Transcription factor 21 (Tcf21) promotes proinflammatory interleukin 6 expression and extracellular matrix remodeling in visceral adipose stem cells

Takeshi Akama1,2 and Tae-Hwa Chun1,2

1. Department of Internal Medicine, Division of Metabolism, Endocrinology & Diabetes, University of Michigan Medical School, Ann Arbor, MI 48109-2800
2. Biointerfaces Institute, University of Michigan, Ann Arbor, MI 48109-2800

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To whom correspondence: Tae-Hwa Chun, MD, PhD, 2800 Plymouth Rd, Ann Arbor, MI 48109; Tel: 734-330-0562; E-mail: taehwa@umich.edu

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ABSTRACT

The visceral (VIS) and subcutaneous (SQ) fat pads are developmentally distinct white adipose tissue depots and contribute differently to inflammation and insulin resistance associated with obesity. The basic helix-loop-helix transcriptional regulator transcription factor 21 (TCF21) is a marker gene for white adipose tissues and is abundantly expressed in VIS-derived adipose stem cells (ASCs), but not in SQ-derived ASCs. However, TCF21’s role in regulating fat depot-specific gene expression and function is incompletely understood. Here, using siRNA-mediated Tcf21 knockdowns and lentiviral gene transfer of TCF21 in mouse ASCs and, we demonstrate that TCF21 is required for the VIS ASC-specific expression of interleukin 6 (IL6), a key cytokine that contributes to the proinflammatory nature of VIS depots. Concurrently, TCF21 promotes MMP-dependent collagen degradation and type IV collagen deposition through the regulation of the extracellular matrix (ECM) modifiers, matrix metalloproteinase-2 (MMP2), MMP13, and tissue inhibitor of MMP 1 (TIMP1) as well as collagen type IV alpha 1 chain (COL4A1) in VIS ASCs. We also found that although IL6 mediates the expression of Mmp13 and Timp1 in VIS ASCs, the TCF21-dependent expression of Mmp2 and Col4a1 is IL6–independent. These results suggest that TCF21 contributes to the proinflammatory environment in VIS fat depots and to active ECM remodeling of these depots by regulating IL-6 expression and MMP-dependent ECM remodeling in a spatiotemporally coordinated manner.

The visceral (VIS) and subcutaneous (SQ) fat pads are two distinct white adipose tissue (WAT) depots that develop and function differently (1,2). In obesity, VIS WAT is prone to inflammatory tissue damage relative to SQ WAT as exemplified by increased macrophage infiltration and extracellular matrix (ECM) remodeling (3). Among a cohort of adipokines secreted from expanding adipose tissues, the expression of interleukin-6 (IL6) from VIS WAT is associated with chronic inflammation in obesity (4). Moreover, the level of adipose IL6 expression is correlated with the degree of insulin resistance in humans (5,6), suggesting a potential role for VIS WAT-derived IL6 in adipose tissue inflammation, and insulin resistance associated with visceral adiposity.

Adipose tissue ECM remodeling is another key pathological process observed in obesity (7,8). Transcriptomic profiling of VIS and SQ WAT-derived stem cell antigen-1 (Sca1)-high adipose-derived stem cells (ASCs), demonstrate the differential expression of inflammatory cytokines,
matrix metalloproteinases (MMPs) and tissue inhibitor of MMPs (TIMPs) (9). VIS ASCs display increased expression of MMP8 and MMP13 as well as TIMP1, all of which are highly induced under inflammatory and obeseogenic conditions (10-13). Elevated VIS ASC expression of MMP8 and -13 was associated with the expression of proinflammatory cytokines, including IL6 and CXCL1 (9). Despite these observations, the transcriptional regulation underlying VIS ASC-specific expression of inflammatory cytokines and MMP/TIMP family members has not been well established.

To define the transcriptional network of VIs ASCs responsible for the co-expression of IL6 and a subset of MMPs and TIMPs, we focused on transcription factor 21 (TCF21), a basic helix-loop-helix (bHLH) transcription factor abundantly expressed in VIS ASCs but barely in SQ ASCs. A human TCF21 gene variant is associated with larger pericardial fat mass (14), and TCF21 promotes epithelial-mesenchymal transition (EMT) of epicardial cells to cardiac fibroblasts (15). Gene targeting of Tcf21 results in perinatal lethality of mice, demonstrating its indispensable role in organ development (16,17).

