Therefore, both IFN-α and IFN-γ directly controlled the expressions of PSMB9 and type I collagen, but not versican V1.

These data led us to determine the detailed mechanism of versican overexpression in the dermis of DM and SLE skin. We hypothesised that PSMB9 is induced by IFN in the epidermis, which stimulates versican V1 expression in the dermis. Previous reports indicated that several cytokines (e.g., TGF-β2 and TGF-β3) stimulate versican expression.[24] When PSMB9 was knocked down by PSMB9 siRNA in keratinocytes (Fig. 4a), versican expression was not affected. On the other hand, PSMB9 knockdown led to the significant suppression of TGF-β2 and TGF-β3, but not TGF-β1 (Fig. 4b). Taken together, our hypothetical model is that increased PSMB9 due to IFN signaling induces TGF-β2/3 in epidermal keratinocytes of DM/SLE, which results in the stimulation of versican synthesis in dermal fibroblasts (Fig. 5). Consistently, TGF-β3 mRNA levels in vivo were significantly upregulated in both DM and SLE skin compared to NS skin and skin of other diseases (Fig. 4c). Of note, TGF-β2 mRNA levels were upregulated only in DM skin. Immunohistochemical findings confirmed that TGF-β2 protein expression was increased only in the epidermis of DM, but not SLE skin (Fig. 4d).

GAGs analysis in DM and SLE skin

We also determined the amount of GAGs in DM and SLE skin. The CS levels were significantly increased in SLE skin compared to NS skin (Fig. 6a). On the other hand, no significant differences were found in the amount of HA or HS between the skin of DM, SLE and NS.

Next, the composition of 6 disaccharides of HS was quantitated by HPLC. We found that the ΔDiHS-diS1 levels were significantly increased only in DM skin compared to SLE and NS (Fig. 6b).

We then determined the mechanism underlying the increased levels of ΔDiHS-diS1 only in DM skin. The stimulation of normal human dermal fibroblasts with TGF-β2 resulted in the significant increase of ΔDiHS-diS1 secretion levels (Fig. 6c). Accordingly, the difference of ΔDiHS-diS1 levels between DM and SLE skin may be associated with the difference of TGF-β2 expression between them.

Discussion

This is the first report of a proteomic analysis using skin samples of DM patients. In the present study, we identified common molecular changes in DM skin and SLE skin and specific molecular changes to DM skin.

Based on the LC-MS/MS results, we focused on the increase of PSMB9, a subunit of immunoproteasome, as common molecular change in DM and SLE skin because of the similarity between Nakajo-Nishimura syndrome (caused by mutations of PSMB8, another immunopreteasome subunit) and DM/SLE.[23] Proteasomes are components of the ubiquitin-proteasome system (UPS), where immunoproteasomes degrade intracellular proteins into peptide fragments, improves bindings of the fragments to major histocompatibility complex (MHC) class I molecules, and induces immune responses effectively. The UPS activation by PSMB overexpression may play a role in the pathogenesis of autoimmune diseases such as DM and SLE. Consistently, bortezomib, an inhibitor of PSMB8, is found to be effective for SLE patients and currently under clinical trial.[25]

In addition, PSMB9 is encoded by MHC class II region, and recent genome-wide association studies (GWAS) indicated MHC as the major genetic region associated with DM or SLE.[26,27] Furthermore, PSMB9 is also known as the target gene of IFN.[28,29] Type I IFN signature in DM and SLE has been well investigated. Meanwhile, several studies have also suggested the involvement of IFN-γ in these diseases.[30,31] Taken together, PSMB9 is a key molecule associated with both MHC and IFN in DM and SLE. PSMB9 overexpression is highly specific to these two diseases, and therefore it may be useful as a diagnostic tool and new therapeutic target.

Collagens and proteoglycans are the main components of the extracellular matrix in skin dermis. Versican is a core protein that mainly interacts with HA and CS, and controls mucin deposition. Furthermore, previous studies suggested that versican also binds to HS indirectly.[32-34] Versican expression was increased, while type I collagen expression was decreased in DM and SLE skin compared to NS skin. As the mechanism of increased cutaneous mucin deposition in DM and SLE, versican upregulation and collagen downregulation may accelerate it.

Chang *et al.* reported that both HA and CS were significantly elevated in discoid LE (DLE) by specific stains, while DM lesional dermis accumulated mainly CS but not HA.[35] Kim and Werth also revealed increased accumulation of chondroitin-4-sulfate in DLE and DM lesions compared to control skin by immunohistochemical analyses.[36] Our study is

the first to focus on HS in these diseases, and identified TGF-β2-ΔDiHS-diS1 pathway as the specific molecular changes in DM skin. TGF-β2 expression was increased only in the epidermis of DM skin, and TGF-β2 induced ΔDiHS-diS1 in cultured dermal fibroblasts. The difference in this pathway may also be useful to distinguish DM from SLE. Furthermore, because different GAGs species have distinct immunomodulatory effects, the TGF-β2-ΔDiHS-diS1 pathway may play a role in the difference between the skin features in DM and SLE (e.g. predilection sites). Further studies are needed to identify upstream events of TGF-β2 increase in DM skin in the future.

