisense); G3PDH 5'-ACCACAGTCCATGCCATCAC-3' (sense), 5'- TCCACCACCCTGTTGCTGTA-3' (antisense).

Immunohistochemistry

Synovial tissues were obtained from 4 OA and 8 RA patients at total knee arthroplasty. The expression of WISP-2 and 3 in synovial tissues at the protein level were determined immunohistochemically. Briefly, the harvested tissues were frozen in liquid nitrogen and stored at -80°C. Six µm-thick serial sections were acetone fixed on slides and blocked with 1% normal rabbit serum. Sections were incubated overnight with 10µg/ml polyclonal anti-WISP-2 (N-15) or anti-WISP-3 (N-18) antibodies (Santa Cruz Biotechnology, Inc, Santa Cruz, CA) at 4°C and then reacted with biotinylated anti-goat IgG (Vector Laboratories, Inc, Burlingame, CA) for 60 min at room temperature. Slides were washed with PBS between incubations. Sections were incubated with diaminobenzidine tetrahydrochloride (DAB) substrate solution for 5 min, counterstained with hematoxylin, and embedded in balsam. Negative control sections were treated by nonimmune serum as a substitute for the primary antibody, or in

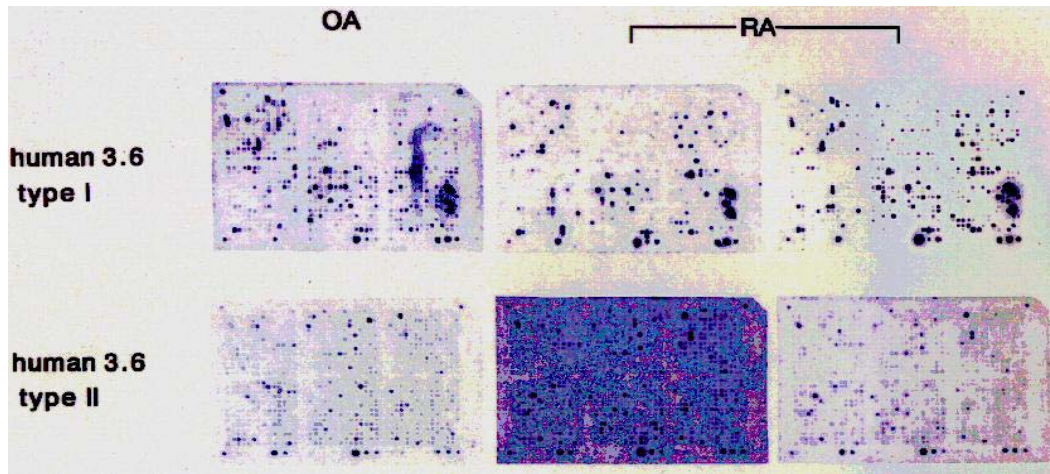


Fig. 1 DNA microarray analysis. Atlas human 3.6 microarray was used for hybridization. The three arrays above in the figure are 3.6 type I arrays and another three arrays below are 3.6 type II arrays. The two arrays in the left are hybridized with probes prepared from OA synoviocytes and another four in the right are hybridized with probes prepared from RA synoviocytes.

the same fashion but excluding the primary antibody. They were then examined under a light microscope.

Results

cDNA microarray analysis

We utilize cDNA microarray technology to compare multiple gene expression profiles representative of human adult OA synoviocytes (n=2 individuals) and RA synoviocytes (n=3 individuals). Total RNA was isolated from primary cultures of OA or RA synoviocytes and then hybridized to Atlas array filters. Fig. 1 showed typical hybridization patterns of the filters. Comparing the density of each spot between the same type of filter hybridized with RNA from OA or RA, genes differentially expressed between OA and RA were isolated. Table 1 showed the ratio and signal intensity of these isolated genes analyzed by ArrayGage software. The intensity values between two filters were normalized by using the values of control (house-keeping) genes on each filter, and direct comparisons were made between all OA and RA for making expression profiles based on a designation for each hybridization dot. Pairwise comparisons between all OA and RA samples demonstrated a similar expression profile of approximately 1,000 known genes present on the microarray. Several genes coding for growth factors, transcription factors and others showed differential expression between OA and RA synoviocytes (Table 1). Among these genes, we focused on WISP genes of the CCN family (Fig. 2) which showed increased expression in RA synovial cells.

