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Angiopoietin-like protein 2, a chronic inflammatory mediator, is a new target induced by TGF-β1 through a Smad3-dependent mechanism

Hee Jae Lee [a,1], Jun Hwan Kim [a,1], Joon-Hyung Kim [a], Ryan Dennis Martinus [b], Seok Hee Park [a,*]

[a] Department of Biological Sciences, Sungkyunkwan University, Suwon 440-746, Republic of Korea
[b] Department of Biological Sciences, Faculty of Science and Engineering, The University of Waikato, Hamilton, New Zealand

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ABSTRACT

Angiopoietin-like protein 2 (Angptl2) levels are increased by obesity and obesity-related pathological conditions, and it is considered to be an important adipocyte-derived inflammatory mediator. In contrast, the multifunctional cytokine TGF-β1 has been reported to be augmented in obesity of rodents and humans, but inhibits adipocyte differentiation *in vitro*. Here we demonstrate that TGF-β1 induces expression of the Angptl2 gene through a Smad3-dependent pathway in RAW264.7 macrophage cells, primary peritoneal macrophages, and differentiated 3T3-L1 adipocytes. Transcriptional induction of the Angptl2 gene by TGF-β1 was dependent on the Smad3 protein which binds to the Smad Binding Element (SBE) region located on the Angptl2 promoter. Macrophages with Smad3 knocked down by small interfering RNA showed reduction of TGF-β1-induced Angptl2 expression. These findings may provide insight into the molecular mechanisms of the increased expression of Angptl2 and TGF-β1 in obesity.

1. Introduction

Angiopoietin-like protein 2 (Angptl2), a member of the Angptl protein family structurally similar to the angiopoietins, is a secreted protein known to be a key inflammatory mediator in obesity and related metabolic diseases [1–3]. Among the Angptl family, Angptl2 seems to have unique properties in cellular function. The Angptl2 protein is mainly expressed in adipose tissue and expression is increased in obesity and obesity-related pathological conditions, including hypoxia and endoplasmic reticulum (ER) stress [4,5]. In addition, the increased level of circulating Angptl2 was reported to be closely related to adiposity, systemic insulin resistance, and inflammation in both mice and humans [2]. Deletion of the Angptl2 gene ameliorated adipose tissue inflammation and systemic insulin resistance in diet-induced obese mice [2]. Furthermore, treatment of pioglitazone, a PPARγ agonist with unique antidiabetic activity, showed that circulating Angptl2 levels decreased in parallel with reduction of visceral fat in obese diabetic patients [2,6,7]. These findings indicate that Angptl2 is an important target for the treatment of obesity and related metabolic diseases.

TGF-β1 is a multifunctional cytokine involved in a variety of cellular functions including cell growth, differentiation, and development, depending on the cellular context [8,9]. TGF-β1 signal transduction pathways can be divided into two main pathways, the canonical Smad-dependent or non-canonical Smad-independent pathway, depending on availability of the intracellular signal transducer Smad proteins [9,10]. In the canonical pathway, both TGF-β type II and type I receptors initiate the signaling process with serine–threonine kinase activity upon TGF-β1 binding, and then the activated type I receptor transmits intracellular signals through the phosphorylation of the receptor-activated Smad proteins (R-Smads) Smad2 and Smad3. The phosphorylated R-Smads form heteromeric complexes with a common partner Smad4 (Co-Smad) and translocate into the nucleus to modulate expression of target genes [9,11]. This canonical pathway is negatively regulated by the inhibitory Smads (I-Smad) Smad6 and Smad7 [12,13]. In contrast, the non-canonical TGF-β pathway is independent of R-Smad activation and utilizes various cellular signaling components such as p38 and JNK mitogen-activated protein kinases, PI3 kinase, TRAF6, and TAK1 [10,14].

Although TGF-β1 is known to be inhibitory to adipogenesis, its physiological role remains unclear. *In vitro* studies indicated that TGF-β1 inhibits the differentiation of 3T3-L1 preadipocyte cells into mature adipocytes during the early stages of differentiation [15]. TGF-β1 was also shown to increase the proliferation of 3T3-F442A preadipocytes [16]. However, studies in humans make it difficult to understand the role of TGF-β in adipogenesis. A study of obese women revealed that circulating TGF-β1 is decreased [17] whereas another study showed increased TGF-β1 expression in adipose tissues [18], similar to obese mice [19]. In addition, circulating TGF-β1 levels were reported to positively correlate with obesity, body mass index, and leptin levels in hypertensive patients [20].