Of note, PSMB9 knockdown resulted in the significant suppression of TGF-β2 and TGF-β3 expressions, but not TGF-β1 expression. In spite of their structural similarities, previous studies have shown that TGF-β1 and TGF-β2/3 can be differentially regulated: for example, Mężyk-Kopeć *et al.* reported that silencing of a disintegrin and metalloprotease 17 (ADAM17) induced the levels of TGF-β2 and TGF-β3 in human dermal lymphatic endothelial cell line, but did not affect the TGF-β1 expression.[37] Furthermore, Olave *et al.* revealed that endothelin-A receptor antagonism up-regulated TGF-β2 and TGF-β3 synthesis, but not TGF-β1 in mice.[38] Clarification of detailed mechanism of TGF-β2/3-specific regulation by PSMB9 is necessary in the future. As the limitation of this study, the study design may be rather story-oriented, and further studies are needed to prove our hypothetical model.

Acknowledgements

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Figure legends

Figure 1. Comparison of gene expression between dermatomyositis skin and normal skin

(a,b) Total RNA was extracted from involved skin tissues of dermatomyositis (DM) patients and control skin of normal subjects (NS). A subset of samples (8 DM skin and 8 NS skin) was randomly chosen from 25 DM skin and 15 NS skin. mRNA expression of indicated molecules was analyzed by real-time PCR. Bars show the means. The mean values in NS were set at 1. *P <0.05, **P <0.01, ***P <0.001.

Figure 2. PSMB9 expression in DM skin

(a) Total RNA was extracted from skin tissues derived from NS (n=15) and patients with DM (n=25), systemic lupus erythematosus (SLE, n=20), psoriasis (PSO, n=3), atopic dermatitis (AD, n=3), lichen planus (LP, n=10) and erythema exsudativum multiforme (EEM, n=10). PSMB9 mRNA expression was analyzed by real-time PCR. Bars show the means. The mean value in NS was set at 1. *P<0.05.

(b) Total RNA was extracted from skin tissues derived from DM (n=9), SLE (n=5) and NS (n=10): A subset of samples was randomly chosen from 25 DM skin, 20 SLE skin and 15 NS skin. PSMB8 mRNA expression was analyzed by real-time PCR.

(c-e) Total RNA was extracted from skin tissues derived from NS (n=15) and patients with DM (n=22) and SLE (n=20): A subset of samples was randomly chosen from 15 NS skin, 25 DM skin and 20 SLE skin. mRNA expression of indicated genes was analyzed by real-time PCR. Samples of 3 AD patients and 10 LP patients were also included as disease controls.

(f) Cell lysates obtained from skin tissues of indicated diseases were subjected to immunoblotimg with antibodies for PSMB9 or type I collagen. β-actin was used as the loading control. The 2 representative results for 5 samples of each disease randomly chosen from 25 DM skin, 20 SLE skin and 15 NS skin are shown. Two scleroderma (SSc) samples were also included as a disease control.

(g) Relative collagen content in paraffin-embedded sections of NS (n=6) and DM skin (n=8) was determined using the assay kit: A subset of samples was randomly chosen from 25 DM skin and 15 NS skin. *P<0.05.

Figure 3. The effects of interferon (IFN) on the expression of PSMB9, collagen and versican V1

(a) Paraffin sections were subjected to immunohistochemical analyses for PSMB9. *(left)* NS skin, *(middle)* DM skin, and (*right*) SLE skin. Original magnification x200 (upper panels) and x400 (lower panels). A subset of samples for each disease (n=15) was randomly chosen from 25 DM skin, 20 SLE skin and 15 NS skin. The representative results were shown.

(b) Paraffin sections were subjected to immunohistochemical analysis for versican V1. *(left)* NS skin, *(middle)* DM skin, and (*right*) SLE skin. Original magnification x200 (upper panels) and x400 (lower panels). A subset of samples for each disease (n=8) was randomly chosen from 25 DM skin, 20 SLE skin and 15 NS skin. The representative results were shown.

(c) Cultured normal human epithelial keratinocytes were incubated in the presence or absence of IFN-α *(left)* or IFN-γ *(right)* for 48 hours. Cell lysates were subjected to immunoblotting using antibody for PSMB9. β-actin was used as the loading control.

(d) Cultured normal human dermal fibroblasts were stimulated with indicated dose of IFN-α or IFN-γ for 72 hours. Cell lysates were subjected to immunoblotting using antibodies for type I collagen or versican V1. β-actin was used as the loading control.

Figure 4. Functional analysis of PSMB9 in keratinocytes

(a) HaCaT cells were transfected with control or PSMB9 siRNA. After 48 hours, cell lysates were obtained and subjected to immunoblotting with antibodies for PSMB9 or versican V1. β-actin was used as the loading control.

(b) HaCaT cells were transfected with control or PSMB9 siRNA, and total RNA was extracted after 24 hours. mRNA expression levels of indicated genes were determined by real-time PCR (n=3). The mean values in control cells were set at 1. *P <0.05.

(c) Total RNA was extracted from skin tissues derived from NS (n=5) and patients with DM (n=5) and SLE (n=5): A subset of samples was randomly chosen from 15 NS skin, 25 DM skin and 20 SLE skin. Samples of psoriasis (PSO, n=3), atopic dermatitis (AD, n=3), lichen planus (LP, n=10) and erythema exsudativum multiforme (EEM, n=10) were also included as the disease controls. mRNA levels of TGF-β2 and TGF-β3 were analyzed by real-time PCR. Bars show the means. *P<0.05.

(d) Paraffin sections were subjected to immunohistochemical analysis for TGF-β2. *(left)* NS skin, *(middle)* DM skin, and (*right*) SLE skin. Original magnification x200 (upper panels) and x400 (lower panels). A subset of samples for each disease (n=8) was randomly chosen from 25 DM skin, 20 SLE skin and 15 NS skin. The representative results were shown.