Confirmation of differentially expressed genes

Among the several genes that were differentially expressed between OA and RA synoviocytes on microarray filters, we picked up WISP genes. They are belonged to the CCN family (Fig. 2) containing connective tissue growth factor (CTGF) which play

important roles in growth and differentiation of chondrocytes¹⁹⁾ and also upregulated in OA cartilage²⁰⁾. To confirm their differential expression between OA and RA synoviocytes, we performed RT-PCR and semiquantitated the expression levels of four CCN genes (Cyr61, CTGF, NOV and WISP-1) in both synoviocytes (Table 2, Fig. 3). When comparing the expression of CCN genes between OA and RA synoviocytes, all four genes were up-regulated in RA synoviocytes. Among them, WISP-1 gene showed the most characteristic pattern of increasing its expression in RA synoviocytes for more than 35-fold comparing with the expression in OA synoviocytes. The expression of other CCN genes (Cyr61, CTGF and NOV) were expressed in RA synoviocytes less than 10-fold higher than in OA synoviocytes.

Detection of a novel truncated form of WISP-3 related to abnormal cellular proliferation

We also analyzed the expression of an alternatively spliced variant of WISPs which is related to abnormal cellular proliferation to produce hepatocellular carcinoma cells²¹⁾. The expression of one of such variant which lacks TSP and CT modules of WISP-3 in synovial cells was analyzed by RT-PCR with specific primers amplifying the cDNA region of IGF-BP and VWC modules (Fig. 4a). The results of RT-PCR in gel electrophoresis (Fig.4b) showed the amplicons of this type of variant in both OA and RA synovial cells. Of note that shorter size of DNA fragments were also detected in only RA synovial cells indicating that novel truncated form of WISP-3 was dominantly expressed in RA synoviocytes. The results of sequence analysis showed that the part of VWC module was deleted in this novel form of mutant (data not shown). The expression of this truncated form of WISP-3 may have some relation to the abnormal proliferation of RA synoviocytes and contribute to the ongoing of RA both in not only quantitative but in qualitative manner.

Table 1 Highly expressed genes in RA synovial cell

RA-I		RA-II	
A01g	Transforming protein rhoA	A01b	CD81
A07K	CDK inhibitor 1	A02d	annexin V
A07I	40S ribosomal protein S19	A09h	forkhead-related transcription factor FREAC-9
A09h	fte-1 40S ribosomal protein S3A	C02h	elastin precursor
A10h	IGFR II	C04g	annexin II
A14E	PDGFR α	D04g	glutamate dehydrogenase 1 precursor
A14I	RCL growth-related c-my responsive gene	D09i	amyloid A4 protein precursor (protease nexin-II)
C02i	calpain	E01h	GABAR β 3
C12d	ems-1 oncogene	E05m	CTGF-L (WISP-2)
D14j	RNA polymerase II coactivator p15	E07	osteopontin (OPG)
E08e	Gs α	E06k	natriuretic peptide precursor β
E12n	glutathione S-transferase Pi		
F02m	MMP-1		
F04m	MMP3		
F05b	HSP27		
F08f	FGF7		

Histochemical analysis

Next, we analyzed the distribution of these WISP proteins in synovial joints of OA and RA isolated in the course of joint replacement. Rheumatoid arthritic synoviocytes in an extensive fibrotic area showed abnormal growth. Fig. 5 showed the results of immunohistochemistry using anti-WISP-2 and anti-WISP-3 antibodies. Both WISP-2 and WISP-3 proteins highly distributed in rheumatoid arthritic synoviocytes of an extensive fibrotic area, and distributed in synoviocytes from OA joints with low density. The staining was performed using synovial joints of later stage in RA. These results of immunohistochemistry showed up regulation of these WISP proteins in rheumatoid arthritic synoviocytes which consistent with the results of microarray analysis in Table 1 and semiquantitative RT-PCR analysis in Table 2.

