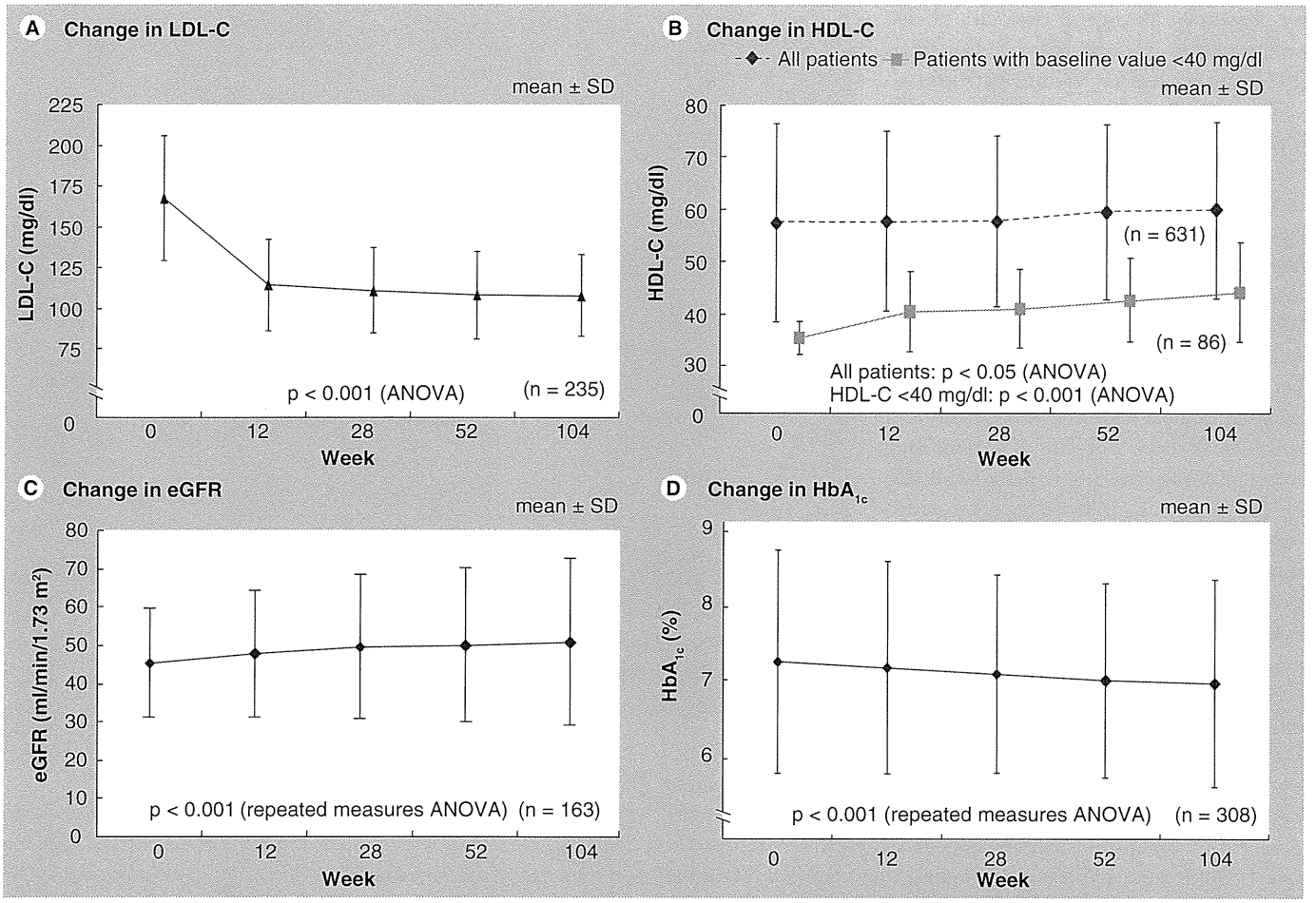

Figure 1. Time course changes in clinical factors in the Livalo Effectiveness and Safety study.
ANOVA: Analysis of variance; EGFR: EGF receptor; HbA_{1c}: Glycohemoglobin A_{1c}; HDL-C: HDL-cholesterol; LDL-C: LDL-cholesterol; SD: Standard deviation.
Adapted with permission from [4,12,13].

improving eGFR may be related to its effect on HDL-C elevation, but not its effect on LDL-C lowering. It is unclear whether pitavastatin increases the eGFR directly or is potentially mediated by effects on HDL-C, for example, improvement of endothelial function, anti-inflammatory and antioxidative function [19,28–30]. Elucidating this mechanism may be the key to understanding the renoprotective effect of pitavastatin. At any rate, the favorable effect of pitavastatin on eGFR is notable, since renal function would otherwise gradually deteriorate with age alone.