Even though TCF21 has been recognized as a WAT-specific marker gene (18), its potential role in regulating VIS WAT-specific inflammatory gene expression and ECM remodeling has not been fully explored. Here, we identify a novel role for TCF21 in driving the expression of IL6 as well as a subset of MMP/TIMP family members (MMP2, MMP13, and TIMP1) and type IV collagen in IL6-dependent and -independent manners. TCF21-dependent regulation of IL6 expression and ECM remodeling may contribute to the unique characteristics of visceral WAT.

RESULTS

**TCF21 is co-expressed with IL6 and specific ECM remodeling factors in visceral adipose stem cells**

Our previous work showed the depot-specific transcriptomes of mouse WAT Sca1high ASCs using whole-genome RNA sequencing (RNA-seq) (9). The gene encoding basic helix-loop-helix transcription factor Tcf21 was among the most differentially expressed; its transcript present at high level in visceral (VIS) ASCs, but nearly undetectable in subcutaneous (SQ) ASCs (Fig 1A). TCF21 is an established regulator of epicardial fibroblast EMT and development of kidney, lung and spleen (16,17); however, its function in WAT is presently undefined.

The mRNA levels of Il6 and Timp1 were also preferentially expressed in VIS ASCs, while those of Mmp2 and Mmp14 were similar between ASCs of both depots (Fig 1A). Notably, Mmp13 transcript level in VIS ASCs was more than double the level observed in SQ ASCs (Fig. 1A).

Real-time qPCR confirmed our RNA-seq findings, showing VIS ASC expression of TCF21 180-fold, and expression of IL6 50-fold that seen in SQ ASCs (Fig. 1B). The mRNA analysis of human preadipocytes derived from VIS and SQ WAT revealed a similar pattern (Fig. 1C). In agreement with gene expression, immunocytochemistry of cultured ASCs revealed nuclear TCF21 staining in VIS ASCs, but not in SQ ASCs (Fig. 1D). IL6 staining was similarly restricted to VIS ASCs and showed substantial co-expression with TCF21 among VIS WAT-derived stromal cells (Fig. 1E). Indeed, IL6 was detected in 89% of TCF21-positive, but only in 34% of TCF21-negative VIS WAT-derived vascular stromal cells (Fig. 1E, table).

Within mouse VIS (peri-gonadal) adipose depot, TCF21 protein was seen exclusively in cells positive for PDGFRα, a cell-surface marker of fibroblast-adipocyte progenitors (Fig. 1F) (19, 20). On the contrary, TCF21 was undetectable in SQ (inguinal) depots isolated from the same animals (Fig. 1F). TCF21 and IL6 were expressed in the same cells specifically in VIS fat depot (Fig. 1F). Taken together, these data show that, in WAT, TCF21 is restricted to ASCs of VIS depot and mostly co-expressed with IL6 in these cells. Genes encoding specific ECM remodeling factors, MMP13 and TIMP1, were also preferentially expressed in VIS ASCs relative to SQ ASCs.

**TCF21 is necessary for IL6 expression in VIS ASCs**

We hypothesized that TCF21 could transcriptionally regulate genes expressed in VIS ASCs, including IL6. To test this, we examined the effect of siRNA-mediated TCF21 knockdown on IL6 transcription and protein expression in isolated mouse VIS ASCs. Two independent siRNA
oligonucleotides (designed within Tcf21 coding regions) suppressed mRNA levels by 40% and 75%, respectively (Fig. 2A). Correspondingly Il6 transcript levels declined to 36% and 46% of baseline (Fig. 2B). We next evaluated the impact of Tcf21 knockdown on levels of IL6 protein in the culture medium. Western blotting revealed progressive accumulation of IL6 protein over a 3-day culture period in control siRNA-treated group, and time-dependent accumulation of IL6 was comparable to that of type I collagen (Fig. 2C). IL6 protein content was decreased in the media of VIS ASCs treated with Tcf21 siRNAs at each time point, whereas no significant effect was observed on type I collagen (Fig. 2C). IL6 protein was nearly undetectable in the medium of cultured SQ ASCs (Fig. 2C). Together, these data demonstrate that Tcf21 is necessary for IL6 expression specifically in mouse VIS ASCs and the minimal expression of Tcf21 accounts for the very low IL6 expression in SQ ASCs. Conversely, when we overexpressed human TCF21 in mouse VIS ASCs using lentiviral gene transfer, we observed that IL6 expression increased in parallel with TCF21 expression (Fig. 2D).