Discussion

In the present study, we compared representative gene expression profiles between OA and RA samples, using the Atlas cDNA microarray filter systems. This microarray (chip) technology offers the advantage of comparing multiple profiles generated from different subjects although it still has some technical difficulties⁸⁾. During the analysis, pairwise comparisons were performed between OA and RA samples from several different individuals to minimize the variation between each sample. Concerning its quantitative aspects, repeated experiments

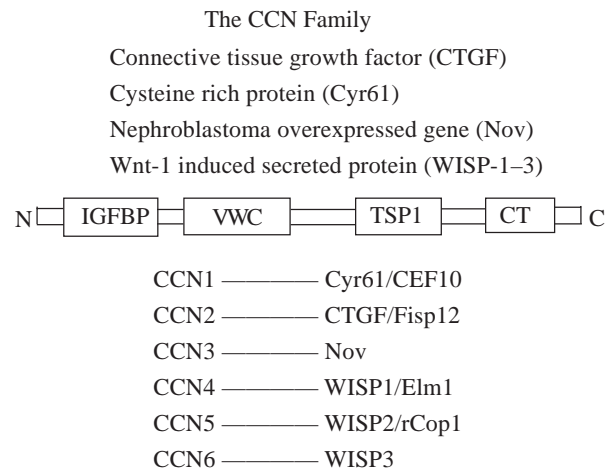


Fig 2 Schematic representation of the CCN family. IGFBP: insulin-like growth factor binding protein-like domain, VWC: von Willbrand factor-like domain, TSP1: thrombospondin-like domain, CT: cysteine knot containing family of growth regulators-like domain.

showed reproduced consistent data of microarray analysis. The sensitivity was relatively low comparing with Northern blot, and sometimes failed to detect the difference between two samples when Northern blot could detect such difference²²⁾. Nevertheless, data obtained with microarrays show almost the same and consistent results comparing with Northern blot or RT-PCR in some cases (also in this case), and this method is thought to be one of the powerful tools for integral analysis of gene expression.

Table 2 Expression level of CCN genes quartified by using TaqMan real time PCR

	OA to RA		RA to OA	
		%		%
CCN1(CYR61)	0.22	22.2	4.50	450
CCN2(CTGY)	0.14	13.8	7.23	723
CCN3(NOV)	0.76	76.1	1.34	131
CCN4(WISP-1)	0.03	2.53	39.6	3960

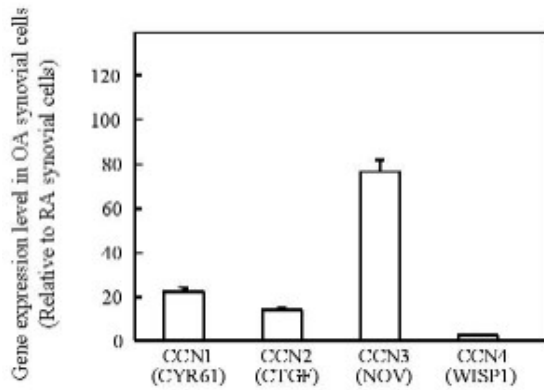


Fig. 3 RT-PCR analysis of the expression of four CCN genes in OA and RA synovial cells. The ordinate shows relative expression level (the expression level in OA synoviocytes relative to the expression level in RA synoviocytes). The value 100 means the case that the expression levels in both cells are the same.