It is well established that lowering LDL-C with statins reduces the CVD risk in patients with diabetes mellitus. At the same time, it is debated whether statins affect glucose tolerance, since some reports have pointed out that statin treatment may be associated with impaired glycemic control (reviewed in [31]). The LIVES study subanalysis on hypercholesterolemic subjects with diabetes mellitus demonstrated that better glycemic control could be achieved as evaluated by the HbA_{1c} levels during a 2-year period when the subjects were on a daily dose of pitavastatin 1–4 mg. Recently, the results of meta-analyses of randomized trials suggested that statin treatment increases the risk of new-onset diabetes mellitus, especially in elderly subjects [32,33]. Further clinical investigation is warranted; however, a currently ongoing prospective trial named Japan Prevention Trial of Diabetes by Pitavastatin in Patients with Impaired Glucose Tolerance (J-PREDICT; NCT00301392) may provide an answer. The trial aims to clarify the influence of pitavastatin on glucose metabolism in 1240 patients with impaired glucose tolerance.

Five-year view

Pitavastatin, after initially being launched in Japan, has since been used in other Asian countries, the USA and Europe. Therefore, the LIVES study data on approximately 20,000 Japanese hypercholesterolemic patients provides useful information for clinicians and patients all over the world.

While there is good evidence concerning the safety and efficacy of pitavastatin in lipid lowering, solid data are still lacking on its effectiveness in preventing cardiovascular events. However, the results of surrogate marker studies suggest that pitavastatin is as effective as other statins already on the market. For example, the Japan Assessment of Pitavastatin and Atorvastatin in Acute Coronary Syndrome (JAPAN-ACS) study showed the noninferiority of pitavastatin 4 mg daily compared with atorvastatin 20 mg daily in promoting coronary plaque regression. This was evaluated

by percentage change in coronary plaque volume using intravascular ultrasound in 307 acute coronary syndrome patients [34].

The LIVES study – initially planned for 2 years – has been extended for 3 more years with the name of the LIVES study extension (Figure 2). The aim of the extension study is to examine the incidence rates of cerebrovascular and cardiovascular events as well as sudden death in patients treated with pitavastatin. It will examine nearly 7000 patients during a follow-up period of 5 years. The relationship between these events and lipid parameters or other clinical factors will be assessed in this investigation. The rate of adverse drug reaction during the 5 year period will also be evaluated. The follow-up period ended on March 31, 2010, and the results will be reported in the near future; these results will possibly provide the first pieces of outcome evidence for the effectiveness of pitavastatin.

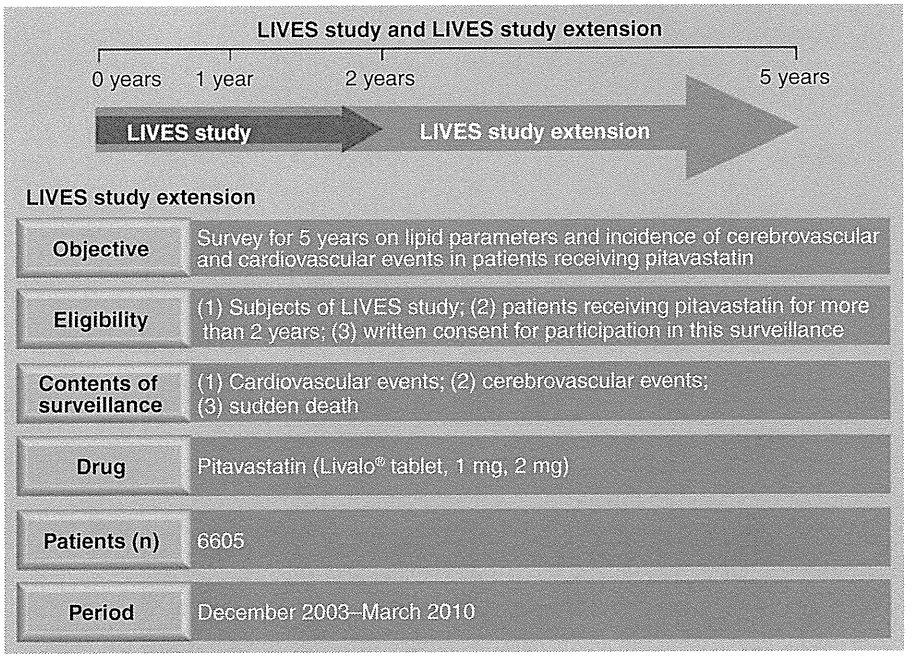

Figure 2. LIVES study and LIVES study extension.
LIVES: Livalo Effectiveness and Safety.
Adapted with permission from [4].

Conclusion

The LIVES study provided the rationale for the safety and efficacy of newly developed pitavastatin based on the data of approximately 20,000 Japanese hypercholesterolemic patients treated for 2 years. In addition to its potent effect on LDL-C lowering, pitavastatin treatment resulted in significant elevation of HDL-C in all study subjects and eGFR in the CKD subgroup. The ongoing LIVES study extension is expected to further provide data on cardiovascular outcomes in subjects treated with pitavastatin. The LIVES study was an observational study based on approximately 20,000 Japanese hypercholesterolemia patients treated with pitavastatin for 2 years.

