Inhibition of each module of connective tissue growth factor as a potential therapeutic target for rheumatoid arthritis

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Running head: Inhibition of each module of CTGF in RA

Abstract

We previously reported the importance of connective tissue growth factor (CTGF) in rheumatoid arthritis (RA). CTGF contains 4 distinct modules connected in tandem, namely insulin-like growth factor-binding protein-like, von Willebrand factor type C repeat, thrombospondin type 1 repeat, and carboxyl-terminal (CT) modules. The relationships between each of these modules of CTGF and RA remain unknown. Here, we analyzed how inhibition of each CTGF module affects the pathophysiology of RA. We conducted stimulation and suppression experiments on synovial cells (MH7A) obtained from patients with RA. Moreover, we examined angiogenesis by means of a tube-formation assay performed using human umbilical vein endothelial cells (HUVECs), and we used tartrate-resistant acid phosphatase staining to analyze osteoclastogenesis. Our results showed that M-CSF/RANKL-mediated osteoclastogenesis was enhanced when CTGF was added, but the effect of CTGF was neutralized by mAbs against CTGF modules 1–4. Furthermore, CTGF treatment of HUVECs induced formation of tubular networks, which resulted in acceleration of the angiogenesis of RA synoviocytes, and quantification showed that this tubular-network formation was also disrupted by anti-CTGF module 1–4 mAbs. Lastly, TNF-α enhanced the expression of CTGF and matrix metalloproteinase-3 in MH7A cells, and this

enhancement was potently neutralized by mAbs against CTGF modules 1, 3, and 4.

Thus, our results indicate that not only an mAb against CTGF but also mAbs against

each specific module of CTGF might serve as potential therapeutic agents in the

treatment of RA.

Introduction

Connective tissue growth factor (CTGF) is a member of the CCN family (CCN2) that was discovered because of its cross-reactivity with an antiserum against platelet-derived growth factor. CTGF is a single 38-kDa polypeptide secreted by cultured human umbilical vein endothelial cells (HUVECs), and it functions in several biological processes such as fibrosis, tumorigenesis, angiogenesis, and endochondral ossification. CTGF contains 4 distinct modules connected in tandem: the insulin-like growth factor-binding protein (IGFBP)-like, von Willebrand factor (vWF) type C repeat, thrombospondin type 1 (TSP-1) repeat, and C-terminal (CT) modules [1–3].

Rheumatoid arthritis (RA) is an autoimmune disease that causes chronic inflammation of synovial joints and eventually results in destruction of cartilage and bone [4]. The growing metabolic demand imposed by synoviocyte proliferation leads to the development of blood vessels that nourish and oxygenate the synovial pannus and promote its invasion and degradation of adjacent cartilage and bone. The neovessels exacerbate inflammation by further facilitating the ingress of inflammatory cells and mediators into the joint [5,6].

Previously, we reported that CTGF was related to the pathogenesis of RA, and we found that blockade of CTGF prevents the progression of arthritis in mice with

collagen-induced arthritis [7,8]. Moreover, we recently reported that inhibition of the

IGF-I/IGFBP complex, including one of the modules of CTGF, ameliorates RA

pathogenesis *in vitro* [9]. However, the relationships between the 4 distinct modules of

CTGF and RA remain unknown. Here, we investigated how inhibiting each module of

CTGF affects the pathophysiology of RA.

Materials and methods

Materials and cell lines

The human synovial fibroblast cell line MH7A (Riken Cell Bank, Ibaraki, Japan), which

was isolated from the knee joint of a patient with RA, was provided by Dr. Miyazawa

[5]. HUVECs were obtained from Lonza (Walkersville, MD). Monoclonal antibodies

that neutralize each CTGF module (anti-module-1 mAb: 30D2; anti-module-2 mAb:

2-3; anti-module-3 mAb: 3-54; and anti-module-4 mAb: 4-69) were kindly provided by

Nihon Nosan Corporation, as noted previously [10,11].

Osteoclast differentiation

Peripheral blood monocytes (PBMCs) obtained from healthy donors were isolated using

Ficoll-gradient centrifugation (Ficoll-Paque PLUS; GE Healthcare, Chafont St. Giles,

UK). PBMCs were purified into a $CD14^+$ population by using anti-CD14 MACS

microbeads (Miltenyi Biotec, Auburn, CA) according to the manufacturer's protocol. A

flow-cytometry analysis performed using a phycoerythrin-conjugated mouse anti-CD14

mAb (Miltenyi Biotec) showed that the purity of the $CD14^+$ monocytes was >98% in

each experiment. The purified $CD14^+$ monocytes (5×10^4 cells/well) were cultured in

96-well plates in Alpha Minimum Essential Medium (Invitrogen, Grand Island, NY)

supplemented with 10% fetal bovine serum (FBS), and then incubated with M-colony

stimulating factor (M-CSF) (25 ng/ml) and soluble receptor activator of NF-κB ligand (RANKL) (sRANKL; 40 ng/ml; Millipore, Billerica, MA) in addition to mAbs against CTGF modules (module 1, 15 μg/ml; module 2, 1 μg/ml; module 3, 2.5 μg/ml; module 4, 5 μg/ml). Next, bioactive recombinant CTGF (10 μg/ml) (Biovender Laboratory Medicine Inc., Brno, Czech Republic) was added to the cultures at 60 min, and 3 days later, the medium was replaced; after incubation for 7 days, the cells were stained for tartrate-resistant acid phosphatase (TRAP) expression by using a commercial kit (Cell Garage, Tokyo, Japan). Lastly, under a light microscope, the TRAP-positive multinucleated cells (MNCs) in 3 randomly selected fields (100× magnification) in each well were counted and the total number of TRAP-positive MNCs per well was considered to represent the total number of osteoclasts.