Some group have tried to analyze gene expression in RA using microarray technique²²⁾. Heller et al. randomly selected 1,000 human genes from a peripheral human blood cell library and also selected genes significant in inflammation, and designed microarrays with 96 elements. They compared gene expression of macrophages, chondrocytes and synoviocytes of human RA tissue, and showed known participants of TNF, IL-1, IL-6, IL-8, G-CSF and VCAM and novel participation of the IL-3, C-X-C chemokine Gro alpha and the matrix metallo-elastase.

In this paper, we used three types of microarrays with 1,024 human universal genes on each array. We prepared isotope-labeled cDNA probes from total RNA isolated from synoviocytes of OA- or RA-derived articular tissue, and hybridized these probes to different arrays of the same type. As a result, we succeeded to show known participants which were mainly related to inflammation and novel participants for RA. Some of the known participants were already showed in previous studies in relation to inflammation or abnormal growth of synoviocytes. (*cdc25B*, *rhoA*, PDGF receptor alpha, FGF7, MMP-1, *c-fos*, and *c-jun*), but we also found additional new participants for RA (*WISP-2*). The gene *cdc25B* is well known as a control factor of cell proliferation and signaling, and GTP binding protein *rhoA* is related to the control of cell-cell adhesion, increasing motility

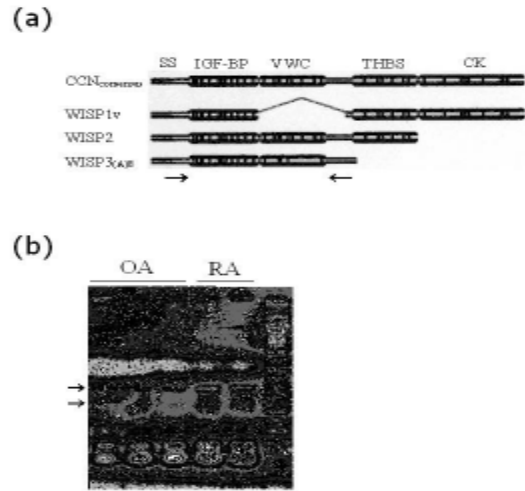


Fig. 4 RT-PCR analysis of the expression of WISP truncated forms. (a) Schematic representation of alternatively spliced WISP truncated forms. Arrows indicate the position of two primers. (b) RT-PCR analysis of the expression of one WISP truncated form. Amplicons with longer size (arrow) contain cDNA region coding for IGF-BP and VWC in WISP-3. Amplicons with shorter size (arrow) indicate the novel truncated form of WISP-3. The three samples in the left of the gel were amplified from OA synovial cells and another two samples in the right were amplified from RA synovial cells.

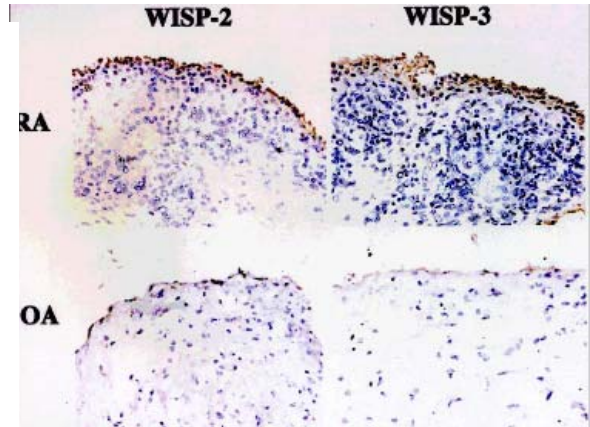


Fig. 5 Immunohistochemical analysis of the distribution of WISP-2 and WISP-3 in synovial joints using anti-WISP-2 or anti-WISP-3 antibodies. RA means the distribution of WISP-2 and WISP-3 in paraffin-embedded slices from RA joints, and OA means the distribution of WISP-2 and WISP-3 in paraffin-embedded slices from OA joints.

and activating beta1 integrins to form stress fibers. It also down regulates cadherin and catenin. PDGF and FGF are known growth factors for fibroblasts. MMP-1 is one of the matrix metalloproteases related to degradation of type I, II, III, VII, VIII and X collagen that promotes cell growth and movement. Transcription factors of *c-fos* and *c-jun* are the component of AP-1 transcription factor and stimulate cell growth or differentiation. Over expression or up-regulation of all these factors are thought to be involved in the abnormal promotion of synovial cell proliferation in RA.

