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BMP-TAK1 (MAP3K7) induces adipocyte differentiation through PPARγ signaling

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Abstract

BMPs have been shown to promote adipocyte differentiation through SMAD-dependent signaling. However, the role of TGF-β-activated kinase 1 (TAK1) in non-canonical BMP signaling in adipocyte differentiation remains unclear. Here, we show that TAK1 inhibition decreases lipid accumulation in C3H10T1/2 mesenchymal stem cells (MSCs) induced to differentiate into adipocytes. TAK1 knockdown by siRNA further confirms that TAK1 is required for adipocyte commitment of MSCs. Additionally, TAK1 knockdown inhibits adipogenesis of 3T3-L1 preadipocytes, indicating that TAK1 is not only needed for adipocyte commitment, but also required for adipocyte terminal differentiation. Furthermore, TAK1 ablation specifically in adipocytes reduced high fat diet-induced weight gaining and improved glucose tolerance. Mechanistically, we demonstrate that TAK1 is required for PPARγ transactivation and promotes PPARγ transcriptional activity synergistically with TAK1 binding protein 1 (TAB1). Collectively, our results demonstrate that TAK1 plays an essential role in BMP-mediated adipocyte differentiation.

Keywords

TAK1; BMP; PPARγ; Adipocyte; Obesity

Obesity increases the risk of multiple types of human diseases such as type 2 diabetes, liver fibrosis and coronary heart disease [Flier, 2004]. Obese patients present with excess adipose

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tissue, which functions to accumulate fatty acid and store energy [Stephens, 2012]. Adipose tissue is generated through adipogenic differentiation of mesenchymal stem cells [Huang et al., 2009; Rosen and MacDougald, 2006; Tang et al., 2004]. Subsequent to the first phase of commitment of mesenchymal stem cells (MSCs) into preadipocytes, a second phase of terminal differentiation leads to the production of mature adipocytes [Tzameli et al., 2004]. Hence, the rate of adipocyte differentiation determines the amount of fat deposition in the body [Rosen and Spiegelman, 2000]. An in-depth study of the mechanisms controlling adipocyte differentiation provides opportunities to develop therapeutics to treat obesity.

Adipocyte differentiation is coordinated by a panel of transcription factors, and the central transcription factor controlling this process is peroxisome proliferator-activated receptor gamma (PPARγ). PPARγ ablation disrupts adipocyte differentiation, and PPARγ$^{-/-}$ mice fail to develop adipose tissue [Barak et al., 1999; Rosen et al., 2002]. PPARγ transactivates gene transcription as a heterodimer with retinoid X receptor (RXR) by binding to the PPAR-responsive element (PPRE) in gene promoters [Kliewer et al., 1992]. The targeting of PPARγ to PPRE induces various genes such as fatty acid binding protein 4 (FABP4), CD36 and phosphoenolpyruvate carboxykinase (PEPCK) to enhance fatty acid accumulation [Ahmadian et al., 2013; Tontonoz and Spiegelman, 2008]. Since PPARγ functions as a master regulator in adipogenesis, a group of PPARγ agonists, the thiazolidinediones (TZDs), have been synthesized [Rosen and Spiegelman, 2000; Tontonoz and Spiegelman, 2008]. A member of the TZD family, Rosiglitazone, binds directly to PPARγ to induce adipocyte differentiation [Ninomiya et al., 2010]. Owning to the critical role of PPARγ in adipocyte tissue production, dissecting the molecular mechanisms in control of PPARγ transactivation process will be significant to cure obesity.