* Corresponding author. Fax: +82 31 290 7015.
E-mail address: parks@skku.edu (S.H. Park).
[1] These authors equally contributed to this work.

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In this study, we demonstrate that Angptl2 protein, an obesity-related inflammatory mediator, is a new target induced by TGF-β1 in a Smad3-dependent manner, suggesting a possible scenario for how increased TGF-β1 in adipose tissue contributes to obesity.

2. Materials and methods

2.1. Reagents, cell culture, transfection and reporter assay

Recombinant human TGF-β1 was purchased from R&D Systems. RAW264.7 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal bovine serum (Gibco). Peritoneal primary macrophages from C57BL/6 mice were prepared as previously described [21]. Peritoneal cavities were lavaged 3 days after intraperitoneal injection of 3% thioglycollate. The animal protocols for preparation of primary peritoneal macrophages were approved by the Institutional Animal Care and Use Committee at the Department of Biological Sciences, Sungkyunkwan University (Suwon, Korea). All plasmids were transfected using lipofectamine LTX™ with Plus™ Reagent (Invitrogen). Luciferase activities were measured by a dual luciferase reporter assay kit (Promega) and normalized based on *Renilla* luciferase expression to adjust for variations in transfection efficiency. The data for luciferase assays represent the mean ± S.D. of three independent experiments.

2.2. 3T3-L1 differentiation

Murine 3T3-L1 preadipocyte cells (American Type Culture Collection), were maintained in DMEM with 10% bovine calf serum. The differentiation of 3T3-L1 preadipocytes to adipocytes were performed as previously described [22]. Briefly, after cells reached confluence, cells were stimulated with DMEM supplemented with 10% FBS plus DMI cocktail solution (1 μM dexamethasone, 0.5 mM isobutyl-1-methylxanthine, and 10 μg/ml insulin) for two days. After stimulation for two days, the medium was repleted with DMEM containing 10% FBS and 10 μg/ml insulin every other day until 7–8 days had passed. Quantitative real time RT-PCR analysis of adipocyte-specific genes such as the adipocyte fatty acid-binding protein (aP2), CD36, PPARγ, and 36B4, was used to confirm the differentiation of 3T3-L1 cells to adipocytes.

2.3. Plasmids

Mouse Angptl2 (mAngptl2) promoter, longer than the one previously reported [23], was amplified from mouse genomic DNA by polymerase chain reaction (PCR) and subcloned into the *Nhe*I and *Bgl*II sites of the pGL3-basic vector. Serial deletion mutants of the Angptl2 promoter (Mutant 1–Mutant 4) were amplified from the full-length promoter (wild-type) region by PCR and subcloned into *Nhe*I and *Bgl*II sites of the pGL3-basic vector. Specific mutations (mAngptl2 mSBE1 and mAngptl2 mSBE2) for the predicted Smad Binding Elements, SBE1 and SBE2, in the mAngptl2 promoter were performed using the Quik Change Site-directed Mutagenesis kit (Stratagene). The sequences of the PCR-generated portions of all constructs were verified by DNA sequencing. The primers for PCR and site-directed mutagenesis are in Supplementary Table 1.

2.4. RNA extraction, RT-PCR, and quantitative real-time RT-PCR

Total RNA was isolated with TRIZOL reagent (Invitrogen). M-MLV reverse transcriptase (Takara) was used for reverse transcription. Primer sequences for the amplification of mAngptl2 and mouse *Gapdh* genes are described in Supplementary Table 1 and PCR amplifications were performed under the following conditions; for mAngptl2, 29 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; for *Gapdh*, 25 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. For quantitative RT-PCR, an iCycler real-time PCR machine and iQ SYBR Green Supermix (Bio-Rad) were used to measure the expression of genes under the following conditions; 45 cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. Quantitative real-time RT-PCR experiments were performed essentially as described [24], and independently repeated at least three times to ensure reproducibility.

2.5. Antibodies and immunoblot analysis

Mouse anti-Angptl2, rabbit monoclonal anti-Smad3, mouse anti-β-actin antibodies were purchased from R&D Systems, Cell Signaling, and Sigma, respectively. Immunoblot analysis for endogenous proteins was performed after lysing cells with NETN buffer (20 mM Tris–Cl (pH8.0), 1 mM EDTA, 100 mM NaCl, 0.5% (v/v) Nonidet P-40, and protease inhibitor cocktail). For immunoblot analysis for secreted proteins, culture supernatants were precipitated by methanol. Coomassie brilliant blue staining was used as a loading control to quantify the secreted proteins.

