

Decoy receptor 3 regulates the expression of various genes in rheumatoid arthritis synovial fibroblasts

KOJI FUKUDA¹, YASUSHI MIURA^{1,2}, TOSHIHISA MAEDA¹, MASAYASU TAKAHASHI³, SHINYA HAYASHI¹ and MASAHIRO KUROSAKA¹

¹Department of Orthopaedic Surgery, Kobe University Graduate School of Medicine, Chuo, Kobe, Hyogo 650-0017; ²Division of Orthopaedic Science, Department of Rehabilitation Science, Kobe University Graduate School of Health Sciences, Suma, Kobe, Hyogo 654-0142; ³Department of Orthopaedic Surgery, Kohnan Hospital, Higashinada, Kobe, Hyogo 658-0064, Japan

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Abstract. Decoy receptor 3 (DcR3), a member of the tumor necrosis factor (TNF) receptor (TNFR) superfamily, lacks the transmembrane domain of conventional TNFRs in order to be a secreted protein. DcR3 competitively binds and inhibits members of the TNF family, including Fas ligand (FasL), LIGHT and TNF-like ligand 1A (TL1A). We previously reported that TNF α -induced DcR3 overexpression in rheumatoid arthritis fibroblast-like synoviocytes (RA-FLS) protects cells from Fas-induced apoptosis. Previous studies have suggested that DcR3 acting as a ligand directly induces the differentiation of macrophages into osteoclasts. Furthermore, we reported that DcR3 induces very late antigen-4 (VLA-4) expression in THP-1 macrophages, inhibiting cycloheximide-induced apoptosis and that DcR3 binds to membrane-bound TL1A expressed on RA-FLS, resulting in the negative regulation of cell proliferation induced by inflammatory cytokines. In the current study, we used cDNA microarray to search for genes in RA-FLS whose expression was regulated by the ligation of DcR3. The experiments revealed the expression profiles of genes in RA-FLS regulated by DcR3. The profiles showed that among the 100 genes most significantly regulated by DcR3, 45 were upregulated and 55 were downregulated. The upregulated genes were associated with protein complex assembly, cell motility, regulation of transcription, cellular protein catabolic processes, cell membrane, nucleotide binding and glycosylation. The downregulated genes were associ-

ated with transcription regulator activity, RNA biosynthetic processes, cytoskeleton, zinc finger region, protein complex assembly, phosphate metabolic processes, mitochondrion, ion transport, nucleotide binding and cell fractionation. Further study of the genes detected in the current study may provide insight into the pathogenesis and treatment of rheumatoid arthritis by DcR3-TL1A signaling.

Introduction

Rheumatoid arthritis (RA) is an inflammatory joint disease characterized by hyperplasia of the synovial tissue and formation of pannus, which grows invasively into the cartilage, causing cartilage and bone destruction. Analyses of hyperplastic synovial tissue of patients with RA have revealed a number of features of transformed long-living cells, such as the presence of somatic mutations, expression of oncogenes and resistance to apoptosis (1-3).

We previously reported that the decoy receptor 3 (DcR3)/TR6/M68/tumor necrosis factor receptor (TNFR) superfamily member 6 (TNFRSF6b) is expressed in rheumatoid fibroblast-like synoviocytes (RA-FLS), and that DcR3 expression induced in RA-FLS by TNF α protects cells from Fas-induced apoptosis (4). DcR3, a member of the TNFR superfamily, lacks the transmembrane domain of conventional TNFRs and thus can be a secreted protein (5). DcR3 is typically overexpressed in tumor cells, including lung and colon cancers (5), gliomas, gastrointestinal tract tumors (6) and virus-associated leukemia (7). In addition, as previous studies have demonstrated, DcR3 is expressed in some normal tissues, including the colon, stomach, spleen, lymph nodes, spinal cord, pancreas and lungs (5,6). However, DcR3 is not expressed in NIH3T3 human fibroblast cells (8). DcR3 has 3 ligands, Fas ligand (FasL), LIGHT and TNF-like ligand 1A (TL1A), which are members of the TNF superfamily (9). The overexpression of DcR3 may benefit tumors by helping them avoid the cytotoxic and regulatory effects of FasL (5,10), LIGHT (11) and TL1A (12). In a previous study, we suggested that DcR3 is one of the key molecules that regulate the proliferation of RA-FLS (4).