So far, a myriad of signaling pathways have been discovered to regulate adipocyte differentiation through PPARγ signaling. Hedgehog (HH) and wingless (WNT)/β-CATENIN signaling, for example, repress adipocyte differentiation by reducing PPARγ signaling [Christodoulides et al., 2009; Suh et al., 2006]. In contrast, bone morphogenetic proteins (BMPs) including BMP2, BMP4 and BMP7 increase adipocyte differentiation [Huang et al., 2009; Tang et al., 2004; Tseng et al., 2008]. Interference of BMP signaling components, BMPR1A and SMAD4, result in a reduction of adipogenesis [Huang et al., 2009], whereas overexpression of BMP6 inhibits adipocyte differentiation [Hata et al., 2003]. In addition to induction of SMAD signaling, binding of BMPs to receptors also leads to phosphorylation of TAK1, which forms a complex with TAB1 to further phosphorylate and activate Jun amino-terminal kinase (JNK) and p38 [Anderson and Darshan, 2008; Yamaguchi et al., 1995]. Prior studies revealed that inhibition of p38 and its downstream transcription factor ATF2 suppresses adipogenesis [Bost et al., 2005; Engelman et al., 1998; Hata et al., 2003]. Overexpression of TAK1 and TAB1 promotes PPARγ transcriptional activity [Hata et al., 2003]. However, whether TAK1 is directly involved in adipocyte differentiation and how TAK1 regulates PPARγ signaling is still unknown.

To determine the role of TAK1 in BMP-mediated adipocyte differentiation, we performed adipocyte differentiation assays using C3H10T1/2 mesenchymal progenitor cells and 3T3-L1 preadipocytes. We found that TAK1 is required for adipocyte differentiation of both cell types suggesting an important role for TAK1 in differentiation of preadipocytes from MSCs

as well as in the differentiation of terminally mature adipocytes from preadipocytes. Additionally, TAK1 inhibition reduces expression of adipocyte markers and suppresses high-fat diet-induced weight gain *in vivo*. Finally, we demonstrated that TAK1 signaling is necessary and sufficient to increase PPARγ transactivation activity.

Materials and Methods

Animal Studies

All animal studies were performed according to the protocol approved by the University of Rochester Committee on Animal Resources. All mice were housed in a room using Microisolator Technology kept at 70–73 degrees F. They had free access to food (LabDiet 5010) and water (Hydropac) at all times. 15 mg/kg TI-2 (5Z-7-oxozeaenol, LLZ1640-2, BioAustralis, Smithfield, NSW, Australia) was injected into 8-week-old C57BL/6J male mice (The Jackson Laboratory) daily via intraperitoneal administration. RNA was isolated from visceral fat 6 days post drug administration. To generate adipocyte-specific Tak1 knockout (TAK1-KO) mice, *Tak1* floxed mice (*Tak1*$^{fx/fx}$) [Liu et al., 2006] were crossed with *aP2-Cre* mice [He et al., 2003]. To induce obesity, five-week-old mice were placed on high fat diet (60% kcal) and weight was measured once weekly. For glucose tolerance tests, mice were fasted for 6 hours and injected intraperitoneally with glucose (2 g/kg body mass). Blood glucose was measured using a glucometer [Villanueva et al., 2011].

Adipocyte differentiation and Oil Red O Staining

Adipocyte differentiation of C3H10T1/2 and 3T3-L1 was adapted from prior studies [Huang et al., 2009; Villanueva et al., 2011]. Briefly, C3H10T1/2 and 3T3-L1 cells were cultured in DMEM containing 10% FBS, 1 μM dexamethasone, 0.5 mM isobutylmethylxanthine (IBMX), and 10 mg/mL insulin for 2 days, followed by 10 mg/mL insulin alone. For BMP2 and TI-2 treatment, C3H10T1/2 cells were treated with BMP2 (R&D Systems) or/and TI-2 for 4 days before being subjected to differentiation medium (Fig. 1A). Following differentiation, intracellular lipids were visualized by Oil Red O staining.

Western blotting

Western blotting was performed as previously described [Dao et al., 2012]. Primary antibodies were used as follows: TAK1 (Cell Signaling Technology, Danvers, MA, USA) and β-actin (Sigma-Aldrich, St. Louis, MO, USA).

Quantitative real-time RT-PCR

The procedure was conducted as previously reported [Zhang et al., 2016]. Briefly, RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reversely transcribed into cDNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Primers (Supplementary Table 1) specific to adipocyte differentiation genes were used to perform Real-time PCR on a Rotor-Gene 6000 real-time DNA amplification system (Qiagen, Valencia, CA) using the PerfeCTa SYBR Green SuperMix (Quanta BioSciences, Inc., Gaithersburg, MD).