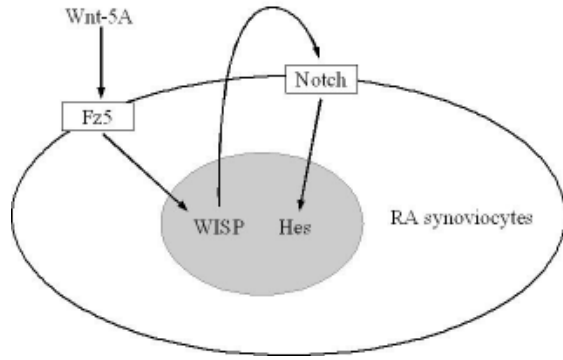


Fig. 6 Schematic representation of Wnt-Notch signaling mediated by WISP-3 in relation to the abnormal proliferation of RA synoviocytes.

As novel participants, we found WISP-2²³, which belong to the CCN gene family¹⁰. CCN gene family contains six members, and two genes, WISP-1²³ and WISP-2 of which expression are induced in Wnt-1 transformed cells, are identical to the previously described Elm1 and rCOP1 genes, respectively. Elm-1 gene was isolated by differential display method between low and high metastatic tumors, and Elm1 gene was selectively expressed in low metastatic tumors¹⁴ indicating that Elm-1 negatively affects the activity of cell growth. rCOP1 was also isolated by differential display technique between normal and transformed fibroblasts, and rCOP1 gene was down-regulated in transformed cells¹⁵ indicating that rCOP1 also negatively affects the activity of cell growth as Elm-1.

Despite the previous research, recent study showed contradictory function of WISP genes. For example, overexpression of WISP-1 induced accelerated growth and transformation of fibroblasts²⁴. Furthermore, Cyr61 and CTGF as well as Wisp-1 and Wisp-2 are now thought to be progression markers of breast cancers²⁵. For about Wisp-3, it was significantly overexpressed in human colorectal carcinoma²⁶. These recent studies indicate that WISP genes are also important for promotion of cell growth in fibroblasts.

Wnt signaling via Frizzled receptor is thought to be one of the important pathways for control of limb bud formation, connective tissue homeostasis and hematopoiesis. Recently, this process is thought to be important for signaling in synoviocytes of RA²⁷. In this point of view, up-regulation of Wnt-inducible proteins, WISP-1, WISP-2 and WISP-3 in RA synoviocytes, which we found with in this paper shows most important aspects of abnormal growth in RA synoviocytes, and the regulation of these WISP factors may be useful for control of their proliferation (Fig. 6).

In addition, Fig. 4 showed that the expression of one novel isoform of WISP-3 in RA synoviocytes may also contribute to the ongoing of RA in the respect of accumulation of abnormal

WISP-3 that promotes abnormal proliferation of synoviocytes after the stimulation by some signaling (Fig. 6). Progressive pseudorheumatoid dysplasia caused by the mutation of Wisp-3²⁸ indicates that some dysfunction of WISP-3 may activates proliferation signal of synoviocytes (ie. Notch signaling) and then induces abnormal growth of the cells and inflammatory phenomena in the joint tissues.

Further investigation for analysis of direct effects of WISP factors in the process of Wnt-induced signaling in RA synoviocytes is undergoing.