Tube-formation assay

Tube formation was analyzed using HUVECs cultured in the medium from the EGM-2 BulletKit set (Lonza), which contained 0.1% R3-IGF-1, 0.1% vascular endothelial growth factor, 0.4% human fibroblast growth factor-2, 0.1% human epidermal growth factor, 0.04% hydrocortisone, 0.1% ascorbic acid, 0.1% heparin, 0.1% GA-100, and 2% FBS; HUVECs from the third passage were used in the analysis. We placed 30 μl of phenol red-free Growth Factor-Reduced Matrigel Matrix (BD, Franklin Lakes, NJ) in

each well of 96-well plates, and then into each well we added recombinant CTGF (1 μg/ml) and anti-CTGF antibodies (module 1, 15 μg/ml; module 2, 1 μg/ml; module 3, 2.5 μg/ml; module 4, 5 μg/ml). After the Matrigel had solidified sufficiently at 37°C, we added 50 μl of the HUVEC culture (concentration adjusted to 4×10^5 cells/ml) to each well and incubated the plates overnight. The number of junctions in 3 fields in each well was determined under a light microscope, and the average number calculated from the 3 fields was used for comparisons.

Total RNA extraction and real-time RT-PCR

MH7A cells were stimulated with or without recombinant TNF-α (20 ng/ml; R&D Systems, Minneapolis, MN) and anti-CTGF antibodies (module 1, 15 μg/ml; module 2, 2.5 μg/ml; module 3, 2.5 μg/ml; module 4, 5 μg/ml) for an appropriate time (6 h) and then used in subsequent experiments. Total RNA was extracted from the MH7A cells by using the RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions, and cDNA strands were synthesized by using the PrimeScript RT reagent kit (Takara, Shiga, Japan) with 0.5 μg of total RNA. Real-time PT-PCR was performed using SYBR Premix Ex Taq (Perfect Real Time; Takara) to quantify CTGF, matrix metalloproteinase-3 (MMP3), interleukin (IL)-23a, IL-6, and IL-1b mRNAs. The following primers were designed for the human isoforms of the

molecules: CTGF: 5′-CTTGCGAAGCTGACCTGGAA-3′ (forward) and

5′-AGCTCAAACTTGATAGGCTTGGAGA-3′ (reverse); MMP3:

5′-CTGGGCCAGGGATTAATGGAG-3′ (forward) and

5′-CAATTTCATGAGCAGCAACGAGA-3′ (reverse); IL-23a:

5′-GCAGATTCCAAGCCTCAGTC-3′ (forward) and 5′-CCTTGAGCTGCTGCCTTTAG-3′

(reverse); IL-6: 5′- AAGCCAGAGCTGTGCAGATGAGTA-3′ (forward) and

5′-TGTCCTGCAGCCACTGGTTC-3′ (reverse); and IL-1b:

5′-TGAAGCCCTTGCTGTAGTGGTG-3′ (forward) and

5′-GCTGATGGCCCTAAACAGATGAA-3′ (reverse). Quantitative real-time RT-PCR was

performed in a 20-μl volume containing 500 ng of cDNA in SYBR Premix Ex Taq Kit

reaction mixture (Takara); per the manufacturer's instructions, the following

amplification steps were used: initial amplification at 95°C for 5 s, followed by 45

cycles of 95°C for 5 s and 60°C for 30 s, and a single final cycle of 95°C for 5 s, 60°C

for 30 s, and 95°C for 15 s. To quantify the expression of transcripts, sample loading

was monitored and the levels were normalized relative to the expression of β-actin

transcripts.

Statistical analysis

Experimental data were compared using an un-pared Student's *t* test; $P < 0.05$ was

considered statistically significant.

Results

Prevention of M-CSF/RANKL-mediated osteoclastogenesis by mAbs that block each module of CTGF

First, we tested how antibodies directed against the 4 modules of CTGF affect osteoclastogenesis to determine how inhibiting each module functionally influences RA-related bone destruction. As expected, M-CSF/RANKL-mediated osteoclastogenesis was enhanced when CTGF was added, but this enhancement effect was neutralized by mAbs against CTGF modules 1–4 (Figure 1A, 1C, 1E, and 1G).

Inhibition of angiogenesis by anti-CTGF module 1–4 mAbs

Next, to examine the inhibitory effect of antibodies against each CTGF module on angiogenic activity *in vivo*, we performed tube-formation assays. CTGF treatment of HUVECs induced the formation of tubular networks (Figure 2B-B, 2D-B, 2F-B, and 2H-B), which were quantified (Figure 2A, 2C, 2E, and 2G), and the formation of these networks was found to be disrupted by anti-CTGF module 1–4 mAbs (Figure 2A-C, 2C-C, 2E-C, and 2G-C).

Inhibition of CTGF and MMP3 production in synovial fibroblasts by antibodies against CTGF modules

We previously reported that CTGF was upregulated by TNF-α in a human synovial fibroblast cell line (MH7A). This finding indicates that excessive TNF-α-induced CTGF

production by synovial fibroblasts in patients with RA can promote aberrant activation of osteoclasts in combination with RANKL/M-CSF and result in bone destruction [7]. Moreover, aberrant CTGF expression leads to cartilage damage through an increase in the levels of the mRNA encoding degrading enzymes such as MMP3 [12]. Therefore, we lastly investigated whether the mAbs against each CTGF module suppress the production of CTGF and MMP3 in synovial fibroblasts; CTGF and MMP3 expression was measured in MH7A cells stimulated with recombinant TNF-α. The results of quantitative real-time PCR revealed that TNF-α enhanced the expression of CTGF and MMP3 in MH7A cells and that this enhancement was potently neutralized by mAbs against CTGF modules 1, 3, and 4 (Figure 3A and 3B).