We identified multiple E-box consensus sequences (CANNTG) in IL6 promoter region (Fig. 2E). We sought to determine whether TCF21 could regulate IL6 promoter activity. We examined the effect of TCF21 overexpression (Fig. S1) on a reconstituted IL6 promoter activity, using its 5’ untranslated region (approximately ~1.5 kb), in Chinese hamster ovary (CHO) cells, which express no endogenous TCF21 (data not shown). IL6 promoter activity in CHO cells was increased 10-fold by co-expressed TCF21 (Fig. 2F). Deletion of a putative bHLH domain (aa 78 – 132) from TCF21 (21) significantly attenuated TCF21-dependent IL6 promoter activity (Fig. 2F). These results suggest that TCF21 acts as a transcriptional regulator of IL6 promoter.

**TCF21 is required for the expression of collagenolytic MMPs and TIMP1 in VIS ASCs**

RNA-seq identified unique expression profiles of ECM remodeling factors (MMPs and TIMPs) in mouse VIS and SQ ASCs (9). Among collagenolytic MMPs, Mmp13 was highly expressed in VIS ASCs, whereas transcripts encoding gelatinolytic MMP2 and MMP9 were more abundant in SQ ASCs. Tcf21 knockdown by siRNA in VIS ASCs markedly suppressed MMP13 mRNA (Fig. 3A). On the contrary, expression of the gene encoding MMP14 (MT1-MMP), a major pericellular collagenase equally expressed in VIS and SQ ASCs (Fig 1A), was unaffected by Tcf21 siRNA (Fig. 3A). Expression of tissue inhibitor of matrix metalloproteinase-1 (Timp1), abundant in VIS ASCs but not in SQ ACSs, was suppressed by Tcf21 knockdown (Fig. 3A). By contrast, Tcf21 siRNA treatment did not change the expression of Timp2 or Timp3, which showed no predominance in VIS ASC over SQ ASC in RNA-seq data (Fig. 3A). VIS ASC-specific expression of Mmp8 was not impacted by Tcf21 knockdown (Fig. 3A). Unexpectedly, Tcf21 was found to be required also for the expression of Mmp2 in VIS ASCs and to a lesser extent in SQ ASCs (Fig. 3A and supplemental Fig. S2). Consistent with the effect on Mmp2 expression, Tcf21 siRNA reduced MMP2 protein level as detected by gelatin zymography (Fig. 3B).

**TCF21 is responsible for type IV collagen deposition by VIS ASCs**

In cultured VIS ASCs, Tcf21 knockdown specifically reduced the expression of Col4a1, but had no impact on Col1a1 or Col3a1 (Fig. 4A). This suggests a specific role for Tcf21 in regulation of basement membrane structure. Type IV collagen deposition by VIS ASCs detected by immunohistochemistry was not much changed with Tcf21 knockdown (Fig. 4B). Nonetheless, while type IV collagen fibers appeared robust and well organized in control siRNA-treated samples, they appear diffuse and finer in Tcf21 siRNA-treated samples (Fig. 4B, high magnification). The Tcf21 knockdown also reduced the amount of degraded type I collagen products (Fig. 4C), suggesting that TCF21 mediates both degradation of type I collagen fibers and the deposition of a basement membrane component, type IV collagen. TCF21-dependent degradation of type I collagen was MMP-dependent as shown by the suppression of the collagen degradation by a MMP inhibitor, GM6001 (Fig. 4D). We next assessed the impact of TCF21 overexpression on ASC collagen deposition. Lentiviral gene transfer of TCF21 resulted in enhanced TCF21 signals in VISC ASCs and novel presence of TCF21 in SQ ASCs (supplemental Fig. 3A).
Elevated TCF21 levels markedly enhanced the deposition of type IV and I collagens in VIS ASCs (Fig. 4E). SQ ASCs, which demonstrate a low staining of type IV collagen at baseline, also showed considerably increased deposition of both type I and IV collagens with TCF21 overexpression (Fig. 4E). The effect of TCF21 was collagen type-selective and no discernable effect was observed on the level of type VI collagen (Fig. 4E). TCF21-dependent accumulation of type IV collagen was pronounced more in VIS ASCs than in SQ ASCs (Fig. 4E). Not only the deposition of collagens, TCF21 overexpression augmented the degradation of type I collagen by VIS ASCs (Fig. 4F). Together, these findings suggest that TCF21 actively engages in type I collagen turnover and type IV collagen deposition by VIS ASCs through the regulation of MMP activities.