Luciferase reporter assays

C3H10T1/2 cells were cotransfected with 100 ng PTK-3XPPRE luciferase, 100 ng pCMX-PPARγ, 20 ng pCMX-RXR and 5 ng SV40-Renilla using X-tremeGENE Transfection Reagents (Roche). Plasmids were provided by Dr. Peter Tontonoz lab [Villanueva et al., 2011]. 48 hours after transfection, cells were treated with DMSO or 1 μM Rosiglitazone (Cayman Chemical) for another 24 hours. Luciferase activity was determined with the Dual-Promoter Luciferase Assay Kit (Promega, Madison, WI, USA) and normalized to Renilla luciferase [Gao et al., 2013].

Statistics

Data are presented as the mean ± s.e.m. Statistical significance was determined by Student's t-test and p values of less than 0.05 were considered significant.

Results

TAK1 inhibition represses adipocyte commitment of MSCs

To test the role of TAK1 in adipocyte commitment, we first treated C3H10T1/2 MSCs with BMP2 and/or a TAK1 inhibitor, TI-2 (5Z-7-oxozeaenol) during adipocyte differentiation (Fig. 1A). Consistent with previous reports, BMP2 induced adipocyte differentiation evident by the presence of a high level of lipid droplets in the cultures. Cells treated with TI-2, however, had much less lipid droplet accumulation, suggesting that TAK1 is required for BMP2-induced adipocyte differentiation (Fig. 1B). To further confirm the role of TAK1 in adipocyte differentiation, we used *Tak1* siRNA to knockdown TAK1, the efficiency of which was determined by Western blotting (Fig. 2A). Consistent with the effect of TI-2, TAK1 deficiency reduced BMP2-mediated lipid production (Fig. 2B).

TAK1 is required for terminal differentiation of preadipocytes and adipocyte homeostasis

Subsequent to the finding that TAK1 is required for adipocyte commitment of MSCs, we further investigated whether TAK1 is necessary for terminal differentiation of preadipocytes. Lipid droplets accumulated in a BMP2 concentration dependent manner (Fig. 3A). TAK1 knockdown via siRNA, however, inhibited droplet production at all concentrations of BMP2. The inhibition was maintained until 13 days of differentiation (Fig. 3B). The expression of *Pparγ*, the master regulator of adipocyte differentiation, was significantly decreased by TAK1 ablation (Fig. 3C). These results collectively revealed that TAK1 is also required for adipocyte terminal differentiation. To determine whether TAK1 functions in adipocyte maintenance *in vivo*, we injected TI-2 into mice and found that TAK1 inhibition reduced the expression of adipocyte markers, *Pparγ*, *Cebpα* and *Fabp4* in visceral fat six days following injection (Fig. 3D). Furthermore, we also generated adipocyte-specific TAK1-KO mice (*aP2-Cre*$^{+/-}$; *Tak1*$^{fx/fx}$). When subjected to a high fat diet, *Tak1-KO* mice not only displayed reduced weight gaining but also increased glucose tolerance (Fig. 4). These results suggest that TAK1 also plays an important role in fat deposition *in vivo*.

TAK1 signaling is necessary and sufficient to promote PPARγ transcriptional activity

While previous studies have shown that co-overexpression of TAK1 and TAB1 in C3H10T1/2 increases PPARγ transcriptional activity [Hata et al., 2003], detailed regulation is still unclear. Here, we transfected C3H10T1/2 cells with *Tak1* siRNA, and assayed for PPARγ transcriptional activity in the presence or absence of PPARγ activator, Rosiglitazone. Although TAK1 knockdown only slightly, but not significantly, decreased PPARγ activity, TAK1 knockdown reduced PPARγ activity more than two-fold in the presence of Rosiglitazone (Fig. 5A). We next transfected cells with TAK1 or/and TAB1 overexpression plasmids. TAK1 alone was sufficient to enhance PPARγ activity in both the absence and presence of Rosiglitazone (2.8- and 4.9-folds, respectively). In contrast, TAB1 significantly promoted PPARγ activity only in the presence of Rosiglitazone treatment (2.6-fold). Intriguingly, co-expression TAK1 and TAB1 synergistically promoted PPARγ activity with or without Rosiglitazone (17.7- and 48-folds, respectively; Fig. 5B).