Figure 5. Our hypothetical model

IFN signaling in DM and SLE skin reduces the expression of collagen, but not versican V1 in dermal fibroblasts. IFN also stimulates PSMB9 expression and subsequently induces TGF-β2 or β3 in keratinocytes, which may increase versican V1 expression in dermal fibroblasts. According to previous reports, TGF-β2 and -β3 are not likely to affect on collagen expression in dermal fibroblasts.

Figure 6. Glycosaminoglycan analyses in DM, SLE and NS skin

(a) Hyaluronic acid (HA), chondroitin sulfate (CS) and heparin sulfate (HS) were extracted from skin tissues of DM (n=4), SLE (n=3) and NS (n=3): A subset of samples was randomly chosen from 25 DM skin, 20 SLE skin and 15 NS skin. The amount of each content /1mg of sample was shown on the ordinate. Bars show the means. *P<0.05.

(b) High performance liquid chromatography (HPLC) for various types of disaccharides composing HS was performed using skin tissues of DM (n=4), SLE (n=3) and NS (n=3). A subset of samples was randomly chosen from 25 DM skin, 20 SLE skin and 15 NS skin. The amount of each content corrected by total amount of HS was shown on the ordinate. *P<0.05.

(c) Normal human dermal fibroblasts were incubated in the presence or absence of TGF-β2 for 72 hours (n=3). HS was extracted from culture media, and was subject to HPLC for ΔDiHS-diS1. The results were normalized for cell numbers. *P<0.05.

Table 1. Comparison of protein expression between DM and normal skin determined by LC-MS/MS.

Gene symbol	Protein	DM1 /NS1	DM2 /NS2	DM1 /NS2	DM2 /NS1
DCD	Dermcidin	0.19	0.28	0.50	0.11
MAP1A	Microtubule-associated protein 1A	0.21	0.41	0.58	0.15
PIP	Prolactin-inducible protein	0.26	0.35	0.44	0.20
SPTAN1	Isoform 2 of Spectrin alpha chain, brain	0.30	0.52	0.59	0.27
GSTM2	Glutathione S-transferase Mu 2	0.36	0.30	0.29	0.38
COL1A2	Collagen alpha-2(I) chain	0.36	0.28	0.42	0.24
LONRF3	Isoform 3 of LON peptidase N-terminal domain and RING finger protein 3	0.42	0.21	0.49	0.18
SFRP1	Secreted frizzled-related protein 1	0.43	0.56	0.62	0.39
GIT1	GTPase-activating protein	0.43	0.44	0.41	0.46
COL1A1	Collagen alpha-1(I) chain	0.45	0.35	0.53	0.29
CKMT1	Isoform 1 of Creatine kinase U-type, mitochondrial	0.45	0.56	0.55	0.46
NOL10	Nucleolar protein 10	0.46	0.62	0.57	0.50
GSTT1	Glutathione S-transferase theta-1	0.47	0.63	0.67	0.44
CAMLG	Calcium signal-modulating cyclophilin ligand	0.47	0.48	0.46	0.49
PI16	Isoform 1 of Peptidase inhibitor 16	0.47	0.49	0.49	0.48
EPHX2	Epoxide hydrolase 2, cytoplasmic	0.48	0.57	0.55	0.50
GPLD1	Isoform 1 of Phosphatidylinositol-glycan-specific phospholipase D	0.49	0.19	0.26	0.36
EFCAB4B	EF-hand calcium-binding domain-containing protein 4B	0.51	0.58	0.47	0.63
RNF214	RING finger protein 214	0.53	0.56	0.46	0.65
GBAS	Protein NipSnap homolog 2	0.54	0.61	0.55	0.60
CCDC80	Isoform 1 of Coiled-coil domain-containing protein 80	0.55	0.39	0.59	0.36
GSTM1	Isoform 1 of Glutathione S-transferase Mu 1	0.56	0.45	0.52	0.48
PEBP1	Phosphatidylethanolamine-binding protein 1	0.56	0.55	0.61	0.50
APOH	Beta-2-glycoprotein 1	0.58	0.45	0.50	0.52

WDR16	Isoform 3 of WD repeat-containing protein 16	0.59	0.35	0.36	0.58
HSPA14	Heat shock 70 kDa protein 14	0.61	0.42	0.41	0.63
NDRG3	N-myc downstream-regulated gene 3 protein	0.62	0.33	0.34	0.62
CD109	Isoform 3 of CD109 antigen	0.62	0.46	0.56	0.51
HEBP2	Heme binding protein 2 (Fragment)	0.63	0.30	0.35	0.54
-	Conserved hypothetical protein	0.64	0.62	0.62	0.63
FLAD1	Flavin adenine dinucleotide synthetase 1	0.65	0.44	0.48	0.59
FBLN1	Isoform D of Fibulin-1	0.66	0.51	0.60	0.55
COL3A1	Isoform 1 of Collagen alpha-1(III) chain	0.67	0.46	0.63	0.48
IARS2	Isoleucyl-tRNA synthetase, mitochondrial	0.67	0.42	0.47	0.59
LOC402160	Uncharacterized protein	1.51	2.67	2.43	1.66
UGT2A3	UDP-glucuronosyltransferase 2A3	1.52	2.34	1.70	2.10
EIF2AK2	Interferon-induced, double-stranded RNA-activated protein kinase (Fragment)	1.53	1.61	1.50	1.65
-	V1-17 protein	1.55	2.79	1.65	2.62
CD209	Isoform 10 of CD209 antigen	1.57	2.58	2.48	1.63
ERAP2	Endoplasmic reticulum aminopeptidase 2	1.57	1.59	1.26	1.99
MANF	Mesencephalic astrocyte-derived neurotrophic factor	1.61	1.74	1.52	1.84
RPS10-NUDT3	RPS10-NUDT3 protein	1.63	1.71	1.67	1.68
FAM21C	Isoform 3 of WASH complex subunit FAM21C	1.69	2.06	2.18	1.59
IFIT3	Interferon-induced protein with tetratricopeptide repeats 3	1.73	2.10	2.31	1.57
PF4	Platelet factor 4	1.74	1.85	1.76	1.83
CCPG1	Isoform 4 of Cell cycle progression protein 1	1.77	1.98	1.80	1.95
ARPC1B	Actin-related protein 2/3 complex subunit 1B	1.78	1.97	1.72	2.04