2.6. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed essentially as described [25]. Briefly, formaldehyde was added to cells to a final concentration of 1% and cells were incubated at 37 °C for 10 min. After cross-linking and cell lysis, lysates were sonicated to shear DNA to an average size between 500 bp and 1000 bp. The sonicated chromatin was immunoprecipitated with anti-Smad3 antibody (Abcam; ab28379). Rabbit IgG was used as a negative control for the ChIP assay. The regions containing SBE1 and SBE2 within the mAngptl2 promoter were amplified from DNA isolated from ChIP experiments under the following PCR conditions; 29 cycles of 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s. PCR products were analyzed by 2% agarose/ethidium bromide gel electrophoresis. The primers used for ChIP assays are described in Supplementary Table 1.

2.7. Small hairpin RNA for Smad3 and lentiviral infection

A lentiviral plasmid expressing a Smad3-specific shRNA (5′-CCGGCATCCGTATGAGCTTCGTCAACTCGAGTTGACGAAGCTCATACGGATGTTTTTG-3′) in the pLKO.1-puro vector was purchased from Sigma. Lentiviruses expressing the Smad3-specific shRNA (sh-Smad3) were produced essentially as described [21]. Lentiviruses expressing a GFP-specific shRNA (sh-GFP) were used as a negative control.

2.8. Statistical analysis

All data are shown as mean values ± S.D. and are representative of the results of three or more experiments. Statistical analysis was performed using the Student's *t*-test for paired data. Differences were considered significant at $P < 0.05$.

3. Results and discussion

To identify new target genes regulated by TGF-β1, we performed microarray experiments using mRNA from peritoneal macrophages treated with/without TGF-β1. Through three independent microarray analyses, we isolated Angiopoietin-like protein 2 (Angptl2) as a new candidate gene induced by TGF-β1. Angptl2 is known as an adipocyte-derived inflammatory mediator that links obesity to systemic insulin resistance [2] whereas the role of TGF-β1 in obesity has not been clearly addressed. Therefore, we hypothesized that TGF-β1 and its related signaling pathway

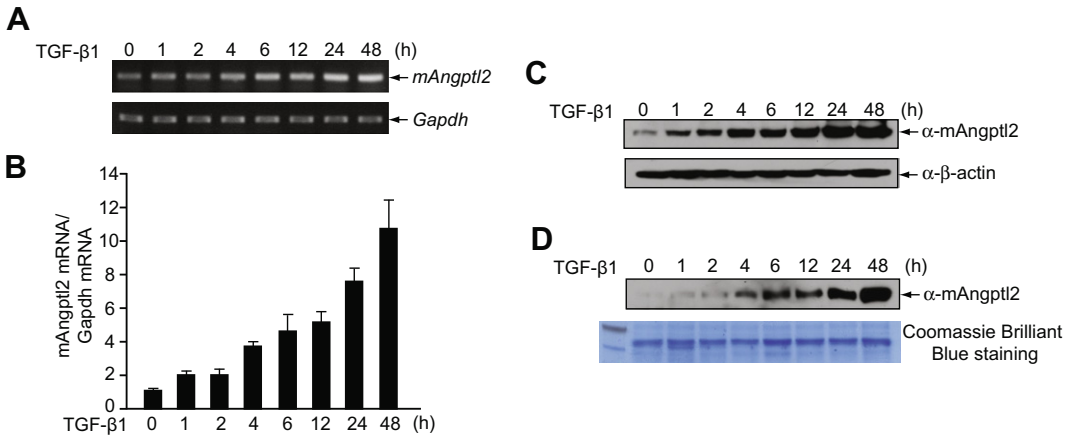

Fig. 1. TGF-β1 increases expression of the mAngptl2 gene in RAW264.7 macrophage cells. RT-PCR (A) and quantitative real time RT-PCR (B) analysis of the mAngptl2 gene were performed in RAW264.7 macrophage cells treated with 5 ng/ml TGF-β1 for the indicated time. GAPDH expression was used as a loading control and for normalization. Immunoblot analysis of mAngptl2 expression in cell extracts (C) and culture supernatants (D) were performed in RAW264.7 macrophages treated with TGF-β1 for the indicated time. β-actin was used as a loading control in immunoblot analysis of cell extracts. Coomassie brilliant blue staining for culture supernatants was used as a loading control to detect secreted mAngptl2 protein. All data are representative results of at least three independent experiments.

may be involved in the progression of obesity through increasing Angptl2 gene expression.