Correspondence to: Dr Yasushi Miura, Division of Orthopaedic Science, Department of Rehabilitation Science, Kobe University Graduate School of Health Sciences, 7-10-2 Tomogaoka, Suma, Kobe, Hyogo 654-0142, Japan
E-mail: miura@kobe-u.ac.jp

Key words: rheumatoid arthritis, fibroblast-like synoviocytes, decoy receptor 3, microarray assay, gene expression profile

Previous studies have suggested that DcR3 directly induces osteoclast formation from monocytes (13), and that DcR3 triggers the enhanced adhesion of monocytes via reverse signaling (14). We have also reported that DcR3 induces very late antigen-4 (VLA-4) expression in THP-1 macrophages, inhibiting cycloheximide-induced apoptosis (15). As for RA-FLS, in a recent study, we reported that DcR3 binds to membrane-bound TL1A expressed on RA-FLS, resulting in the negative regulation of cell proliferation induced by inflammatory cytokines (16). Therefore, we hypothesized that DcR3 plays a role in the pathogenesis of RA, not only as a decoy receptor, but also as a ligand via TL1A on RA-FLS. However, the function of DcR3 as a ligand in RA-FLS is not yet well understood. In the current study, we searched for genes in RA-FLS whose expression was regulated by the ligation of DcR3 using cDNA microarray. The gene expression profiles may reveal the possible target molecules that play a significant role in the DcR3-TL1A signaling pathway in the pathogenesis of RA.

Materials and methods

Isolation and culture of synovial fibroblasts. RA-FLS were obtained during total knee replacement surgery from 4 patients (samples 1-4) with RA who fulfilled the 1987 criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (17), who had never been treated with biological drugs. Synovial samples were collected from the patients who provided written consent in order to participate in this study in accordance with the World Medical Association Declaration of Helsinki Ethical Principles for Medical Research Involving Human Subjects. The protocol, including consent procedures, was approved by Kobe University Graduate School of Medicine Ethics Committee. Tissue specimens were minced and digested in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grand Island, NY, USA) containing 0.2% collagenase (Sigma, St. Louis, MO, USA) for 2 h at 37°C with 5% CO₂. The dissociated cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Walkersville, MD, USA) and 100 U/ml of penicillin/streptomycin. Following overnight culture, the non-adherent cells were removed, and the adherent cells were subsequently incubated further in fresh medium. All experiments were conducted using cells from passages 3 to 4 (4).

RNA extraction. Four individual lines (samples 1-4) of primary cultured RA-FLS (2x10⁶ cells/well) were incubated with 1.0 µg/ml of recombinant DcR3-Fc protein or control human IgG1 (R&D Systems, Minneapolis, MN, USA) for 12 h at 37°C with 5% CO₂. Following incubation, RNA was extracted using a QIAshredder and the RNeasy Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Extraction of total RNA was performed for each sample separately.

Gene expression profiling and data analysis. Gene expression was detected by microarray (Human Genome U133 Plus 2.0, GeneChip® 3' Expression Array; Affymetrix, Santa Clara, CA, USA). The labeling of RNA probes, hybridization and washing were carried out according to the manufacturer's instructions.

Avadis 3.3 Prophetic software (Strand Life Sciences, Bangalore, India) was used for statistical analysis. Differentially expressed genes were extracted by a paired t-test, with a P-value <0.05 considered to indicate a statistically significant difference, and fold change >1.4, and ordered into hierarchical clusters using the Euclidean algorithm as the distance measure, and the complete algorithm as the linkage method.