We also investigated whether mAbs against the distinct CTGF modules suppressed the production of inflammatory cytokines (IL-1b, IL-6, IL-23a) by synovial fibroblasts. TNF-α enhanced the production of these inflammatory cytokines in MH7A cells, but this enhancement was not blocked by mAbs against CTGF modules (Figure 3C, 3D, and 3E).

Discussion

This study investigated the inhibitory effects of antibodies against the 4 modules of CTGF on the pathogenesis of RA. First, CTGF addition enhanced M-CSF/RANKL-mediated osteoclastogenesis, but this CTGF effect was neutralized by anti-CTGF module 1–4 mAbs (Figure 1). Second, CTGF induced the formation of tubular networks in HUVECs, which resulted in acceleration of the angiogenesis of RA synoviocytes, and this tubular-network formation was also disrupted by mAbs against CTGF modules 1–4 (Figure 2). Lastly, TNF-α enhanced the expression of CTGF and MMP3 in MH7A cells, and this enhancement was effectively neutralized by mAbs against CTGF modules 1, 3, and 4 (Figure 3). The pro-inflammatory cytokines (IL-1b, IL-6 and IL-23a) that act downstream of TNF-α were not affected by the mAbs for each CTGF module. These data suggest that CTGF and MMP-3 strongly participate in CTGF signaling.

In relation to module 1, the IGFBP-like module, in CTGF, a previous study by Fernihough and colleagues [13] revealed a strong correlation between C-reactive peptide (CRP) and serum IGFBP-3 levels in patients with RA. More recently, Neidel [14] examined 53 clinically active patients with RA and 51 control study participants and found that serum IGFBP-2 and IGFBP-3 levels were higher in patients with RA than in controls. Moreover, our immunohistochemical studies revealed that IGF-I and

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IGFBP-3 are produced by RA macrophages, and we reported that inhibition of the IGF-IGFBP signaling pathway could potentially exert beneficial effects in the treatment of RA [9].

Changes in inflammatory activity in patients with RA are among the most likely reasons for endothelial dysfunction, which has been shown to be related to diverse events such as oxidative stress-induced metabolic abnormalities, genetic predisposition, polymorphisms, and cardiovascular disorders [15]. Several studies have investigated vWF in patients with RA as a biomarker of endothelial activity and have examined the role of vWF in platelet adhesion. A previous study evaluating the relationship between endothelial-activity markers and inflammatory parameters in patients with RA indicated that inflammatory markers such as IL-8, CRP, RF, and vWF are correlated positively [16]. Moreover, Gurol *et al.* identified a positive correlation between vWF and disease activity in patients with RA [17]. Collectively, these findings suggest that inhibition of vWF could represent a therapeutic target for RA.

TSP-1 is a multifunctional glycoprotein expressed in cells derived from multiple lineages [18]. TSP-1 is frequently detected in the serum of patients with RA, but not in the serum of patients with other rheumatic diseases or in the serum of healthy people [19]. Suzuki *et al.* reported that TSP-1 expression in synovial tissues was considerably

higher in RA than in osteoarthritis, and further that expression of TSP-1 was evident in the lining and sublining layers of rheumatoid synovial tissues where active inflammation is detected, which suggests that TSP-1 might be involved in rheumatoid synovitis [20]. TSP-1 is recognized as an activating factor for the latent form of transforming growth factor (TGF)-β1. Suzuki *et al.* also found that production of TSP-1 in fibroblast-like synovial cells was increased following TGF-β1 stimulation, and the correlation observed between variations in plasma TSP-1 levels and variations in disease-activity score [28] indicated that TSP-1 may play a role in active RA disease and could be a novel biomarker of RA [20].

Regarding receptors, the signal transduction and function of each domain of CTGF is primarily mediated through interaction with cell adhesion receptors, including integrins and heparin sulfate proteoglycans. For instance, $\alpha_v\beta_3$, $\alpha_v\beta_5$, and $\alpha_{IIb}\beta_3$ integrin interact with the vWC domain, $\alpha_6\beta_1$ integrin interact with the TSP domain and $\alpha_v\beta_3$, $\alpha_6\beta_1$, $\alpha_5\beta_1$ integrin and HSPG interact with the CT domain. Moreover, the vWC domain also binds to bone morphogenetic protein and transforming growth factor β, and the TSP and CT domains bind vascular endothelial growth factor (VEGF). The angiogenetic activity is regulated through interactions with VEGF, and osteogenesis is regulated through interactions with integrin and HSPG [21]. Little is known about the signaling of CTGF.

Chen et al. showed that a neutralizing antibody against $\alpha_v\beta_3$ integrin significantly attenuated CTGF-mediated extracellular signal-regulated kinase (ERK) 1/2 activation and cellular migration in human breast cancer cells [22]. We also reported that CTGF could enhance osteoclastic function through activation of $\alpha_v\beta_3$ integrin signal transduction pathways with ERK 1/2 and focal adhesion kinase as downstream targets [7].

In conclusion, the results of our study indicate that not only antibodies against CTGF but also mAbs against each specific module of CTGF might serve as potential therapeutic agents in the treatment of RA.

Declaration of interest

References

1. Grotendorst, G. R. 1997. Connective tissue growth factor: a mediator of TGF-beta action on fibroblasts. *Cytokine Growth Factor Rev.* 8: 171–179.

2. Perbal, B. 2004. CCN proteins: multifunctional signalling regulators. *Lancet* 363: 62–64.

3. Takigawa, M., T. Nakanishi, S. Kubota, and T. Nishida. 2003. Role of CTGF/HCS24/ecogenin in skeletal growth control. *J. Cell. Physiol.* 194: 256–266.

4. Feldmann, M., F. M. Brennan, and R. N. Maini. 1996. Rheumatoid arthritis. *Cell* 85: 307–310.

5. Itoh, Y., H. Hayashi, K. Miyazawa, S. Kojima, T. Akahoshi, and K. Onozaki. 2007. 17β-estradiol induces *IL-1α* gene expression in rheumatoid fibroblast-like synovial cells through estrogen receptor α (ERα) and augmentation of transcriptional activity of Sp1 by dissociating histone deacetylase 2 from ERα. *J. Immunol.* 178: 3059–3066.