To verify whether the mouse Angptl2 (mAngptl2) gene is upregulated by TGF-β1, we examined the expression of the mAngptl2 gene upon TGF-β1 treatment in RAW264.7 macrophage cells. RT-PCR and quantitative real time RT-PCR analysis showed that the mAngptl2 gene transcriptionally increased in a time-dependent manner (Fig. 1A and B). Since the mAngptl2 protein is known to be secreted from cells [26], increased expression of the mAngptl2 protein was confirmed in both RAW264.7 cell extracts and culture supernatant by immunoblot analysis (Fig. 1C and D). A similar increase in expression of the mAngptl2 gene by TGF-β1 was also observed in primary peritoneal macrophages (Supplementary Fig. S1).

Next, we examined TGF-β1-induced expression of the mAngptl2 protein in differentiated 3T3-L1 adipocyte cells. After confirming the complete differentiation of 3T3-L1 preadipocyte cells by expression of adipocyte-specific genes such as aP2, CD36 and PPARγ (Fig. 2A), the differentiated 3T3-L1 adipocytes were treated with TGF-β1 for the indicated time periods (Fig. 2B). Similar to what was observed for RAW264.7 macrophages and primary peritoneal macrophages, mAngptl2 protein was increased upon TGF-β1 treatment in 3T3-L1 adipocytes, showing maximum expression at 24 h (Fig. 2B). These results strongly indicate that TGF-β1 is a crucial factor upregulating the mAngptl2 protein in macrophages and adipocytes which are key sources related to obesity.

These findings prompted us to examine mAngptl2 promoter activity in the presence of TGF-β1. Based on a previous report [23], a roughly 3 kb fragment, including 5′ flanking sequence and the transcription start site of the mAngptl2 gene, was cloned into the pGL3-basic reporter plasmid (Fig. 3A) and subsequently transfected into RAW264.7 cells, followed by TGF-β1 treatment for 24 h. The full length promoter of the mAngptl2 gene was significantly induced by TGF-β1 (Fig. 3B). Although the Angptl2 promoter

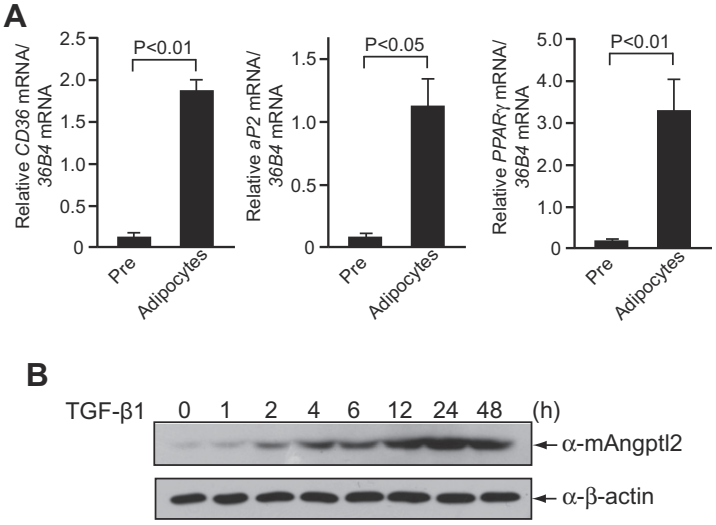

Fig. 2. Expression of the mAngtpl2 gene increased in differentiated 3T3-L1 adipocytes upon TGF-β1 treatment. (A) Expression analysis of the adipocyte markers CD36, aP2, and PPARγ, by quantitative real time RT-PCR was used to confirm the differentiation of 3T3-L1 preadipocytes. Expression of 36B4 mRNA, coding an acidic ribosomal phosphoprotein, was used to normalize quantitative real-time RT-PCR results in 3T3-L1 adipocytes. All data represent the mean ± S.D. of three independent experiments. Statistical analyses were performed using a *t*-test. (B) Immunoblot analyses of mAngptl2 protein were performed in differentiated 3T3-L1 adipocytes treated with 5 ng/ml TGF-β1 during the indicated time. β-actin was used as a loading control. Data are representative results of at least three independent experiments.