Microarray data have been deposited in NCBI's Gene Expression Omnibus (GEO) and are accessible through GEO series accession no. GSE45665.

Results

Microarray analysis (gene expression profiling of RA-FLS stimulated by DcR3-Fc). Microarray data analysis revealed that DcR3 upregulated or downregulated the expression of various genes in RA-FLS. We identified the 100 most differentially regulated genes in the DcR3-stimulated group compared with the control IgG1-stimulated group. Among these, 45 genes were upregulated (Table I) and 55 genes were downregulated (Table II).

Hierarchical clustering analysis. The upregulated and downregulated genes were classified into 7 and 10 categories according to their biological functions, respectively (Fig. 1). The upregulated genes were associated with protein complex assembly, cell motility, regulation of transcription, cellular protein catabolic processes, cell membrane, nucleotide binding and glycosylation. The upregulated genes belonging to each cluster are listed in Table III. The downregulated genes were associated with transcription regulator activity, RNA biosynthetic processes, cytoskeleton, zinc finger region, protein complex assembly, phosphate metabolic processes, mitochondrion, ion transport, nucleotide binding and cell fractionation. The downregulated genes belonging to each cluster are listed in Table IV.

Discussion

Among the 3 ligands of DcR3, TL1A (TNFSF15) is expressed by endothelial cells (12), macrophages (18,19), T cells (20,21), monocytes (22,23), dendritic cells (23), chondrocytes (24) and synovial fibroblasts (24), and contributes to the pathogenesis of cancer and autoimmune diseases via the apoptotic, stress, mitogenic and inflammation pathway by binding death receptor 3 (DR3) and DcR3 (12,25). The 3 ligands of DcR3 have been reported to contribute to the pathogenesis of RA (4,24,26,27). In these studies, DcR3 was considered a decoy receptor for ligands. We previously demonstrated that DcR3 binds to membrane-bound TL1A expressed on RA-FLS when it acts as a ligand in the pathogenesis of RA (16).

Genome-wide gene expression cDNA microarray is a powerful technique used to investigate the pathophysiology of a variety of diseases, including tumors (28-30), immune-mediated diseases (31,32) and inflammatory diseases (33-35). Using microarray, Chang *et al.* revealed that genes characteristically expressed by tumor-associated macrophages were upregulated by DcR3 (30). In the current study, we first demonstrated the expression profiles of genes in RA-FLS regulated by DcR3.

We demonstrated that DcR3 regulates the expression of genes that are mainly associated with the upregulation of the

Table I. The 45 genes upregulated by DcR3.