Financial & competing interests disclosure

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Key issues

- The Livalo Effectiveness and Safety (LIVES) study was an observational study based on approximately 20,000 Japanese hypercholesterolemia patients treated with pitavastatin for 2 years.
- No significant problems concerning safety were observed in association with pitavastatin treatment.
- The demographic characteristics of patients that affect incidence of adverse drug reactions became clear.
- Pitavastatin showed a potent and stable lowering of LDL-cholesterol.
- HDL-cholesterol continuously increased during the 2 years of follow-up upon treatment with pitavastatin.
- Pitavastatin treatment was associated with an increase in estimated glomerular filtration rate in patients with chronic kidney disease.
- Pitavastatin treatment did not adversely affect glycemic control as evaluated using the glycohemoglobin A_{1c} levels in patients with diabetes mellitus and hypercholesterolemia.

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The roles of transforming growth factor-β and Smad3 signaling in adipocyte differentiation and obesity

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ABSTRACT

We aimed at elucidating the roles of transforming growth factor (TGF)-β and Smad3 signaling in adipocyte differentiation (adipogenesis) and in the pathogenesis of obesity. TGF-β/Smad3 signaling in white adipose tissue (WAT) was determined in genetically obese (ob/ob) mice. The effect of TGF-β on adipogenesis was evaluated in mouse embryonic fibroblasts (MEF) isolated both from WT controls and Smad3 KO mice by Oil red-O staining and gene expression analysis. Phenotypic analyses of high-fat diet (HFD)-induced obesity in Smad3 KO mice compared to WT controls were performed. TGF-β/Smad3 signaling was elevated in WAT from ob/ob mice compared to the controls. TGF-β significantly inhibited adipogenesis in MEF, but the inhibitory effects of TGF-β on adipogenesis were partially abolished in MEF from Smad3 KO mice. TGF-β inhibited adipogenesis independent from the Wnt and β-catenin pathway. Smad3 KO mice were protected against HFD-induced insulin resistance. The size of adipocytes from Smad3 KO mice on the HFD was significantly smaller compared to the controls. In conclusion, the TGF-β/Smad3 signaling pathway plays key roles not only in adipogenesis but also in development of insulin resistance.

1. Introduction

Obesity, defined as an excess amount of body fat, is associated with metabolic disorders, such as type 2 diabetes, dyslipidemia, and hypertension [1], which eventually increase morbidity and mortality all over the world. Previously, the adipose tissue was considered as a storage organ for excessive energy; however, recent scientific progress has shed light on the crucial roles of adipocytes in not only whole body insulin sensitivity but also energy homeostasis [2].

White adipose tissue (WAT), a predominant type of fat distributed throughout the body, secretes a number of molecules that are now defined as adipokines [3]. In obesity, adipocytes undergo hypertrophy, which leads to dysregulation of WAT-mediated glucose and lipid disposal and an imbalanced secretion of adipokines, contributing to the development of hyperglycemia, insulin resistance, and dyslipidemia.

Adipocyte differentiation is controlled by a complex network of transcriptional factors, including members of the CCAAT/enhancer-binding protein (C/EBPs) and peroxisome proliferator-activated receptor γ (PPAR γ) family [4]. Indeed, elucidating the molecular mechanisms underlying adipogenesis is crucial for the development of more effective therapies for obesity in order to prevent metabolic diseases.

The transforming growth factor beta (TGF-β) plays important roles in the progression of a variety of diseases such as diabetic complications [5,6], atherosclerosis [7], and cancer [8]. TGF-β exerts its biological functions mainly through its downstream signaling molecules, the Smads [9]. It has been reported that TGF-β has a broad spectrum of biological functions and actions in a variety of cell types, but its role in the process of adipogenesis has not been fully elucidated.

In the present study, we aimed to investigate the pathophysiological roles of TGF-β/Smad3 signaling in adipocyte differentiation. For this purpose, we first examined the expression of TGF-β signaling in adipose tissue of genetically obese mice (ob/ob mice). We then investigated the role of TGF-β in adipogenesis both *in vitro* and *in vivo* using Smad3 knockout (KO) mice.

2. Materials and methods

2.1. Reagents, animals and tissue preparation

Reagents used are described in the expanded Materials and Methods section.

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2.2. Real-time quantitative PCR and reverse transcription PCR

Real-Time quantitative PCR (real-time PCR) and reverse transcription PCR (RT-PCR) were performed as described previously [10]. See expanded Materials and Methods section for details.

2.3. Immunohistochemistry and immunocytochemistry

See the expanded Materials and Methods section.

2.4. Nuclear extraction and immunoblotting

See expanded Materials and Methods section for details.

2.5. Cell culture

Mouse embryonic fibroblasts (MEF) cells were established from E13.5 embryos. See expanded Materials and Methods section for details.

2.6. Measurement of triglyceride (TG) contents in MEF

The TG content of MEF was measured with a Triglyceride Quantification kit (BioVision, USA) according to the manufacturer's instructions.

2.7. Retroviral infection

Immortalized white pre-adipocyte HW cells were kindly provided by Prof. Masayuki Saito (Tenshi College, Sapporo, Japan) and differentiated into mature adipocytes as described previously [11]. See expanded Materials and Methods section for details.

2.8. Insulin tolerance test (ITT)

Eight-week-old male Smad3 KO and littermate controls were placed on a HFD for 8 weeks. An intraperitoneal ITT using 5 units of insulin/kg was performed in mice fasted for 16 h. Blood samples were collected at 0, 15, 30, 60, 90, and 120 min after insulin injection.