**IL6 is responsible for the expression of MMP13 and TIMP1 but not for collagen IV and MMP2 and ECM remodeling**

Given positive regulation of IL6 by TCF21, we sought to determine whether TCF21-dependent ECM remodeling requires IL6 effector function. To test this, we incubated VIS and SQ ASCs with IL6 neutralizing antibody with and without recombinant IL6. We found that mRNA levels of *Mmp13* and *Timp1* were reduced in the presence of anti-IL6, and that the effect was rescued by the presence of excess recombinant IL6 (Fig. 5A). On the contrary, expression of *Col4a1* and *Mmp2* were unaffected by either IL6 neutralizing antibody or IL6 protein (Fig. 5A). To determine whether TCF21 regulates type IV collagen deposition and type I collagen degradation through IL6 and its potential effectors, i.e., MMP13 and TIMP1, VIS ASCs transfected with siTcf21 or control siRNA were cultured in the presence and absence of IL6 neutralizing antibody. Despite the inhibitory effects on the expression of MMP13 and TIMP1, the presence of IL6 neutralizing antibody did not change either type IV collagen deposition or type I collagen degradation (Fig. 5B), suggesting that TCF21 promotes collagenolytic ECM remodeling in a IL6-independent manner.

**Discussion**

In this report, we have demonstrated for the first time that TCF21, a bHLH transcription factor, drives the expression of IL6 concurrently of MMP2 and type IV collagen in VIS ASCs. TCF21 has been known to regulate the EMT of epicardial fibroblast progenitors (15) and to be essential for the development of the heart, kidney, and spleen (17). While TCF21 was recognized as a gene highly expressed in white adipose tissues (18), its role in adipose tissue biology, particularly in WAT depot-dependent gene expression, had been unknown.

While WAT actively expands in response to excess calorie intake, the cellular mechanisms underlying the expansion of two WAT depots, i.e., visceral and subcutaneous WAT, appear different (22,23). Visceral WAT expansion involves not only hypertrophy of adipocytes but ASC proliferation and adipogenesis, particularly in male mice (22,23). Our previous work suggests that a cohort of transcription factors along with cytokines and ECM remodeling genes are differentially expressed between VIS and SQ ASCs (9). Jeffery E et al. suggest that visceral WAT provides a microenvironment permissive for ASC proliferation and adipogenesis under a HFD condition (23). Nonetheless, it is unclear whether the pro-adipogenic microenvironment of VIS WAT is conferred by paracrine cytokines, ECM molecules, or physical interaction between stromal cells and other cell types. Our data suggest that TCF21, which is specifically expressed in VIS ASCs, is responsible for the expression of IL6 along with ECM remodeling genes in VIS ASCs. It is likely that the heightened expression of IL6 coupled with type IV collagen deposition and type I collagen degradation contributes to a unique VIS WAT microenvironment permissive for inflammatory adipose tissue expansion *in vivo* (22,23).

IL6 is expressed most abundantly in adipose tissues, followed by the heart, kidney, and spleen (24). In parallel, TCF21 expression is found not only in adipose tissues, but in the heart, kidney, and spleen. TCF21 plays an indispensable role for the development of these organs (15-17). Our study shows the role of TCF21 in regulating IL6 expression and ECM remodeling in white adipose tissues; however, it is conceivable that TCF21 may play a similar role in regulating IL6 expression and ECM remodeling in the vital organs, such as the heart and kidney. The potential role for TCF21 in...
defining pro-inflammatory environment and ECM remodeling may need to be further investigated in these organs.