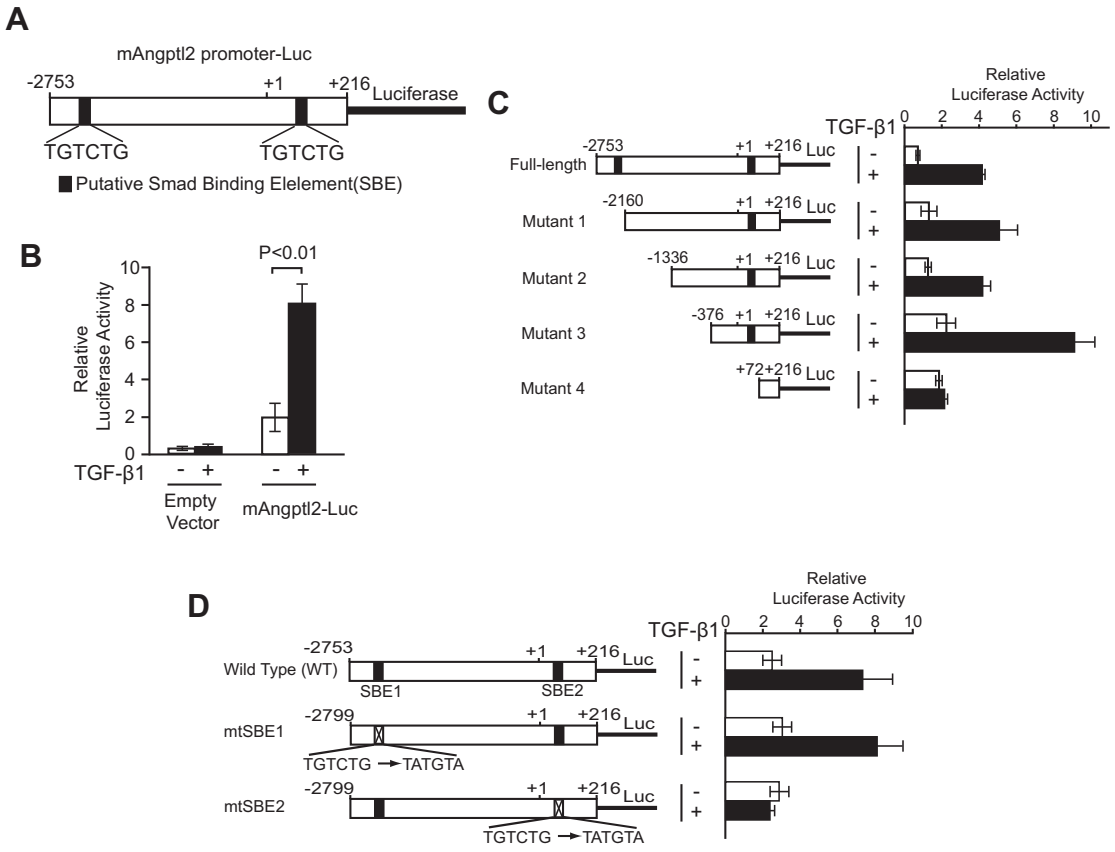

Fig. 3. Smad Binding Element 2 (SBE2), located on +46 to +51 of the mAngptl2 promoter, is critical for TGF-β1-induced activation of the mAngptl2 promoter. (A) Schematic representation of an mAngptl2 promoter-fused reporter plasmid. The promoter contains two putative SBE regions, SBE1 and SBE2. (B) A reporter plasmid containing the full-length mAngptl2 promoter was transiently transfected into RAW264.7 macrophage cells. (C) Serial deletion mutants of the mouse Angptl2 promoter were transiently transfected into RAW264.7 cells. (D) Site-directed mutants for the SBE1 and SBE2 sequences were transfected into RAW264.7 cells. In all experiments, TGF-β1 was treated or not for 24 h after transfection and luciferase activity was subsequently measured. The luciferase activity was normalized on the basis of *Renilla* luciferase expression to adjust for variation in transfection efficiency. All data represent the mean ± S.D. of three independent experiments.