Gene symbol	Representative public ID	P-value	Fold change	Gene title
---	AW612461	0.002695443	2.0972981	---
CDH2	NM_001792	0.0327261	1.9276143	Cadherin 2, type 1, N-cadherin (neuronal)
AGPAT9	BC006236	0.006782195	1.9184313	1-Acylglycerol-3-phosphate O-acyltransferase 9
LOC440944	BC036698	0.039432783	1.8932389	Hypothetical protein LOC440944
BIVM	BC039587	0.012760683	1.6798534	Basic, immunoglobulin-like variable motif-containing protein
ZC3H3	D63484	0.02914655	1.6767992	Zinc finger CCCH-type containing 3
IL12B	NM_002187	0.008306135	1.6463093	Interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40)
NUB1	AK026433	0.045851827	1.6456505	Negative regulator of ubiquitin-like proteins 1
LTV1	AW236214	0.002476914	1.6453595	LTV1 homolog (<i>S. cerevisiae</i>)
DMRT2	AF284225	0.033502746	1.6123677	Doublesex and mab-3 related transcription factor 2
SUZ12P	AI820796	0.021593563	1.5686668	Suppressor of zeste 12 homolog pseudogene
ZBTB1	BU950380	0.039786864	1.5227357	Zinc finger and BTB domain containing 1
---	N29716	0.00277571	1.5223095	---
---	AK090762	0.034467466	1.5185958	---
---	BG398977	0.015083793	1.5135463	---
RANBP17	NM_022897	0.021518266	1.5035642	RAN binding protein 17
GAS5	BF336936	0.041366957	1.499078	Growth arrest-specific 5 (non-protein coding)
---	BC031996	0.011258653	1.4903411	---
REPS2	AI984607	0.012121066	1.4882914	RALBP1 associated Eps domain containing 2
LOC645158	BC018088	0.013921799	1.4854323	Hypothetical protein LOC645158
---	AI332454	0.012677074	1.4752275	---
CCDC138	AU152965	0.022518823	1.4711432	Coiled-coil domain containing 138
hCG_1749898	BC012486	0.002276273	1.471024	KRTAP2-4 protein
---	AI693281	0.0196566	1.4607961	---
TUBB2B	AL533838	0.006628409	1.4584572	Tubulin, beta 2B
SLC9A9	AA029791	0.004298657	1.4567653	Solute carrier family 9 (sodium/hydrogen exchanger), member 9
---	AA505135	0.023416178	1.4560933	---
SLC16A6	AI873273	0.032126337	1.4534916	Solute carrier family 16, member 6 (monocarboxylic acid transporter 7)
---	AL080112	0.035647828	1.4486992	---
LOC100128988	AI761436	0.035967685	1.4464797	Similar to hCG2018847
ZNF252	AU145662	0.015798416	1.4447424	Zinc finger protein 252
FGFR1OP2	R91766	0.021976791	1.4398756	FGFR1 oncogene partner 2
---	R26931	0.03931969	1.4398652	---
ZBTB10	BG483802	0.006168698	1.4324573	Zinc finger and BTB domain containing 10
C3AR1	U62027	0.031616967	1.4275972	Complement component 3a receptor 1
ZER1	NM_006336	0.000529948	1.4270489	Zer-1 homolog (<i>C. elegans</i>)
THRB	BF431989	0.027043225	1.4229552	Thyroid hormone receptor, beta (erythroblastic leukemia viral (v-erb-a) oncogene homolog 2, avian)
FLJ35220	AI311040	0.008202907	1.422941	Hypothetical protein FLJ35220
CDH10	NM_006727	0.018871933	1.4211183	Cadherin 10, type 2 (T2-cadherin)
PEX13	BC040953	0.00670011	1.4182297	Peroxisomal biogenesis factor 13
C7orf58	NM_024913	0.04462639	1.4087468	Chromosome 7 open reading frame 58
DOK3	BC004564	0.039809346	1.4068991	Docking protein 3
---	CA776505	0.038875684	1.4046313	---
EGR3	NM_004430	0.008657368	1.4038599	Early growth response 3
ZNF681	BG281940	0.009279283	1.4019198	Zinc finger protein 681

Table II. The 55 genes downregulated by DcR3.