Discussion

Using mesenchymal progenitor cells and preadipocytes, we provide data to demonstrate that TAK1 is required to mediate BMP2-induced adipocyte commitment and terminal differentiation. Additionally, we provide evidence to support that, *in vivo,* TAK1 is necessary for adipocyte maintenance. Furthermore, we provide molecular data to support that TAK1 is required for PPARγ transcriptional activity and that TAB1 synergizes with TAK1 to further activate PPARγ.

To determine the role of TAK1-dependent BMP2 signaling in adipocyte determination and terminal differentiation, we conducted adipocyte differentiation assays in C3H10T1/2 mesenchymal progenitor cells and 3T3-L1 preadipocytes, respectively. First, inhibition of TAK1 activity reduced BMP2-induced adipocyte commitment of C3H10T1/2 cells. Additionally, loss of TAK1 via RNA interference repressed adipogenesis of these cells at different BMP2 concentrations, even though lipids accumulated in a BMP2 concentration-dependent manner. These results collectively suggested that TAK1 plays an essential role in adipocyte commitment of MSCs. Prior findings revealed that inhibition of p38-ATF2 signaling blocked adipocyte differentiation of C3H10T1/2 cells [Huang et al., 2009; Maekawa et al., 2010]. Since p38-ATF2 signaling is activated downstream of TAK1 [Yamaguchi et al., 1995], we asked whether TAK1 regulates adipocyte terminal differentiation. To do this, we ablated TAK1 through siRNA in 3T3-L1 preadipocytes and found that TAK1 is also required for adipogenesis, consistent with the role of p38 in terminal differentiation [Engelman et al., 1998]. Collectively, our findings support that TAK1 is crucial for the phases of commitment and terminal differentiation during adipogenesis [Rosen and MacDougald, 2006]. Finally, blocking TAK1 in mice downregulated the expression of adipocyte differentiation markers including *Pparγ*, *Cebpa* and *Fabp4* indicating that TAK1 is not only critical for adipogenesis *in vitro*, but also significant for the maintenance of the adipocyte *in vivo*. Intriguingly, p38 inhibitors were found to attenuate high fat diet-induced adipocyte size, insulin resistance and hyperlipidemia, symptoms of obesity [Maekawa et al., 2010]. The phenotype is similar to the phenotypes of Tak1-KO which showed reduced weight gaining on high fat diet and also

increased glucose tolerance. Whether these mice also display a decreased risk of obesity-related diseases including insulin resistance, hyperglycemia and liver fibrosis will be a focus of future research. Of note, our *in vivo* findings support those recently published by Sassmann-Schweda, *et. al.* where acute, postnatal, ablation of *Tak1* using the *Adipoq-CreER^{T2}* transgene resulted in reduced weight gain and increased glucose tolerance following exposure to a high-fat diet [Sassmann-Schweda et al., 2016].

Mechanistically, our data demonstrate that TAK1 is necessary and sufficient to promote PPARγ transcriptional activity. Even though TAK1 interference through siRNA only led to a slight reduction in PPARγ activity, TAK1 interference disrupts PPARγ activation induced by Rosiglitazone. Furthermore, our findings not only support that TAK1 or TAB1 alone are sufficient to increase PPARγ activity, but also that TAK1 and TAB1 synergistically contribute to PPARγ activation. This synergistic effect is possibly because overexpressed TAB1 directly binds and activates TAK1 [Kishimoto et al., 2000; Shibuya et al., 1996], which further amplifies downstream cellular signaling and eventually leads to PPARγ activation. Interestingly, activation of p38, has been shown to be required for maintaining PPARγ protein stability, but proteasome inhibitor MG-132 reverses the reduction [Schild et al., 2006], which suggests that TAK1 possibly promotes PPARγ activity through p38-mediated PPARγ protein stabilization. Beyond the significant role in adipogenesis, PPARγ activation also leads to myriad benefits such as insulin sensitization in skeletal muscle [Mayerson et al., 2002], inflammation reduction in the heart [Ahmadian et al., 2013] and gluconeogenesis in the liver [Gavrilova et al., 2003]. Whether activating TAK1 serves as an alternative approach to produce these beneficial effects is open to questions.