2.9. Statistical analysis

Results were presented as mean ± SEM. Statistical analyses used a 2-tailed unpaired Student *t*-test.

3. Results

3.1. TGF-β/Smad3 signaling pathway is activated in the WAT from ob/ob mice

In order to investigate the roles of TGF-β signaling in obesity and adipogenesis, we initiated our study by analyzing the expression of TGF-β in WAT from genetically obese mice (ob/ob mice). Epididymal fat pad were dissected from 12-week-old ob/ob mice and WT control mice and subjected to real-time PCR and immunohistochemisty. As shown in Fig. 1A, the expression of TGF-β mRNA was 2.3-fold higher in ob/ob mice than in the WT controls. We could also localize the TGF-β protein to the crown-like structure seen in ob/ob mice but not in WT controls, as confirmed by immunohistochemistry (Fig. 1B). On the other hand, there was no significant difference in the mRNA expression of TGF-β type 1 receptor (Alk-5) and type 2 receptor in WAT between ob/ob mice and controls (Fig. 1C). Next, we examined the phosphorylation of Smad3 (p-Smad3) in order to evaluate the activation of TGF-β signaling in WAT. The nuclear fractions of WAT were extracted and subjected to immunoblotting using a specific antibody against p-Smad3 protein. As shown in Fig. 1D, p-Smad3 was 8-fold higher in WAT of ob/ob mice compared to WT controls. These results indicated that activation of TGF-β/Smad3 signaling might play a role in the pathogenesis of obesity and/or adipogenesis.

3.2. TGF-β/Smad3 signaling inhibits adipogenesis in vitro

Next, we examined the effects of TGF-β signaling on adipogenesis. For this purpose, we isolated MEF both from WT and Smad3 KO mice. Adipocyte differentiation was then induced in these cells by a hormonal stimulus in the presence or absence of 1 ng/mL TGF-β. Eight days after hormonal stimulation, the adipocyte differentiation was evaluated by Oil Red-O staining. As shown in Fig. 2A, TGF-β completely inhibited the accumulation of lipids in MEF from WT, whereas the inhibitory effects of TGF-β on lipid accumulation were attenuated by the lack of Smad3 in MEF. Consistent with this observation, TGF-β profoundly decreased the amounts of TG contents in MEF from WT after the induction of adipocyte differentiation; however, the inhibitory effects of TGF-β on the accumulation of TG in MEF was significantly abolished by the lack of Smad3 as shown in Fig. 2B. These results indicated that TGF-β inhibited adipogenesis partially through the Smad3-dependent pathway. Next, we examined the expression of transcriptional factors that have been reported to regulate adipogenesis, such as C/EBPα, C/EBPβ, C/EBPδ, PPARγ and aP2 by RT-PCR. C/EBPβ and C/EBPδ are expressed in earlier phases of adipogenesis and cooperate in inducing expression of C/EBPα, PPARγ, and aP2, which are known to be involved in terminal differentiation. As shown in Fig. 2C, the expression of C/EBPα, PPARγ and aP2 were significantly suppressed in the presence of TGF-β in WT controls, while the expression of neither C/EBP β nor C/EBP δ was changed (data not shown). In the Smad3 KO MEF, the inhibitory effects of TGF-β on the expression of C/EBPα, PPARγ, and aP2 were attenuated significantly.

3.3. TGF-β inhibits adipogenesis independent from Wnt and β-catenin signaling

Among several pathways known to inhibit adipogenesis, we examined the functional relationship between the Wnt/β-catenin pathway and TGF-β signaling, since the cross-talk between TGF-β/Smad3 and Wnt/β-catenin signaling pathways had been reported during chondrocyte development [12]. Wnts are a family of proteins that affect cell fate and differentiation, including myogenesis, neurogenesis, and mammary development [13]. When Wnt signaling is activated, the kinase activity of glycogen synthase kinase 3 (GSK3) is inhibited, which allows cytosolic β-catenin to accumulate and translocate to the nucleus and activate transcription of Wnt target genes.

At first, we evaluated the effects of TGF-β on the translocation of β-catenin in MEF. As shown in Fig. 3A and B, the cytoplasmic β-catenin translocated into the nucleus in the presence of 1 ng/mL TGF-β in MEF from WT controls but not from the Smad3 KO mice. Next, we examined the effects of TGF-β on adipocyte differentiation in the presence of Chibby, which has been reported to inhibit β-catenin-mediated transcriptional activation [14]. Over-expression of Chibby in HW cells was confirmed by RT-PCR (Fig. 3C). Next, Chibby-infected HW cells were induced to differentiate in the presence or absence of TGF-β, and the adipocyte differentiation was evaluated by Oil Red-O staining. TGF-β significantly inhibited HW cell differentiation regardless of the presence of Chibby (Fig. 3D). These results indicated that the TGF-β/Smad3 pathway might physically interact with β-catenin in the course of its translocation into the nucleus; however, TGF-β inhibited adipocyte differentiation independent from the Wnt/β-catenin pathway.