Acknowledgments

This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Science, Sports, and Culture of Japan (to T.N.), by grants-in-aid from the Ministry of Health and Welfare (to T.N.), and by grants from the Japan Private School Foundation (to T.N.), and the Ryobi Teien Foundation (to H.M.). The authors wish to thank Drs. Hirokato Matsuo and Junko Kato for helpful suggestions, and Ms. Shiho Kawai for secretarial assistance.

This work was also partially supported by grants in aid for scientific researches from the Ministry of Education, Culture, Sports, Science and Technology (#15209060, 17406027, 17591910, 17591911). This work was also supported by grant-in aid from Chugoku Industrial Innovation Center (#1039908059 and 1039908061).

References

1. Firestein GS. Rheumatoid arthritis: Rheumatoid synovitis and pannus: Rheumatology, second edition. Mosby: London, 1998,13, pp1-24.
2. Monier S, Reme T, Cognot C, Gao QL, Travaglio-Encinoza A, Cuchacovich M, Gaillard JP, Jorgensen C, Sany J, Dupuy d'Angeac A. Growth factor activity of IL-6 in the synovial fluid of patients with rheumatoid arthritis. Clin Exp Rheumatol 12:595-602, 1994
3. Badolato R, Oppenheim JJ. Role of cytokines, acute-phase proteins, and chemokines in the progression of rheumatoid arthritis. Semin Arthritis Rheum 26:526-38, 1996
4. Peichl P, Pursch E, Broll H, Lindley IJ. Anti-IL-8 autoantibodies and complexes in rheumatoid arthritis: polyclonal activation in chronic synovial tissue inflammation. Rheumatol Int 18:141-145, 1999
5. Harada S, Yamamura M, Okamoto H, Morita Y, Kawashima M, Aita T, Makino H. Production of interleukin-7 and interleukin-15 by fibroblast-like synoviocytes from patients with rheumatoid arthritis. Arthritis Rheum 42:1508-1516, 1999
6. Yumoto K, Ishijima M, Rittling SR, Tsuji K, Tsuchiya Y, Kon S et al. Noda M. Osteopontin deficiency protects joints against destruction in anti-type II collagen antibody-induced arthritis