EIF3M	Eukaryotic translation initiation factor 3 subunit M	1.80	1.64	1.55	1.91
VCAN	Isoform V1 of Versican core protein	1.81	2.30	2.49	1.67
SPRR1B	Cornifin-B	1.88	1.60	1.92	1.56
THBS1	Thrombospondin-1	1.95	1.62	1.76	1.79
CCT7	Chaperonin containing TCP1, subunit 7	1.98	1.56	1.70	1.82
MAN1A1	Mannosyl-oligosaccharide 1,2-alpha-mannosidase IA	2.09	1.53	1.90	1.68
STAT1	Isoform Alpha of Signal transducer and activator of transcription 1-alpha/beta	2.46	2.02	2.05	2.41
PSMB9	Isoform LMP2.S of Proteasome subunit beta type-9	2.57	2.77	2.51	2.83
EIF1AX	Eukaryotic translation initiation factor 1A, X-chromosomal	3.06	2.12	3.29	1.97
TYMP	Thymidine phosphorylase	3.35	1.50	1.53	3.28
HLA-B	HLA class I histocompatibility antigen, B-54 alpha chain	3.92	1.55	1.60	3.80
CTSL1	Cathepsin L1	4.25	1.92	2.36	3.46
FCER1G	High affinity immunoglobulin epsilon receptor subunit gamma	4.25	1.71	2.42	3.00
GBP1	Interferon-induced guanylate-binding protein 1	5.44	1.59	1.65	5.25

Protein samples were extracted from DM skin (n=2) and normal skin samples (NS, n=2), and were analyzed by LC-MS/MS. Proteins whose expression ratio of DM1/NS1, DM2/NS2, DM1/NS2 and DM2/NS1 was always >1.5 or ≦0.67 are listed.