contained binding sites for transcription factors such as NF-κB, C/EBP and Sp1 [23], it remains unknown whether intracellular signal transducers of the TGF-β1 signaling pathway, Smads, are involved in induction of the mAngptl2 promoter. Homology analysis to analyze transcription factor binding sites indicated that two Smad Binding Elements (SBE), SBE1 (−2184 to −2176) and SBE2 (+46 to + 51), are located within the mAngptl2 promoter (Fig. 3A). To investigate which SBE element is required for activation of the mAngptl2 promoter by TGF-β1, serial deletion mutants of the mAngptl2 promoter were transfected into RAW264.7 cells and subsequently treated with TGF-β1 for 24 h. Deletion of −376 to + 73 in the mAngptl2 promoter abolished TGF-β1-mediated induction of the Angptl2 promoter (Fig. 3C), indicating that the SBE2 element residing in this region may be important for induction of the mAngptl2 promoter by TGF-β1. To confirm whether the SBE2 element is critical for TGF-β1-mediated induction of the mAngptl2 promoter, we generated site-directed mutants of each SBE element and transfected them into RAW264.7 cells, followed by TGF-β1 treatment. Mutation of the SBE2 element abolished induction of the mAngptl2 promoter by TGF-β1 whereas that of the SBE1 element did not (Fig. 3D). These results suggest that Smad proteins binding to the SBE2 element may be crucial for induction of the mAngptl2 promoter by TGF-β1.

To test whether a receptor-activated Smad (R-Smad), Smad2 or Smad3, binds to the SBE2 element in the mAngptl2 promoter in the presence of TGF-β1, we performed chromatin immunoprecipitation (ChIP) assays in RAW264.7 cells. ChIP analysis showed that the Smad3 protein binds to SBE2, but not SBE1, within the mAngptl2 promoter (Fig. 4A). Smad2 protein did not bind to either element (data not shown). Next, we generated Smad3 knock-down primary peritoneal macrophages by infection of lentiviruses expressing Smad3-specific shRNA (Fig. 4B). Expression of the mAngptl2 gene was not significantly increased by TGF-β1 treatment in Smad3 knock-down primary peritoneal macrophages compared to wild-type cells expressing control shRNA (sh-GFP) (Fig. 4C and D). These results reveal that mAngptl2, an inflammation mediator in obesity, is induced by TGF-β1 through a Smad3-dependent pathway.

Accumulating evidence emphasizes an inflammatory role of Angptl2 in diverse chronic diseases. A knock-out mice study indicated that the Angptl2 protein acts as a key adipocyte-derived inflammatory mediator and promotes chronic adipose tissue inflammation and obesity-related systemic insulin resistance [2]. In addition, Angptl2, which is derived from synoviocytes, has been reported to contribute to synovial chronic inflammation in rheumatoid arthritis [27] and the pathogenesis of dermatomyositis [28]. Moreover, Angptl2 protein levels in lung tissues extracted from primary tumor sites in patients with non-small cell lung carcinoma correlated with poor disease-free survival and promoted metastasis in an autocrine/paracrine manner [29]. Also, Angptl2 has been reported to be highly correlated with tumor cell metastasis in a chemically induced skin squamous cell carcinoma (SCC) mouse model [30].

In spite of the important role of Angptl2 gene in these diseases, the molecular mechanism underlying the regulation of Angptl2 gene expression has not been clearly addressed. Recent studies