The role of IL6 in obesity has been somewhat controversial. While visceral WAT-derived IL6 is correlated with the presence of insulin resistance (4), skeletal muscles respond to IL6 favorably by improving whole-body glucose metabolism (25). In mice, genetic loss of IL6 paradoxically increases the risk of age-dependent obesity (26), insulin resistance, and steatohepatitis (27). Our findings suggest that a bHLH transcriptional factor, TCF21, is essential for IL6 expression in VIS ASCs. The expression of TCF21 and IL6 in visceral WAT did not change during a short-term high fat diet challenge in mice (data not shown); however, macrophages infiltrating WAT in chronic obesity express high levels of IL6 in the late stage of HFD-induced obesity (28). Despite these findings, the specific contribution of stromal versus macrophage-derived IL6 to insulin resistance in the early and late stages of obesity remains unclear. Our study has not addressed whether TCF21-dependent expression of stromal IL6 expression is metabolically beneficial or detrimental for glucose metabolism in obesity. Nonetheless, as TCF21 and IL6 are expressed in VIS WAT under both physiological and obesogenic conditions, it is likely that TCF21-dependent IL6 expression and ECM remodeling may play an active role in defining visceral WAT function in both development and obesity.

Experimental procedures

Animals

C57BL/6J and 129SvEv male mice of age 8–12-week-old were purchased from the Jackson laboratory (Bar Harbor, ME) and Taconic (Rensselaer, NY), respectively. Animals were housed in a pathogen-free environment with 12-hour light-dark cycle and free access to food and water. Animal studies and procedures were approved by the University of Michigan Institutional Animal Care and Use Committee.

Isolation of Sca1<sup>high</sup> ASCs

Mouse primary vascular stromal (VS) cells were isolated as described previously (9). VIS and SQ WATs were isolated, minced, and digested by 5 mg/ml type 3 collagenase (Worthington Biochemical Corp., Freehold, NJ) in Hank’s Balanced Salt Solution (HBSS) with calcium and magnesium (Invitrogen/Thermo Fisher Scientific, Pittsburgh, PA) at 37 °C for 20 minutes. DMEM containing 10% FBS was added to the cell suspension to inactivate collagenase, then cells were passed through a 100 μm cell strainer. After centrifugation for 10 minutes at 1,500 rpm (412.5 xg), the pellet was re-suspended in 5 ml distilled water for 30 seconds to lyse red blood cells. After adding growth medium (DMEM containing 10% FBS, 20 mM glucose, 100 U/ml penicillin, 100 U/ml streptomycin, and 250 ng/ml amphotericin B), the cell suspension was passed through a 100 μm cell strainer again and centrifuged for 10 minutes at 1,500 rpm and seeded onto a culture plate. Cultured primary cells were applied to MACS separator (Miltenyi Biotec, Auburn, CA) to enrich for Sca1<sup>high</sup> ASCs as previously reported (9,29).

Cell culture

Isolated mouse ASCs and human primary preadipocytes from VIS and SQ WAT (Lonza, Fair Lawn, NJ) were cultured in DMEM containing 10% FBS.

Immunostaining

Cultured cells were fixed in 4% paraformaldehyde (PFA)/PBS and permeabilized with 0.2% Triton X-100 in PBS for 20 minutes. To stain extracellular collagen, this permeabilization was skipped and detergents were excluded during all subsequent steps. Adipose tissue was fixed in 4% PFA/PBS overnight and cut into small pieces (< 2 mm square). After permeabilization in 0.5% Triton X-100 in PBS for three hours, tissue chunks were washed three times with 0.1% Triton X-100 in PBS (PBST) and blocked with 10% nonimmune goat serum in PBS for one hour. Rabbit anti-TCF21 antibody (#32981, 1:100, Abcam, Cambridge, MA), rat anti-IL6 antibody (#MAB406, 1:100, R&D Systems, Minneapolis, MN), mouse anti-PDGFα antibody (#sc-398206, 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-collagen IV antibody (#600-401-106, 1:100, Rockland Immunochemicals Inc., Limerick, PA), rabbit anti-
collagen I (#600-401-103, 1:200, Rockland Immunochemicals Inc.), rabbit anti-collagen VI (#600-401-108, 1:100, Rockland Immunochemicals Inc.), and C1,2C (staining degraded collagen) (#50-1035, 1:200, IBEX Pharmaceuticals Inc, Montreal, Canada) were used for immunostaining. After a subsequent wash in PBS, samples were incubated with donkey Alexa Fluor 594 anti-rabbit IgG (#A11072, 1:1000, Invitrogen), donkey Alexa Fluor 488 anti-rabbit IgG (#A11070, 1:1000, Invitrogen) or donkey Alexa Fluor 488 anti-mouse IgG (#A11017, 1:1000, Invitrogen), Alexa Fluor 488 phallodin (Invitrogen) for F-actin, and DAPI (Invitrogen) for nuclear staining. Samples were mounted in ProLong Gold Antifade Reagent (Invitrogen) and fluorescent images were obtained with Nikon A1si confocal microscope and NIS element 4.0 software (Tokyo, Japan). Signal intensity in five images from each sample was quantified using NIH Image (NIH, Bethesda, MD). Each experiment was repeated at least twice.