Gene symbol	Representative public ID	P-value	Fold change	Gene title
TPH1	NM_004179	0.022398373	2.4520018	Tryptophan hydroxylase 1
TREML4	AK090633	0.028559439	1.9765018	Triggering receptor expressed on myeloid cells-like 4
CEP70	AI285884	0.038745213	1.8690827	Centrosomal protein 70 kDa
CCNB2	AK023404	0.040380865	1.8138049	Cyclin B2
CCDC121	NM_024584	0.023371078	1.8025432	Coiled-coil domain containing 121
ZNF563	NM_145276	0.01811626	1.7816929	Zinc finger protein 563
PANK2	AV703394	0.025317192	1.769196	Pantothenate kinase 2
---	N46436	0.047841128	1.7669531	---
ZFP28	AW590434	0.011407225	1.7393188	Zinc finger protein 28 homolog (mouse)
LOC284926	BG828817	0.04181046	1.7324702	Hypothetical protein LOC284926
SLC24A1	AF026132	0.001774447	1.7150456	Solute carrier family 24 (sodium/potassium/calcium exchanger), member 1
---	AU146924	0.04154287	1.699999	---
SOS2	L20686	0.019266233	1.6804754	Son of sevenless homolog 2 (<i>Drosophila</i>)
UBE3B	AL096740	0.048379965	1.6700492	Ubiquitin protein ligase E3B
ELL	AL521391	0.03850427	1.6655054	Elongation factor RNA polymerase II
SEZ6L2	AF131749	0.008593195	1.6527929	Seizure related 6 homolog (mouse)-like 2
MB	NM_005368	0.024074124	1.650637	Myoglobin
---	BC040628	0.049794715	1.6501505	---
MCOLN3	NM_018298	0.030422723	1.6494503	Mucolipin 3
---	AK021551	0.04994646	1.6114371	---
---	BF062156	0.03806778	1.5880637	---
FBXL17	AW002273	0.003356424	1.5799221	F-box and leucine-rich repeat protein 17
ZNF117	BF107006	0.048431396	1.5662925	Zinc finger protein 117
C1orf230	AV746331	0.007892164	1.5605123	Chromosome 1 open reading frame 230
---	AA650017	0.049496956	1.5536357	---
VKORC1L1	NM_173517	0.01724287	1.5391829	Vitamin K epoxide reductase complex, subunit 1-like 1
MIR155HG	BG231961	0.026413728	1.5365719	MIR155 host gene (non-protein coding)
KCNAB1	L39833	0.024623908	1.534346	Potassium voltage-gated channel, shaker-related subfamily, beta member 1
---	AI870634	0.048582062	1.5243825	---
YTHDC2	AW975818	0.008661097	1.5151424	YTH domain containing 2
CCNO	BC004877	0.012114909	1.5041811	Cyclin O
C5orf24	AW068615	0.029783456	1.5023562	Chromosome 5 open reading frame 24
---	AV648424	0.032394256	1.5009412	---
ADRBK2	NM_005160	0.034059193	1.4894675	Adrenergic, beta, receptor kinase 2
PIH1D2	AI744716	0.0245486	1.477943	PIH1 domain containing 2
---	BF224218	0.036218014	1.4755102	---
---	BF591554	0.042058036	1.474276	---
GRIN2A	N48896	0.006276551	1.4638788	Glutamate receptor, ionotropic, N-methyl D-aspartate 2A
RABL2A//RABL2B	NM_007082	0.00963415	1.4549305	RAB, member of RAS oncogene family-like 2A//RAB, member of RAS oncogene family-like 2B
MYH14	BC000676	0.009328251	1.4530606	Myosin, heavy chain 14
LOC727820	AW340595	0.018635018	1.4494766	Hypothetical protein LOC727820
---	AW074143	0.04609772	1.448378	---
ATP5G2	X69909	0.005548685	1.4429137	ATP synthase, H ⁺ transporting, mitochondrial F0 complex, subunit C2 (subunit 9)
MDM4	AW269813	0.031021323	1.4353052	Mdm4 p53 binding protein homolog (mouse)
C11orf84	AI866590	0.02536254	1.433334	Chromosome 11 open reading frame 84
STYX	AW968935	0.000515506	1.4238193	Serine/threonine/tyrosine interacting protein

Table II. Continued.

Gene symbol	Representative public ID	P-value	Fold change	Gene title
S100A14	NM_020672	0.03042584	1.4226245	S100 calcium binding protein A14
---	BE549780	0.020434508	1.4224106	---
NEURL4	AL136870	0.03654641	1.4173305	Neuralized homolog 4 (<i>Drosophila</i>)
---	AI803010	0.009107939	1.4132907	---
ETV7	AF218365	0.007053576	1.4130081	Ets variant 7
RBBP9	AL121893	0.01381168	1.4109538	Retinoblastoma binding protein 9
---	AI298755	0.048068948	1.4096705	---
LOC100130855	AK093077	0.008585751	1.4052048	Hypothetical protein LOC100130855
TSFM	AI796813	0.005840706	1.4036938	Ts translation elongation factor, mitochondrial

DcR3, decoy receptor 3.