Figure 1

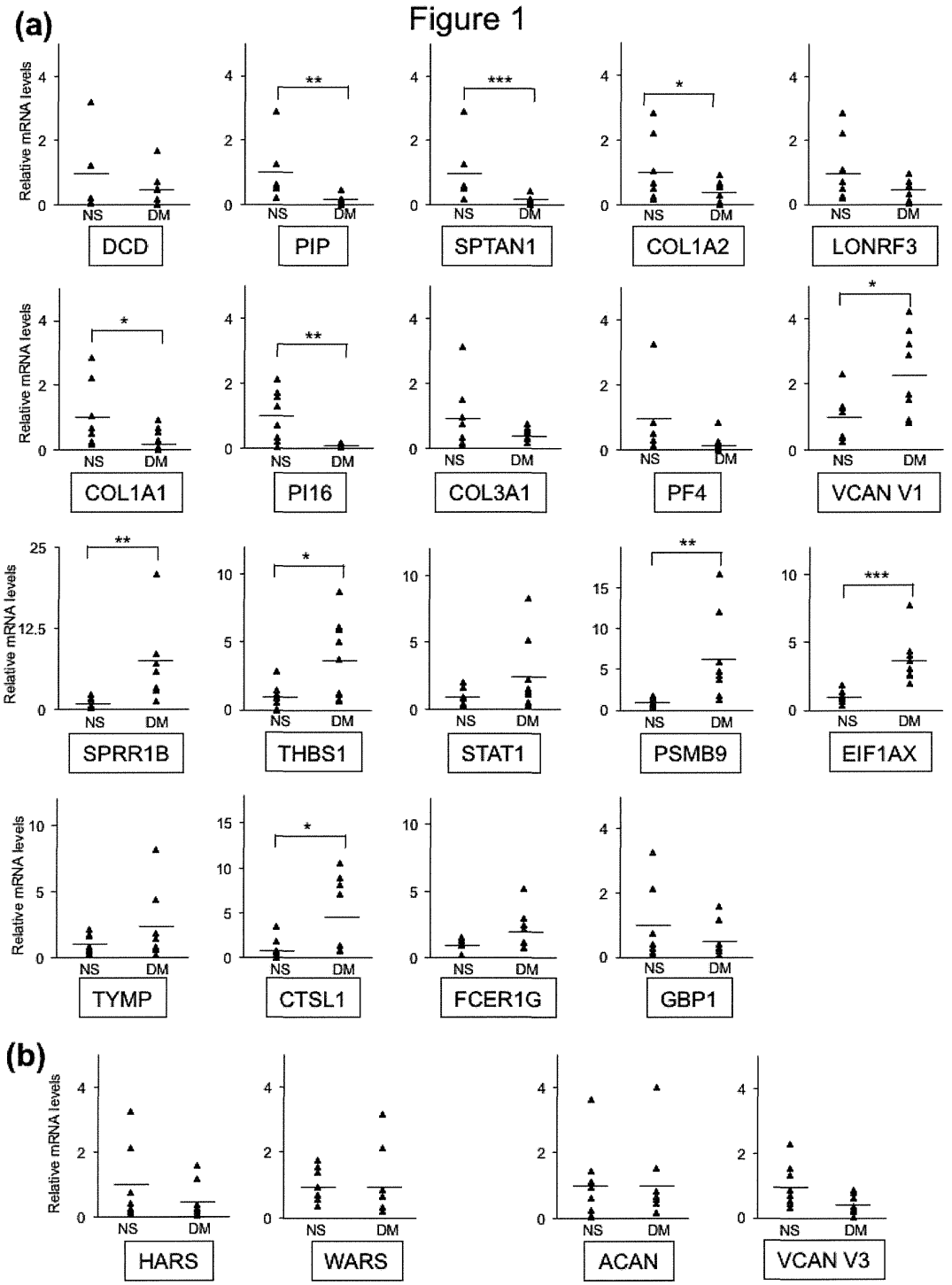


Figure 2

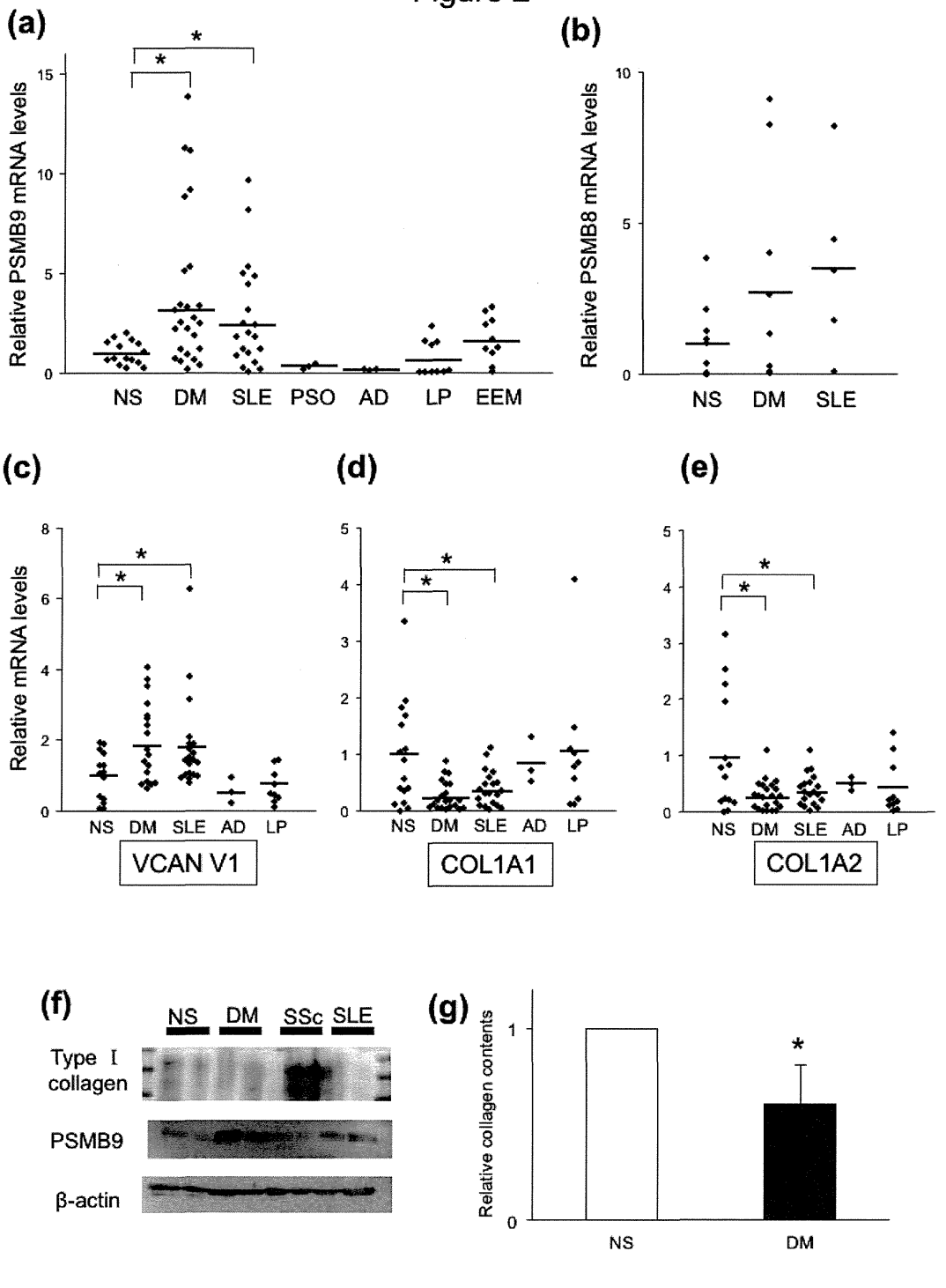