- in mice. *Proc Natl Acad Sci USA* 99: 4556-4561, 2002
7. Tsuchiya K, Kimura A, Kondo M, Nishimura Y, Sasazuki T. Combination of HLA-A and HLA class II alleles controls the susceptibility to rheumatoid arthritis. *Tissue Antigens* 58:395-401, 2001
 8. Nakanishi T, Oka T, Akagi T. Recent advances in DNA microarrays. *Acta Med Okayama* 55: 319-328, 2001
 9. Risch N, Merikanges K. The future of genetic studies of complex human diseases. *Science* 273: 1516-1517, 1996
 10. Bork P. The modular architecture of a new family of growth regulators related to connective tissue growth factor. *FEBS Lett* 327: 125-130, 1993
 11. Brigstock DR. The connective tissue growth factor/ cysteine-rich 61 /nephroblastoma overexpressed (CCN) family. *Endocrine Rev* 20: 189-206, 1999
 12. Bradham DM, Igarashi A, Potter RL, Grotendorst, GR. Connective tissue growth factor: a cysteine-rich mitogen secreted by human vascular endothelial cells is related to the SRC-induced immediate early gene product CEF-10. *J Cell Biol* 114: 1285-1294, 1991
 13. Joliot V, Martinerie C, Dambrine G, Plassiart G, Brisac M, Crochet J, Perbal B. Proviral rearrangements and overexpression of a new cellular gene (nov) in myeloblastosis-associated virus type 1-induced nephroblastomas. *Mol Cell Biol* 12: 10-21, 1992
 14. Hashimoto Y, Shindo-Okada N, Tani M, Nagamachi Y, Takeuchi K, Shiroishi T, Toma H, Yokota J. Expression of 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* 187: 289-296, 1998
 15. Zhang R, Averboukh L, Zhu W, Zhang H, Jo H, Dempsey PJ, Coffey R J, Pardee AB, Liang P. Identification of rCop-1, a new member of the CCN protein family, as a negative regulator for cell transformation. *Mol Cell Biol* 18: 6131-6141, 1996
 16. Pennica D, Swanson TA, Welsh JW, Roy MA, Lawrence DA, Lee J, Brush J, Taneyhill LA, Deuel B, Lew M, Watanabe C, Cohen RL, Melhem MF, Finley GG, Quirke P, Goddard AD, Hillan KJ, Gurney AL, Botstein D, Levine AJ. 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* 95: 14717-14722, 1998
 17. Perbal B. NOV (nephroblastoma overexpressed) and the CCN family of genes: structural and functional issues. *Mol Pathol* 54: 57-79, 2001
 18. Kumar S, Hand AT, Connor JR, Dodds RA, Ryan PJ, Trill JJ, et al. Identification and cloning of a connective tissue growth factor-like cDNA from human osteoblasts encoding a novel regulator of osteoblast functions. *J Biol Chem* 274: 17123-17131, 1999
 19. Nakanishi T, Nishida T, Shimo T, Kobayashi K, Kubo T, Tamatani T, Tezuka K, Takigawa M. Effects of CTGF/Hcs24, a product of a hypertrophic chondrocyte-specific gene, on the proliferation and differentiation of chondrocytes in culture. *Endocrinology* 141: 264-273, 2000
 20. Omoto S, Nishida K, Yamaai Y, Shibahara M, Nishida T, Doi T, Asahara H, Nakanishi T, Inoue H, Takigawa M. Expression and localization of connective tissue growth factor (CTGF/Hcs24/CCN2) in osteoarthritic cartilage. *Osteoarth Cart* 12: 771-778, 2004
 21. Cervello M, Giannitrapani L, Labbozzetta M, Notarbartolo M, D'Alessandro N, Lampiasi N, Azzolina A, Montalto G. Expression of WISPs and their novel alternative variants in human hepatocellular carcinoma cells. *Ann N Y Acad Sci* 1028: 432-439, 2004
 22. Heller RA, Schena M, Chai A, Shalon D, Bedilion T, Gilmore J, Woolley DE, Davis RW. Discovery and analysis of inflammatory disease-related genes using cDNA microarrays. *Proc Natl Acad Sci U S A* 94:2150-2155, 1997
 23. Pennica D, Swanson TA, Welsh JW, Roy MA, Lawrence DA, Lee J, Brush J, Taneyhill LA, Deuel B, Lew M, Watanabe C, Cohen RL, Melhem MF, Finley GG, Quirke P, Goddard AD, Hillan KJ, Gurney AL, Botstein D, Levine AJ. 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 U S A* 95:14717-14722, 1998
 24. 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* 61:8917-8923, 2001
 25. 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* 228:99-104, 2001
 26. Thorstensen L, Diep CB, Meling GI, Aagesen TH, Ahrens CH, Rognum TO, Lothe RA. WNT1 inducible signaling pathway protein 3, WISP-3, a novel target gene in colorectal carcinomas with microsatellite instability. *Gastroenterology* 121:1275-1280, 2001
 27. Sen M, Chamorro M, Reifert J, Corr M, Carson DA. Blockade of Wnt-5A/frizzled 5 signaling inhibits rheumatoid synoviocyte activation. *Arthritis Rheum* 44:772-781, 2001
 28. Hurvitz JR, Suwairi WM, Van Hul W, El-Shanti H, Superti-Furga A, Roudier J, Holderbaum D, Pauli RM, Herd JK, Van Hul EV, Rezaï-Delui H, Legius E, Le Merrer M, Al-Alami J, Bahabri SA, Warman ML. Mutations in the CCN gene family member WISP3 cause progressive pseudorheumatoid dysplasia. *Nat Genet* 23:94-98, 1999