6. Paleolog, E. M. 2009. The vasculature in rheumatoid arthritis: cause or consequence? *Int. J. Exp. Pathol.* 90: 249–261.

7. Nozawa, K., M. Fujishiro, M. Kawasaki, H. Kaneko, K. Iwabuchi, M. Yanagida,

F. Suzuki, K. Miyazawa, Y. Takasaki, H. Ogawa, K. Takamori, and I. Sekigawa. 2009. Connective tissue growth factor promotes articular damage by increased osteoclastogenesis in patients with rheumatoid arthritis. *Arthritis Res. Ther.* 11: R174.

8.   Nozawa, K., M. Fujishiro, Y. Takasaki, and I. Sekigawa. 2014. Inhibition of rheumatoid arthritis by blocking connective tissue growth factor. *World J. Orthop.* 5: 653–659.

9.   Suzuki, S., S. Morimoto, M. Fujishiro, M. Kawasaki, K. Hayakawa, T. Miyashita, K. Ikeda, K. Miyazawa, M. Yanagida, K. Takamori, H. Ogawa, I. Sekigawa, and Y. Takasaki. 2014. Inhibition of the insulin-like growth factor system is a potential therapy for rheumatoid arthritis. *Autoimmunity* 48: 251–258.

10.   Chujo, S., F. Shirasaki, M. Kondo-Miyazaki, Y. Ikawa, and K. Takehara. 2009. Role of connective tissue growth factor and its interaction with basic fibroblast growth factor and macrophage chemoattractant protein-1 in skin fibrosis. *J. Cell. Physiol.* 220: 189–195.

11.   Ikawa, Y., P. S. Ng, K. Endo, M. Kondo, S. Chujo, W. Ishida, F. Shirasaki, M. Fujimoto, and K. Takehara. 2008. Neutralizing monoclonal antibody to human

connective tissue growth factor ameliorates transforming growth factor-β-induced mouse fibrosis. *J. Cell. Physiol.* 216: 680–687.

12.   Blaney Davidson, E. N., E. L. Vitters, F. M. Mooren, N. Oliver, W. B. Berg, and P. M. van der Kraan. 2006. Connective tissue growth factor/CCN2 overexpression in mouse synovial lining results in transient fibrosis and cartilage damage. *Arthritis Rheum.* 54: 1653–1661.

13.   Fernihough, J. K., M. E. Billingham, S. Cwyfan-Hughes, and J. M. Holly. 1996. Local disruption of the insulin-like growth factor system in the arthritic joint. *Arthritis Rheum.* 39: 1556–1565.

14.   Neidel, J. 2001. Changes in systemic levels of insulin-like growth factors and their binding proteins in patients with rheumatoid arthritis. *Clin. Exp. Rheumat.* 19: 81–84.

15.   Klimek, E., A. Skalska, B. Kwasny-Krochin, A. Surdacki, J. Sulicka, M. Korkosz, D. Fedak, I. Kierzkowska, B. Wizner, and T. K. Grodzicki. 2014. Differential associations of inflammatory and endothelial biomarkers with disease activity in rheumatoid arthritis of short duration. *Mediators Inflamm.* 2014: 681635.

16.   Foster, W., G. Y. Lip, K. Raza, D. Carruthers, and A. D. Blann. 2012. An

observational study of endothelial function in early arthritis. *Eur. J. Clin. Invest.* 42: 510–516.

17. Gurol, G., I. H. Ciftci, H. Harman, E. Karakece, A. Kamanli, and I. Tekeoglu. 2015. Roles of claudin-5 and von Willebrand factor in patients with rheumatoid arthritis. *Int. J. Clin. Exp. Pathol.* 8: 1979–1984.

18. Lawler, J. 1986. The structural and functional properties of thrombospondin. *Blood* 67: 1197–1209.

19. Ohyama, K., A. Kawakami, M. Tamai, M. Baba, N. Kishikawa, and N. Kuroda. 2012. Serum immune complex containing thrombospondin-1: a novel biomarker for early rheumatoid arthritis. *Ann. Rheum. Dis.* 71: 1916–1917.

20. Suzuki, T., N. Iwamoto, S. Yamasaki, A. Nishino, Y. Nakashima, Y. Horai, S. Y. Kawashiri, K. Ichinose, K. Arima, M. Tamai, H. Nakamura, T. Origuchi, C. Miyamoto, M. Osaki, K. Ohyama, N. Kuroda, and A. Kawakami. 2015. Upregulation of thrombospondin 1 expression in synovial tissues and plasma of rheumatoid arthritis: role of transforming growth factor-β1 toward fibroblast-like synovial cells. *J. Rheumatol.* 42: 943–947.

21. Chen, CC, and Lau LF. 2009. Functions and mechanisms of action of CCN matricellular proteins. *Int. J. Biochem Cell Biol.* 41: 771-783.

22. Chen, PS. WANG, MY. Wu, SN. Su, JL. Hong, CC. Chuang, SE. Chen, MW. Hua, KT. Wu, YL. Cha, ST. Babu, MS. Chen, CN. Lee, PH. Chang, KJ and Kuo, ML. CTF enhances the motility of breast cancer cells via an integrin-alphavbeta3-ERK1/2-dependent S100A4-upregulated pathway. 2007. *J. Cell Sci.* 120: 2053-2065.

Figure legends

Figure 1. Synergetic effect of CTGF on M-CSF/sRANKL-mediated osteoclastogenesis and inhibition of this effect by anti-CTGF module 1–4 mAbs. Numbers of TRAP-positive cells (A, C, E, and G) and images of TRAP staining (B, D, F, and H). CTGF increased the number and the size of differentiated osteoclasts when added in combination with M-CSF/sRANKL. This effect was neutralized following the addition of mAbs against each module of CTGF. Bars = SDs.