Small interfering RNA (siRNA) treatment, lentiviral transduction, IL6/IL6 neutralizing antibody treatment, gene expression analysis

For TCF21 knockdown, ASCs were transfected with control siRNA (siControl), CCUGCAGUAGUACCAUCAACCAA, siRNA against Tcf21 (siTcf21) #1, CCUCAGCGAUAGAAGAGCCUCAA, or siTcf21 #2, CCGGCAAAACCAGAAATGACCTGAAA. Briefly, siRNA (10 nM) was incubated with Lipofectamine RNAiMAX (Invitrogen) in OptiMEM (Gibco/Thermo Fisher Scientific) and added to cell suspension at the beginning of cell culture as per manufacturer’s recommendation. For TCF21 overexpression, lentivirus carrying TCF21 gene in pLenti-GIII-CMV-GFP-2A-Puro vector (Applied Biological Materials Inc, Richmond, BC) was transduced with 8 μg/ml polybrene (American Bioanalytical, Natick, MA) for 8 hours twice. GM6001 (10 μM final, MilliporeSigma, Burlington, MA) was added to culture for two days. VIS and SQ ASCs were incubated in the presence or absence of 100 ng/ml IL6 neutralizing antibody (#MAB406, R&D systems) and 30 ng/ml recombinant mouse IL6 (Cell Signaling) in serum-free DMEM supplied 1 μg/ml. RNA was extracted from cultured cells using RNeasy mini kit (Qiagen, Valencia, CA) and subjected to reverse transcription with SuperScript II (Invitrogen). cDNA samples were then subjected to qPCR by using either Power SYBR green or Universal Taqman Mastermix in StepOnePlus (Applied Biosystems/Thermo Fisher Scientific). Relative cDNA quantities were normalized to the expression of a housekeeping gene, 36B4 (Rplp0), and are shown as fold-change relative to control. Sequences of primers and Taqman probes are shown in supplemental Table S1.

Western blot

Conditioned medium from cultured cells was mixed with 4x Laemmli sample buffer (Bio-Rad, Richmond, CA) and heat denatured for 5 minutes at 95 °C. Protein samples were then separated by standard SDS-PAGE in 13% acrylamide gel followed by transfer to nitrocellulose membrane (Bio-Rad). Primary antibodies recognizing anti-IL6 antibody (#12912, 1:1000, Cell Signaling Technology, Danvers, MA), anti-Mmp13 antibody (#IM78, 1:400, EMD Millipore, Billerica, MA), anti-mCherry antibody (#632543, 1:1000, TaKaRa Clontech), and anti-TCF21 antibody (1:1000) were used for immunoblotting. Membranes were treated with horseradish peroxidase (HRP)-conjugated secondary antibodies—anti-rabbit IgG antibody (#170-6515, 1:5000, Bio-Rad), anti-mouse IgG antibody (#170-6516, 1:5000)–and chemiluminescent signal was developed using ECL western blotting substrate (Pierce/Thermo Fisher Scientific) for detection with FluorChem M (Proteinsimple, Santa Clara, CA).