Table III. Functional categories of the 45 upregulated genes classified into 7 categories.

Functional category	Genes
Protein complex assembly	CDH2, RANBP17, REPS2, TUBB2B, PEX13
Cell motility	CDH2, IL12B, PEX13
Regulation of transcription	CDH2, ZC3H3, NUB1, DMRT2, ZBTB1, REPS2, SLC9A9, ZBTB10, THRB, CDH10, EGR3, ZNF681
Cellular protein catabolic processes	ZER1
Cell membrane	CDH2, AGPAT9, RANBP17, SLC9A9, SLC16A6, C3AR1, CDH10, PEX13, DOK3
Nucleotide binding	RANBP17, TUBB2B,
Glycosylation	CDH2, IL12B, SLC9A9, C3AR1, CDH10, C7orf58

Table IV. Functional categories of the 55 downregulated genes classified into 10 categories.

Functional category	Genes
Transcription regulator activity	ZNF563, ZFP28, ELL, ZNF117, CCNO, MDM4, ETV7, TSFM
RNA biosynthetic processes	ELL, ZNF117, ETV7, TSFM
Cytoskeleton	Cep70, CCNB2, GRIN2A, MYH14
Zinc finger region	TPH1, ZNF563, ZFP28, SLC24A1, SOS2, ELL, MB, ZNF117, KCNAB1, GRIN2A, MDM4, S100A14, ETV7, RBBP9, TSFM
Protein complex assembly	MDM4
Phosphate metabolic processes	ADRBK2, ATP5G2, STYX
Mitochondrion	PANK2, ATP5G2, TSFM
Ion transport	TREML4, SLC24A1, SEZ6L2, MB, MCOLN3, VKORC1L1, KCNAB1, GRIN2A, ATP5G2
Nucleotide binding	PANK2, YTHDC2, ADRBK2, RABL2A//RABL2B, MYH14
Cell fractionation	CCNB2, SLC24A1, GRIN2A

protein complex assembly, cell motility and the regulation of transcription, and the downregulation of transcription regulator activity, RNA biosynthetic processes and cytoskeleton. We then focused on the following genes: cadherin 2, type 1,

N-cadherin (neuronal) (CDH2), interleukin 12B (natural killer cell stimulatory factor 2, cytotoxic lymphocyte maturation factor 2, p40) (IL12B), tryptophan hydroxylase 1 (TPH1), centrosomal protein 70 kDa (Cep70) and Zinc finger proteins