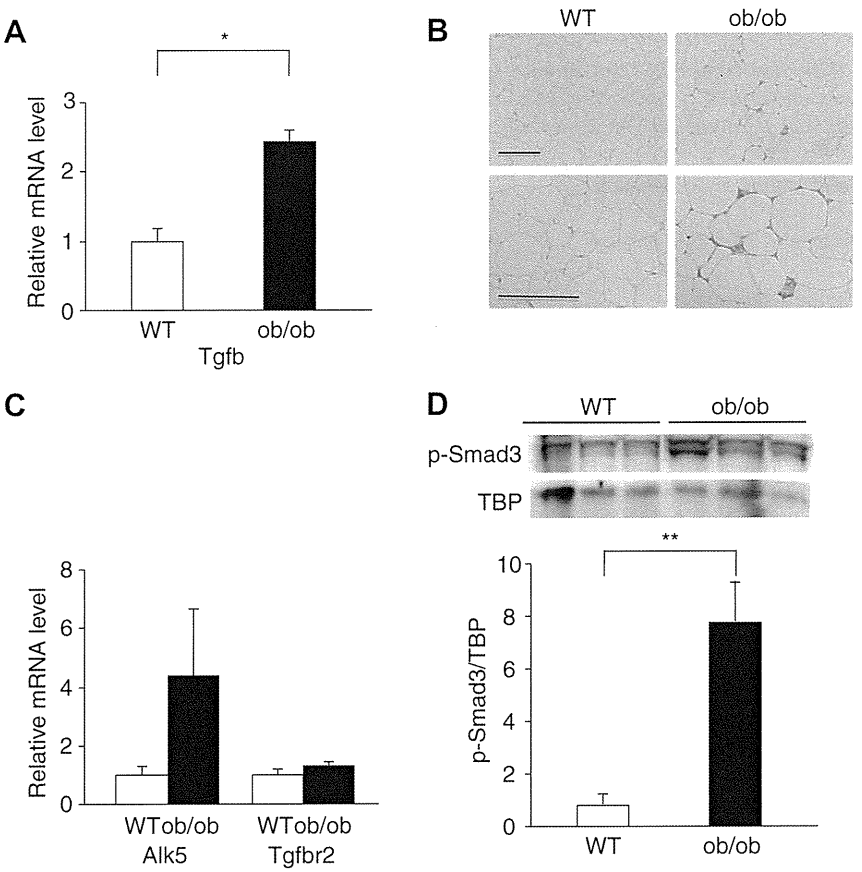

Fig. 1. TGF-β/Smad3 signaling is increased in WAT from ob/ob mice. Epididymal fat fad were dissected from 12-week-old ob/ob mice and control mice and subjected to gene expression analysis (A and C) and immunohistochemistry using an anti-TGF-β-specific antibody (B). The nuclear fractions of WAT were extracted and subjected to immunoblotting using a specific antibody against phospho-Smad3 (D). Anti TATA box binding protein (Tbp) was used as a control for estimating sample loading. Bars, 200 μM. $^{*}p < 0.01$, $^{**}p < 0.05$.

3.4. Smad3 KO mice improve insulin sensitivity on HFD and show smaller-size adipocytes compared to WT

Finally, in order to investigate the roles of TGF-β/Smad3 signaling in obesity and adipogenesis *in vivo*, 8-week-old WT and Smad3 KO mice were placed on a HFD for 8 weeks. Body weight and the amounts of food intake were measured every week. Smad3 KO mice were smaller in size than the littermate controls. As shown in Fig. 4A, the net body weight gain was significantly increased in Smad3 KO mice and caught up to the same body weights compared to the controls after 8-week-HFD, while there was no difference in the amounts of food intake between the 2 groups (data not shown).

We evaluated insulin sensitivity with an ITT and found that insulin sensitivity, especially at 30 min, was significantly better in Smad3 KO mice than in the controls (Fig. 4B). In order to investigate how Smad3 KO mice improved insulin sensitivity on HFD, we dissected WAT out from both Smad3 KO and controls, analyzed it histologically, and examined gene expression by RT-PCR. The weighs of WAT were similar in Smad3 KO and WT (data not shown). Histological analysis revealed that adipocytes from Smad3 KO mice were significantly smaller in size compared to the controls (Fig. 4C and D). However, mRNA expression of adipocyte markers related to differentiation was not changed significantly in the 2 groups (data not shown).

4. Discussion

In the present study, we showed that the expression of p-Smad3 was increased in WAT from ob/ob mice. TGF-β inhibited adipogenesis partially through the Smad3-dependent pathway and independent from the Wnt/β-catenin pathway. Further, we showed that Smad3 KO mice were protected against HFD-induced insulin resistance and the adipocytes from Smad3 KO mice were smaller than the WT controls when they were fed HFD *in vivo*.

TGF-β is a multi-functional growth factor. We have previously reported the important roles of TGF-β/Smad3 signaling in the development of atherosclerosis [7] and diabetic nephropathy [5,6]. In this report, we further analyzed the roles of TGF-β/Smad3 signaling in adipogenesis. The expression of TGF-β mRNA and protein were significantly increased in WAT from ob/ob mice. Moreover, we showed that p-Smad3 was significantly elevated in WAT from obese mice, confirming that the elevated expression of TGF-β functionally activated Smad3 within the obese fat tissues.