Figure 2. Each CTGF-module mAb inhibits angiogenesis in *in vitro* models. HUVECs were plated on Matrigel and the effects of treatment with recombinant CTGF and mAbs against each CTGF module on tube formation were analyzed (A, C, E, and G). Representative tubular-network photomicrographs are shown in panels B, D, F, and H. Bars = SDs.

Figure 3. Effect of TNF-α on the production of CTGF (A), MMP3 (B), and inflammatory cytokines (C: IL-23a; D: IL-6; and E: IL-1b) in synovial fibroblasts. Expression of CTGF, MMP3, and inflammatory cytokines in the human synovial fibroblast cell line MH7A stimulated with or without TNF-α was evaluated using
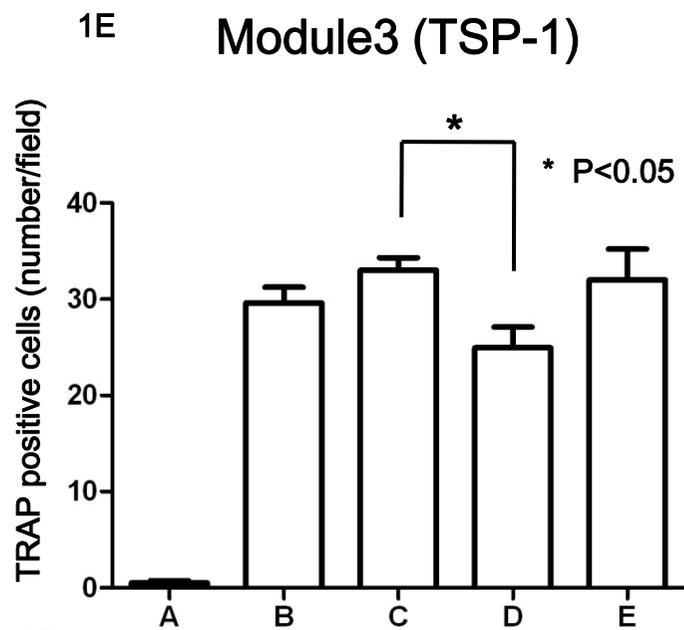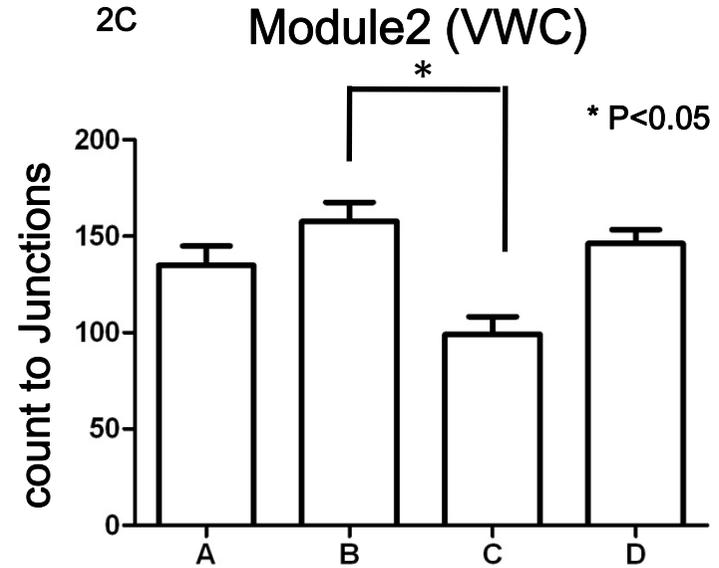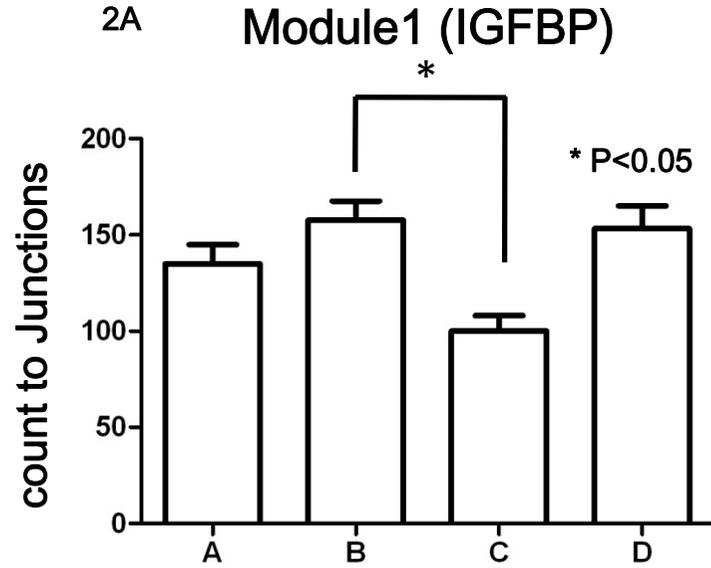
quantitative real-time PCR.