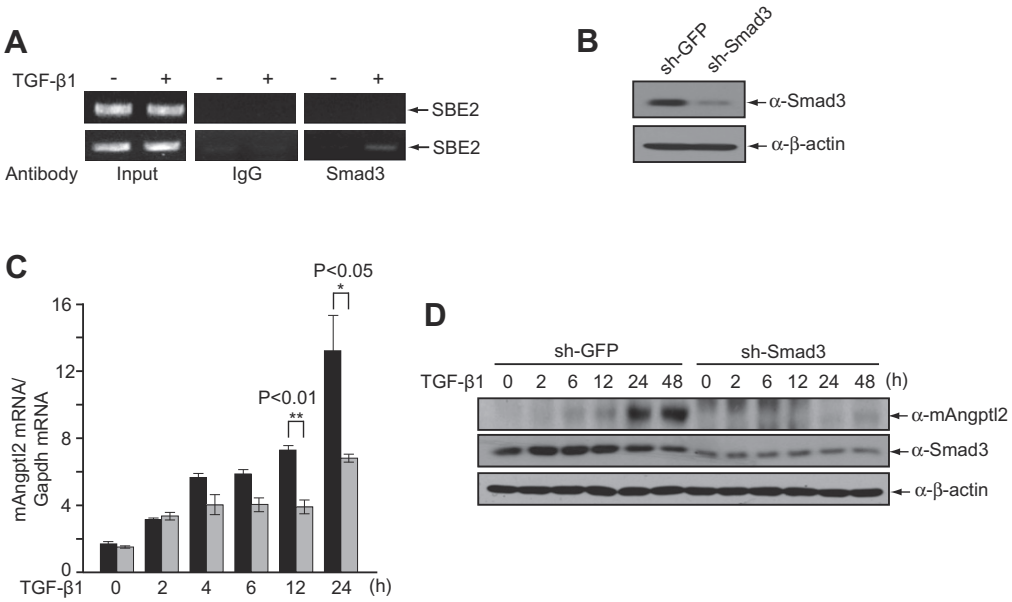

Fig. 4. TGF-β1-mediated induction of the mAngptl2 gene requires Smad3. (A) Chromatin immunopreciptation (ChIP) assays were performed in RAW264.7 cells after treatment of TGF-β1 for 24 h. Sonicated chromatin fragments were immunoprecipitated by an antibody against endogenous Smad3 and the immunoprecipitated regions of SBE1 and SBE2 were analyzed by PCR, respectively. Non-immune IgG was used as a negative control for ChIP assays. (B) Lentiviruses expressing Smad3-specific shRNA (sh-Smad3) were infected into primary peritoneal macrophages and expression of the Smad3 protein was observed by immunoblot analysis. Lentiviruses expressing green fluorescent protein (GFP)-specific shRNA (sh-GFP) were used as a negative control. (C) Quantitative real time RT-PCR analysis of mAngptl2 gene expression in Smad3 knock-down and Smad3-expressing primary peritoneal macrophages. TGF-β1 was treated for the indicated time. Expression of the GAPDH gene was used for normalization. All data represent the mean ± S.D. of three independent experiments. Statistical analyses were performed using a *t*-test. (D) Immunoblot analysis of the mAngptl2 protein in Smad3 knock-down and Smad3-expressing primary peritoneal macrophages. TGF-β1 was treated for the indicated time. All data are representative results of at least three independent experiments. β-actin was used as a loading control.

indicated that tumor necrosis factor (TNF)-α increases Angptl2 gene through Foxo1 activation in 3T3-L1 adipocytes [23] and the NFATc transcription factor is involved in Angptl2 gene expression in tumor cells [29].

In this study, we demonstrate that TGF-β1, a multifunctional cytokine, is an important key modulator of Angptl2 gene expression through a Smad3-dependent mechanism in both macrophages and adipocytes. Although TGF-β1 has been involved in a variety of cellular functions such as cell growth, apoptosis, and immune suppression, its role and molecular mechanisms in obesity still remain obscure. *In vitro* studies showed that TGF-β1 inhibits the early stage of differentiation of 3T3-L1 preadipocytes [15], while some reports indicated that TGF-β1 is increased in obese humans [18] and circulating TGF-β1 positively correlates with obesity in hypertensive patients [20]. Our present study may provide an important clue in addressing the role of TGF-β1 in obesity.

Based on our findings that TGF-β1 increases expression of the Angptl2 gene at the transcriptional level in macrophages as well as adipocytes, and previous results showing increased TGF-β1 expression in obese women and mice [18,19], TGF-β1 may contribute to obesity through increasing Angptl2 protein in macrophages and adipocytes in an autocrine/paracrine manner (Supplementary Fig. S2). Sequence analysis of the Angptl2 promoter and a ChIP assay indicated that the Angptl2 promoter contains a functional SBE element and that the Smad3 protein, which is an intracellular mediator of TGF-β signaling, binds to this SBE element. These experimental evidences strongly supports a connection between TGF-β signaling and Angptl2 pathophysiology. This relationship may also extend into a new scenario for tumor metastasis. That is, TGF-β1, which is increased in a tumor microenvironment, may upregulate Angptl2 expression in an autocrine/paracrine manner and indirectly contribute to Angptl2-mediated metastasis. This speculation can be supported by the findings that Angptl4, another member in the Angptl family, is induced by TGF-β1 and contributes to the metastasis of breast cancer to lung tissue [31], although the mechanisms regarding TGF-β1-mediated activation of the Angptl4 gene was not described.

Our present results, together with previous reports, strongly suggest that TGF-β1-mediated activation of Angptl2 may be a new axis in obesity progression. Therefore, modulation of this TGF-β1-Angptl2 axis in cellular signaling may be a promising target in obesity and related human diseases.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.127.

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