Figure 3

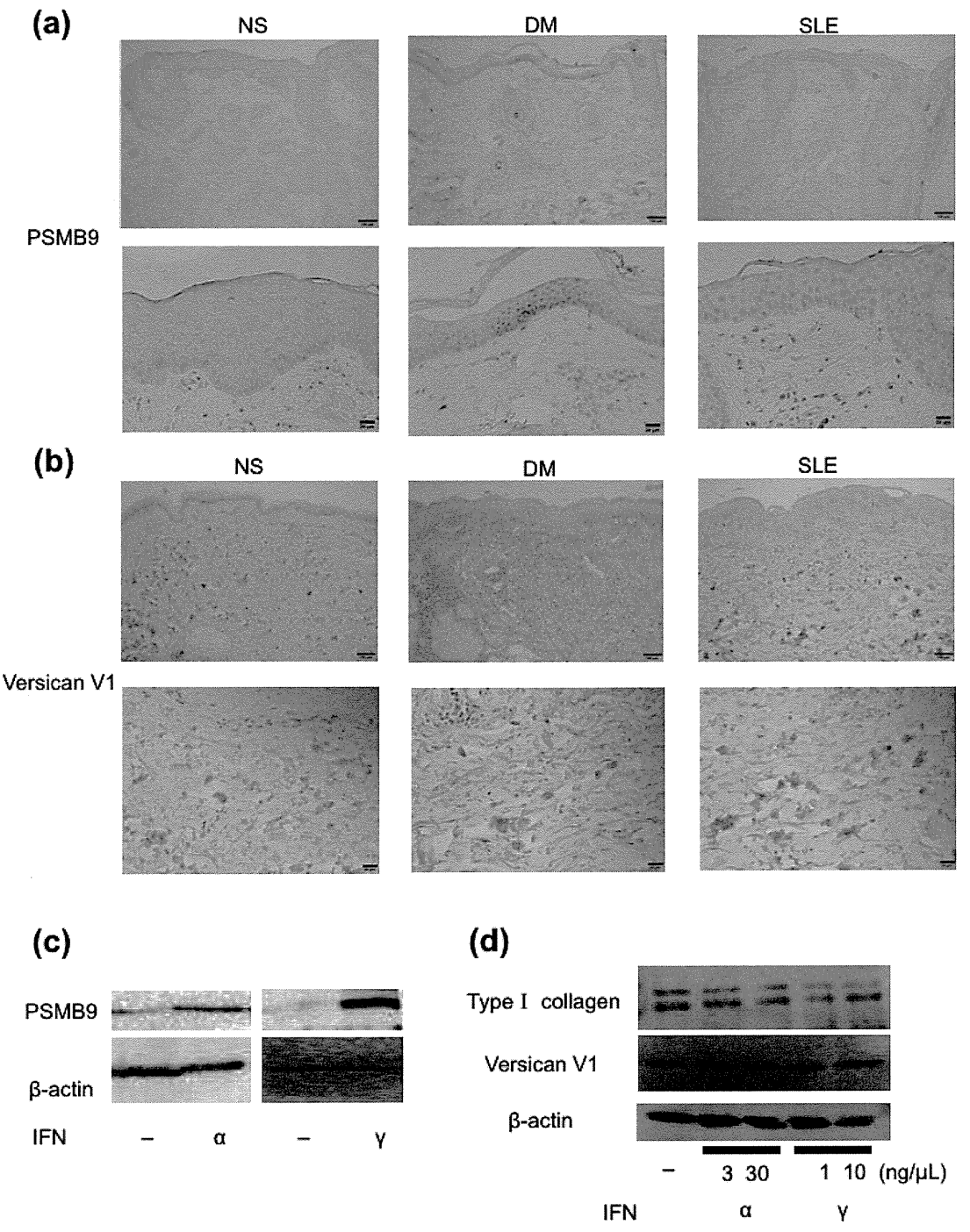


Figure 4

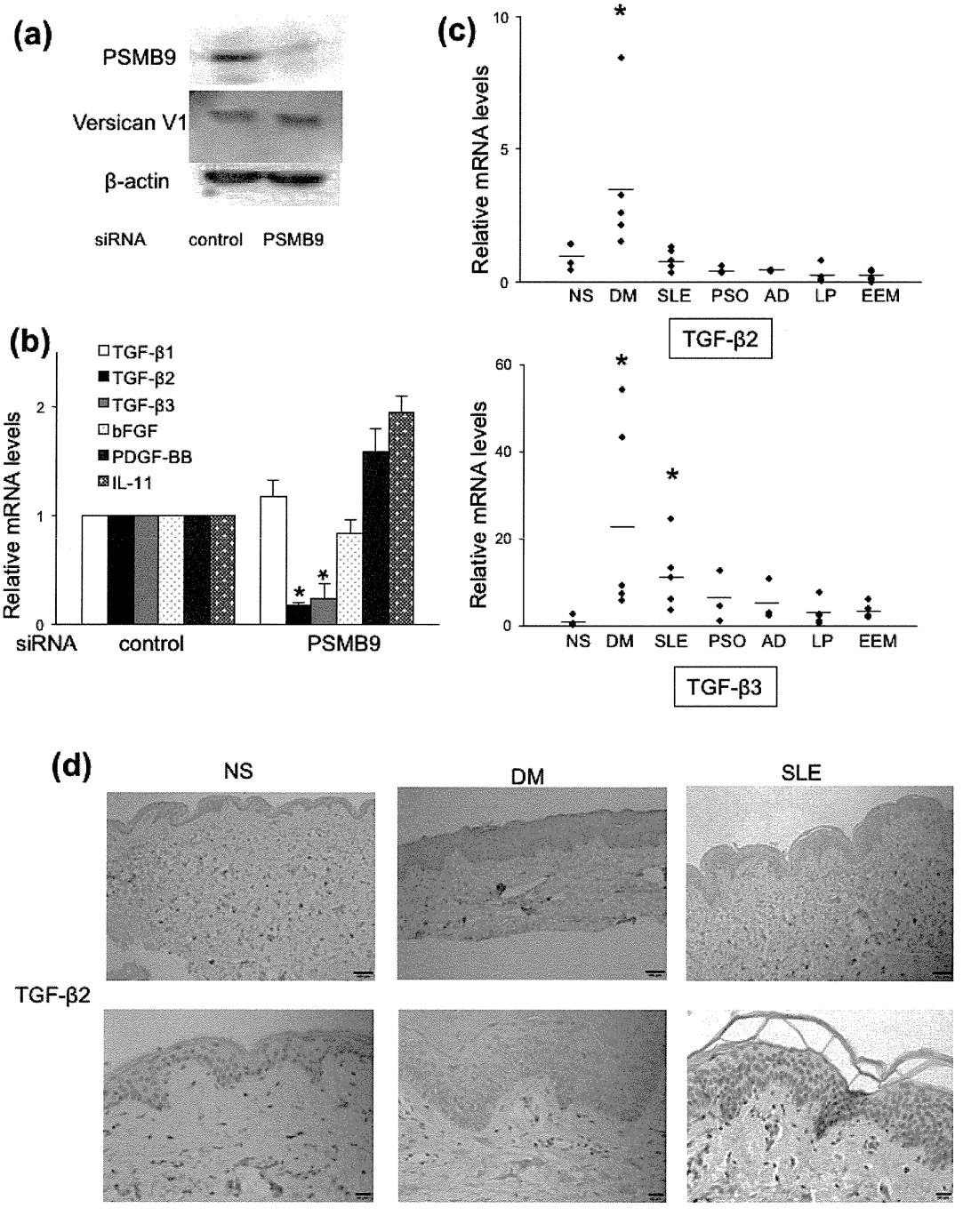