1A Module1 (IGFBP)

* P<0.05
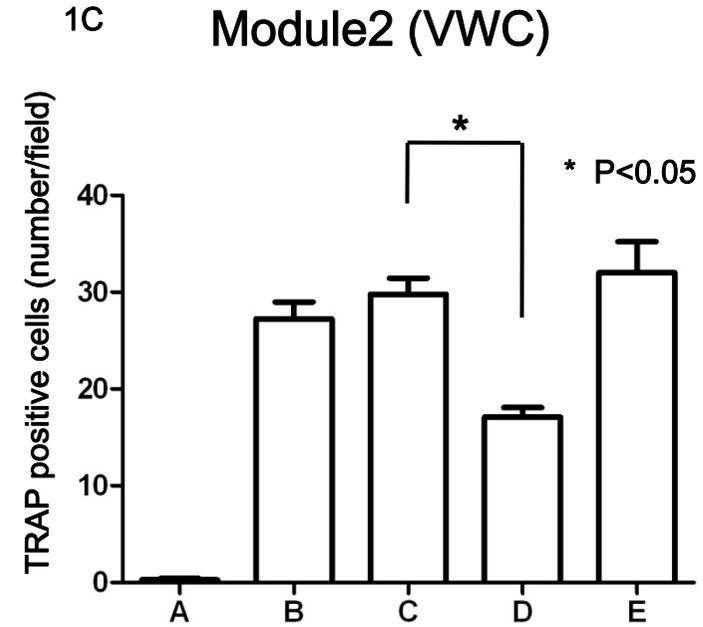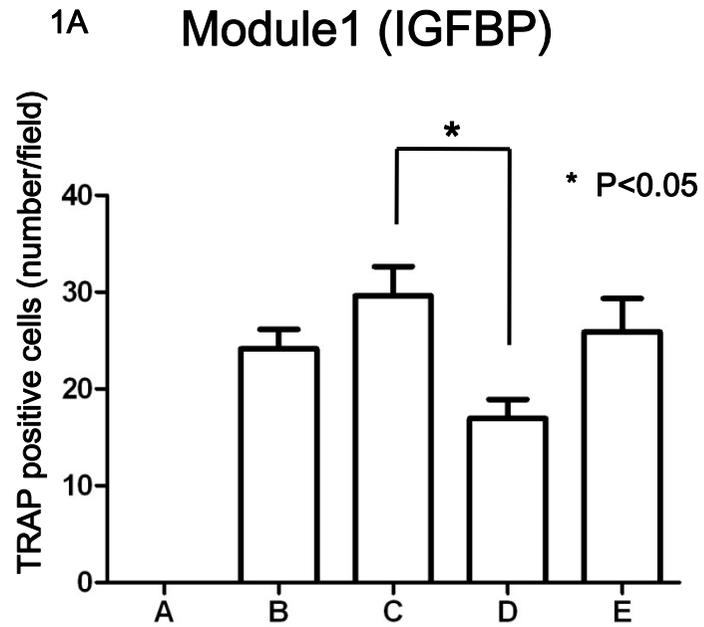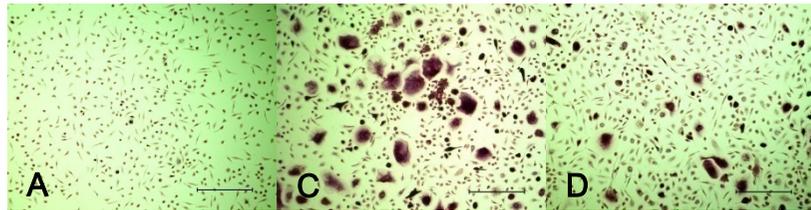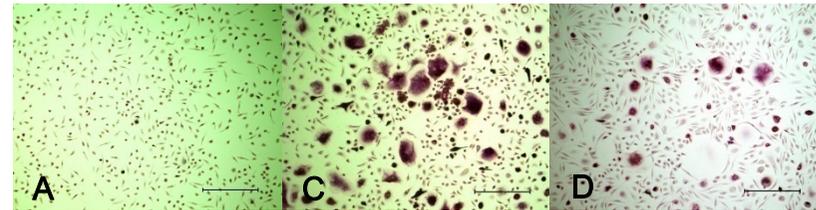
A: M-CSF 25 ng/ml
B: M-CSF 25 ng/ml + RANKL 40 ng/ml
C: B + rCTGF 10 ng/ml
D: C + anti-module1 Ab (15 μg/ml)
E: B + mouse IgG (15 μg/ml)

1C Module2 (VWC)

* P<0.05

A: M-CSF 25 ng/ml
B: M-CSF 25 ng/ml + RANKL 40 ng/ml
C: B + rCTGF 10ng/ml
D: C + anti-module2 Ab (1 μg/ml)
E: B + mouse IgG (1 μg/ml)

1E Module3 (TSP-1)
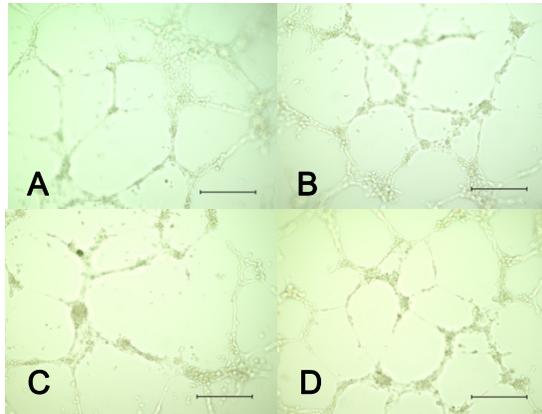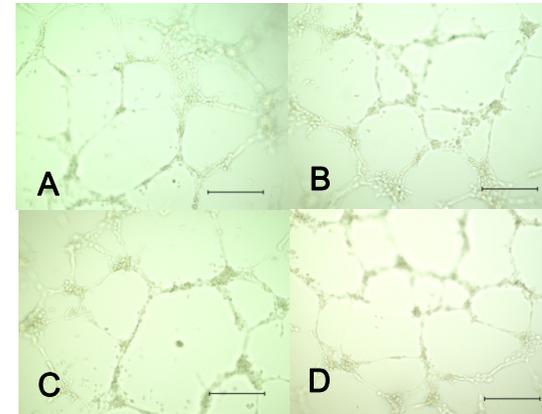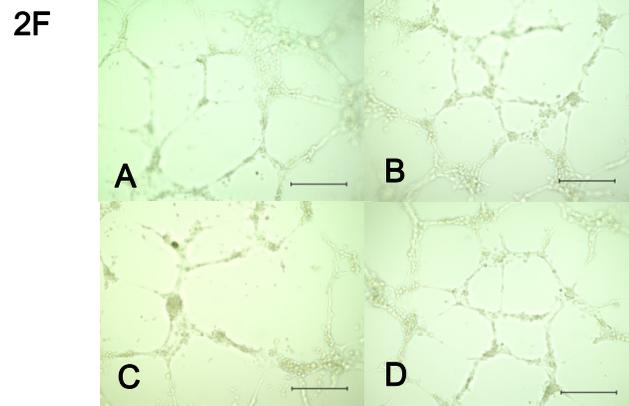
1G Module4 (C-terminal)