In conclusion, our findings support that TAK1 is a key player in BMP-mediated adipogenesis. Our findings lead us to suggest the model depicted in Fig. 5C. BMPs bind to BMPRI and BMPRII heteromeric complexes resulting in the phosphorylation of SMAD1/5/8 [Hata et al., 2003; Huang et al., 2009] which then forms a complex with SMAD4 to enhance gene expression and adipogenesis. Meanwhile, TAK1 is also phosphorylated and forms a complex with TAB1. The p-TAK1/TAB1 complex upregulates PPARγ transcriptional activity, and contributes to adipogenesis. Collectively, our findings support that TAK1 serves as a novel therapeutic target for obesity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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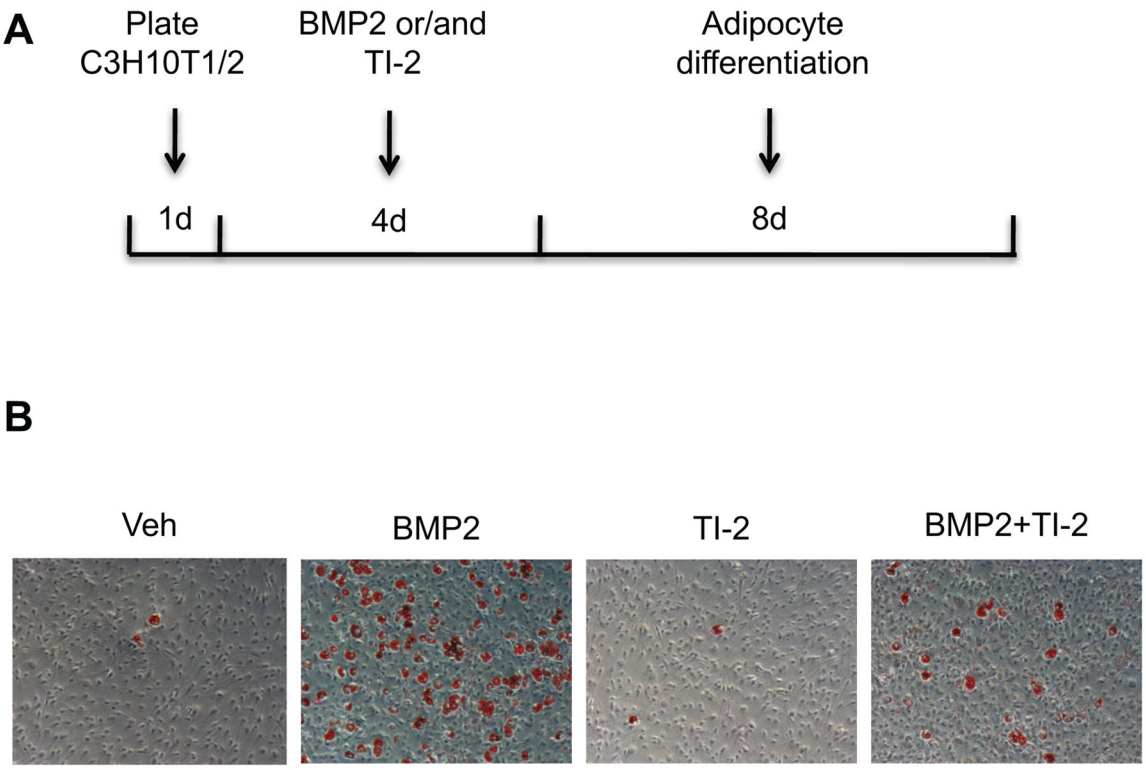

Fig. 1.