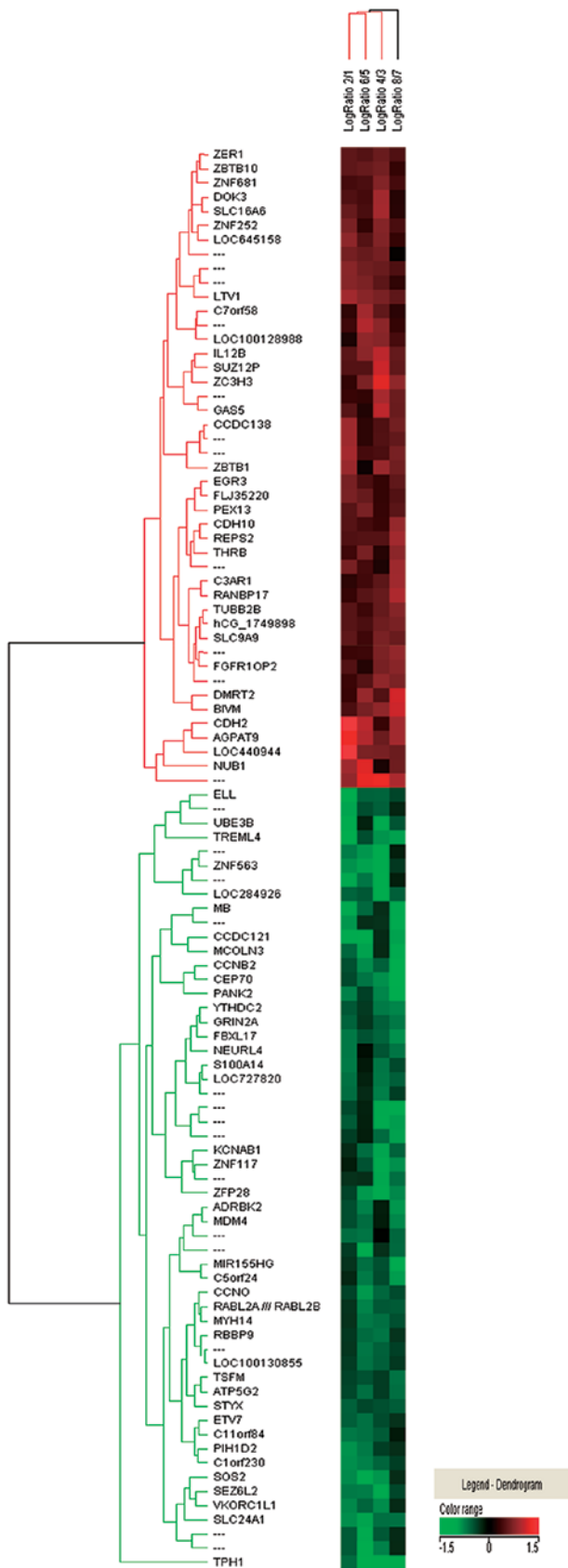


Figure 1. Cluster analysis and heat map of 100 significantly regulated probe sets. The heat map illustrates the expression values mapped to a color gradient from low (green) to high expression (red). Experiments are arranged according to a hierarchical clustering dendrogram. The horizontal dendrogram illustrates the similarity of functions between neighboring genes. The vertical dendrogram shows similarities in gene expression between neighboring samples.

as these genes were highly regulated, either upregulated or downregulated, and belonged to major functional clustering categories.

As for each gene, CDH2 has been reported to be associated with cell attachment and migration (36), metastatic potential (37), osteoblast differentiation (38) and the proliferation of RA-FLS (39).

IL12B encodes the IL-12B p40 subunit of IL-12 and IL-23 cytokines. IL-12 induces Th1 immune responses, and is thus linked with autoimmune diseases (40), while IL-23 is linked with autoimmune diseases via Th17 immune responses (41). IL-12 (42) and IL-23 (43,44) have also been reported to be involved in the pathogenesis of RA.

TPH1 is a rate-limiting enzyme involved in the synthesis of serotonin, and has been reported to be associated with the pathogenesis of RA through the inflammatory pathway (45) and bone biology (46-48).

Cep70 was discovered in a proteomic study of the centrosome (49). Centrosomal activity is indispensable for the execution of cytokinesis and the progression of the cell cycle (50). Cep70 is crucial for mitotic spindle assembly (51) and promotes microtubule polymerization by increasing microtubule elongation (52).

Zinc finger proteins are involved in a broad range of biological activities, including double-stranded DNA binding, single-stranded DNA and RNA recognition, as well as coordinating protein-protein interactions (53).

In the current study, we first reported the expression profiles of genes in RA-FLS regulated by DcR3. Combined with our previous findings that DcR3 serves as a ligand by binding to membrane-bound TL1A on RA-FLS, our data demonstrate that DcR3 may regulate the gene expression of various key molecules in RA-FLS by binding to TL1A, thus affecting the pathogenesis of RA, such as proliferation, apoptosis, inflammation and bone biology. Further studies on the genes detected in the current study may provide a deeper understanding of the pathogenesis and treatment of RA by DcR3-TL1A signaling.

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