Figure 5

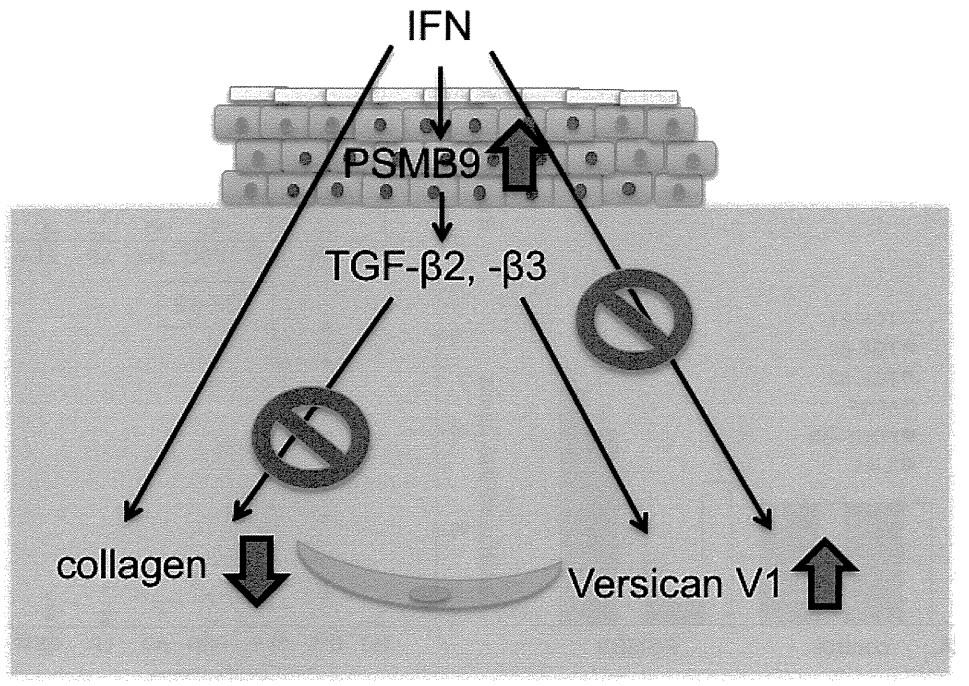
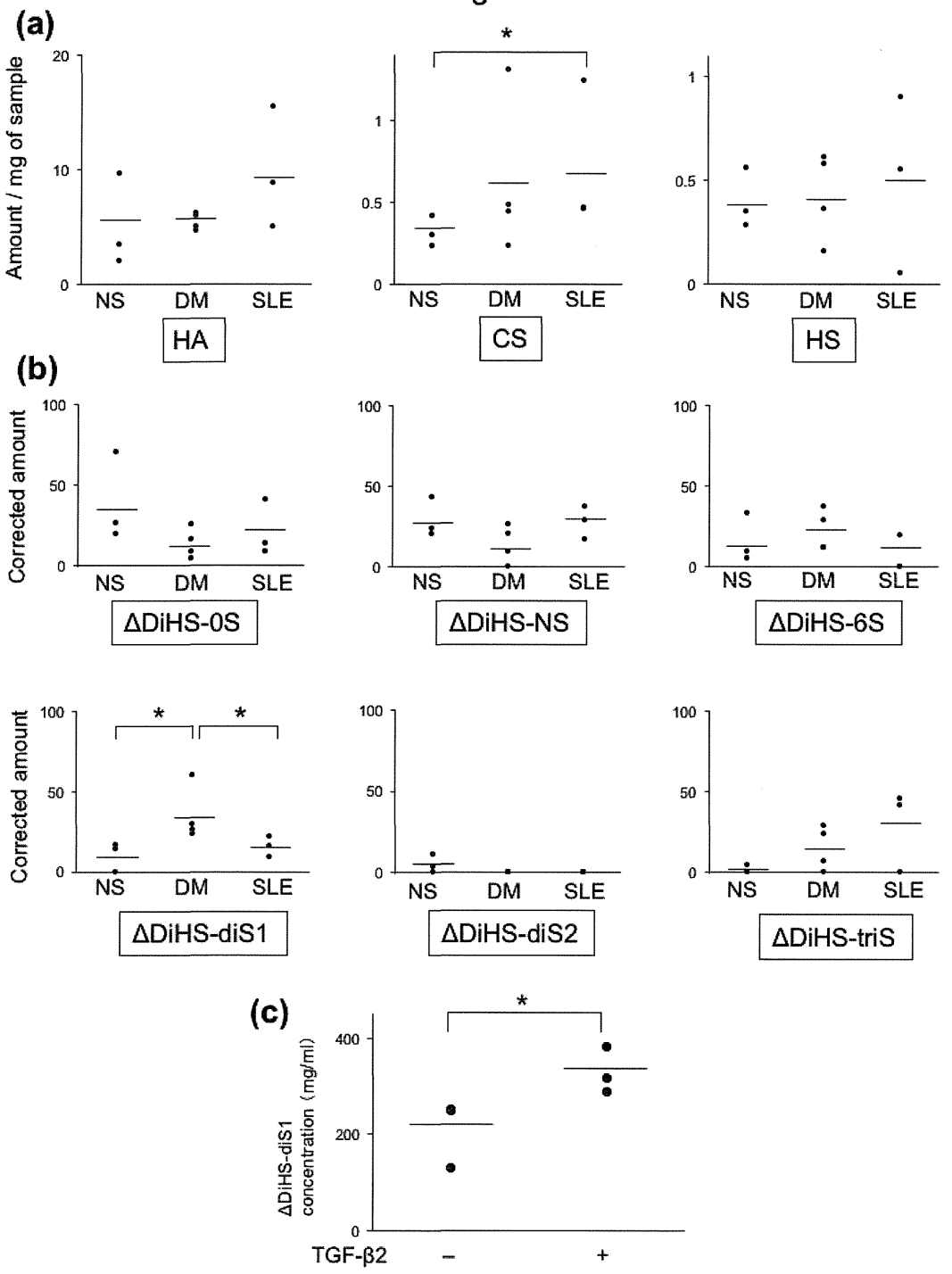