Inhibition of TAK1 reduces BMP2-mediated adipocyte differentiation of mesenchymal progenitor cells. (A) C3H10T1/2 cells were plated on Day 1 and then treated with 50 ng/mL BMP2 and/or 0.5 μM TI-2 for 4 days. Cells were then cultured in adipocyte differentiation medium for 8 days and harvested following differentiation. (B) C3H10T1/2 cells were stained with Oil Red O after 8 days of differentiation. Veh, vehicle.

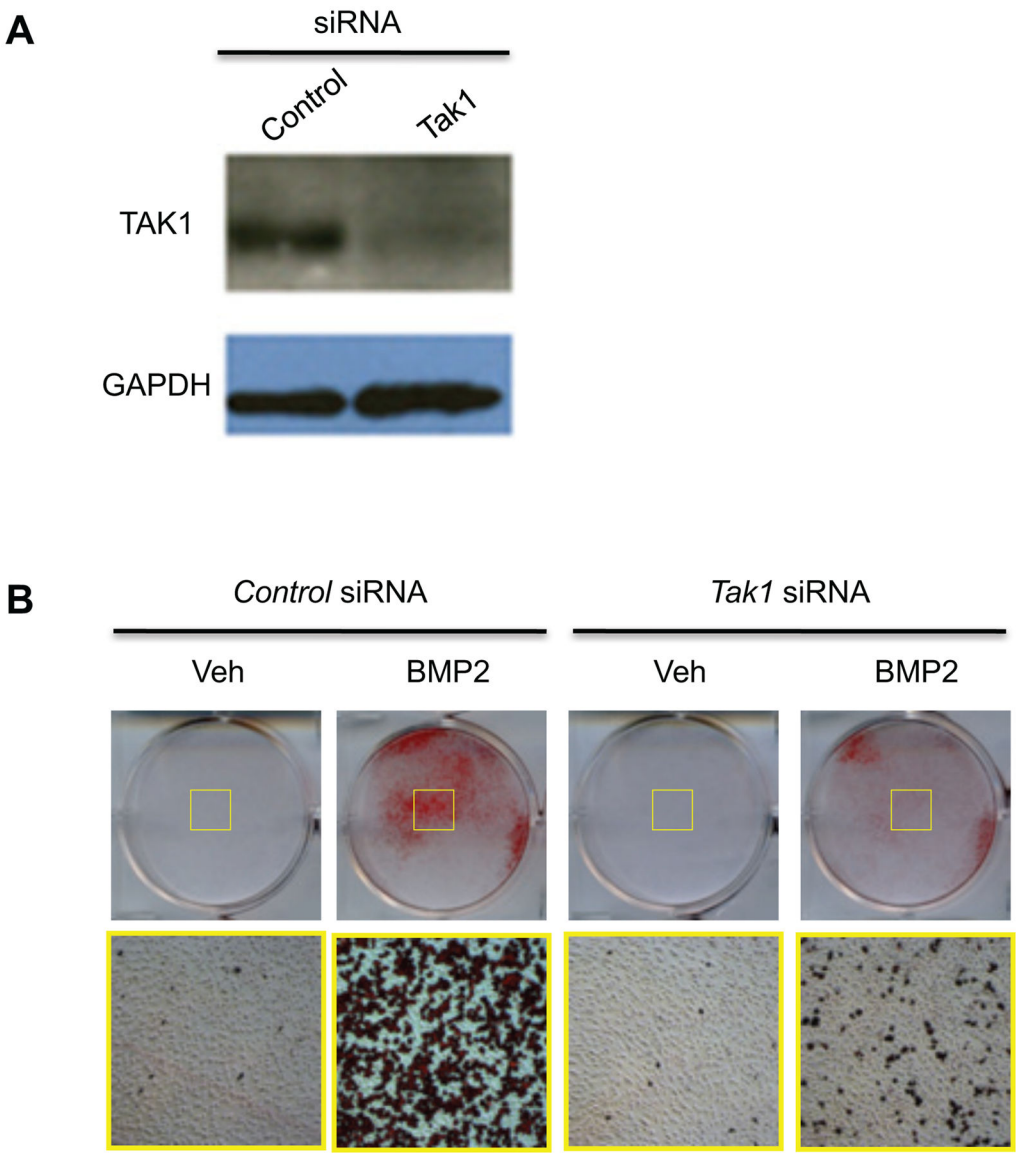

Fig. 2.