Promoter activity assay

Synthesized human IL6 promoter region (-1479 - +21) was cloned into pGL3-basic plasmid (Promega, Madison, WI). Synthesized human TCF21 ORF (Genbank Accession number NM_198392) was cloned into pmCherry-C1 (TaKaRa Clontech, Kyoto, Japan). Amino acid 78 – 132 in TCF21 was predicted as basic helix-loop-helix by BLAST and was eliminated by inverse PCR using FW 5’-GGGGAAGCAGCAGATCCTGGCTAAGCA-3’ and RV 5’-CCAGGATCTGCTGTTCCCCCTGGCTGA-3’ primers and QuikChange site-directed mutagenesis kit (Agilent Technologies, Palo Alto, CA). mCherry-tagged protein expression was
confirmed by transfecting the plasmid into COS7 cells followed by western blotting with the cell lysates. For promoter activity assay, 500 ng of pmCherry-TCF21 and 500 ng of pGL3-IL6 promoter, or their empty vector were transfected together with 20 ng of pRL-TK Renilla luciferase plasmid (Promega) and 2.5 μl of Lipofectamine 2000 (Invitrogen) into Chinese hamster ovary cells in each well of 12-well plate. Two days later, Firefly and Renilla luciferase activity was assayed by using Dual-Luciferase Reporter Assay System (Promega) and Synergy NEO microplate reader (BioTek Instruments, Inc., Winooski, VT).

**Gelatin zymography**

Conditioned medium from cultured cells was mixed with 2x sample buffer (125 mM Tris, pH6.8, 20% glycerol, 4% SDS, and 0.005% bromophenol blue) and incubated for ten minutes at room temperature. Then samples were separated by 10% SDS-PAGE gel containing 1 mg/ml gelatin (type A from porcine skin, Sigma, St Louis, MO). Gels were incubated in 2.5% Trion X-100 for 30 minutes at room temperature, then in developing buffer (50 mM Tris, 200 mM NaCl, 6.7 mM CaCl₂, and 0.02% Brij35 (Sigma)) at 37 °C overnight. Collagenolytic bands were visualized by 0.5% Coomassie R-250 (Sigma) dissolved in 5% methanol and 10% acetic acid.

**Statistical analysis**

All data were statistically analyzed using Student’s t-test or paired t-test, one-way ANOVA with post hoc multiple comparison by Tukey’s procedure, or two-way ANOVA with post hoc multiple comparison by Sidak’s procedure as specified in figure legends.

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**Conflict of interest**

The authors declare that they have no conflicts of interest with the contents of this article.
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Figure 1. TCF21 is expressed predominantly in visceral WAT ASCs

A, B: Vascular stromal cells were isolated from visceral and subcutaneous adipose depots, then subjected to magnetic cell sorting using anti-Sca1 antibody to enrich Sca1-high ASCs. A: A volcano plot of gene expression in visceral (VIS) and subcutaneous (SQ) ASCs. B: Total RNA samples were prepared from VIS and SQ ASCs and analyzed with RT-qPCR. (n = 3 independent biological replicates, mean ± SEM, *: P < 0.05, ***: P < 0.0001 by Student’s t-test) C: RT-qPCR with human primary preadipocytes. n = 3 independent biological replicates, mean ± SEM, *: P < 0.05 by Student’s t-test. D: Immunostaining of endogenous TCF21 (red), nucleus (DAPI, blue), and F-actin (green) in VIS and SQ ASCs. Bar = 50 μm. E: Immunostaining of endogenous TCF21 (red), IL6 (green), and DAPI (blue) in unfractionated vascular stromal cells. Bar = 20 μm. The table shows the percentage of each group of cells per total vascular stromal cells, means ± SEM. F: Left, immunostaining of endogenous TCF21 (red), PDRGFRα (green), nucleus (DAPI, blue); Right, TCF21 (red), IL6 (green), nucleus (DAPI, blue) in VIS and SQ fat depots in vivo. Cells expressing both TCF21 and PDGFRα or IL6 were indicated by arrows. Bar = 20 μm.
Figure 2. TCF21 drives IL6 expression in VIS ASCs.

A, B: The siRNA oligos targeting Tcf21 and control siRNA were transfected to mouse VIS ASCs and cultured for three days. Total RNA was extracted to assess Tcf21 and Il6 expression by RT-qPCR. (n = 4 independent biological replicates, mean ± SEM, *: P < 0.05, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001 by one-way ANOVA)

C: IL6 and type I collagen (COL1) protein levels in the conditioned media of siRNA transfected VIS and SQ ASCs. Conditioned media were collected at indicated time points for western blotting. Independent experiments were performed twice.