Adipogenesis is a complex process dependent on the interplay between extracellular signals and transcriptional cascades. Some factors act promoting adipogenesis, while the others act anti-adipogenic. We showed that TGF-β inhibited adipogenesis partially through the Smad3-dependent pathway. It has been reported that Smad3, which is activated by TGF-β, binds to C/EBPβ and C/EBPδ, inhibits their transcriptional activity. This in turn leads to decreased transcription of PPARγ, a master regulator of adipogenesis, and resulting in inhibition of the process of adipogenesis [15,16]. Since TGF-β was still able to inhibit adipogenesis in Smad3 KO MEF, we assumed that another pathway was involved in the inhibition of adipogenesis independent from the physical interaction of Smad3 and C/EBPs. TGF-β activates not only Smad3 but also Smad2; however, it has already been confirmed that TGF-β inhibited adipogenesis independent from Smad2 [16].

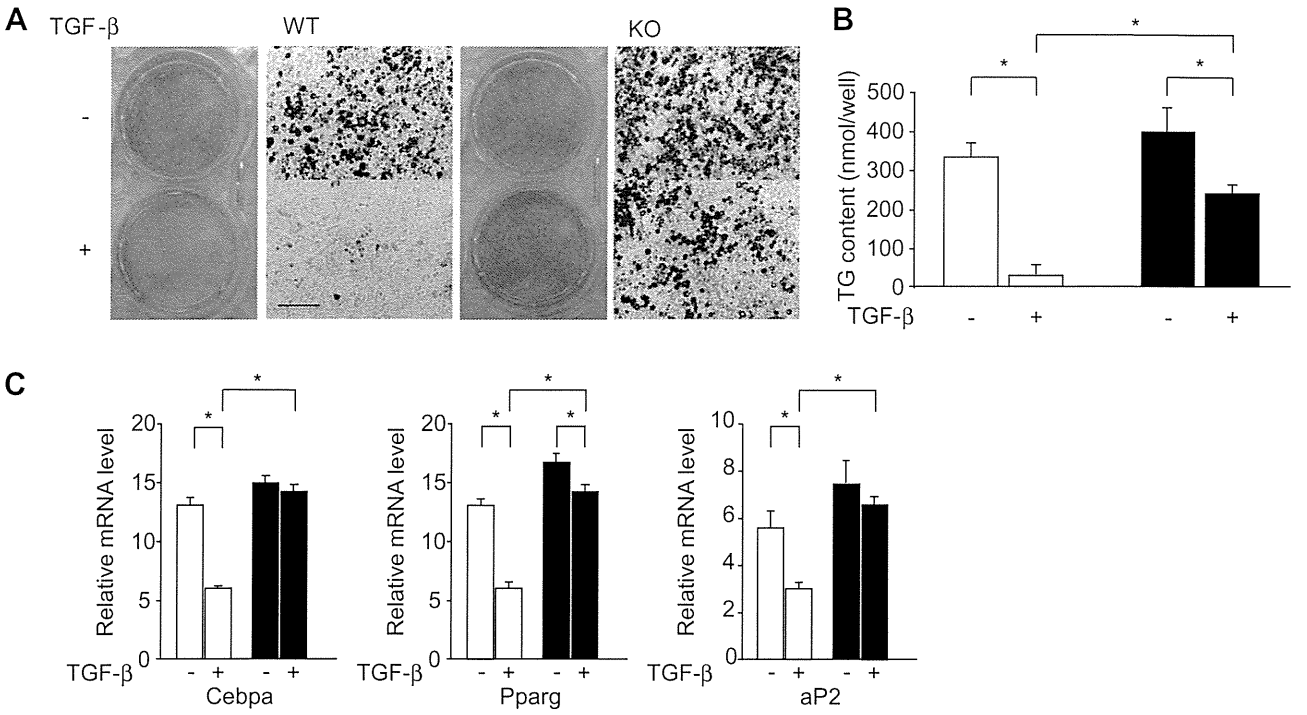

Fig. 2. TGF-β/Smad3 signaling inhibits adipocyte differentiation in MEF. Primary MEF were isolated both from Smad3 KO mice and WT controls. MEF were induced to differentiate by hormonal stimulation in the presence or absence of TGF-β. Lipid accumulation was evaluated by Oil Red-O staining (A) and the TG contents were evaluated (B) The expression of adipocyte differentiation markers was also evaluated by RT-PCR at day 8 after the initiation of adipogenesis (C). Open squares indicated MEF from WT and closed squares indicated MEF from Smad3 KO (B and C). $^{*}p < 0.01$.