TAK1 knockdown reduces BMP2-mediated adipocyte differentiation of C3H10T1/2. (A) C3H10T1/2 cells were transfected with control or *Tak1* siRNA. 3 days later, TAK1 protein levels were determined by Western blotting. (B) C3H10T1/2 cells were transfected with control or *Tak1* siRNA and cultured in differentiation medium with vehicle or 50 ng/mL BMP2 for 8 days. Cells were then stained with Oil Red O.

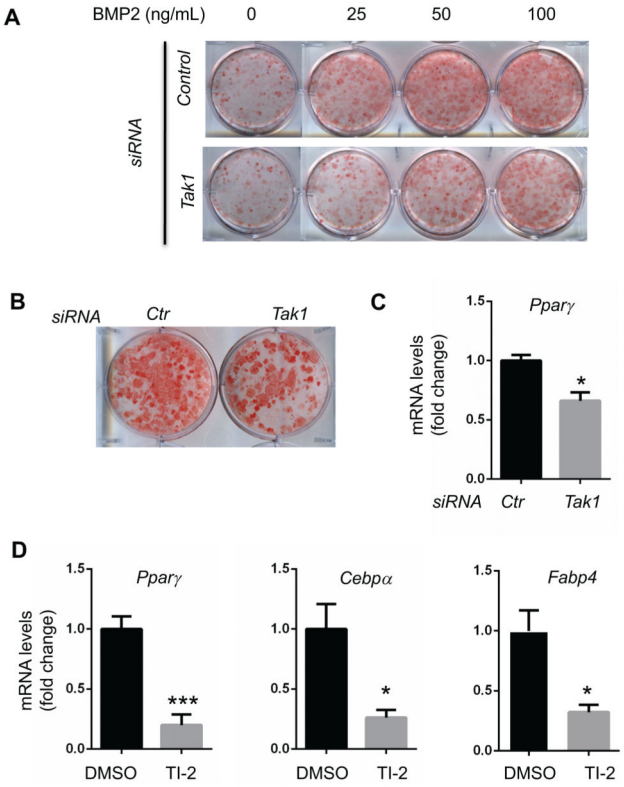

Fig. 3.

TAK1 knockdown reduces differentiation of 3T3-L1 preadipocytes. (A) 3T3-L1 cells were transfected with control or *Tak1* siRNA, and then cultured in differentiation medium at 0, 25, 50 and 100 ng/mL BMP2. Following 8 days of differentiation, cells were stained with Oil Red O. (B, C) 3T3-L1 cells were transfected with control or *Tak1* siRNA, and stained with Oil Red O after 13 days of adipocyte differentiation. Pparγ mRNA was also measured. (D) Wild type mice were administrated with control DMSO (n=4) or TI-2 (n=3) for 6 consecutive days. Visceral fat was collected and mRNA for the indicated genes was measured. Ctr, control; $*P < 0.05$; $***P < 0.001$.