* P<0.05

1F

1H
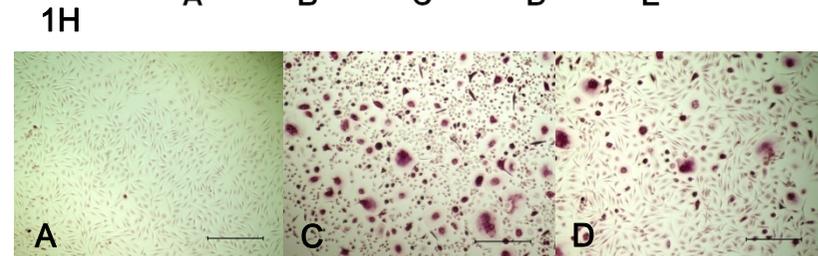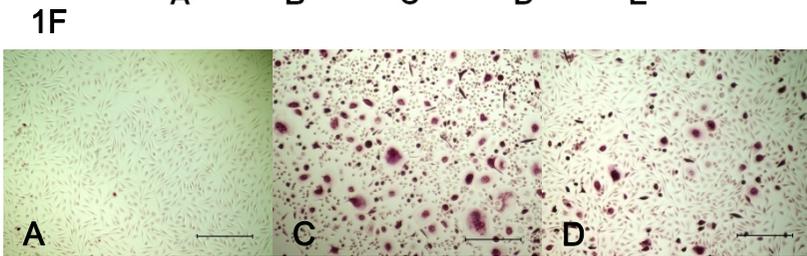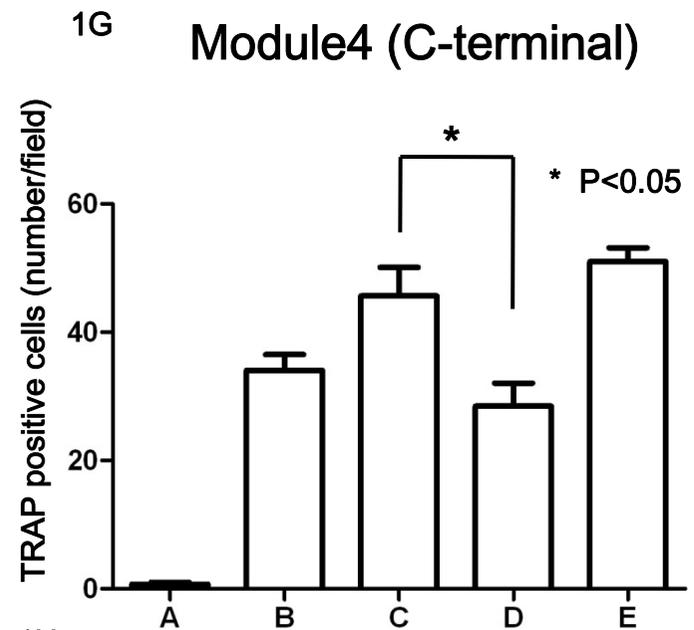
A: M-CSF 25 ng/ml
B: M-CSF 25 ng/ml + RANKL 40 ng/ml
C: B + rCTGF 10 ng/ml
D: C + anti-module3 Ab (2.5 μg/ml)
E: B + mouse IgG (2.5 μg/ml)

A: M-CSF 25 ng/ml
B: M-CSF 25 ng/ml + RANKL 40 ng/ml
C: B + rCTGF 10 ng/ml
D: C + anti-module4 Ab (5 μg/ml)
E: B + mouse IgG (5 μg/ml)

2A

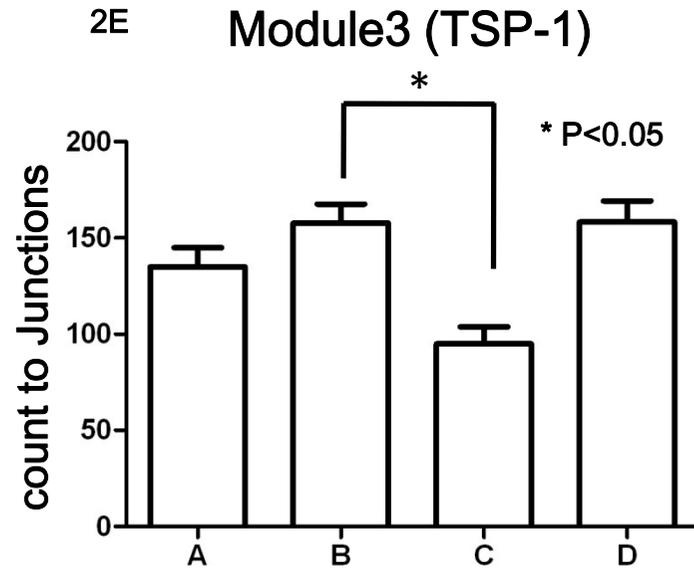## Module1 (IGFBP)

* P<0.05

count to Junctions

A   B   C   D

2B

A: control
B: rCTGF (1 μg/ml)
C: B + anti-module1 Ab (15 ng/ml)
D: B + mouse IgG (15 ng/ml)

2C

Module2 (VWC)