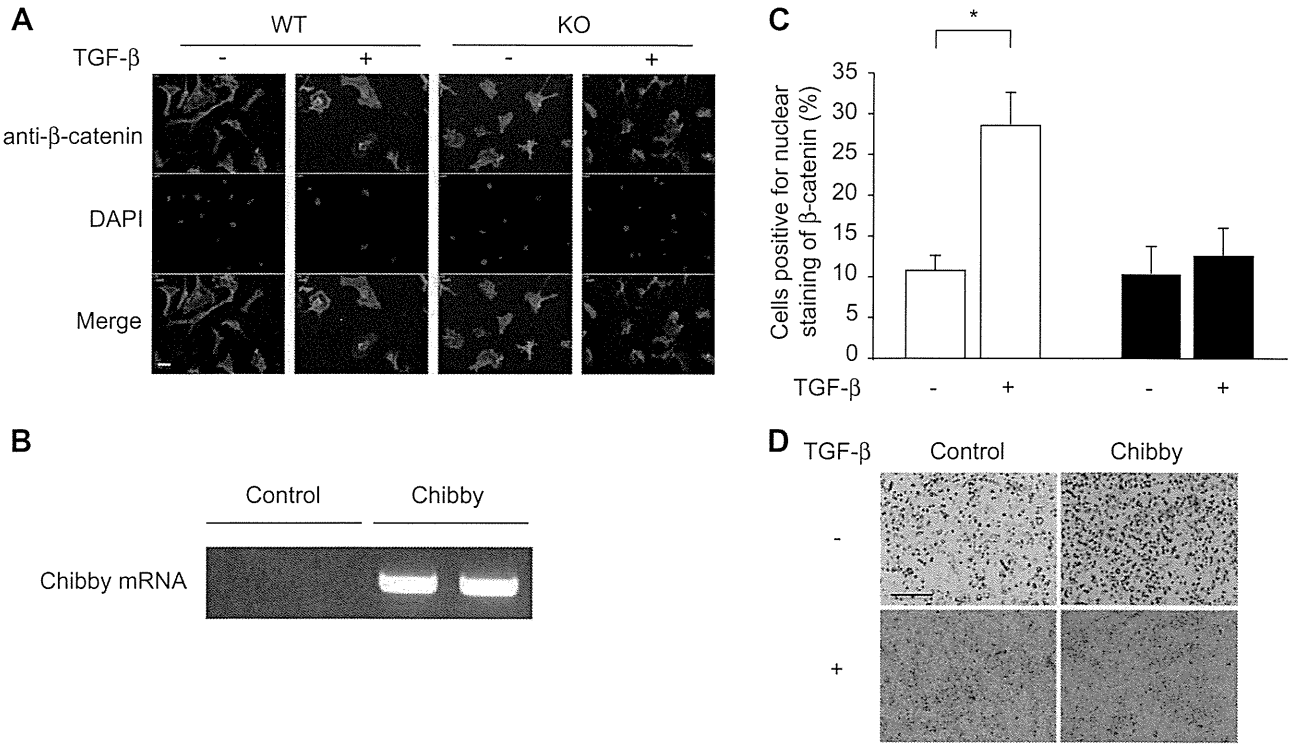

Fig. 3. TGF-β/Smad3 inhibits adipogenesis independent from the Wnt-β/catenin signaling pathway. (A) The effect of TGF-β on the translocation of β-catenin was evaluated in WT MEF and Smad3 KO MEF. The cells were stimulated with 1 ng/mL TGF-β for 1 h, fixed with ice-cold methanol, and subjected to immunocytochemistry using an anti-β-catenin-specific antibody. Cells with nuclear β-catenin were counted and compared among the groups (B). A pre-adipocyte cell line, HW cell, was transfected with Chibby, an inhibitor of β-catenin signaling (C), and differentiation was induced in the presence or absence of 1 ng/mL TGF-β for 8 days. Lipid accumulation was evaluated by Oil Red-O staining (D).