Figure 6



Original Article

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Serum concentrations of Flt-3 ligand in rheumatic diseases

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Summary **Fms-like tyrosine kinase 3 (Flt-3) is a cytokine receptor expressed on the surface of bone-marrow progenitor of hematopoietic cells. Flt-3 ligands are produced by peripheral blood mononuclear cells, and found in various human body fluids. Flt-3 signal is involved in the regulation of vessel formation as well as B cell differentiation, suggesting that Flt-3 signal contributes to the pathogenesis of vascular abnormalities and immune dysregulation in rheumatic diseases. The aim of the present study is to examine serum Flt-3 ligand levels in patients with various rheumatic diseases, and to evaluate the possibility that serum Flt-3 ligand levels can be a useful disease marker. Sera were obtained from 20 dermatomyositis (DM) patients, 36 systemic sclerosis (SSc) patients, 10 systemic lupus erythematosus (SLE) patients, 10 scleroderma spectrum disorder (SSD) patients, 4 mixed connective tissue disease (MCTD) patients, and 12 normal subjects. Flt-3 ligand levels were determined with ELISA. Serum Flt-3 ligand levels were significantly elevated in patients with DM, SSc, SSD and MCTD compared to those in normal subjects. DM patients with elevated Flt-3 ligand levels were accompanied with significantly increased CRP levels and increased frequency of heliotrope rash than those with normal levels. In addition, SSc patients with elevated Flt-3 ligand levels showed significantly reduced frequency of nailfold bleeding. Serum Flt-3 ligand levels can be a marker of cutaneous manifestation in DM and a marker of microangiopathy in SSc. Clarifying the role of Flt-3 ligand in rheumatic diseases may lead to further understanding of these diseases and new therapeutic approaches.**

Keywords: Flt-3, dermatomyositis, systemic sclerosis, systemic lupus erythematosus

1. Introduction

Immune dysfunction and vascular abnormalities are thought to be the common features in rheumatic diseases including rheumatoid arthritis (RA), polymyositis/dermatomyositis (PM/DM), systemic sclerosis (SSc), systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD) or Sjögren's syndrome (SjS) (*1,2*). For example, autoantibodies, Raynaud's phenomenon, nailfold bleeding (NFB) and skin ulcers are frequently seen in various rheumatic diseases. These vascular abnormalities are characterized by uncontrolled regeneration of the vasculature and vascular losses due to defective maintenance of the vasculature (*3*). However, the mechanism is hardly understood, and needs to be clarified.

Fms-like tyrosine kinase 3 (Flt-3), also known as CD135, is a cytokine receptor expressed on the surface of bone marrow-derived hematopoietic progenitor cells. Activation of the receptor has been reported to regulate vessel formation as well as the blood cell differentiation. For example, Flt-3 signal is thought to play roles in the angiogenesis of malignant tumors, the process of B cell differentiation, immune response to virus infection, and aging (*4-7*). Furthermore, Flt-3 mutations are found in patients with leukemia (*8*). Thus, there is a possibility that Flt-3 signal is involved in the pathogenesis of vascular abnormalities and immune dysregulation in rheumatic diseases as well as similar dysregulation of immunity and angiogenesis such as malignancy, infection and aging.

On the other hand, Flt-3 ligands, approximately 30 kDa transmembrane glycoproteins, are produced

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