D: Mouse VIS ASCs were transduced with human TCF21 cDNA by lentiviral gene transfer. Total RNA was extracted for RT-qPCR analysis of TCF21 and Il6 expression. (n = 3 independent biological replicates, mean ± SEM, *: P < 0.05 by ratio paired t-test)

E: Schematic diagram of human IL6 promoter region cloned into pGL3-basic reporter plasmid. Known cis-elements (black boxes) and predicted TCF21-binding E-box sites (hatched boxes) are shown along with respective sequences.

F: IL6 promoter in CHO cells regulated by co-transfected full-length and mutant TCF21. Reconstituted IL6 promoter activity (firefly luciferase) was normalized by co-transfected thymidine kinase promoter activity (Renilla luciferase). n = 7, mean ± SEM, ****: P < 0.0001 by ANOVA. The experiment was independently repeated.
Figure 3. TCF21 regulates the expression of a subset of MMPs and TIMPs

A-C: The siRNA oligos targeting Tcf21 and control siRNA were transfected to mouse VIS ASCs and cultured for three days. A: Total RNA was extracted for RT-qPCR analysis. n = 4, mean ± SEM, **: P < 0.01, ***: P < 0.001, ****: P < 0.0001 by one-way ANOVA. B: To assess MMP2 activity, the media of transfected cells were changed to serum-free DMEM a day after transfection; conditioned media were collected three days later and subjected to gelatin zymography. Representative figure, n = 3, mean ± SEM, ****: P < 0.0001 by one-way ANOVA.
TCF21 mediates type IV collagen deposition by VIS ASCs

A-C: Mouse VIS ASCs were transfected with siRNA oligos targeting Tcf21 and control oligo and then cultured for three days. A: RT-qPCR analysis of collagen gene expression. n = 4, mean ± SEM, *: P < 0.05, **: P < 0.01 by one-way ANOVA. B: Extracellular type IV collagen was detected (red) and quantified, normalized by the number of nuclei (blue). Bar = 50 μm (low mag) and 10 μm (high mag). n = 5, mean ± SEM, not significant per two-way ANOVA. C: Extracellular degraded collagen was stained (red) and quantified per nuclei (blue). Bar = 50 μm. n = 5, mean ± SEM, not significant per two-way ANOVA.

D: ASCs isolated from VIS WAT were cultured in the presence or absence of 10 μM GM6001 for two days, stained for degraded collagen (red), and quantified per nuclei (blue). Bar = 50 μm. n = 5, mean ± SEM, *: P < 0.05 by Student t-test.

E, F: ASCs isolated from VIS and SQ WATs were transduced with TCF21 cDNA by lentiviral gene transfer. Cells were cultured three days, analyzed by immunostaining for collagens type IV, I, VI, and degraded type I collagen (all in red) along with DAPI (nuclei, blue). Signal intensities of staining were quantified and normalized by nucleus number (n = 5, mean ± SEM, *: P < 0.05, **: P < 0.01, ****: P < 0.001 by two-way ANOVA (E) or by Student t-test (F)).
Figure 5. IL6 is responsible for the expression of Mmp13 and Timp1 but not for type IV collagen deposition and type I collagen degradation.

A: VIS and SQ ASCs were incubated in the presence and absence of IL6 neutralizing antibody (IL6 Ab, 100 ng/ml) with and without 30 ng/ml recombinant mouse IL6 for 24 hours. Total RNA was isolated 24 hours later and analyzed by RT-qPCR. \( n = 3, \) mean ± SEM, *: \( P < 0.05 \) by two-way ANOVA.

B: Mouse VIS ASCs were transfected with siRNA oligos targeting Tcf21 and control oligo and then treated with IL6 neutralizing antibody (100 ng/ml) for three days. Extracellular deposition of type IV collagen and degraded collagen products were shown in red, nuclei in blue. Bar = 50 \( \mu \)m. Signal quantification on the right (\( n = 5, \) mean ± SEM, **: \( P < 0.01 \) by two-way ANOVA).
Transcription factor 21 (Tcf21) promotes proinflammatory interleukin 6 expression and extracellular matrix remodeling in visceral adipose stem cells
Takeshi Akama and Tae-Hwa Chun

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