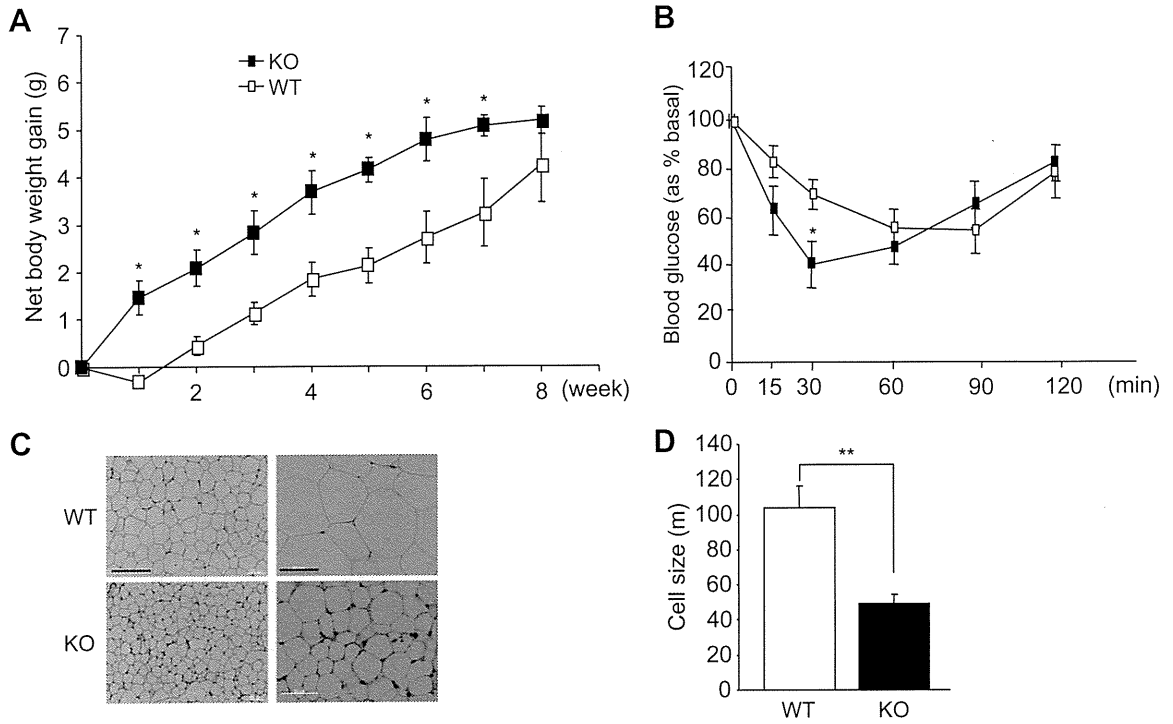

Fig. 4. Smad3 KO mice are protected against HFD-induced insulin resistance and show smaller-size adipocytes after administration of the HFD. Eight-week-old Smad3 KO mice ($n = 5$) and WT controls ($n = 5$) were placed on the HFD for 8 weeks. The body weights (A) was evaluated every week. After 8 weeks on the HFD, ITTs were performed (B). After 8 weeks on the HFD, the epididymal fat fad were dissected out and subjected to histological and gene expression analysis. C: H&E staining. The size of adipocytes was estimated under the light microscope. At least 10 high power fields were selected at random in each animal, the size was measured (D). Bar, 200 μM (C, left) and 50 μM (C, right). $^{*}p < 0.05$, $^{**}p < 0.01$.

Many transcriptional factors have been reported to repress adipogenesis, including GATA2, ETO/MTG8, GLIZ, DIPA, CHOP10, KLF2, and FOXO1 [4]. There are also extracellular signalings, such as Wnt [17] and sonic hedgehog [18], which inhibit adipogenesis. In human mesenchymal stem cells, TGF-β induces nuclear translocation of β-catenin, a major downstream molecule of canonical Wnt signaling, in a Smad3-dependent manner [19]. Thus, we examined the involvement of the Wnt/β-catenin pathway in TGF-β/Smad3 signaling-dependent anti-adipogenesis. In the presence of Chibby, TGF-β was still able to inhibit adipogenesis completely. This result indicated that the Wnt/β-catenin pathway was not involved in anti-adipogenic effects induced by the TGF-β/Smad3. It has been reported that retinoic acid, which is known as a strong inhibitory factor of adipocyte differentiation, also acts in cooperation with Smad3 in adipocytes [20]. Furthermore, a Smad3-independent TGF-β signaling pathway has also been reported [21]. Therefore, TGF-β may be able to inhibit adipogenesis independent from Smad3.

Increased expression of TGF-β in obese mice suggested that the TGF-β/Smad3 signaling contributes to insulin resistance in obesity. Therefore, we investigated the roles of TGF-β/Smad3 signaling in adipogenesis and insulin resistance *in vivo* using Smad3 KO mice. It has been reported that fasting blood glucose levels do not differ between Smad3 KO and WT [5]. However, under HFD conditions, the Smad3 KO mice tended to gain more body weight showing better insulin sensitivity compared to the controls. Histological analysis revealed that the size of the adipocytes was smaller than the control. Consistent with previous reports, small-size adipocytes are more insulin-sensitive than large-size adipocytes [22]. Moreover, it is known that administration of pioglitazone, a PPARγ ligand, improves insulin sensitivity and weight gain in humans and rodents. It is reasonable to speculate that the lack of inhibitory cue, TGF-β/Smad3, made adipocytes differentiate further and improved insulin sensitivity. Surprisingly, while the Smad3 KO mice showed higher insulin sensitivity and smaller adipocytes, there was no significant difference in adipocyte markers between the Smad3 KO and control. Because even with the lack of Smad3 some adipocytes showed inhibited differentiation in the presence of TGF-β, the HFD-induced WAT from Smad3 might be heterogeneous. This might make it difficult to detect a difference in adipocyte markers when we analyzed whole adipose tissues. It has also been reported that C/EBPβ and C/EBPα double KO mice exhibited impairment of fat tissue development, whereas there were no changes in the differentiated adipocyte markers [23]. Therefore, there might be a discrepancy between the adipocyte morphology and its marker expressions. It has been reported that TGF-β increased the pre-adipocyte proliferation in many species [24,25]. However, we were not able to detect proliferation marker expression (Ki-67) in neither Smad3 KO nor WT controls under the HFD conditions (data not shown).

There are some limitations to the present study. First, we still do not know whether the elevated TGF-β signaling in obese mice causes obesity and insulin resistance as a primary or a secondary effect. Second, we do not completely understand the molecular mechanism by which TGF-β/Smad3 signaling inhibited adipogenesis.

Nonetheless, we showed that Smad3 KO mice exhibited improvement of HDF-induced insulin sensitivity when they were fed HFD *in vivo*. Taken together with the *in vitro* data, the inhibition of TGF-β/Smad3 might be a new drug target to prevent obesity and improve insulin resistance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.02.106.

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