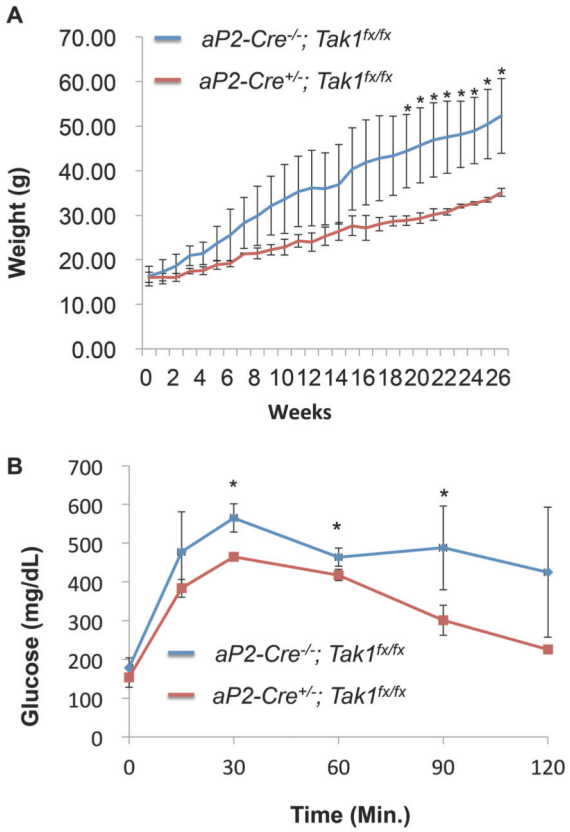

Fig. 4.

TAK1 LOF leads to reduced body weight gaining and glucose tolerance. (A) 5-week old mice were fed with high fat diet (60% kcal) and body weight was measured every week. (B) After 26 weeks of diet feeding, glucose tolerance test were performed. * $P < 0.05$.

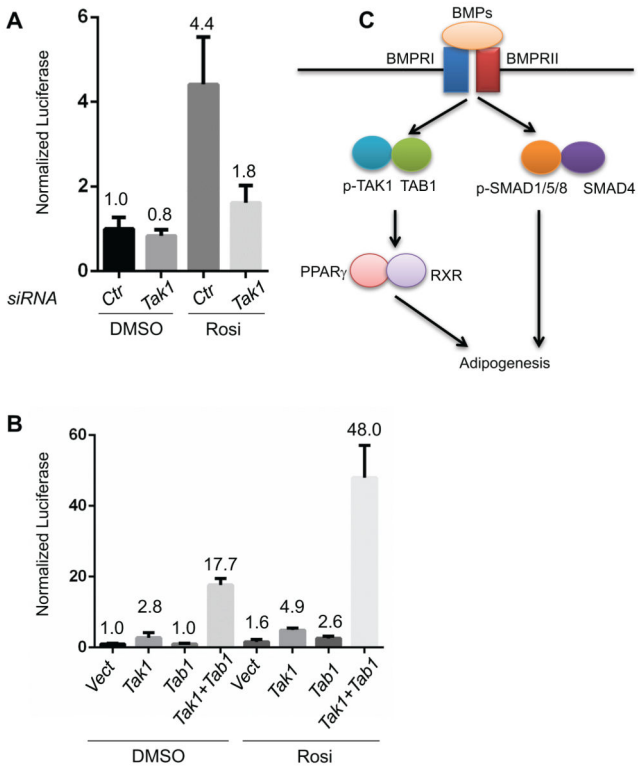

Fig. 5.

TAK1 and TAB1 synergistically upregulate PPARγ transactivation activity. (A) C3H10T1/2 cells were transfected with control or *Tak1* siRNA, and then transfected with PTK-3XPPRE-luciferase, pCMX-PPARγ, pCMX-RXR, and Renilla control vector. 48 hours later, cells were treated with DMSO or Rosiglitazone for twenty hours. Firefly luciferase activity was normalized to Renilla luciferase. (B) C3H10T1/2 cells were transfected with control, TAK1, TAB1 or TAK1 combined with TAB1 vectors. Firefly luciferase activity was measured. (C) Model for BMP signaling in adipocyte differentiation. Binding of BMPs to BMPRI and BMPRII leads to the phosphorylation of SMAD1/5/8 (p-SMAD1/5/8) and TAK1 (p-TAK1). p-SMAD1/5/8 forms a complex with SMAD4 to induce adipocyte differentiation; p-TAK1 and TAB1 complex upregulates pCMX-PPARγ transcriptional activity, and hence adipogenesis. Ctr, control; Vect, empty vector; Rosi, Rosiglitazone.