* P<0.05

count to Junctions

A   B   C   D

2D

A: control
B: rCTGF (1 μg/ml)
C: B + anti-module2 Ab (1 ng/ml)
D: B + mouse IgG (l ng/ml)

2E Module3 (TSP-1)

* P<0.05

count to Junctions
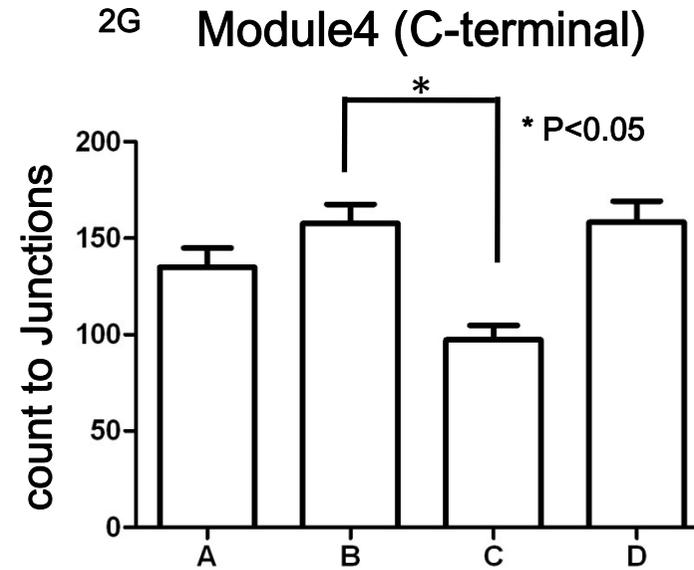
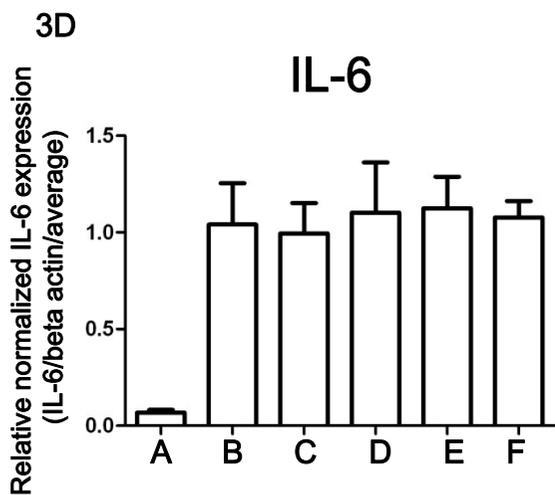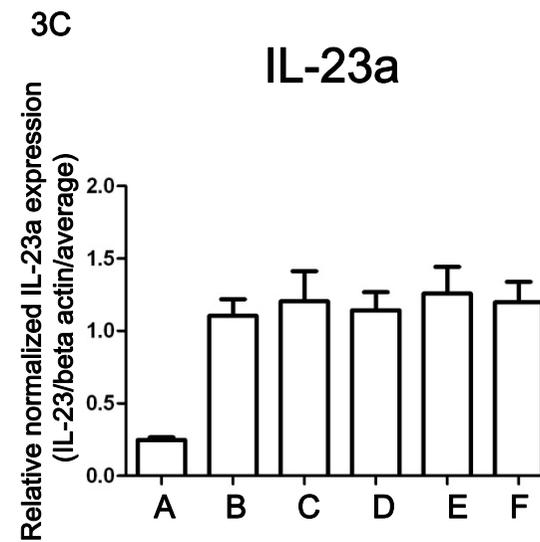A: control
B: rCTGF (1 μg/ml)
C: B + anti-module3 Ab (2.5 ng/ml)
D: B + mouse IgG (2.5 ng/ml)

2G Module4 (C-terminal)

* P<0.05

count to Junctions
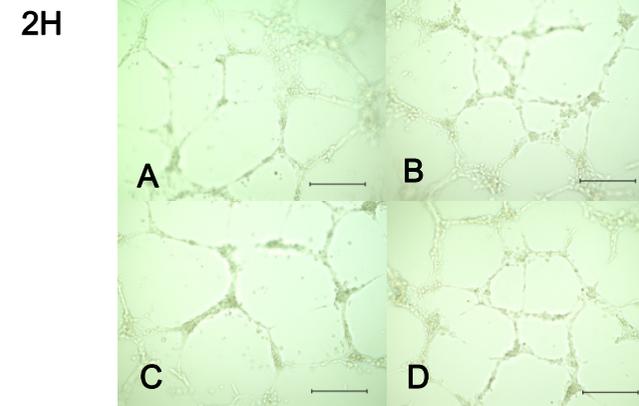
A: control
B: rCTGF (1 μg/ml)
C: B + anti-moudle4 Ab (2.5 ng/ml)
D: B + mouse IgG (2.5 ng/ml)

3A

CTGF

Relative normalized CTGF expression (CTGF/beta actin/average)

A   B   C   D   E   F

3B

MMP3

Relative normalized MMP3 expression (MMP3/beta actin/average)

A   B   C   D   E   F

3C

IL-23a

Relative normalized IL-23a expression (IL-23/beta actin/average)

A   B   C   D   E   F

3D

IL-6

Relative normalized IL-6 expression (IL-6/beta actin/average)

A   B   C   D   E   F

3E

IL-1b

Relative normalized IL-1b expression (IL-1b/beta actin/average)

A   B   C   D   E   F

* $P < 0.05$

A; control
B; TNF-α (20 ng/ml)
C; TNF-α (20 ng/ml) + anti-module1 Ab (15 μg/ml)
D; TNF-α (20 ng/ml) + anti-module2 Ab (2.5 μg/ml)
E; TNF-α (20 ng/ml) + anti-module3 Ab (2.5 μg/ml)
F; TNF-α (20 ng/ml) + anti-module4 Ab (5 μg/ml)